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Novel Roles of Cell Cycle Regulator E2f1 in the Cns: Implications for Synaptic Damage in HIV-Associated Neurocognitive Disorders

Abstract

HIV-associated neurocognitive disorders (HAND) are comprised of a host of cognitive, motor, and behavioral impairments affecting approximately 30-50% of HIV-infected individuals. Despite the development of combination antiretrovirals as a therapy to suppress viral replication, there is currently no effective treatment for HAND-associated neurological symptoms. Advanced understanding of HAND neuropathogenesis is necessary to identify novel therapeutic targets that mediate the neuronal damage and neuronal death associated with this disease. One of the molecular pathways implicated in HAND progression is the aberrant activation of the cell cycle machinery. Activation of the cell cycle machinery leads to the coordinated disinhibition of the transcription factor E2F1, which initiates the irreversible entry from G1 to S phase in cycling cells, leading to neuronal dysfunction and death. Although E2F1 is well-studied in the context of neuronal death, little is known regarding its physiologic role in the healthy central nervous system (CNS). In the present dissertation work, we aimed to elucidate the role of E2F1 in the healthy CNS, providing implications for E2F1 contribution to HAND neuropathogenesis. We first provided data to suggest that E2F1 does not mediate neuronal damage through its classical functions as a transcriptional regulator of apoptotic and cell cycle-related genes in vitro model of HIV-associated toxicity. Next, we characterized the age-dependent behavioral deficits and synaptic disruptions and the impairment in adult neurogenesis in mice with E2F1 gene mutation. Furthermore, we verified that E2F1 can regulate the expression of postsynaptic density protein-95 and neuronal morphology independently from of its effects on neurogenesis. We also found that E2F1 in the brain, unlike in other peripheral tissues, is regulated through alternative splicing. Lastly, we presented evidence that mice classically described as E2F1 null animals do indeed express residual mutant E2F1 mRNA transcript and protein. Taken together, our results suggest that E2F1 plays additional roles in the CNS and that E2F1 is regulated by unique mechanisms in the CNS. Understanding these new functions of E2F1 and how they are regulated will provide new insights into CNS development, disease and therapeutic intervention.

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**NOVEL ROLES OF CELL CYCLE REGULATOR E2F1 IN
THE CNS: IMPLICATIONS FOR SYNAPTIC DAMAGE IN
HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS**

Jenhao Harry Ting

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Degree of Doctor of Philosophy

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NOVEL ROLES OF CELL CYCLE REGULATOR E2F1 IN THE CNS: IMPLICATIONS FOR
SYNAPTIC DAMAGE IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

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Jenhao Harry Ting

DEDICATION

This body of work is dedicated to my grandmother, Suchin Ting-Yang, whose bravery inspired my pursuit of scientific excellence.

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First and foremost, I would like to thank my mentor, Dr. Kelly Jordan-Sciutto, for providing me the most incredible environment of support during my thesis years at the University of Pennsylvania. Her continuous guidance and perpetual patience have been pivotal for the completion of my work. Despite periodic setback in my research project, her endless kind and gentle words of optimism, encouragement, and confidence allowed me to rally and carry forward. Without her nurturing support, this work would not have been possible. I am fortunate enough to have her as my thesis advisor and have had the most wonderful thesis years with her.

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ABSTRACT

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Jenhao Harry Ting
Dr. Kelly L. Jordan-Sciutto

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characterized the age-dependent behavioral deficits and synaptic disruptions and the impairment in adult neurogenesis in mice with E2F1 gene mutation. Furthermore, we verified that E2F1 can regulate the expression of postsynaptic density protein-95 and neuronal morphology independently from of its effects on neurogenesis. We also found that E2F1 in the brain, unlike in other peripheral tissues, is regulated through alternative splicing. Lastly, we presented evidence that mice classically described as E2F1 null animals do indeed express residual mutant E2F1 mRNA transcript and protein. Taken together, our results suggest that E2F1 plays additional roles in the CNS and that E2F1 is regulated by unique mechanisms in the CNS. Understanding these new functions of E2F1 and how they are regulated will provide new insights into CNS development, disease and therapeutic intervention.

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CHAPTER 1 - Introduction

HIV-Associated Neurocognitive Disorders

According to the latest epidemiological data from World Health Organization in 2011, there are approximately 35 million individuals infected with the human immunodeficiency virus (HIV) and 1.6 million reported deaths due to HIV/acquired immunodeficiency syndrome (AIDS) worldwide (Organization 2012). HIV is a retrovirus that infects host cells expressing CD4 receptor and one of the two co-receptors: CXCR4-chemokine receptor 4 or CXCR5-chemokine receptor 5 (Dalglish *et al.* 1984, Deng *et al.* 1996, Feng *et al.* 1996). After infection, the virus replicates within the host cell and spreads to other susceptible cells, often destroying the host cell in the process. Acute infection of HIV is followed by a latency period and, without any therapeutic intervention, the development of AIDS (Stevenson 2003). Along with the destruction of the host immune system, HIV infection can also lead to central nervous system (CNS) complications. In fact, prior to the development of combination anti-retroviral therapy (cART) as an efficient method for controlling viral replication, as many as 20~30% of HIV-infected individuals developed a neurological disorder known as HIV-associated dementia (HAD) (Kaul *et al.* 2005). In the post-cART era, most neurological dysfunctions associated with HIV infection are clustered together as HIV-associated neurocognitive disorder (HAND). Clinically, HAND is characterized by cognitive impairments (forgetfulness, poor attention, weak executive functioning), behavioral abnormalities (apathy, social withdrawal, agitation), and motor deficits (leg weaknesses,

tremor, ataxia) (Gonzalez-Scarano & Martin-Garcia 2005, Boisse *et al.* 2008). Given that HAND onset correlates well with plasma viral load, it is not surprising that the use of cART designed to block viral replication has also reduced the incidence of HAND (Childs *et al.* 1999, Robertson *et al.* 2007). However, the prevalence of individuals living with HAND has increased significantly due to the increased average lifespan of HIV-infected individuals (Dore *et al.* 1999, Sacktor *et al.* 2001). In addition, the brain pathology in the era of cART has shifted to from neuronal death to severe synaptodendritic damage, which has not been a target of treatment (Ellis *et al.* 2007). The lack of a treatment for HAND creates a pressing need for a greater understanding of HAND pathogenesis and for the development of novel therapeutic strategies for these patients.

Pathologically, the post-mortem examination of brains of HAND patients reveal exhibit classic hallmarks of neuroinflammation that including astrocytosis, myelin pallor, activated microglia, and multinucleated giant cells (McArthur *et al.* 2003). Although overt neuronal death is no longer a prominent feature of HAND neuropathology in the era of cART treatments, there is evidence of persistent neuronal damage in these patients as evidenced by reduced synaptic density, dendritic simplification, and neuronal loss (Boisse *et al.* 2008, Everall *et al.* 2005). Neuronal damage in HAND begins with HIV infiltration into the CNS. Although some studies suggest HIV can cross the blood brain barrier (BBB) by transcytosis of endothelial cells, the low amount of infected endothelial cells observed do not reflect this interpretation (Bomsel 1997, Banks *et al.* 2001). Instead, the virus resides in infected peripheral monocytes that replenish the perivascular macrophages in the CNS, and thus gaining entry into the CNS (Haase 1986, Gonzalez-

Scarano & Martin-Garcia 2005). Once inside the CNS, the virus replicates in infected macrophages and subsequently spreads to other macrophages and resident microglia in the brain parenchyma. Though infection of astrocytes have been documented *in vivo*, this infected cell population is not thought to contribute to the viral reservoir since HIV infection in these cells has not been conclusively shown to lead to viral integration and replication (Churchill & Nath 2013). Importantly, despite evidence of neuronal damage, neurons themselves are not susceptible to HIV infection as they lack CD4 receptors necessary for viral entry (Gonzalez-Scarano & Martin-Garcia 2005, Dalgleish et al. 1984). Rather, the neuronal damage is likely due to soluble factors released from other infected cells in the CNS such as cytokines, chemokines, reactive oxygen species, excitotoxins, quinolinic acid, and viral proteins (Lipton *et al.* 1991, Achim *et al.* 1993, Bukrinsky *et al.* 1995, Hesselgesser *et al.* 1998, Ohagen *et al.* 1999, Kaul & Lipton 1999). As a result, this change in extracellular milieu often leads to the activation of molecular pathways that respond to excitotoxicity and neuroinflammation mediating neurodegeneration (Kaul & Lipton 2004). One such molecular pathway that has been implicated in HAND is the dysregulation of different components of the cell cycle machinery that leads to the damage and death of fully differentiated post-mitotic neurons (Jordan-Sciutto *et al.* 2002a, Jordan-Sciutto *et al.* 2002b, Silva *et al.* 2003, Garden *et al.* 2004, Strachan *et al.* 2005a, Wang *et al.* 2007b, Shimizu *et al.* 2007, Patrick *et al.* 2011, Akay *et al.* 2011, Colacurcio *et al.* 2013).

Role of cell cycle machinery in neurodegeneration

In dividing cells, for cell division to occur, the coordinated activation of cell cycle-related proteins, such as cyclin:cyclin dependent kinase (CDK) complexes, retinoblastoma protein (pRb) and adenovirus E2 promoter binding factor (E2Fs) must be tightly controlled (Kovesdi *et al.* 1986, Helin *et al.* 1992, Kaelin *et al.* 1992). Typically, E2Fs are bound by pRb or related pocket binding proteins p107 and p130 in the nucleus which prevent the transcriptional activation of E2Fs target genes that are necessary for cell cycle progression (Bagchi *et al.* 1991, Chellappan *et al.* 1991). In response to mitogenic signal and the activation of the CyclinD:CDK4/6 and CyclinE:CDK2 complexes, pRb is phosphorylated and undergoes conformational changes, which disrupts its interaction with E2Fs and, thereby, disinhibits E2Fs transcriptional activity (Giacinti & Giordano 2006). E2F1 is the most well characterized family members amongst all E2Fs and the upregulation of its transcriptional targets such as Cyclin A, E, and DHFR is necessary for the cycling cells to enter the S phase to begin DNA replication in preparation for cell division (DeGregori & Johnson 2006). In the absence of incorrect DNA replication or mitotic segregation-led cell cycle arrest, the precursor cell rapidly progresses through the rest of the cell cycle yielding two progeny cells. In contrast, the majority of adult neurons in the CNS are post-mitotic and fully differentiated. The failure to inhibit entry into S phase in neurons has been shown to lead to neuronal death (Herrup & Yang 2007). Therefore, blocking aberrant activation of cell cycle machinery is a potential therapeutic strategy in neurodegenerative diseases including HAND.

Evidence for the re-activation of the cell cycle machinery in neurodegenerative diseases, particularly in Alzheimer's disease (AD), has been well documented (Herrup &

Arendt 2002, Arendt & Bruckner 2007, Kim & Tsai 2009, Arendt 2012, Herrup 2012). The earliest indication came from the observations that a number of cell cycle components typically associated with cells undergoing division including CDK1, CDK4, Cyclin B, Cyclin D, proliferating cell nuclear antigen (PCNA), p16, and Ki-67 are aberrantly expressed in neurons of AD brains (Smith & Lippa 1995, Arendt *et al.* 1996, Vincent *et al.* 1997, Busser *et al.* 1998). Moreover, neurons with greater than twice the normal chromosomal DNA content are found in AD brains, suggesting that these neurons have re-entered the cell cycle to replicate their DNA (Yang *et al.* 2001). Although neuronal aneuploidy can also be observed in normal brains, the frequency of such neurons is significantly greater in AD, and is accompanied by the expression of mitotic marker cyclin B (Mosch *et al.* 2007). Importantly, these types of cell cycle related events are observed in early stages of AD before the onset of other neuronal pathologies, suggesting that entry into the cell cycle precedes neuronal death in AD (Yang *et al.* 2003). Furthermore, *in vivo* mouse models recapitulating AD pathology and *in vitro* models mimicking A β neuronal toxicity reveal clear evidence of activation of cell cycle machinery (Andorfer *et al.* 2005, Yang *et al.* 2006, Varvel *et al.* 2008, Jaworski *et al.* 2009, Bhaskar *et al.* 2009, Judge *et al.* 2011, Bhaskar *et al.* 2014). Together, these studies firmly establish the involvement of cell cycle machinery in the pathogenesis of AD. However, the precise mechanism as to how the activation of cell cycle machinery leads to neuronal death is still unclear. Given that the cell cycle entry requires the inactivation of pRb and the subsequent transcriptional regulation of E2F targets, E2F1 has been well described for these roles in neurodegenerative diseases.

Altered expression of E2F1 has been observed in a number of neurodegenerative

diseases such as AD, Parkinson's (PD), Huntington's (HD), and amyotrophic lateral sclerosis (ALS) (Ranganathan *et al.* 2001, Jordan-Sciutto *et al.* 2002a, Jordan-Sciutto *et al.* 2003, Hoglinger *et al.* 2007, Pelegri *et al.* 2008, Varvel *et al.* 2008). Aberrant E2F1 activity has also been implicated in HAND and their respective *in vivo* and *in vitro* models of toxicity. For example, numerous studies have reported abnormal expression and localization of E2F1 in autopsy brain tissues of HIV encephalitis, HAD patients, and a simian model of lentiviral disease (SIVE) (Jordan-Sciutto *et al.* 2000, Jordan-Sciutto *et al.* 2002b, Shimizu *et al.* 2007, Wang *et al.* 2010). Most notably, E2F1 levels are increased in the hippocampus, basal ganglia, and frontal cortex of encephalitic human brains compared with non-encephalitic, age-matched controls. Several studies have mechanistically linked E2F1 to neuronal death in these diseases. For example, cell cycle related events precede neuronal death in models of retinal degeneration, and the depletion of E2F1 significantly attenuates neuronal death (Zencak *et al.* 2013). The pathogenic gain of function mutation of leucine-rich repeat kinase 2 (LRRK2) found in familial forms of PD antagonizes microRNA (MiR)-184 suppression of E2F1, resulting in neurodegeneration (Gehrke *et al.* 2010). Similarly, MiR-26b, which is found to be upregulated in AD, acts through the Rb/E2F1 pathway to mediate cell cycle re-entry and neuronal death (Absalon *et al.* 2013). Interestingly, however, increased E2F1 expression is not exclusively in the nucleus but also in the cytoplasm, a subcellular localization inconsistent with its transcriptional activities defined in dividing cells (Jordan-Sciutto *et al.* 2002b, Wang *et al.* 2010). These observations suggest that E2F1 may contribute to disease pathogenesis through an unidentified mechanism. Given the unusual localization and increased expression of E2F1 in neurodegenerative diseases, it is imperative to

further investigate its role and regulation in the CNS.

E2F1: structure, function and regulation

E2F1 is the first identified member of the eight E2F family members of transcription factors. E2F1 shares many promoter binding sites with the rest of the E2F family since they share similar consensus target binding sequence and a highly homologous nucleic acid binding domain (DeGregori & Johnson 2006). The transcriptional targets, and the outcomes of E2F binding to these targets are dictated by their interacting partners and other co-factors, as opposed to the specific target sequence. In general, E2Fs are categorized into two groups: E2F1-3a are transcriptional activators, whereas E2F3b-8 are the transcriptional repressors (Blais & Dynlacht 2004, Johnson & Degregori 2006, Iaquinta & Lees 2007). For the relevance of the present work, the discussion will focus specifically on E2F1, its structure, function, and regulation.

Human E2F1 is composed of 437 amino acids (Fig 1). Structural analysis of the DNA binding domain reveals that E2Fs possess a highly homologous winged-helix DNA-binding motif (Zheng *et al.* 1999). Next to the DNA-binding motif is the heterodimerization domain through which E2F1 binds to its dimerization partners differentiation regulated transcription factor polypeptide (DP) 1/2 to become a fully functional transcription factor (Cress & Nevins 1994, Girling *et al.* 1993). The Cyclin A/CDK2 complex interacts with E2F1 near the N-terminus and modulates E2F1 DNA binding activity (Xu *et al.* 1994). Harbored in this region is a nuclear localization signal (NLS) that when unmasked, confers E2F1 nuclear distribution (Muller *et al.* 1997,

Verona *et al.* 1997). The pRb binding site resides in the transactivation domain of E2F1 at the C-terminus (Huang *et al.* 1992). While bound to DNA, E2F1 forms a stable complex with DP1, CyclinA/CDK2, and pRb. In the quiescent state of the cell cycle, the transcriptional activation of E2F1 targets is physically blocked by pRb binding. Upon its phosphorylation by upstream cyclin/CDK complexes, pRb undergoes a conformational change releasing E2F1 from the pRb pocket, and allowing E2F1 to activate its transcriptional targets (DeGregori & Johnson 2006).

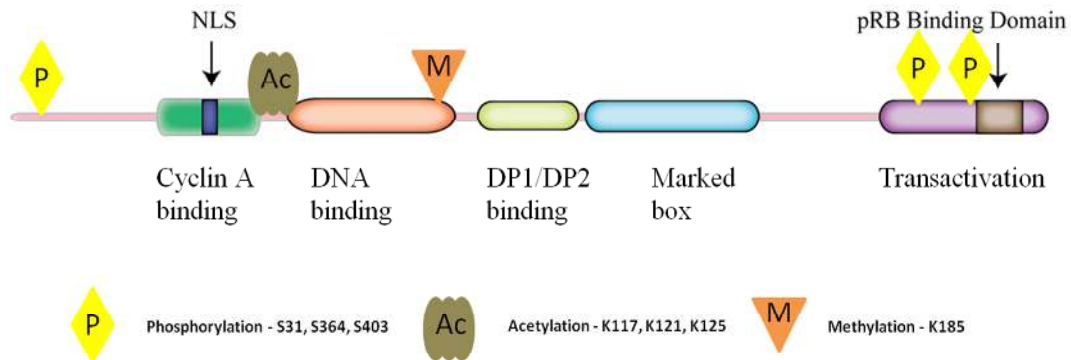


Figure 1. Schematic of the human E2F1 structure. E2F1 has five highly characterized functional domains: Cyclin A binding, DNA binding, DP-1/DP-2 binding, Marked box, and transactivation domains. Also critical in determining E2F1 activity is the nuclear localization signal near the N-terminus and pRb binding domain near the C-terminus. E2F1 is also highly post-translationally modified by phosphorylation (P), acetylation (Ac), and methylation (M).

Given that the ectopic expression of E2F1 initiates S-phase entry in the absence of mitogenic stimuli, E2F1 was originally studied solely as a transcription factor upregulating genes involved in the G₁ to S phase transition in response to mitogenic stimuli (DeGregori & Johnson 2006, Macleod 1999). However, it later became apparent that E2F1 also had tumor suppressive properties as E2F1 mutant mice develop

widespread cell proliferation and tumor growth (Field *et al.* 1996, Yamasaki *et al.* 1996). Indeed, aside from its role in G₁ to S phase regulation, E2F1 can also induce apoptosis via p53-dependent, p53-independent, and transcription-independent mechanisms (Putzer 2007). p53 is a well characterized tumor suppressor that can induce cell cycle arrest and apoptosis through transcriptional regulation but is often disrupted in wide variety of cancers (Polager & Ginsberg 2009). E2F1 can upregulate p14^{arf} (p19^{arf} in rodents), which disrupts p53 interaction with MDM2, an E3 ubiquitin ligase that maintains p53 at a basal level through ubiquitination. Disruption of the MDM2/p53 interaction stabilizes p53 resulting in p53-dependent apoptosis (Bates *et al.* 1998, Sherr 2006). In addition, E2F1 can affect p53 function independently of p14^{arf}/p19^{arf} protein as activation of E2F1 induces p53-dependent apoptosis in p19^{arf} null animals (Tolbert *et al.* 2002). E2F1 can affect the phosphorylation status of p53 or bind directly to p53 to enhance p53 induced apoptosis (Rogoff *et al.* 2002, Hsieh *et al.* 2002). Alternatively, E2F1 can induce apoptosis independently of p53 (Holmberg *et al.* 1998) by directly upregulating apoptotic targets such as APAF1 (Moroni *et al.* 2001), PUMA, NOXA, Bim, Hrk/DP5 (Hershko & Ginsberg 2004), Smac/Diablo (Xie *et al.* 2006), caspase 3,7,8,9, (Nahle *et al.* 2002), SIVA (Fortin *et al.* 2004) and Bik (Real *et al.* 2006). Finally, E2F1 can induce cell death in a transcription-independent manner as ectopic expression of DNA-binding or transcriptional-activating incompetent E2F1 also leads to cell death (Phillips *et al.* 1999, Strachan *et al.* 2005a, Bell *et al.* 2006).

Other than its role in cell proliferation and death, E2F1 can also regulate energy metabolism by gene modulation as demonstrated with mice lacking E2F1 resulting in various metabolic phenotypes including disrupted adaptive thermogenesis and energy

expenditure (Blanchet *et al.* 2011). Following detections of double stranded breaks, E2F1 can also trigger DNA damage response (Biswas & Johnson 2012). For example, upon irradiation-induced DNA damage, E2F1 localizes to regions of damaged DNA and recruits nucleotide excision repair factors (NER) to the sites of repair. This recruitment is not dependent on the DNA binding domain or the transactivation domain of E2F1, indicating that E2F1 function is not restricted to transcriptional regulation (Guo *et al.* 2010). Not surprisingly, given the wide range of E2F1 functions and sometimes paradoxical roles that have been reported, the precise mechanism that determines whether E2F1 signals cell division, arrest, or death in response to different stimuli remains unknown. However, E2F1 expression is highly regulated at multiple levels including transcription, post-transcription, and post-translational modification.

At the transcription level, E2F1 can upregulate its own expression, creating a positive feedback during G₁ to S phase transition (DeGregori & Johnson 2006, Iaquinta & Lees 2007). At the post-transcription level, E2F1 expression is highly subjected to regulation by MiRs following its mRNA transcription. E2F1 expression can be negatively regulated by several MiRs including MiR 17-5p and 20a (O'Donnell *et al.* 2005), MiR-106b (Petrocca *et al.* 2008), MiR-205 (Dar *et al.* 2011). Depending on which MiR cluster is expressed or activated, the apoptotic or the proliferative properties of E2F1 can be selectively suppressed allowing the cells to respond to different stimuli in a highly regulated fashion (Knoll *et al.* 2013).

At the post-translational level, E2F1 activity can be altered by protein modification and subcellular redistribution. Typically, E2F1 is ubiquitinated and targeted for proteasomal degradation by SKP1–CDC53 (cullin)–F-box protein-SKP2 complex

(Marti *et al.* 1999). However, E2F1 can be phosphorylated at serine-31 by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) in response to DNA damage, which increases E2F1 stability (Lin *et al.* 2001). Phosphorylation at serine-31 can lead to the recruitment of 14-3-3tau chaperone protein to further stabilize E2F1 by direct protein:protein interaction and promote E2F1 apoptotic activity (Wang *et al.* 2004). In addition, E2F1 can also be phosphorylated at serine-364 by checkpoint kinase 2 (Chk2) or serine-403 by an unknown kinase in response to DNA damage that leads to E2F1-mediated apoptosis (Stevens *et al.* 2003, Real *et al.* 2010). Furthermore, E2F1 can be acetylated by P/CAF or P300 acetyltransferases, which increases its DNA binding activity, transactivation activity, and stability (Martinez-Balbas *et al.* 2000, Galbiati *et al.* 2005). This modification is countered by the E2F1 transcriptional target deacetylase sirtuin1 which suppresses E2F1 activity in a negative feedback loop (Wang *et al.* 2006). E2F1 is also methylated by Set9 methyltransferase which inhibits E2F1 transcriptional activity (Kontaki & Talianidis 2010). Finally, E2F1 is also regulated by its subcellular distribution as it undergoes constant nuclear-cytoplasmic shuttling (Ivanova & Dagnino 2007, Ivanova *et al.* 2007).

In summary, E2F1 has various functions that determine the fate of cycling cells. Despite extensive study of E2F1 function and regulation, our understanding of E2F1 is largely restricted to the context of cycling cells. In post-mitotic neurons, however, cell cycle proteins like E2F1 may play an entirely different role. For example, components of the origin recognition complex (ORC), which localizes to initiation sites of DNA replication in proliferating cells, are enriched in the synapses of mature neurons and regulate dendritic morphology and spine formation (Huang *et al.* 2005). Therefore, we

hypothesize that E2F1 may also have novel functions in the CNS that are separate from its classic cell cycle/apoptotic functions implicated in neurodegenerative diseases.

E2F1 in the CNS

Given that E2F1 is a crucial player in determining cell fate, E2F1 in the context of neuronal death has been extensively studied *in vitro*. *In vitro*, cell cycle reactivation through the pRb:E2F1 pathway has been implicated in a number of neuronal death models including activity deprivation, beta-amyloid toxicity, and DNA damage (Park *et al.* 1997, Giovanni *et al.* 1999, Giovanni *et al.* 2000, Park *et al.* 2000a, Park *et al.* 2000b, O'Hare *et al.* 2000). Ectopic expression of full length E2F1 results in the death of cerebellar granule neurons (CGN), whereas loss of E2F1 function partially delays potassium deprivation- induced neuronal death (O'Hare *et al.* 2000). However, it is later reported in another study that loss of E2F1 function did not lead to a rescue of deprivation-mediated CGN death, suggesting that E2F1 apoptotic function in neurons may be context dependent (Yuan *et al.* 2007). In support of this, overexpression of E2F1 in mouse CGN induces cell death, while such overexpression in rat CGN has no effect (Yuan *et al.* 2011). Thus, the role of E2F1 in neuronal death may depend on several factors, such as the neuron subtype, neuronal age, and the presence or absence of interacting partners.

Similarly in cycling cells, E2F1 can mediate death in neurons through several mechanisms. First, neuronal death occurs when E2F1 mediates cell cycle re-entry as inhibitors that block cell cycle machinery upstream of E2F1 also block neuronal death

(Park et al. 1997, Giovanni et al. 1999, Hou *et al.* 2002). Alternatively, E2F1 can upregulate pro-apoptotic targets such as SIVA and neuropilin-1 to mediate neuronal death (Fortin et al. 2004, Jiang *et al.* 2007). The fact that E2F1 can mediate neuronal death through different mechanisms, and that its dysregulation can irreversibly change neuronal fate underscores the importance of understanding E2F1 function in neurodegenerative diseases as well as in healthy CNS. Despite this, its physiologic role in the CNS under normal conditions has long been neglected. In the subsequent section, I highlight the scarce literature that has focused on E2F1 physiologic function in the CNS.

Given that neural progenitor are proliferating throughout brain development *in utero*, E2F1 expression is ubiquitous in the brain, particularly in the ventricular regions where the proliferation of neural stem cells takes place (Dagnino *et al.* 1997). However, E2F1 expression does not terminate following brain development. Instead, the mRNA transcript of E2F1 is kept at a basal level similar to what is observed in developing brain (Tevosian *et al.* 1996). Furthermore, the expression of E2F1 protein is upregulated through development and into early adulthood suggesting a role for E2F1 in the adult CNS (Kusek *et al.* 2001). Additionally, E2F1 has been shown to interact with novel protein partners that are not highly expressed in cycling cells in post-mitotic neurons. For example, E2F1 interacts with Necdin, a post-mitotic nuclear factor that suppresses cell cycle and growth, and functions with E2F1 to promote terminal differentiation of neurons (Taniura *et al.* 1998, Kobayashi *et al.* 2002). E2F1 also interacts with neural proliferation differentiation and control protein 1 (NPDC1), another marker for postmitotic neurons; and this interaction reduces E2F1 DNA binding and transcriptional activity (Galiana *et al.* 1995, Sansal *et al.* 2000). Glycogen synthase kinase 3 (GSK3) β

also binds to E2F1 promoting E2F1 degradation that is necessary for the nerve growth factor induced PC12 differentiation (Zhou *et al.* 2008). Similarly, when oligodendrocyte progenitors undergo differentiation, nuclear E2F1 is decreased with corresponding increase in cytoplasmic E2F1 (Magri *et al.* 2014). Expectedly, this compartmental redistribution of E2F1 is accompanied by a decrease in E2F1 binding to its target gene promoters, highlighting that E2F1 does not regulate its canonical targets in differentiated cell types in the brain. Indeed, N-cadherin, important in dendritic and synaptic morphology, is one of the novel targets of E2F1 in neural cells (Kubota *et al.* 2009).

In contrast to its role in cycling cells where it drives G₁ to S phase transition, E2F1 in the brain functions as a cell cycle repressor in mature neurons as the neurons derived from E2F1 deficient brains cycle autonomously (Wang *et al.* 2007a). The concerted regulation of the cell cycle-related genes with CDK5, E2F1, and other E2F family members is required for maintaining the terminal differentiated state of post-mitotic neurons (Zhang *et al.* 2010). On the other hand, in the context of neural progenitors, E2F1 can indeed promote physiologic cell proliferation in adult neurogenesis. Neural progenitor proliferation in the dentate gyrus and the olfactory bulbs are significantly reduced in mice lacking E2F1 function without any change in the total number of neurons in the neocortex (Cooperkuhn *et al.* 2002). This reduction in newly generated cells is accompanied by a reduction of apoptotic death in these mouse brains, indicating that E2F1 may have multiple roles depending on the environmental context. In summary, these studies provide evidence that the traditional E2F1 cell cycle inducing function is suppressed in post-mitotic cell types but not obsolete, as in the context of neurogenesis. The lack of clarity provided by these studies also underscores the necessity

to investigate and enhance our understanding of E2F1 physiologic function in the CNS. Since the removal of E2F1 has been linked to neurogenesis, in the following section, we will briefly summarize our current understanding of adult neurogenesis and in the context of disease.

Neurogenesis in the adult brain

Neurogenesis in the adult brain was first documented nearly half a century ago when mitotic activity was observed in the dentate gyrus of young adult rats (Altman & Das 1965). Despite its recognition, there was much resistance surrounding the functional role of this mitotic activity until it was discovered that these newly generated cells had the ability to differentiate into glia and neurons (Lois & Alvarez-Buylla 1993). Since the acceptance of neurogenesis in the subventricular zone (SVZ) and the dentate gyrus (DG) in the adult brain, there is an increasing, although controversial, expansion of other brain areas with reported neurogenesis, including the neocortex in macaques, substantia nigra in rodents, cerebellum in rabbits, and most recently the striatum in humans (Gould *et al.* 1999, Lie *et al.* 2002, Madsen *et al.* 2003, Ponti *et al.* 2008, Ernst *et al.* 2014). Nevertheless, the persistent production of new neurons and glial cells in the SVZ and DG is now regarded as a crucial part of brain development and maturation and will be the primary focus of discussion.

In the SVZ, the proliferative self-renewing stem cells give rise to neuroblasts that migrate through the rostral migratory stream (RMS) to the olfactory bulbs, where they may be incorporated into the local circuitry as immature, doublecortin (DCX) –

expressing and often inhibitory interneurons (Ming & Song 2011, Carleton *et al.* 2003). In contrast, the majority of the cells generated in the DG that do not rapidly differentiate into neurons and incorporate into the local circuitry are eliminated through apoptosis (Dayer *et al.* 2003). The surviving cells in the DG are typically neuronal marker NeuN-expressing glutamatergic neurons; while a lower percentages of surviving cells express glia marker glial fibrillary acidic protein (GFAP) (Steiner *et al.* 2004, van Praag *et al.* 2002). However, the rate of cellular proliferation and the fate of these cells in both regions depend heavily on intrinsic factors such the cell cycle machinery and on extrinsic factors such as trophic factors (Abrous *et al.* 2005).

Like all cycling cells, neural progenitor cells are strictly regulated by the Rb-E2F pathway at the G₁ to S cell cycle checkpoint (Abrous *et al.* 2005). In transgenic mice, deletion of E2F1 exons 3 and 4 leads to significant deficits in the number of adult-born neurons, while deletion of pRb removal leads to increased apoptotic cells during embryonic development and even in post-mitotic neurons (Cooperkuhn *et al.* 2002, Lee *et al.* 1992, Andrusiak *et al.* 2012). On the other hand, the administration of trophic factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor (TGF), brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) or insulin-like growth factor (IGF) can directly stimulate neurogenesis in the adult brain, while the disruption of these signaling pathways impedes neurogenesis (Kuhn *et al.* 1997, Craig *et al.* 1996, Zigova *et al.* 1998, Zhu *et al.* 2003, Abrous *et al.* 2005). Furthermore, there is some evidence of crosstalk between the intrinsic and the extrinsic factors. Specifically, E2F1 can independently regulate the downstream signaling pathways of the trophic factors, as E2F1 can directly regulate the

expression of FGF and IGF receptors via transcriptional regulation in the proper context (Tashiro *et al.* 2003, Schayek *et al.* 2010). Although the regulatory role of E2F1 on other trophic factor signaling pathways remains to be investigated, these studies highlight the importance of E2F1 on neurogenesis in the adult brain.

Since adult neurogenesis often declines with age and cognitive deficits, neurogenesis in the adult brain is regarded as a target of disruption in neurological disorders (Zhao *et al.* 2008, Rodriguez & Verkhatsky 2011). However, in the context of AD, there is no consensus as to how neurogenesis is affected since neurogenesis is significantly elevated in post-mortem tissue of AD brains but impaired in mouse models of AD (Jin *et al.* 2004, Verret *et al.* 2007). Adult neurogenesis has also been linked to major depressive disorders as effective antidepressants therapies boost neurogenesis (Abrous *et al.* 2005, Villanueva 2013, Malberg *et al.* 2000, Santarelli *et al.* 2003). In post mortem tissue from patients with HIVE or with neuroinflammation, trophic factors such as BDNF, FGF, nerve growth factor (NGF) that normally stimulate neurogenesis are significantly reduced (Fields *et al.* 2014, Woodbury & Ikezu 2014, Everall *et al.* 2001). Consistent with these studies, in mouse models of HAND, adult neurogenesis is significantly impaired but is restored following treatment with the antidepressant paroxetine or trophic factor FGF and BDNF (Okamoto *et al.* 2007, Everall *et al.* 2001, Lee *et al.* 2013).

In summary, adult neurogenesis is dysregulated in various neurological disorders, and the therapeutic strategies utilized in these disorders often rescue the disruption to neurogenesis as well. Given that E2F1 has been shown to regulate neurogenesis in the brain, it is possible that E2F1 may also directly contribute to the pathogenesis of these

diseases through its regulation of adult neurogenesis. Alternatively, E2F1 may have other functions in the brain that remain to be identified. Nevertheless, the loss of the endogenous E2F1 activity can be as damaging to neuronal function as the activation of its cell-cycle inducing or apoptotic function. To examine E2F1 alternate function in the CNS and in disease, as described in Chapter 2, we first determined whether E2F1 contributes to the pathogenesis of HAND through its classic role as a transcriptional regulator in an *in vitro* model of HIV-associated toxicity. In Chapter 3, we sought to identify the novel role of E2F1 in the brain by characterizing the age-dependent behavioral and synaptic disruptions in mice with E2F1 gene mutation. In Chapter 4, we further verified whether E2F1 may have a more direct role in synaptogenesis independent of its effect on neurogenesis. In Chapter 5, we identified and characterized the novel regulation of E2F1 in the brain by alternative splicing. In Chapter 6, we present evidence that mice classically described as E2F1 null animals do indeed express residual mutant E2F1 mRNA transcript and protein. Lastly, in Chapter 7, we discuss our current findings and propose future directions to address the implications of our studies. Taken together, our results suggest that E2F1 plays additional roles in the CNS beyond stem cell and neural progenitor proliferation and cell cycle exit. Further, E2F1 is regulated by unique CNS specific mechanisms. Understanding these new functions of E2F1 and how they are regulated will shed new insight into CNS development, disease and therapeutic intervention.

**CHAPTER 2: E2F1 localizes predominantly to neuronal cytoplasm and
fails to induce expression of its transcriptional targets in Human
Immunodeficiency Virus-induced neuronal damage**

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Abstract

As human immunodeficiency virus (HIV) does not induce neuronal damage in HIV-associated dementia (HAD) by direct infection, the mechanisms of neuronal damage or loss in HAD remain unclear. Interestingly, immunoreactivity of E2F1, a pro-apoptotic, cell cycle regulatory transcription factor, has been reported to increase in neurons with a predominantly cytoplasmic localization in several neurodegenerative diseases including HIV-associated pathologies. Here we confirm that E2F1 localization is predominantly cytoplasmic in postmitotic primary rat cortical neurons in vitro, as well as in cortical tissue derived from HIV-infected individuals. To determine whether E2F1 contributes to neuronal death in HAD by transactivation of its transcriptional targets implicated in cell cycle progression and apoptosis, we assessed the mRNA and/or protein levels of several classical E2F1 transcriptional targets in an in vitro model of HIV-induced neurotoxicity and in patients infected with HIV. Using real-time PCR, we show that mRNA levels of E2F1 transcriptional targets implicated in cell cycle progression (E2F1, Cyclin A, proliferating cell nuclear antigen (PCNA), and dihydrofolate reductase (DHFR)) and apoptosis (caspase 3, 8, 9 and p19^{ARF}) remain unchanged in an in vitro model of HIV-induced neurotoxicity. In addition, our data also show that protein levels of p19^{ARF}, Cyclin A, and PCNA are not altered in either our in vitro model of HIV-induced neurotoxicity or in the cortex of patients with HAD. Our observation that E2F1 is predominantly cytoplasmic in neurons may account for the lack of E2F1 target transactivation in neurons responding to HIV-induced neurotoxicity. Collectively, our data suggest that E2F1 does not contribute to neuronal death in HIV-associated

neurotoxicity via the previously described, transcription-dependent mechanisms.

Keywords: E2F1, HIV-associated dementia, transcription factor

Human immunodeficiency virus (HIV)-associated dementia (HAD) is a common neurological disorder associated with HIV infection. Pathologic studies of the brains of patients with HAD suggest an inflammatory mechanism in the progression of this disease, as evidenced by astrogliosis, microgliosis, and perivascular macrophage infiltration (Garden 2002, Ghorpade *et al.* 2003, Kaul *et al.* 2001). Although neuronal death, dendritic loss and synaptic loss are features of HAD, there is little evidence of direct HIV infection of neurons. Instead, neuronal dysfunction and death likely result from the release of various neurotoxic factors from activated macrophages and microglia, such as reactive oxygen species and excitatory amino acids (Genis *et al.* 1992, Wasmuth *et al.* 2004, Gonzalez-Scarano & Martin-Garcia 2005). Correlative evidence suggests that neuronal damage in HAD may result from several mechanisms, including decreased neuronal autophagy (Alirezaei *et al.* 2008), NMDA receptor activation (O'Donnell *et al.* 2006, Aksenova *et al.* 2009), abnormal CDK5 kinase activity (Wang *et al.* 2007b), activation of the p38 mitogen-activated protein kinase (MAPK) cascade (Ullrich *et al.* 2000), caspase activation, inhibition of the nuclear factor- κ B survival pathway via glycogen synthase kinase-3 α activation (Sui *et al.* 2006), and/or aberrant cell cycle regulation involving E2F1 (Jordan-Sciutto *et al.* 2002b, Shimizu *et al.* 2007).

E2F1 is a member of the E2F family of transcription factors, which play a pivotal role in cell differentiation, proliferation, and apoptosis through transcriptional regulation. In non-neuronal cells, E2F1 functions as a transcription factor that regulates genes necessary for RNA and DNA synthesis, such as Dihydrofolate reductase (DHFR) and proliferating cell nuclear antigen (PCNA) and those necessary for cell cycle progression,

such as Cyclin A (DeGregori *et al.* 1995) and E2F1 itself (Johnson *et al.* 1994). In addition, E2F1 regulates genes involved in apoptosis, including p19^{ARF}, an initiator of p53-dependent apoptosis (Bates *et al.* 1998), as well as other apoptotic genes that are independent of the p53 pathway, including APAF1, BID, caspase 2, 3, 7, 8, and 9 (Stanelle & Putzer 2006). Finally, E2F1 has been shown to induce cell death independently of gene transactivation through inhibition of anti-apoptotic signaling, NF- κ B pathway (Hou *et al.* 2001) or through induction of the calcium activated, cysteine protease, calpain (Strachan *et al.* 2005a).

Using *in vitro* models of neurodegeneration, E2F1 has been reported to contribute to neuronal damage and death (Fortin *et al.* 2004, Hou *et al.* 2002, Jiang *et al.* 2007, Smith *et al.* 2003, Hou *et al.* 2000, O'Hare *et al.* 2000, Giovanni *et al.* 2000). These studies speculate that E2F1 mediates neuronal death via activation of its transcriptional targets (Jordan-Sciutto *et al.* 2002a). However, several reports have observed cytoplasmic localization of E2F1 in postmitotic neurons of patients with neurodegenerative diseases including HIV encephalitis, SIV encephalitis, Alzheimer Disease, Parkinson Disease, Huntington Disease, and amyotrophic lateral sclerosis (Jordan-Sciutto *et al.* 2002a, Jordan-Sciutto *et al.* 2002b, Jordan-Sciutto *et al.* 2000, Chu *et al.* 2007, Pelegri *et al.* 2008). In these diseases, E2F1 immunoreactivity and/or protein levels were also reported to increase (see review (Chu *et al.* 2007)). To resolve the paradox that E2F1 induces cell death in a transcription-dependent manner *in vitro*, but is increased in the cytoplasm in HIV and SIV encephalitis (Jordan-Sciutto *et al.* 2002b, Jordan-Sciutto *et al.* 2000, Strachan *et al.* 2005a), we determined the localization of E2F1 in primary cortical neurons and in neurons from human cortex and assayed the expression of several classic

E2F1 targets in an in vitro model of HIV-induced neurotoxicity and in cortical autopsy tissue from 14 HIV-positive individuals.

As an in vitro model of HIV-induced neurotoxicity, we used a previously described model in which supernatants from HIV-infected primary monocyte-derived macrophages are used to treat primary rat cortical neurons (O'Donnell et al. 2006). Briefly, primary blood mononuclear cells were isolated from healthy volunteers as previously described (Chen *et al.* 2002), in accordance with protocols approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Cells were differentiated for 7 days in macrophage media (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin, and 1% non-essential amino acids) in six-well plates (2.5×10^6 cells per well). On day 7, cultures were infected with cell-free HIV-1 inoculum (100 ng of p24 per well) and incubated for 24 h. At which point, cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh macrophage media. Supernatants from HIV-infected monocyte-derived macrophages (HIV MDM) were monitored for productive infection by p24 ELISA (NEN, Boston, MA, USA). HIV infection of macrophages was confirmed by reverse transcriptase activity over time in each case, and only HIV MDM supernatants exhibiting productive infection, as determined by reverse transcriptase activity were used to treat neuronal cultures. Previous studies (O'Donnell et al. 2006, Wang et al. 2007b) have demonstrated that the NMDA receptor, at least partially, mediates neuronal death in this model.

Primary rat cortical cultures were prepared from embryonic day 17 Sprague-Dawley rat pups. Cells were plated at a density of 2×10^6 cells per 60 mm dish pre-

coated with poly-l-lysine (Peptides International, Louisville, KY, USA) and maintained in neurobasal media (Invitrogen) with B27 supplement (Invitrogen) at 37°C and 5% CO₂. Half of the media was replenished every 7 days, and cultures were utilized at 21 days in vitro (DIV) unless indicated otherwise.

For western blotting of human brain tissue, fresh frozen tissue samples from midfrontal cortex of HIV-infected patients suffering from neurocognitive impairments (n = 13) and control patients with normal neurocognitive status (n = 5) were obtained from the tissue banks of National NeuroAIDS Tissue Consortium (Morgello *et al.* 2001). For protein extraction, tissue was homogenized on ice in two tissue volumes of Lysis Buffer [50 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 2 mmol/L EGTA, 2 mmol/L EDTA, 5 mmol/L NaF, 0.1 mmol/L phenylmethane-sulphonylfluoride, 1 µg/mL leupeptin, 1 mmol/L DTT, and protease inhibitor cocktail]. Cell lysates were separated by 10% SDS-PAGE gel and transferred onto PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies against p19^{ARF} (Abcam, MA, USA), PCNA, DHFR and Cyclin A (Santa Cruz, CA, USA).

Quantitative real-time polymerase chain reaction (Q-PCR) was performed as described previously (Wang *et al.* 2007b). Expression levels of E2F1, p19^{ARF}, PCNA, caspase 3, 8, 9 and the internal controls of 18S, or TATA box binding protein (TBP) were quantified using the LightCycler Amplification Kit SYBR Green I (Roche Applied Science, Indianapolis, IN, USA). Results were normalized to either 18S or TBP RNA levels and were reported using arbitrary units. Primers used were: Cyclin A, forward CCGTTGCATTTATAGCCTTTG, reverse GAGTCAAAGTCCAGGTAAGGTGA; E2F1, forward CTGCAACTGCTTTCTGAGGA, reverse GATCTTGGCAGGTCACATAGG;

proliferating cell nuclear antigen (PCNA), forward GCGCAGGTGACTGGTTATC, reverse AAGGTACCCCGACTCACGAT; dihydrofolate reductase (DHFR), forward TTCCCAGAAATTGATTTGGAG, reverse AGAGAGGACGCCTGGGTATT; P19ARF, forward GTGTTGAGGCCAGAGAGGAT, reverse TTGCCATCATCATCACCT; caspase3, forward GAATGTCAGCTCGCAATGGTAC, reverse AGTAGTCGCCTCTGAAGAACTAG; caspase 8, forward AGTTTCTGTTTTGGATGAGGTGAC, reverse TCTTCATTGGTAAACGTTGTCTGA; caspase 9, forward GAGGGAAGCCCAAGCTGTTC, reverse GCCACCTCAAAGCCATGGT; TBP, forward CCCACCAGCAGTTCAGTAGC, reverse CAATTCTGGGTTTGATCATTCTG and 18S, forward GAGAAACGGCTACCACATCC, reverse GGGTCGGGAGTGGGTAAT.

Immunofluorescent staining was described previously (Lindl *et al.* 2007). The tyramide amplification system (New England Biolabs, USA) was used to detect E2F1. DNA was visualized by DAPI staining. Neurons were marked by staining for the neuronal marker, MAP2. The dilutions used were as follows: E2F1 (1:50, Santa Cruz, CA, USA), DAPI (5mM, Molecular Probes, CA, USA), MAP2 (1:200, Covance, USA). Slides were mounted and analyzed by laser confocal microscopy.

The MAP2 cell-based ELISA assay used in the current studies was described previously (Wang *et al.* 2007b). Briefly, following treatments, cells were fixed for 30 min with 4% para-formaldehyde in 4% sucrose. After blocking for 1 h with 5% normal goat serum in PBS, the plates were incubated with mouse anti-MAP2 antibody (1:1000 dilution) overnight at 4°C and were then washed with PBS-T. The plates were then incubated for 30 min with goat anti-mouse secondary antibody conjugated to beta-

lactamase TEM-1 (1:500), washed, and incubated at 23°C for 60 min with 0.5 µg FluorocillinTM Green (Invitrogen, CA, USA) substrate per well. Fluorescence intensity was measured using a Fluoroskan Ascent fluorimeter plate reader (Thermo Electron, Waltham, MA, USA) with excitation at 485 nm and emission at 527 nm. Relative changes in the intensity of the fluorescent MAP2 signal were used to estimate neuronal damage.

Values were expressed as mean ± SEM. Data with multiple categories was analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test using Prism software (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

E2F1 is well-characterized as a transcription factor that functions to control cell cycle progression. Its nuclear activity is regulated primarily by its interaction with DP1, pRB, and Cyclin A:cdk2. However, in 3 week-old rat cortical neurons left untreated (Fig 1, top row), we observed E2F1 (green) predominantly in the neuronal cytoplasm; a compartment inconsistent with its defined role as a transcription factor. In the mid-frontal cortices of uninfected, normal brains, we observed the same pattern of localization (Fig 1, bottom row). It is worth noting that the cytoplasmic localization is not altered by the ages of rat cortical cultures nor by different neurotoxic treatments, such as mock-infected MDM (mock MDM) and HIV MDM. Moreover, the cytoplasmic localization of E2F1 in cortical brain tissue of HIV-infected individuals is not dependent on the neurocognitive status of the patient (data not shown). Taken together, these data suggest that, in neurons in vitro and in the brains of HIV-infected individuals, E2F1 is

predominantly localized in a subcellular compartment that is inconsistent with its well-characterized role as a transcriptional activator.

As an *in vitro* model of HIV-induced neurotoxicity, we use a previously described model of neuronal toxicity in which supernatants from HIV-infected monocyte-derived macrophages (HIV MDM) are used to mirror the neuroinflammatory insult observed in patients. In this model, it has been shown that death of primary rat hippocampal neurons is partly mediated by NMDA receptors (O'Donnell et al. 2006, Wang et al. 2007b) particularly NR2B/NR2B and NR2A/NR2B containing NMDARs (O'Donnell et al. 2006). As these NMDARs are not present on primary neurons until 21 days in culture, we use primary rat cortical neurons at this age for our studies. Using this model, we observed 50 - 80% neuronal death depending on the dilutions of HIV-MDM (Fig 2A). For our analysis of E2F1 target transactivation, we used a dilution of HIVMDM that resulted in 50% neuronal loss at 20 hours to capture a point in which neurons were in the process of undergoing cell death.

To determine if HIV MDM-induced neuronal death is mediated by activation of classical E2F target genes, we assessed mRNA levels of select E2F target genes from each of the following categories: cell cycle progression, RNA and DNA synthesis, or apoptosis. E2F1, an indicator of G1 to S progression, and cyclin A, a regulator of S phase exit, were assessed in our model of HIV-induced toxicity as examples of E2F targets that regulate cell cycle progression. To determine if E2F1 is upregulating genes whose products are necessary for DNA and RNA synthesis, we assessed PCNA and DHFR respectively. Finally, E2F1 has been shown to induce apoptosis by transactivation of several pro-apoptotic gene products which act in distinct apoptotic cascades. Thus, we

assessed the mRNA levels of targets from each of these pathways (intrinsic apoptosis – caspase 9, extrinsic apoptosis – caspase 8, effector caspase – caspase 3, and p53-dependent apoptosis – p19^{ARF}) in neurons responding to HIV MDM. Three week-old primary cortical cultures were treated with mock MDM or HIV MDM or were left untreated. As NMDA activation partially mediates neuronal death in this model, we also included a set of cultures pretreated with MK801, an NMDA receptor antagonist, to determine if any observed changes were NMDA-dependent. By Q-PCR, we found that the mRNA levels of all analyzed target genes were similar in neurons treated with HIV MDM, mock MDM, MK801-pretreated cultures, and in cultures left untreated (Fig 2B-I). When we selectively compared the protein levels of DHFR, PCNA, Cyclin A, and p19^{ARF} by immunoblot, we were again unable to detect a difference in the expression of these targets across different treatments (Fig 3A). Our Q-PCR results are consistent with our previous observation that neither caspase 3 protein levels nor cleaved-caspase 3 are altered by HIV MDM treatment (Wang et al. 2007b).

Consistent with our findings in vitro, we did not observe differences in expression of the E2F1 targets, PCNA, Cyclin A, and p19^{ARF}, at the protein level in cortical tissue from patients with HAD, as compared with cognitively normal, HIV-infected cortex (Fig 3B, C). We also did not see significant differences in these same target genes in cortex from HIV-infected patients, as compared with uninfected patients. However, we did observe an increase in DHFR in cortical tissue of HIV-infected patients (Fig 3B, C). These data suggest that any role for E2F1 in this model of neurodegeneration may mediate death via a transcription-independent mechanism.

E2F1 is upregulated in neurons subjected to apoptotic stimuli (Verdaguer *et al.*

2002, Hou et al. 2001) and is elevated in neurons of patients with Alzheimer's disease (Jordan-Sciutto et al. 2002a), Down's syndrome (Motonaga *et al.* 2001), Parkinson's disease (Hoglinger et al. 2007), Huntington's disease (Pelegri et al. 2008), and amyotrophic lateral sclerosis (Ranganathan & Bowser 2003), simian immunodeficiency virus encephalitis (Jordan-Sciutto et al. 2002b). In addition, neurons cultured from mice null for E2F1 are at least partially protected from diverse insults (Hoglinger et al. 2007, Gendron *et al.* 2001, Hou et al. 2000). While transcription-dependent E2F1-induced apoptosis has been implicated in several *in vitro* models of neuronal death (Hoglinger et al. 2007, Verdaguer *et al.* 2004), the role of E2F1 transactivation in neuronal death was not explicitly demonstrated (Shirvan *et al.* 1997, Chen & Wang 2008, McPherson *et al.* 2003, Kuroiwa *et al.* 1999, Arendt *et al.* 1998, Park *et al.* 1998). Surprisingly, in our *in vitro* model of HIV-induced neurotoxicity and in cortical tissues from HIV-infected individuals, we did not observe a change in typical E2F1 target genes (Fig 2B-I and Fig 3), including those implicated in apoptosis. Further, we have shown that E2F1 is predominantly cytoplasmic in 3 week-old primary rat cortical cultures which may partially account for these results. As we have found that E2F1 predominantly resides in the neuronal cytosol independent of culture treatment (Untreated, Mock or HIV MDM), our results suggest that the main function of E2F1 in post-mitotic neurons may not be to regulate its nuclear targets.

E2F1 is able to induce cell death via several distinct pathways. Transcription-dependent E2F1-induced apoptosis can be both dependent on the p53 tumor suppressor protein or independent of p53. In p53-dependent apoptosis, E2F1 transactivates p19^{ARF}, leading to stabilization of p53 by blocking MDM2-mediated p53 degradation. E2F1 can

also induce apoptosis by transactivation of caspase proteins directly. Our results indicate that HIV MDM does not induce p19^{ARF} and/or caspases at the mRNA or protein levels (Fig 2B-I, Fig 3). Further, HIV MDM treatment does not induce caspase 3 protein levels or cleavage of caspase 3 in primary rat cortical neurons, as we have reported previously (Wang et al. 2007b). Among all of the classical targets of E2F1 analyzed here, the protein level of DHFR is the only one to increase in the cortex of HIV-infected patients compared with that of uninfected patients, although it does not change in the in vitro model of HIV-induced neurotoxicity. DHFR has been implicated in the onset and progression of several neurodegenerative disorders and its turnover can be affected by oxidative conditions (Amici *et al.* 2004). Therefore, it is possible that the altered DHFR protein levels we observed in cortical tissue from HIV-infected patients are independent of E2F1 transcriptional regulation. Our data from the in vitro model of HIV-induced neurotoxicity and from cortical tissue from HIV-infected patients suggests that E2F1 may not be mediating its effects via a classical, transcription-dependent mechanism.

The predominant cytoplasmic localization of E2F1 in postmitotic neurons is highly interesting. Other transcription factors have been reported as being abnormally localized in the cytoplasmic compartment of neurons under pathological conditions (see review (Chu et al. 2007)); however, we also observe cytoplasmic E2F1 in neurons that are not exposed to stress as well as in neurons of cognitively normal control cortex. Interestingly, levels of nuclear E2F1 have been reported to be down-regulated during the differentiation of keratinocytes (Ivanova & Dagnino 2007), and our unpublished data also reveal a similar finding during the differentiation of neuronal cell lines (data not shown). These data suggest that nuclear E2F1 may be decreased as cells permanently exit the cell

cycle and undergo differentiation. In both keratinocytes and primary mouse neurons, E2F1 has been shown to shuttle between the nucleus and the cytoplasm in a CrmA-dependent manner (Strachan *et al.* 2005b, Ivanova *et al.* 2007, Ivanova & Dagnino 2007). Based on these observations, it would be interesting to investigate the relationship between nuclear E2F1 and the differentiation process to determine whether a change of predominant E2F1 localization to the cytoplasm triggers differentiation or vice versa. Furthermore, localization or shuttling of E2F1 is affected by its nuclear localization signal, its nuclear export signal and its binding partners, such as Cyclin A and pRb. While we have previously detected pRb in the nuclear compartment of primary neurons (13), we have been unable to co-immunoprecipitate pRb and E2F1 in postmitotic neurons (unpublished data). Further investigation is required to determine the role that these factors play in the cytoplasmic localization and shuttling of E2F1 in postmitotic neurons.

Since postmitotic neuronal E2F1 is predominantly cytoplasmic, it is possible that E2F1 may regulate neuronal death by affecting proteins residing in the cytoplasmic compartment. Recent evidence points towards this possibility, as E2F1 has been shown to induce cell death via transcription-independent pathways, such as via its regulation of NF- κ B and calpain (18, 19). It would be interesting to identify downstream factors that are regulated by cytoplasmic E2F1 in HAD. Thus, the role of E2F1 in neuronal death in our model appears to be transcription-independent, which contrasts with previous data suggesting that E2F1 induces neuronal death via a p53-independent, but caspase-dependent mechanism in other models of neurodegeneration (39). A possible explanation for this discrepancy may lie in differences in the neuronal cultures. p53-independent, caspase-dependent E2F1 induced death was observed in primary mouse neurons cultured

for 3 days in vitro (Giovanni et al. 2000) as opposed to primary rat cortical neurons cultured for 21 days in vitro as was used for this study. A possible explanation may lie in the differential rate of shuttling of E2F1 between the nucleus and the cytoplasm in different ages of neurons or in neurons of different species. Although E2F1 is predominantly cytoplasmic in postmitotic neurons, the rate of E2F1 shuttling between the cytoplasm and the nucleus could be affected by the age of the neurons when exposed to insults. Further experiments are required in order to ascertain the relationship between the rate of E2F1 shuttling between the nucleus and cytoplasm and the age of primary neurons.

To summarize, our data suggest that the two groups of classical targets of E2F1, genes associated with cell cycle progression, such as Cyclin A, PCNA, DHFR, and genes associated with apoptosis, such as p19^{ARF}, caspase 3, caspase 8, caspase 9, are not affected in HIV-induced neurotoxicity which may be explained by the predominantly cytoplasmic localization of E2F1 in postmitotic neurons which we show here. Further studies are needed to understand the role of cytoplasmic E2F1 in normal neurons and neurons responding to neurodegenerative stimuli such as those seen in HAD. As such roles for E2F1 are largely unknown, these studies may lead to novel and unique therapeutic opportunities.

Acknowledgements

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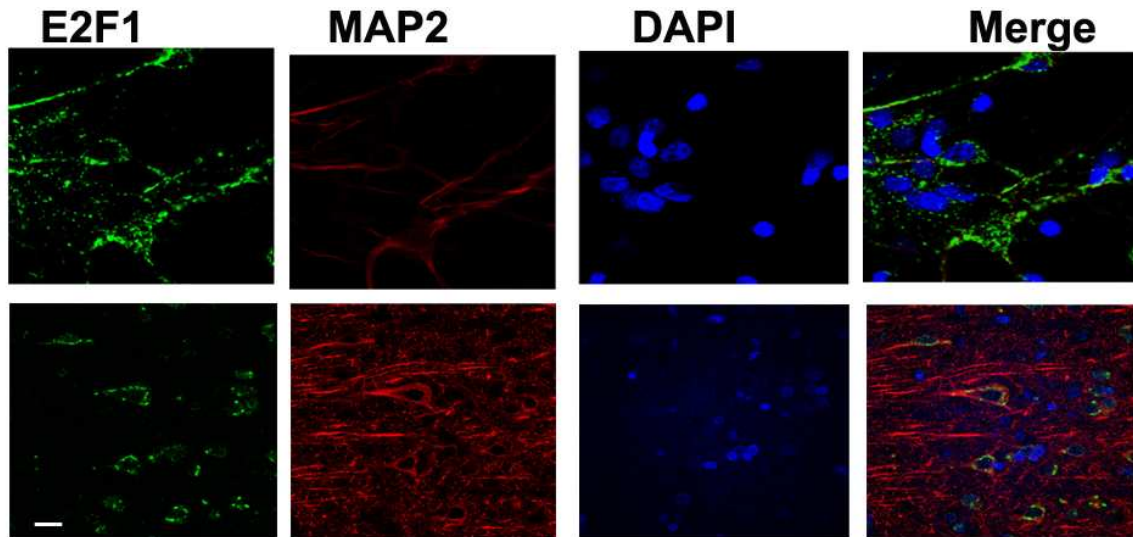


Figure 1. E2F1 is predominantly cytoplasmic in neurons. In 3 week old rat cortical neurons (top panel) and cortical tissues from individuals (bottom panel), immunofluorescent staining for E2F1(green) is predominantly cytoplasmic in neurons (MAP2, red); the nuclei here are stained blue (DAPI). Co-localization of E2F1 and MAP2 appears yellow. Bar = 20 μ m

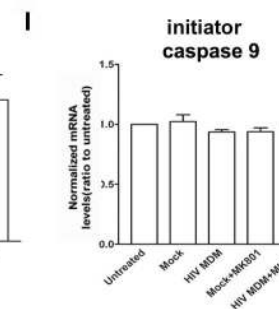
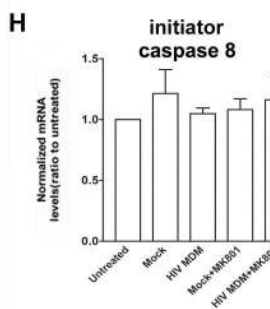
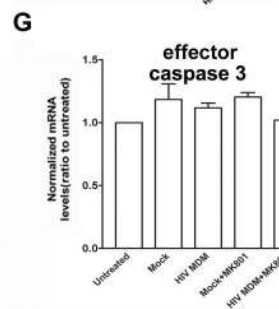
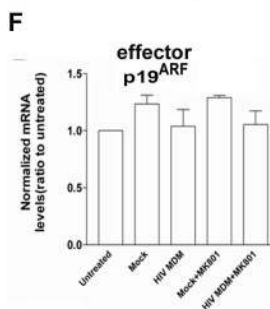
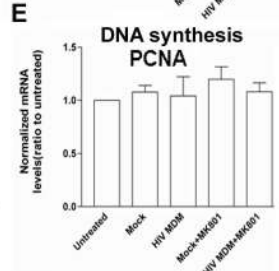
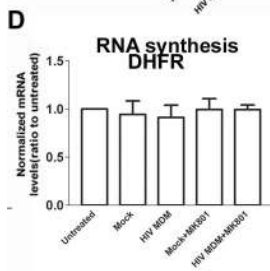
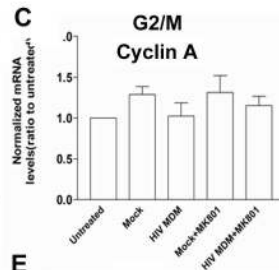
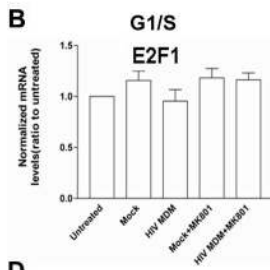
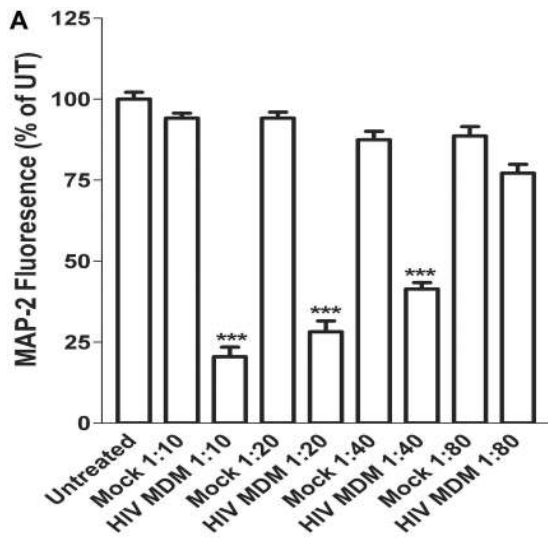


Figure 2. E2F1 targets are not induced in HIV MDM treated primary rat cortical neurons. A) Human immunodeficiency virus-infected, monocyte-derived macrophage (HIV MDM) induces cortical neuron death in a dose-dependent manner. 3 week-old rat cortical cultures treated for 20 h with 3 increasing dilutions of HIV MDM supernatants resulted in a significant decrease in relative microtubule-associated protein 2 (MAP2) fluorescence when assessed by a MAP2 cell-based ELISA, whereas untreated cultures or treatment with Mock-infected MDM supernatant did not result in a detectable loss of MAP2 fluorescence. Values are mean \pm SEM, n = 6, one-way anova, Newman-Keuls, ***p < 0.001 compared with their respective Mock-treated cultures. B – I) E2F1 target genes were not altered at the mRNA levels in HIVMDM-treated rat cortical neurons. Using Q-PCR, mRNA levels of E2F1 target genes involved in cell cycle progression - E2F1 (B) and Cyclin A (C), in RNA and DNA synthesis - DHFR (D) and PCNA (E) and those involved in apoptosis - p19^{ARF} (F), caspase 3 (G), caspase 8 (H) and caspase 9 (I) were not altered by HIVMDM, Mock or MK801 treatment.

model of HIV-induced neurotoxicity or in the cortex of patients with HAD. A) By immunoblotting, protein levels of PCNA, Cyclin A, DHFR and p19^{ARF} were unchanged in HIVMDM, Mock and MK801 treatment. B) Protein levels of PCNA and Cyclin A were not significantly different in the frontal cortex of HIV-infected cases compared with HIV negative cases, nor with 13 cases with neurocognitive impairment as compared with 5 cognitively normal cases, one of which was also infected with HIV. Bands were quantified and normalized to actin. Protein levels of DHFR were significantly increased in HIV-infected patients and in neurocognitive impairment cases. C) PCNA levels were not significantly different ($p>0.05$); similar results were observed for Cyclin A and p19^{ARF} (data not shown). Statistical analysis of DHFR showed significant increases in HIV-infected individuals and those with neurocognitive impairment, ** $p<0.01$.

CHAPTER 3: Targeted gene mutation of E2F1 evokes age-dependent synaptic disruption and behavioral deficits

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Abstract

Aberrant expression and activation of the cell cycle protein E2F1 in neurons has been implicated in many neurodegenerative diseases. As a transcription factor regulating G₁ to S phase progression in proliferative cells, E2F1 is often upregulated and activated in models of neuronal death. However, despite its well studied functions in neuronal death, little is known regarding the role of E2F1 in the mature brain. In the present study, we used a combined approach to study the effect of E2F1 gene disruption on mouse behavior and brain biochemistry. We identified significant age-dependent olfactory and memory related deficits in E2f1 mutant mice. In addition, we found that E2F1 exhibits punctated staining and localizes closely to the synapse. Furthermore, we found a mirroring age-dependent loss of postsynaptic protein-95 in the hippocampus and olfactory bulb as well as a global loss of several other synaptic proteins. Coincidentally, E2F1 expression is significantly elevated at the ages in which behavioral and synaptic perturbations were observed. Lastly, we show that deficits in adult neurogenesis persist late in aged E2f1 mutant mice which may partially contribute to the behavior phenotypes. Taken together, our data suggest that the disruption of E2F1 function leads to specific age-dependent behavioral deficits and synaptic perturbations.

Introduction

E2F1 is a highly conserved cell-cycle related transcription factor that regulates the gene targets that are necessary for the transition from G1 to S phase in dividing cells. Additionally, E2F1 also has the capacity to regulate cell death as it can induce p53-dependent and p53-independent apoptosis as well as transcription-dependent and, transcription-independent cell death (Iaquinta & Lees 2007, Johnson & Degregori 2006). As a result, aberrant E2F1 expression and activity has been investigated as a contributor to neuronal death in various neurodegenerative diseases (Jordan-Sciutto et al. 2002b, Jordan-Sciutto et al. 2002a, Jordan-Sciutto et al. 2003, Hoglinger et al. 2007, Pelegri et al. 2008). However, studies evaluating its role in various neurotoxicity models in vitro have not conclusively provided a precise mechanism as to how E2F1 mediates death in mature neurons (Park et al. 1997, Giovanni et al. 1999, Giovanni et al. 2000, Park et al. 2000a, Park et al. 2000b, O'Hare et al. 2000).

In the developing brain, E2F1 is expressed abundantly in the ventricular regions during progenitor cell proliferation (Dagnino et al. 1997). As the brain matures, E2F1 mRNA levels are maintained, and protein levels of E2F1 increase into adulthood (Tevosian et al. 1996, Kusek et al. 2001). Disruption to E2f1 in mice can lead to increased cell cycle events as well as reduced expression of neuronal marker calbindin (Wang et al. 2007a). Interestingly, in mature neurons, E2F1 is predominantly cytoplasmic, which differs from its nuclear localization in cycling cell types and is inconsistent with its well-characterized role as a transcription factor (Wang et al. 2010,

Strachan et al. 2005b). This cytoplasmic location of E2F1 has also been observed in other terminally differentiated cells, such as myocytes and keratinocytes (Ivanova & Dagnino 2007, Ivanova et al. 2007, Gill & Hamel 2000). While it is logical that terminally differentiated cells would favor a predominantly cytoplasmic localization of E2F1 to ensure that it is not transcriptionally active to trigger cell-cycle re-entry, the exact role of E2F1 in neurons remains poorly understood.

Mice carrying disrupted E2f1 have a substantial reduction in adult neurogenesis at 3 months, even though brain development appears unaffected (Cooperkuhn et al. 2002). To further investigate potential roles for E2f1 in post-mitotic neurons, we assessed the impact of E2f1 disruption on behavior as well as morphologic and biochemical changes in the CNS. In the present study, we demonstrate that mice with E2f1 disruption display age-dependent olfactory and memory related behavioral deficits. In addition, we demonstrate that E2F1 is predominantly cytoplasmic and localizes to synaptic fractions. The disruption of E2f1 results in a significant reduction in the expression of crucial synaptic proteins including postsynaptic density protein-95 (PSD-95), specifically in the hippocampus (HC) and the olfactory bulb (OB). The age in which the synaptic disruptions were observed correlated with that of the olfactory and memory deficits. The synaptic and behavioral effects in the E2f1^{tm1} mice are likely due to the absence of E2F1 function as the age in which E2F1 expression peaks strongly correlates with the onset of these perturbations. Furthermore, the persistent deficits in adult neurogenesis partially contribute to these age-dependent effects. Taken together, our study provides evidence that the disruption of E2F1 function in mice elicits both synaptic and behavioral deficits in an age-dependent manner.

Materials and Methods

Animal Behavior. E2f1^{tm1} (B6;129S4-E2F1^{tm1M^{eg}}/J; strain # 002785) and Wildtype F2 hybrid (B6129SF2/J; strain # 101045) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were described in Field et al. (1996), and are a hybrid of C57BL/6 and SV129 strains (Field et al. 1996). Briefly, mutations in the E2f1^{tm1} mice were generated by disrupting exon3 with the insertion of the neomycin selection marker and by removing the entire exon4. Disruption of exon3 and the deletion of exon4 of E2f1 lead to, at a minimum, disruption of the E2F1 DNA binding and heterodimerization domains. Analysis of RNA from these animals revealed that several E2f1 mRNA species are produced by the disrupted E2f1 locus lacking the coding sequences for the DNA binding region as predicted. The same strain mixture was used for wildtype mice bred for use as controls. All mice were housed at the University of Pennsylvania animal facilities on a 12-hour light/dark cycle and water were provided *ad libitum*. Only males were used in the behavioral and biochemical studies and all experiments in this study were approved by the Institutional Animal Use and Care Committee and in agreement with the ARRIVE Guidelines.

Odor Habituation. Mice were habituated to a standard size mouse cage for a period of 1 hour before testing (Fletcher & Wilson 2002, Fadool *et al.* 2004, Marks *et al.* 2009). Single odorant alcohols differing by the number of carbon atoms (octanol C9/C10) were diluted 1:100 in mineral oil or water and applied to a cotton swab. The cotton swab was

introduced to the mouse through the top of the testing cage and the time of active investigation/smelling of the odor was recorded. Mice were habituated to the first odor of an odor pair combination by repeated stimulation with the odor saturated swab. On the 8th trial, the second odor was presented and the time of exploration was scored. All recorded times were normalized and compared to the animal's original exploration time prior to habituation to minimize the between-animal variance.

General Anosmia. Naive mice were removed from the home cage and placed in a testing cage (29.2 x 19.1 x 12.7 cm) in which a scented cracker or size-matched marble was hidden from view under the bedding. The item to be retrieved was randomly selected and hidden in a different location on each trial. The retrieval time was recorded from the instant the mouse is released until the item was found. Experiments were terminated at 10 minutes and mice were scored for that time duration if the item was not retrieved (Fadool et al. 2004, Marks et al. 2009)

Novel Object Recognition Memory. Mice were habituated to a testing cage for a period of 1 hour before testing (Jeon *et al.* 2003, Marks et al. 2009, Fadool et al. 2004). Two non-identical objects were placed in the chamber and mice were allowed to explore them for a 5 minute interval. The time that each mouse was oriented toward each object within one head length was scored as exploratory time. The mice were then removed from the testing cage for either a 1 or 24-hour delay period. Mice were then reintroduced to the testing cage containing the object 1 (familiar object) and object 3 (novel object) in the same original position and scored for a 5 minute interval.

Light/Dark Box. A light/dark chamber resembling the home cage was modified as follows: A black divider was cut to the exact width and height of the cage and a 7x7 cm square opening was cut in the middle on the bottom edge (Crawley & Goodwin 1980, Marks et al. 2009). The divider was placed in the middle of the cage. One side of the cage was painted black (dark), and the other side painted white (light). Mice were released into the light chamber and the time spent in either chamber was recorded for 300 seconds.

Locomotor Activity. Mice were placed in a testing chamber that was identical to its home cage and set in a photo-beam frame with sensors arranged in an 8-beam array strip (Mackler et al. 2008). Each animal was first habituated to the testing chamber over two 1-hour sessions across two days and the final basal locomotor activity monitored in a 1-hour session. Cumulative number of beam breaks were recorded and quantified into personal computer designed software (Med Associates).

Accelerating Rotarod. Mice were placed on an accelerating rotarod (Med Associates) for four trials a day over four days with a minimum of a 20-minute inter-trial interval. Each trial lasted a maximum of five minutes during which the rotarod accelerated linearly from 3.5 revolutions per minute (RPM) to 35 RPM. The amount of time for the animal to fall from the rod was recorded for each trial and averaged for each day for four days total (Wang et al. 2012).

Tissue Processing: Mice were perfused with phosphate-buffered saline (PBS) (pH 7.4), followed by ice cold 4% paraformaldehyde (PFA) fixation. Tissues were treated of graded cryoprotection in 10%, 20%, and 30% sucrose prepared in PBS. 8-16 μm tissue sections were cut coronally on a Leica CM1850 microtome-cryostat, and sections were stored at -20°C until use.

Cell Culture and transfection: Primary neuroglial cultures were isolated from the brains of embryonic day 17 Sprague Dawley rats (Wilcox et al, 1994). Dissociated cells in suspensions were plated on poly-L-lysine coated coverslips or plates and the cultures are maintained in neurobasal media with B27 supplement [Invitrogen] at 37°C with 5% CO_2 . Transfections of primary rat hippocampal neurons were performed using lipofectamine 2000 [Invitrogen] at 10 days *in vitro* (DIV). Transfection mixture was added to the cells for 2 hours and subsequently replaced with the original, conditioned media. Cells were then fixed 4 days post-transfection using 4% PFA.

Synaptosome fractionation: Synaptic proteins from 21 DIV primary hippocampal cultures were isolated using the Syn-PER extraction buffer according to manufacturer protocol [Pierce]. Synaptoneuroosomes were isolated from mouse brains according to Villasana et al, 2006 with minor modifications. Presynaptic and postsynaptic fractions were isolated according to Gurd et al. 1974., Carlin et al, 1980.

Antibodies and Reagents: The following antibodies were purchased from the indicated vendors: E2F1 KH20 (sc-56662); E2F1 KH95 (sc-251); GAPDH (sc-32233) [Santa Cruz],

Doublecortin (AB2253); vGluT1 (AB5905); Tyrosine hydroxylase (AB152); PSD-95 (MAB1596) [Millipore], OMP (#544-10001) [Wako]. NMDAR1 (#5704); NMDAR2A (#4205); NMDAR2B (#4212); Synapsin (#5297); GluR2 (#2460); ERK1/2 (#4695); E2F1 (#3742); Lamin A/C (#2032); α -tubulin (#2125) [Cell Signaling], Synaptophysin (ab8049); MAP2 (ab5392) [Abcam], actin (A2066) [Sigma]; SV2 [DSHB]. The following chemical reagents were used from the indicated vendors: DAPI [Molecular Probes]; Coomassie (161-0786); Protein assay dye (500-0005), PVDF membrane [BioRad], Fast Green FCF; protease inhibitor cocktail [Sigma], Pageruler plus protein ladder [Thermos], Luminata Forte Western HRP substrate [Millipore]. All HRP conjugated secondary antibodies were obtained from Pierce and all dye conjugated secondary antibodies were obtained from Jackson Immuno-Research.

Immunoblotting: Tissues were harvested from single animals and not pooled. Cultured cells were homogenized in ice cold, whole cell lysis buffer containing 50 mM Tris, 120 mM NaCl, 0.5% NP-40, 0.4mM sodium orthovanadate and protease inhibitor cocktail. Protein concentrations were determined using the Bradford method. Equal amounts of proteins (2-10 ug from cells and 20-50 ug from tissue) were loaded for immunoblotting and confirmed by staining the gel with Coomassie and the membrane with Fast Green. For densitometric analysis, autographs were scanned and cropped using Adobe Photoshop (Adobe Systems). Pixel intensities of each bands of interest were quantified using Image J software (NIH) and normalized to gel Coomassie stain. Immunoblots shown are representative of three independent biological replicates.

Immunostaining: Glass slides containing frozen tissue sections (~8-16 μ m per section) were baked rehydrated, and treated with antigen retrieval solution. Tissues were blocked in 10% normal goat serum and incubated with primary antibodies overnight. Slides were washed with PBS containing 0.1% Tween 20 and mounted in Citifluor for subsequent image acquisition. Cells grown on coverslips were fixed, permeabilized, and blocked at room temperature and incubated with primary antibodies overnight at 4°C and appropriate secondary 30 minutes at room temperature. The coverslips were mounted in Aqua-mount [Thermos]. Tyramide Signal Amplification system [Perkin Elmer] was used according to manufacturer instructions for PSD-95 and E2F1 signal amplification in tissue and endogenous E2F1 in cells (Wang et al, 2010).

Image acquisition and analysis: Images from samples were either captured at 400x or 600x on a laser confocal microscope with Biorad Radiance 2100 (Biorad), or 200x or 400x on a standard epifluorescent microscope (Nikon E400). Total E2F1, PSD-95 pixel intensity and MAP2 area in an image were quantified using Metamorph 6.0 (Universal Imaging) while total intensity of PSD-95 puncta and fractions of maximally saturated puncta were quantified using ImageJ.

Statistical analysis. All data were analyzed by either Prism 5.0 software (GraphPad Software). All data are expressed as mean \pm SEM with values of $p < 0.05$ considered significant. Unless otherwise noted, all asterisks denote statistical significance by comparing the means of E2f1^{tm1} and WT within the same age group using the two-tailed student's t-test.

Results

1) Age-dependent memory and olfactory deficits and elevated anxiety in the E2f1^{tm1} animals

To determine if E2F1 has a role in the CNS, we systematically characterized various behavioral functions in the E2f1^{tm1} mice. We first asked whether the mutant mice have a shorter lifespan since they have been shown to be more susceptible to spontaneous tumor formation. Indeed, E2f1^{tm1} mice display significantly shorter lifespans than their wildtype counterparts (Fig 1A).

Apparent aberrant expression of E2F1 has been observed in several neurodegenerative diseases including AD, PD, and HIVE (Jordan-Sciutto *et al.*, 2002a; Jordan-Sciutto *et al.*, 2002b; Hoglinger *et al.*, 2007). Early clinical signs common to these neurodegenerative diseases include pronounced olfactory dysfunctions followed by their respective disease symptoms (Barresi *et al.* 2012, Doty 2012). Therefore, we first investigated the olfactory function in the E2f1^{tm1} mice by subjecting them to an odor discrimination task in which they are exposed to two odorants differing by a single carbon atom (Fig 1B.a-c). We measured scent discrimination by the length of time that the novel scent is explored in the last trial, with longer times indicating that the animal is able to discriminate the novel odor from the habituated odor. When the mice were repeatedly exposed to the initial odor, C9, mice of both genotypes regardless of age were able to habituate to that odor comparably. Mice of both genotypes at age P40 were also able to discriminate between C9 and C10 as indicated by increased exploratory time

when presented with the novel odor, C10 (Fig 1Bi.). However, E2f1^{tm1} mice from age P90 through P365+ lose the ability to discriminate between the two odors while the WT mice retain this ability (Fig 1Bii, 1Biii.). To verify that the olfactory deficit in the E2f1^{tm1} mice is not specific to ester odors, we subjected the animals to a more general anosmia task. In this task, a scented object (cracker) or unscented object (marble) was buried under the bedding and the time in which the animal retrieved these objects was quantified as a measure of olfactory function. Similar to the odor discrimination task, we observed a marked deficit in olfactory function in the E2f1^{tm1} mice starting at age P90 and persisting through P365+ (Fig 1Biv.). Both WT and E2f1^{tm1} mice retrieved the unscented object at comparable rates suggesting that the difference in performance is specific to olfaction and unrelated to basal exploratory activity.

To assess memory function, we subjected WT and E2f1^{tm1} mice to a novel object recognition task. Briefly, animals were first exposed to two different objects (objects 1 and 2) during the familiarization phase and the mice were subsequently removed. After a one hour (short-term) or 24 hour (long-term) delay, the animals were re-exposed to one of the two previous objects (object 1) and a novel third object (object 3) in the same position. Since mice typically explore objects that they deem as novel, increased exploration of object 3 is indicative of normal recognition memory function as animals retained information regarding object 1 and 2 and thus recognizing object 3 as novel. As expected, we found that E2f1^{tm1} and WT mice showed no bias towards object 1 or 2. However, while WT of all age groups explored the novel object more in the test phase of both short- and long-term paradigms, E2f1^{tm1} mice failed the short-term novel paradigm starting at age P270 and the more difficult long-term paradigm starting at age P180 as indicated by

their equal exploration time of familiar and novel objects (Fig 1Ci, 2Cii.). Importantly, the deficits of the E2f1^{tm1} mice in these paradigms are specific to memory since E2f1^{tm1} mice at P180 can still distinguish between novel object and familiar object after 1 hour delay; however, they fail the task the delay period is increased to 24 hours.

We have also consistently observed that E2f1^{tm1} mice display increased digging behaviors. Since digging behaviors can be correlated to anxiety, we assayed the anxiety levels of E2f1^{tm1} mice using the light/dark box paradigm. In this test, reduction in time spent in the chamber with light is an indicator of increased anxiety as mice prefer the dark (Marks et al. 2009). We found that E2f1^{tm1} mice spent significantly less time in the light chamber of the box compared to the WT starting at age P90 (Fig 1D). To verify that these findings were not due our breeding or housing, we repeated the light/dark box test with P90 WT and E2f1^{tm1} mice purchased directly from Jackson Laboratories and obtained similar results.

Finally we examined “home cage” activity in the E2f1^{tm1} mice as a measurement of basal locomotor and the accelerating rotarod test for defects in motor performance. We found that mice of both genotypes from ages P90 and P365+ exhibited a comparable basal locomotor activity level (Fig 1E). Furthermore, we found no significant difference between the genotypes in either the P90 and P365+ age groups on the accelerating rotarod (Fig 1Fi.-ii.). Together, these data suggest that the disruption to E2f1 gene leads to age-dependent reduced performance in olfactory, memory, and anxiety test paradigms but no changes were seen in basal motor activity or a motor related task.

2) E2F1 exhibits cytoplasmic and punctated staining *in vivo* and *in vitro*

In order to gain further insight into how the disruption of the E2F1 may lead to these

pronounced age-dependent behavioral changes, we examined the localization of E2F1 protein in neurons in the HC. Interestingly, E2F1 expression in hippocampal tissue is predominantly cytoplasmic and exhibits localized puncta where synaptic innervations are formed (Fig 2A). To investigate this localization more closely *in vitro*, primary rat hippocampal cultures were grown to 21 DIV and immunostained with either a C-terminal E2F1 antibody (KH95) or an N-terminal antibody (KH20) or no primary antibody as a negative control (Fig 2B). Immunostaining with either KH95 or KH20 revealed a cytoplasmic punctated staining for E2F1 that co-localized with neuronal cytoplasmic marker MAP2 but not nuclear counter-stain DAPI, consistent with what we observed *in vivo* and previous observations in cortical neurons (Strachan et al 2007, Wang 2010, Jordan-sciutto 2002b). Next we examined the subcellular localization of exogenous E2F1 protein by overexpressing E2F1 in hippocampal neurons and immunostained for E2F1. Using the same KH95 E2F1 antibody without signal amplification, we found that exogenously expressed E2F1 is predominantly cytoplasmic as E2F1 signal is found in neuritic processes (Fig 2C). To determine whether the cytoplasmic localization of E2F1 is specific to neuronal subtype, we also investigated E2F1 localization in primary cortical neurons (Fig 2D). As expected, E2F1 was again predominantly cytoplasmic and exhibiting punctated staining throughout the neuritic processes. Furthermore, MAP2 negative E2F1 expression can be found to co-localize with axonal marker staining growth associated protein 43 suggesting that axonal E2F1 is also abundant in these primary cultures. While these findings confirm previous observations that E2F1 is predominantly cytoplasmic in primary neurons, the presence of E2F1 puncta along the MAP2 positive dendrites suggests it may also localize to the synapse.

3) E2F1 is enriched in synaptic fractions

Because of its intriguing punctated staining pattern, we hypothesized that E2F1 may be associated with the synapses in neurons. We first co-immunostained E2F1 and synaptic marker PSD-95 in primary hippocampal cultures and found that E2F1 can be found to co-localize or be adjacent to the PSD-95 puncta (Fig 3A). However, because E2F1 staining is distributed throughout the neuritic processes, E2F1 staining does not exclusively co-localize with the PSD-95 puncta. Therefore, to determine if E2F1 is enriched in the synapses, we isolated synaptosomes from 21 DIV hippocampal cells and immunoblotted for E2F1 expression (Fig 3B). E2F1, along with two synaptic markers PSD-95 and synapsin are significantly enriched in the synaptosomes isolated from these cultures. To determine if E2F1 is also enriched in the synaptic protein-rich fractions *in vivo*, we isolated synaptoneuroosomes from both cortex and HC using size fractionation. E2F1 as well as PSD-95 and vesicular glutamate transporter (vGluT1) were all enriched in the synaptoneuroosomes isolated from both cortex and HC of adult mice (Fig. 3C).

Finally, to determine whether E2F1 is enriched presynaptically or postsynaptically, we fractionated brain specimens into synaptic vesicle enriched presynaptic fractions and postsynaptic densities enriched postsynaptic fractions and subsequently immunoblotted for E2F1 expression. As shown in figure 3D, E2F1 was enriched in the synaptosomal P2 fraction along with various synaptic markers. However, E2F1 is noticeably absent in the postsynaptic fractions PSDT1, PSDT2, and PSDTS despite enrichment of PSD-95. On the other hand, E2F1 is significantly enriched in the presynaptic SV fraction along with presynaptic proteins SV2, vGluT1, and synaptophysin. Thus, we observed that E2F1 is closely associated with synapses and particularly abundant in the presynaptic fraction.

4) Age-dependent synaptic protein perturbations in the E2f1^{tm1} animals

Because E2F1 is closely associated with synapses *in vitro* and *in vivo*, we hypothesized that the disruption of the E2f1 gene would disrupt the expression of synaptic proteins. As shown in figure 4A, the expression of a subset of synaptic proteins such as PSD-95, N-methyl-D-aspartate receptor 1(NMDAR1) and NMDA receptor subunit 2A (NR2A), Synaptic Ras GTPase activating protein (SynGAP) were reduced at P270 but not P40 in the E2f1^{tm1} mice. A closer examination across all age groups revealed that reduced expression of these proteins in the E2f1^{tm1} mice is age-dependent, manifesting in the animals at P270 and P365+ (Fig 4B). On the other hand, the expression of other synaptic proteins such as synaptophysin and NMDA receptor subunit 2B are unchanged regardless of age. Furthermore, not all components of the synapse are affected similarly across age in the E2f1^{tm1} mice. While PSD-95 and its interacting protein SynGAP are reduced at P1, when the brain is still developing and rewiring its synaptic circuitry, NMDAR1 and glutamate receptor subunit 2 (GluR2) are unchanged at this time. Thus, mice lacking functional E2F1 exhibit reduced expression of a subset of synaptic proteins during brain maturation as well as a more profound reduction of synaptic proteins in aged brains.

5) Age-dependent reduction of PSD-95 expression in hippocampus and olfactory bulbs

Since PSD-95 has been well documented for its crucial role in synapse maturation and connectivity (Kim & Sheng 2004), we assessed changes in PSD-95 expression as a marker of synaptic disruption which we hypothesized would correlate with the age-dependent behavioral phenotype observed in the E2f1^{tm1} mice. Expression of PSD-95

was determined in the brain regions most relevant for the affected behaviors: OB for olfaction and HC for memory. As shown in figure 5A, the expression of PSD-95 in the OBs is significantly reduced in the E2f1^{tm1} mice when compared to the WT starting at age P90 and persisting through P365+. E2f1^{tm1} mice can have as much as 60% reduction in PSD-95 expression in the OB when the animals are as old as one year of age compared to the WT (Fig 5Aiii). Furthermore, the age at which the loss of PSD-95 in the OBs in the E2f1^{tm1} mice corresponds with the age in which the same animals exhibit olfactory deficits: P90-P365+. Interestingly, we observed no differences in the expression of olfactory marker protein or tyrosine hydroxylase in the E2f1^{tm1} mice suggesting that the reduction in PSD-95 is not due to overall protein loss (data not shown).

Since the hippocampal formation has been shown to be involved in object recognition memory (Broadbent *et al.* 2010), we examined the expression of PSD-95 in this brain structure. We observed a reduction of PSD-95 levels starting at P270 and persisting through P365+ (Fig 5Aii.,5Aiii.). As predicted, the age of onset of reduced PSD-95 expression is at P270, which is the same age when the animals fail the 1-hour memory task. Of note, the expression of PSD-95 is unaltered in the cerebellum in P90 and P365+ (Fig 5Bi.-ii.). This lack of change resembles the absence of any significant impairment in the motor function related tasks. To verify the immunoblotting results, we immunostained hippocampal brain sections from WT and E2f1^{tm1} mice at P365+ for PSD-95 and dendritic marker MAP2 (Fig 5C). Both the total intensity level of PSD-95 staining and total number of PSD-95 puncta in the HC of E2f1^{tm1} mice are significantly reduced compared to that of the WT. Together, these data show that PSD-95 expression in E2f1^{tm1} mice is reduced in an age-dependent manner at ages that are coincident with

the behavioral deficits.

6) E2F1 expression increases with age *in vitro* and *in vivo*

Since the synaptic and behavioral phenotypes in the E2f1^{tm1} mice were age-dependent, it is possible that these effects were not due to E2F1 disruption but instead due to an indirect defect in CNS development. Previous research has characterized the neuroanatomy of the adult E2f1^{tm1} mice and found no changes in neocortical anatomy (Cooperkuhn et al. 2002). Similarly, we did not detect any overt structural changes in the hippocampus or the olfactory bulbs suggesting that the age-dependent behavioral and biochemical perturbations are not due to abnormal development (data not shown).

Alternatively, we hypothesized that E2F1 is necessary in maturing neurons and adult brains and that its expression increases during neuronal maturation and through adulthood. Though E2F1 has been shown to be increased in adult CNS compared to embryonic brain, a complete age-dependent expression of E2F1 in the adult CNS has not been examined (Kusek et al. 2001). Therefore, we characterized the E2F1 expression profile in maturing neurons *in vitro* and adult brain *in vivo*. As shown in figure 6A, neuronal E2F1 expression increases as hippocampal neurons mature from 1 to 14 DIV. The maturation of these hippocampal neurons is evident by the increasing elaboration of neurites marked by GAP43 staining. Furthermore, we also collected cytoplasmic and nuclear lysates by subcellular fractionation and found that E2F1 increases in the cytoplasmic fraction as cortical cells mature (Fig 6B). To determine if E2F1 expression also increases in the adult brain *in vivo*, we assayed for its expression in brain lysates collected from P40, P180, P270, P365, and P465 WT mice (Fig 6C). Similarly, E2F1 expression increases late into adulthood and peaks at P270 which is the onset of the

synaptic disruptions in the E2f1^{tm1} mice. Taken together, our data show that the E2F1 expression increases in maturing neurons *in vitro* and in adult brain *in vivo* correlating with the age of onset of the synaptic and behavioral perturbation in the E2f1^{tm1} mice.

7) Impairment of adult neurogenesis in the E2f1^{tm1} persists to 1 year of age.

Deficits in adult neurogenesis have been linked to various defects in mouse behaviors (Zhao et al. 2008). Previous work using BrdU labeling to mark proliferating cells have demonstrated that disruption of the E2f1 in mice leads to a significant reduction proliferating cells in the dentate gyrus of the HC and the OB of 3 month old mice (Cooperkuhn et al. 2002). Here, we asked whether the deficits in the adult neurogenesis can persist in aged E2f1^{tm1} mice and thereby contribute to the synaptic and behavioral defects we observed. To assess adult neurogenesis, we measured the number of cells expressing doublecortin (DCX), a microtubule component used as a marker for newly divided immature neurons, in the OB and the HC of one-year-old mice (Fig 8A) (Rao & Shetty 2004). In both the OB and the HC, E2f1^{tm1} mice had significantly reduced number of DCX-positive cells as compared with WT when the animals are one year of age (Fig 8B). The level of reduction of these immature cells is comparable to what was previously reported by BrdU labeling at 3 months. Our results indicate that the deficit in adult neurogenesis in the E2f1^{tm1} mice in the hippocampus and the olfactory bulbs persists to at least 1 year of age.

Discussion

E2F1 has long been linked to neurodegeneration due to its observed upregulation in

post-mortem brains from various neurodegenerative diseases (Jordan-Sciutto et al. 2002a, Hoglinger et al. 2007, Jordan-Sciutto et al. 2002b)). Indeed, experimental manipulations that leads to the overactivity of the E2F1 in post-mitotic neurons results in significant increase of neuronal death *in vitro* (O'Hare et al. 2000, Giovanni et al. 2000). However, although E2F1 has been studied in the context of neuronal death and neurodegenerative diseases, study focusing on its physiologic role in the developed CNS is lacking. Here we report for the first time the consequence of disrupting E2f1 on the resulting behavior as well as on other biochemical changes that may accompany these behavioral deficits.

In the present study, we characterized several age-dependent behavioral deficits in mice with a disruption in the E2f1 gene. E2f1^{tm1} mice exhibited significant olfactory deficits and elevated anxiety as early as 3 months of age. Furthermore, memory deficits manifest when the animals were significantly older at 6 to 9 months. The behavioral deficits are not due to general impairment across all behavioral domains as E2f1^{tm1} mice show no impairment in two motor-related tasks. Additionally, we have corroborated published results that demonstrate that the neuroanatomical development in the E2f1^{tm1} mice are not disrupted (Cooperkuhn et al. 2002). Given that E2F1 expression is significantly elevated at around the age when the behavioral deficits are most prominent, it is plausible that the behavioral deficits are due to the absence of E2F1 activity in the E2F1^{tm1} mice instead of other confounds.

Furthermore, we observed that the deficits in adult neurogenesis in the E2F1^{tm1} mice persist into one year of age. Deficits in adult neurogenesis have long been linked to behavioral abnormalities (Zhao et al. 2008). Hippocampal dependent memory tasks as well as other learning tasks such as eye-blink conditioning, T maze performance, object

recognition, and contextual fear conditioning are impaired following the ablation of adult neurogenesis (Leuner *et al.* 2006, Saxe *et al.* 2006, Winocur *et al.* 2006, Jessberger *et al.* 2009). Similarly, adult neurogenesis has also been shown to be involved in olfactory physiology (Gheusi *et al.* 2000). Additionally, enriched odor exposure increased OB adult neurogenesis, which is presumably linked to increased olfactory function (Rochefort *et al.* 2002). Likewise, adult neurogenesis is also strongly involved in anxiety-related behaviors (Vaidya *et al.* 2007, Revest *et al.* 2009). Therefore, it is possible that the deficits in adult neurogenesis can partially explain the age-dependent memory and olfactory deficits as well as heightened levels of anxiety that we observed in the E2f1^{tm1} mice.

The age-dependent synaptic perturbations as evidenced by reduced PSD-95 expression in the E2f1^{tm1} mice can also explain the behavioral deficits. PSD-95 has been shown to be one of the crucial scaffolding proteins and complexes with other synaptic proteins and receptors including SynGAP, NMDAR1, NMDAR2A/B, and GluR2 (Lin *et al.* 2004, Dosemeci *et al.* 2007, Sheng & Hoogenraad 2007). Given its importance in synaptic physiology, behavioral impairments are often accompanied by changes to PSD-95 expression (Wakade *et al.* 2010, Sun *et al.* 2009). Training in object-place recognition led to a rapid induction of PSD-95 expression while mice lacking PSD-95 failed to learn simple associations (Soule *et al.* 2008, Nithianantharajah *et al.* 2013). Likewise, changes to SynGAP expression also result in significant behavioral impairment as heterozygous deletion of SynGAP leads to deficits in fear conditioning, working memory and reference memory (Muhia *et al.* 2010, Guo *et al.* 2009). Consistent with these studies, the age-dependent deficits in memory and olfaction are accompanied by

corresponding age-dependent reduction in PSD-95 expression in the HC and OB. The changes to PSD-95 expression as well as other synaptic proteins may have a profound impact on synaptic physiology in the E2f1^{tm1} mice and thereby contribute to the behavioral deficits.

Although the behavioral deficits E2f1^{tm1} mice do not manifest until they are at least 3 months, reduction of PSD-95 and SynGAP expression can be as observed as early as P1 when synaptic innervations are formed and pruned (Fig 4B). Interestingly, E2F1 expression *in vitro* increases as the neurons mature and synaptogenesis takes place (Fig 6). These results may suggest that E2F1 may also be necessary during synaptic development when the synapses form and mature. Additional experiments with more direct temporal control in regulating E2F1 expression are necessary to more precisely define the role of E2F1 in synapse formation.

One of the more intriguing findings of the present study is the subcellular localization of E2F1 in neurons namely, the predominant cytoplasmic localization of E2F1 and its abundance in the synaptic fractions *in vitro* and *in vivo*. Using a subcellular fractionation approach, we have found that E2F1 is overwhelmingly associated with the GAPDH-positive, Lamin A/C negative cytoplasmic fraction (Fig 6B). E2F1 is not the only transcription factor operating outside the nucleus in neurons as another transcription factor Elk-1 also localizes to neuritic processes and can bind to the mitochondrial permeability transition pore complex to induce neuronal apoptosis (Barrett *et al.* 2006a). However, we speculate that E2F1 is unlikely to be exclusively found in the cytoplasm as nuclear E2F1/DP1 complexes have been observed in neurons and proposed to be responsible for the unintended activation of cell cycle machinery in post-mitotic neurons

(Zhang & Herrup 2011, Zhang et al. 2010). Using a more sensitive luciferase reporter assay, E2F1 transcriptional activity in neurons has been described in various neuronal death models, though contribution by other E2F family members could not be excluded by this method (Shimizu et al. 2007, Jiang et al. 2007, Hou *et al.* 2013). Studies that implicate E2F1 in neuronal apoptosis through its transcriptional activity have primarily utilized cerebellar granule neurons, suggesting that E2F1-mediated effects may vary in different neuronal population (O'Hare et al. 2000). Our data from the E2F1^{tm1} mice similarly suggest that the role of E2F1 may be distinct in different neuronal population as PSD-95 expression in cerebellum is unchanged despite robust reductions of PSD-95 expression in the HC and the OB.

Given that E2F1 is a transcription factor that regulates cell cycle progression, its synaptic enrichment, particularly in the presynaptic terminal, is quite surprising. However, E2F1 is not the only cell cycle protein found in the synapse. Components of the origin recognition complex, known to initiate DNA replication in the nucleus, are also enriched in the synapse and their depletion leads to reduced dendritic branching and dendritic spine morphogenesis (Huang et al. 2005). A neuronal co-activator of CDK5 has also been found in the synapse and its regulation of CDK5 activity has been shown to be crucial for synaptic physiology (Humbert *et al.* 2000, Morabito *et al.* 2004). Other transcriptional factors/co-factors such as NF-kappa B, STAT3, and CREB2 can be found in the presynaptic terminal of the synapse and subsequently translocate to the nucleus (Jung *et al.* 2012, Ben-Yaakov *et al.* 2012, Meffert *et al.* 2003, Riccio *et al.* 1997, Lai *et al.* 2008). We speculate that E2F1 may also be present in the synapse and upon stimulation, translocate into the nucleus and regulate its target genes. Alternatively, E2F1

function may be mediated through direct protein to protein interaction in the synapse. E2F1 interaction with NPDC-1 which co-localizes with synaptic vesicle markers in the synapse is one potential target (Sansal et al. 2000, Evrard & Rouget 2005). We have also identified a putative SH3 motif in E2F1 protein sequence that may be important for synaptic protein interactions. Further investigations of the function of E2F1 in the synaptic compartment as well as its potential interacting partners are warranted to understand the role of such localization in neurons.

In the present study, we demonstrate for the first time the behavioral consequence of disrupting the E2F1 gene and report age-dependent deficits in memory and olfaction that correlate with changes in expression of PSD-95 and other components of the synapse. Future investigations to elucidate the role of E2F1 at the synapse and the precise mechanism of regulating the expression of PSD-95 are warranted to gain a broader understanding of the role E2F1 plays in normal neurons and in diseases associated with synaptic damage and loss during aging.

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Abbreviations: DCX, doublecortin; DIV, days in vitro GluR2, glutamate receptor subunit 2; HC, hippocampus; MAP2, microtubule associated protein-2; NMDAR1, N-methyl-D-aspartate receptor-1; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; OB, olfactory bulbs; PBS, phosphate buffered saline; PFA, paraformaldehyde; PSD-95, postsynaptic density protein 95; SynGAP, synaptic ras GTPase activating protein; vGluT1, vesicular glutamate transporter-1.

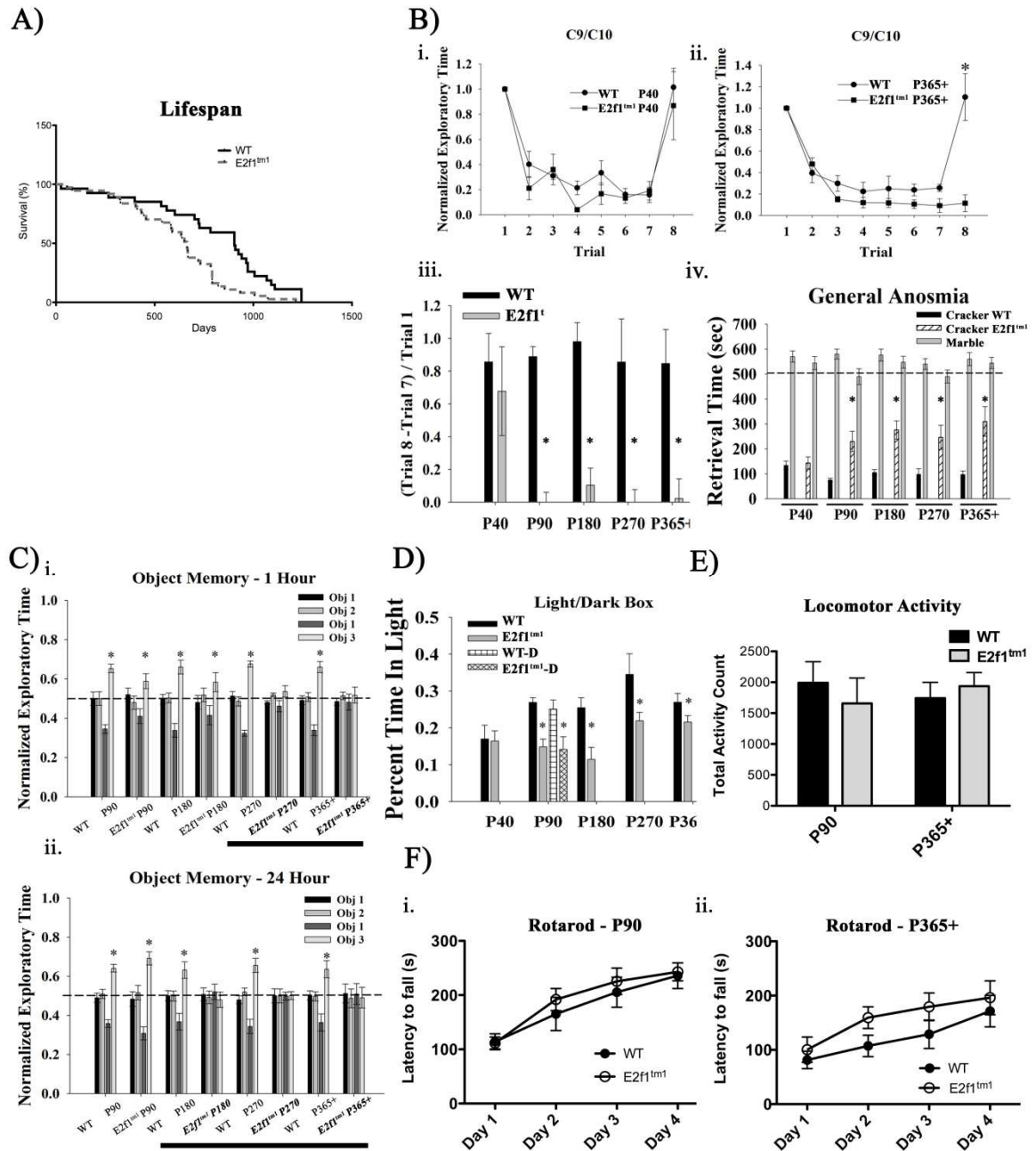


Figure 1. Behavioral phenotyping of $E2f1^{tm1}$ animals. A) Survival curve of $E2f1^{tm1}$ mice compared to WT. The median lifespan of $E2f1^{tm1}$ mice is approximately 25% less than that of WT ($N_{WT}=27$, $N_{TM}=37$, $p<.01$, log-rank and Gehan-Breslow-Wilcoxon tests). B) $E2f1^{tm1}$ mice display age-dependent deficits in olfaction. (i-ii.) Odor habituation paradigms using ethyl heptanoate (C9)- ethyl caprylate (C10) ester odorant pairs on (i.) P40 and (ii.) P365+ postnatal age groups ($N=7$ per group.) Normalized exploratory time is represented as exploratory time in Trial 8/Trial 1. (iii.) Summary of normalized

exploratory index of (Trial 8 – Trial 7)/Trial 1 across age groups (N=7 per group). (iv.) General anosmia test on all age groups reveal the similar age-dependent olfactory deficits (N=7 per group.) Black bars represent the WT retrieval time of scented cracker, hatched bars represent that of E2f1^{tm1}, and gray bars represent that of both genotypes of unscented marble. C) E2f1^{tm1} mice display age-dependent deficits in memory. (i-ii.) Short-term 1-hour delay (i.) and long-term 24 hour delay (ii.) novel object recognition and memory task across all age groups (N=7 per group). * denotes statistical significance in the critical comparison made between the normalized exploratory time of object 3 (light gray bars) and that of object 1 (dark gray bars). Bolded text on the x-axis denotes the age groups of when E2f1^{tm1} mice failed the task. D) E2f1^{tm1} mice display age-dependent elevated anxiety. Light/dark box paradigm (N=7-9 per group) show that E2f1^{tm1} (gray bars) spend less time in the light chamber compared to the WT (black bars) at age P90 and greater. Hatched bars at P90 represent results from mice of both genotypes obtained directly from Jackson laboratory and revealed similar results as mice bred in our colony. E) E2f1^{tm1} mice have comparable basal activity level as WT. Total beam-break counts in activity chamber of WT (black bars) and E2f1^{tm1} (gray bars) on two representative age groups P90 and P365+ (P90 N=5, P365+ N_{WT}=6 and N_{TM}=8). F) E2f1^{tm1} mice do not exhibit any deficits in motor functioning. (i.-ii.) Latency to fall from the accelerating rotarod of WT (closed symbol) and E2f1^{tm1} (open symbol) on two representative age groups P90 (i.) and P365+ (ii.) (P90 N=5, P365+ N_{WT}=6 and N_{TM}=8). All data are represented as mean ± SEM (*, Student's t-test; $\alpha \leq 0.05$).

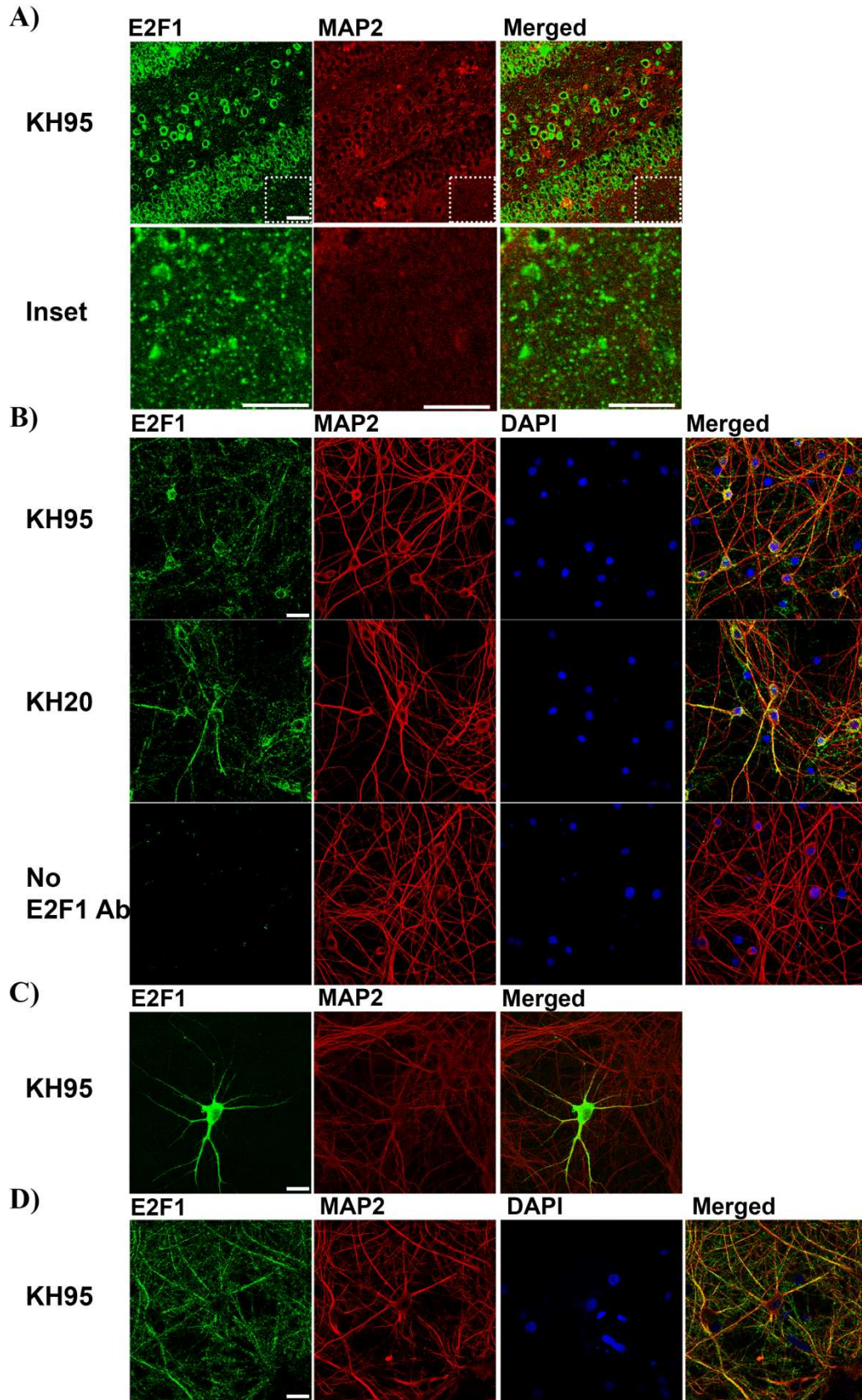


Figure 2. E2F1 is predominantly cytoplasmic *in vivo* and *in vitro*. A) Coronal hippocampal sections from WT P365+ mice were immunolabeled with E2F1 in green and neuronal dendritic marker MAP2 in red (Top). Higher magnification images of the inset areas reveal E2F1 staining can be punctated where neuritic processes are abundant and the cell bodies are absent (Bottom). B) Primary hippocampal neurons at 14 DIV were labeled with E2F1 in green, MAP2 in red, and the nuclei counterstained with DAPI in blue. Two specific E2F1 antibodies KH95 (Top) and KH20 (Middle) were used and produced similar staining patterns. Condition with no E2F1 primary antibodies was included as negative control (Bottom). C) Exogenous E2F1 expression is predominantly cytoplasmic when overexpressed in primary hippocampal neurons. Primary hippocampal neurons were transfected with E2F1 plasmid at 10DIV, fixed at 14 DIV and labeled with E2F1 in green and MAP2 in red. D) Primary cortical neurons at 21 DIV were fixed and labeled with E2F1 in green, MAP2 in red, and nuclei counterstained with DAPI in blue. Scale bar=30 μ m.

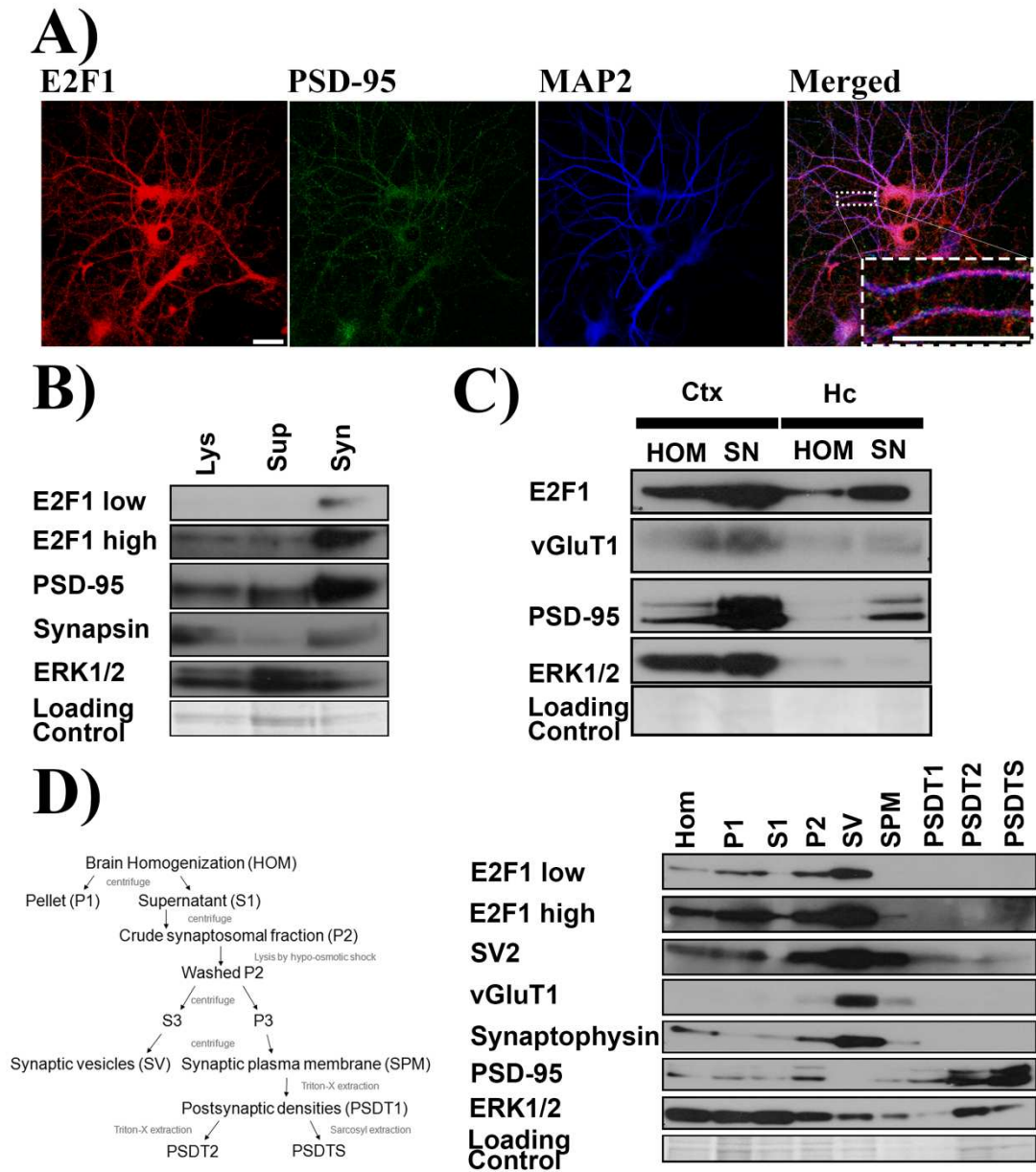


Figure 3. E2F1 is enriched in the synaptic fractions. A) E2F1 puncta can colocalize with synaptic marker PSD-95. Primary hippocampal neurons at 21 DIV were immunostained with E2F1, PSD-95, and MAP2. Higher magnification of boxed neuritic process is shown as inset. B) E2F1 is enriched in the crude synaptosome isolated from primary hippocampal neuron at 21 DIV. Cell lysates (Lys) were collected using synaptic protein extraction reagent and centrifuged to yield the soluble fraction (Sup) and the synaptosomes (Syn). Enriched synaptic markers PSD-95 and Synapsin were used to validate the isolation of synaptosomes. C) E2F1 is enriched in the synaptoneuroosomes isolated from adult mouse cortex and hippocampus. Cortical (Ctx) and hippocampal (Hc) tissues were homogenized (HOM) and the synaptoneuroosomes (SN) were isolated.

Enriched synaptic markers PSD-95 and vGluT1 were used to validate the isolation of synaptoneurosomes D) E2F1 is enriched in the presynaptic fractions. Pre- and postsynaptic fractions were isolated according to the schematic (Left). Postsynaptic markers PSD-95 was enriched in PSDT1, PSDT2, and PSDT3 whereas presynaptic markers SV2, vGluT1, and synaptophysin were enriched in the SV. All of the synaptic markers were enriched in the crude synaptosomes fraction. Immunoblots for ERK 1/2 are shown in each fractionation experiments as non-synaptic protein loading control. Scale bar=30 μ m.

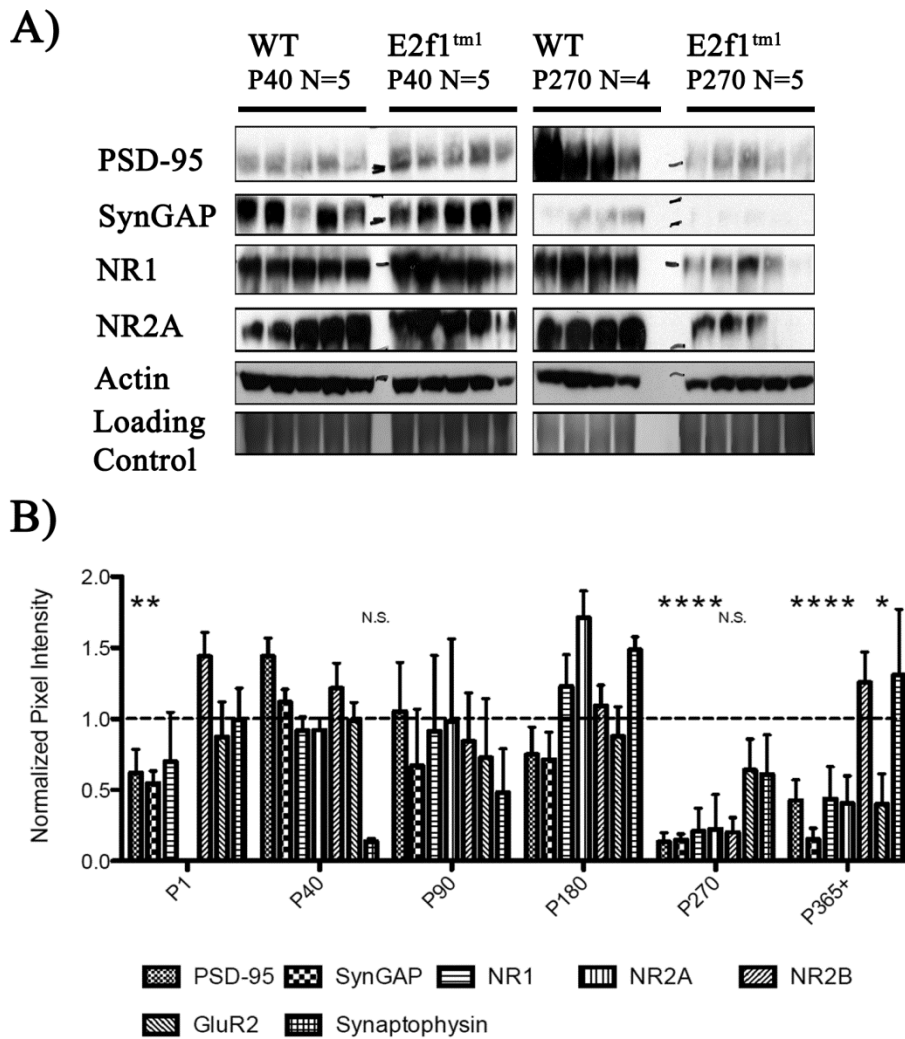


Figure 4. Age-dependent synaptic protein perturbations in the E2f1^{tm1} animals. A) Immunoblots of various synaptic proteins PSD-95, synGAP, NR1, and NR2A in the brains of WT and E2f1^{tm1} mice from two representative age groups P40 and P270. Immunoblots for actin and Coomassie-stained gels are shown as loading controls. B) Quantification of the densitometry analysis of the expression of all tested synaptic proteins displayed as a ratio of the E2f1^{tm1} to the WT. There were significant reductions of PSD-95 and synGAP expression in P1, P270, and P365+, of NR1 and NR2A in P270 and P365+, and of GluR2 in P365+ in the E2f1^{tm1} mutants compared to the WT. All data are represented as mean \pm SEM (*, Student's t-test; $\alpha \leq 0.05$).

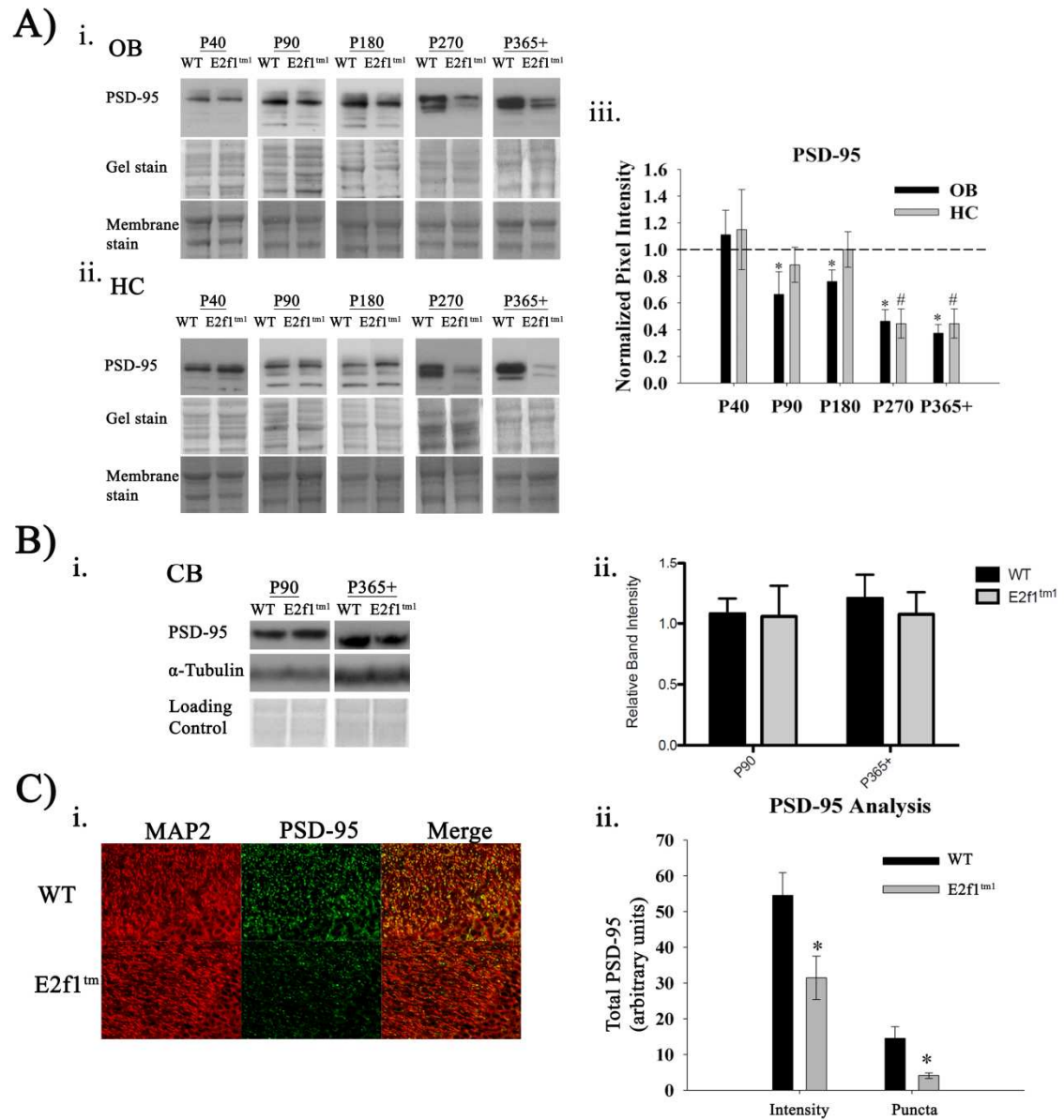


Figure 5. Age-dependent reduction in PSD-95 expression in hippocampus and olfactory bulbs. A) Immunoblots of PSD-95 expression in OB (i.) and HC (ii.) across age groups. Coomassie-stained gels and fast green-stained membranes are shown as loading controls. (iii.) Quantification of the densitometry analysis displayed as a ratio of the E2f1^{tm1} to the WT. (N=6. *, OB; #, HC) B) (i.) Immunoblots of PSD-95 expression in cerebellum across two representative age groups P90 and P365+. (ii.) Quantification of the densitometry analysis (N=5 P90, N=6 P365+). C) (i.) Representative images of coronal WT and E2f1^{tm1} P365+ hippocampal sections immunolabeled with MAP2 (red) and PSD-95 (green) captured at 400x. (ii.) Quantification of the total PSD-95 pixel intensity and the intensity-saturated PSD-95 puncta in the WT and E2f1^{tm1} (N=3 for each genotype, 8 sections each, * Student's *t*-test; $\alpha \leq 0.05$). All data are represented as mean \pm SEM.

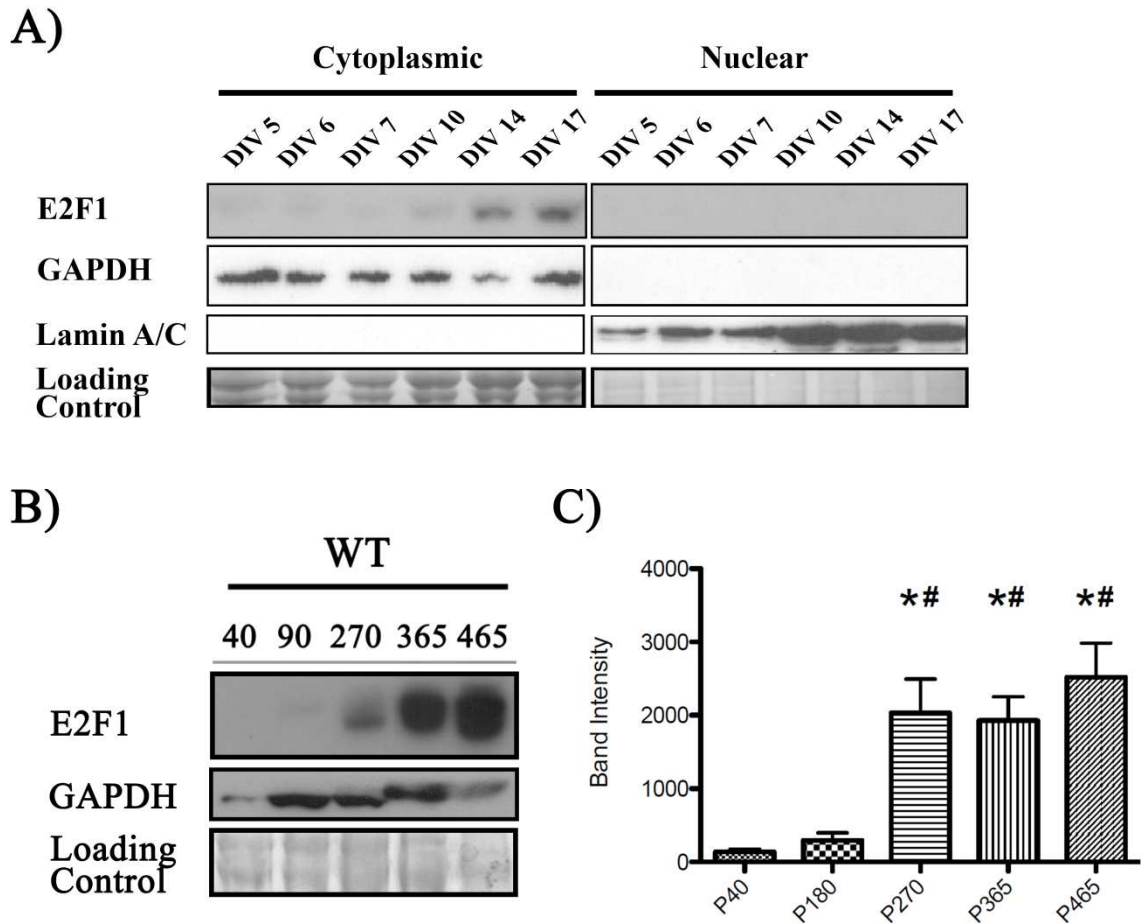


Figure 6. E2F1 expression increases with age *in vitro* and *in vivo*. A) Cytoplasmic and nuclear lysates were collected from primary cortical cultures at various ages *in vitro* by subcellular fractionation. E2F1 expression elevates in the cytoplasmic fraction but is undetectable in the nuclear fraction. GAPDH serves as a cytoplasmic fraction marker while Lamin A/C serve as a nuclear fraction marker. B) Representative immunoblot of cortical lysates collected from postnatal age 40, 180, 270, 365 for E2F1 expression (left). Immunoblots for GAPDH and Coomassie-stained gels are shown as loading controls. C) Densitometry analysis of E2F1 reveals significant increase starting at P270 and persist until P465 (right, N=5 per group). *denotes $p < .05$ compared to P40, # denotes $p < .05$ compared to P180, one way-ANOVA Newman-Keuls post-hoc test.

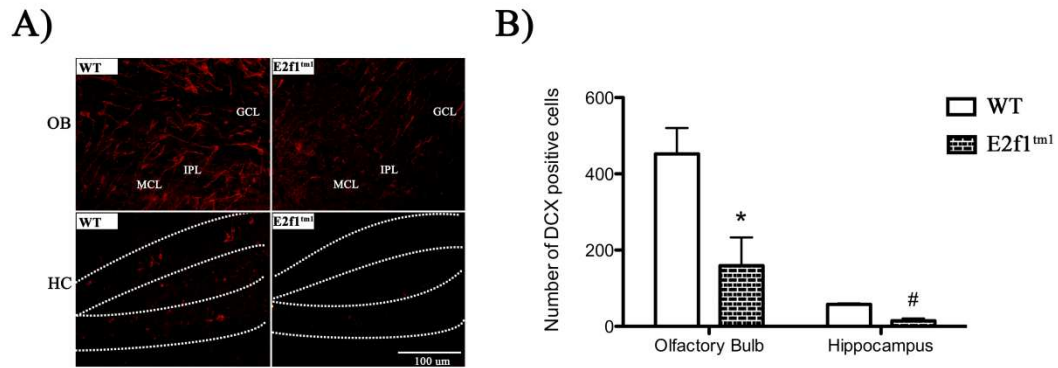


Figure 7. E2f1^{tm1} mice display a significant reduction in the number of doublecortin-positive cells in the OB and dentate gyrus of HC. A) Coronal OB and HC sections from WT and E2f1^{tm1} P365+ animals immunolabeled with DCX in red. In HC sections, the dentate gyrus is outlined with dashed lines. B) Quantification reveals a strong reduction in the number of newly generated DCX-positive neurons. All data are represented as mean \pm SEM. * Student's *t*-test; $\alpha \leq 0.05$.

**CHAPTER 4: E2F1 depletion in primary neurons leads to the loss of
PSD-95 expression and defect in neuritic arborization**

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Abstract

E2F1 has been long characterized for its function as a transcription factor regulating gene expression necessary for transition from G₁ to S phase during the cell cycle. In addition, E2F1 can also regulate apoptosis by transcriptionally regulating a wide range of pro-apoptotic genes, and as such, E2F1 has been well characterized in the context of neuronal death in neurodegenerative diseases and *in vitro* models of neurotoxicity. Despite this, the role of E2F1 in healthy neurons has been largely neglected. In a recent study, we have reported that mice lacking functional E2F1 exhibit age-dependent behavioral deficits and synaptic disruption. Specifically, we observed a significant reduction in PSD-95 expression in the newborn E2f1^{tm1} mice, indicating that E2F1 activity is required for early normal brain maturation. In the present study, we examined the effect of E2F1 depletion in cultured neurons *in vitro* on PSD-95 expression and neuronal morphology. We report that reducing E2F1 expression in primary neurons *in vitro* led to a significant reduction of PSD-95 expression and impairment in neuritic arborization, providing evidence that E2F1 has a physiologic role in neuronal development outside the context of cell cycle and death.

Introduction

E2F1 is the first of eight family members of E2F transcription factors identified in regulating G₀/G₁ to S phase transition during cell cycle progression (DeGregori & Johnson 2006, Iaquina & Lees 2007). Originally classified as a tumorigenic protein due to its role in cell proliferation, E2F1 has subsequently been demonstrated to be critical in tumor suppression through its apoptotic functions (Putzer & Engelmann 2013, Field et al. 1996, Yamasaki et al. 1996) and interaction with tumor suppressor protein, pRb (Chellappan et al. 1991, Hsieh *et al.* 1997). Specifically, E2F1 can mediate cell death through the upregulation of p14^{arf} (p19^{arf} in rodents), which in turn stabilizes the levels of another apoptotic protein p53 by disrupting its ubiquitination by E3 ligase Mdm2 (Bates et al. 1998). Alternatively, E2F1 can also induce cell death by directly increasing expression of other pro-apoptotic genes such as APAF1, Bim, Bik, caspases 3,7,8,9 among others through transcriptional regulation (Moroni et al. 2001, Hershko & Ginsberg 2004, Nahle et al. 2002, Real et al. 2006). Because of the capacity for E2F1 to induce apoptosis, E2F1 has been studied extensively in the context of neuronal death and neurodegenerative diseases. For example, overexpression of E2F1 in cerebellar granular neurons leads to neuronal apoptosis through pro-apoptotic protein Bax, while neurons lacking functional E2F1 are also less vulnerable to B-amyloid induced neurotoxicity (O'Hare et al. 2000, Giovanni et al. 2000). Furthermore, aberrant E2F1 expression and activity have been implicated in several neurodegenerative diseases including

Alzheimer's and Parkinson's disease (Ranganathan et al. 2001, Jordan-Sciutto *et al.* 2001, Hoglinger et al. 2007).

Despite its well characterized roles in apoptosis and cell proliferation in the past, E2F1 has since been demonstrated to have other functions including the induction of DNA damage repair, differentiation, and energy metabolism (Guo et al. 2010, Fajas *et al.* 2002, Blanchet et al. 2011, Fajas *et al.* 2004). Emerging evidence suggests that classic proliferative and apoptotic activity of E2F1 in the nucleus is suppressed in post-mitotic cells; however, its physiologic function in these cell types remain uncharacterized. Evidence for an alternate role for E2F1 during cell differentiation include the redistribution of E2F1 to different subcellular compartments in myocytes and keratinocytes, which is required to maintain the terminal differentiation of the (Gill & Hamel 2000, Ivanova & Dagnino 2007, Ivanova *et al.* 2009). Similarly, the levels of E2F1 expression and promoter binding activity are suppressed in the nucleus of oligodendrocyte precursor cells, thereby permitting their differentiation to mature oligodendrocytes in the CNS (Magri et al. 2014).

Consistent with these reports, we have previously demonstrated that E2F1 expression is predominantly in the cytoplasmic compartments of terminally differentiated post-mitotic neurons both *in vivo* and *in vitro* (Strachan *et al.* 2003, Wang *et al.* 2010). In addition, E2F1 localizes in proximity of synapses, as E2F1 is enriched in synaptosomes isolated from cultured neuroglial cells and from whole brains (Ting *et al.* 2014). Furthermore, mice lacking functional E2F1 exhibit age-dependent reduction in the expression of postsynaptic density protein 95 (PSD-95). Though the loss of synaptic

proteins in the older E2f1^{tm1} mice may be partially attributable to persistent deficits in adult neurogenesis, the reason for the early loss of PSD-95 expression in the newborn E2f1^{tm1} mice remains unclear (Toni & Sultan 2011). Given that E2F1 is enriched in the synaptosomes, it is possible that E2F1 has a more direct link to the expression of PSD-95 during synapse maturation. To test this hypothesis in the present study, we examined the effect of E2F1 depletion on PSD-95 expression and neuronal morphology in cultured neurons *in vitro*, where the confounding effect of E2F1 on neurogenesis is minimal.

Materials and Methods

Animals: E2f1^{tm1} (B6;129S4-E2F1^{tm1Mtg}/J; strain # 002785) and Wildtype (B6129SF2/J; strain # 101045) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Transgenic mice were previously described and housed at the University of Pennsylvania animal facilities on a 12-hour light/dark cycle and water were provided *ad libitum* (Field et al. 1996). All experiments were approved by Institutional Animal Use and Care Committee.

RNA Interference: pSuper system based small hairpin RNA (shRNA) vector was designed and constructed according to manufacturer instructions (Oligoengine) (Brummelkamp *et al.* 2002). Sequences of E2F1 mRNA that the mature processed siRNAs target: 984 - 5' CCACCATCACCTCCCTCCA 3', 1018 - 5' ATCCCAGCCAGTCCCTGTT 3', 1027 - 5' AGTCCCTGTTAGGCCTGGA 3'. BglII and XhoI were the restriction enzymes used for cloning the annealed oligonucleotides

into the empty pSuper plasmids.

Cell culture and transfection: Primary cortical or hippocampal neuroglial cultures were isolated from the brains of embryonic day 17 Sprague Dawley rats or of embryonic day 16.5-17.5 mice as previously described (Wilcox *et al.* 1994). Dissociated cells were plated on poly-L-lysine coated dishes and the cultures are maintained in neurobasal media with B27 supplement at 37°C with 5% CO₂. Rat2 fibroblasts and HEK293T cells were maintained in DMEM supplemented with 10% FBS. Transfections of primary neurons were performed using lipofectamine 2000 [Invitrogen] as previously described (Ting *et al.* 2014). Transfection mixture was added to the cells for 2 hours and subsequently replaced with the original, conditioned media. Cells were then fixed 4 days post-transfection using 4% PFA.

Antibodies and Reagents: The following antibodies were purchased from the indicated vendors: E2F1 KH95 (sc-251) [Santa Cruz]; PSD-95 (MAB1596) [Millipore]; NMDAR1 (#5704), GluR2 (#2460), GFP (#2956), BiP (#3177) [Cell Signaling]; MAP2 (ab5392) [Abcam]; GAP-43 (NB300-143) [Novus Biologicals]. The following chemical reagents were used from the indicated vendors: DAPI [Molecular Probes]; Coomassie (161-0786); Protein assay dye (500-0005), PVDF membrane [BioRad], Pageruler plus protein ladder [Thermos], Luminata Forte Western HRP substrate [Millipore]. All HRP conjugated secondary antibodies were obtained from Pierce and all dye conjugated secondary antibodies were obtained from Jackson Immuno-Research.

Immunoblotting and Immunocytochemistry: Cultured cells were homogenized in ice cold, whole cell lysis buffer containing 50 mM Tris, 120 mM NaCl, 0.5% NP-40, 0.4mM

sodium orthovanadate and protease inhibitor cocktail. Protein concentrations were determined using the Bradford method. Equal amounts of proteins were loaded for immunoblotting and confirmed by staining the gel with Coomassie. For densitometric analysis, autographs were scanned and cropped using Adobe Photoshop (Adobe Systems). Pixel intensities of each bands of interest were quantified using Image J software (NIH) and normalized to gel Coomassie stain. Cells grown on coverslips were fixed, permeabilized, and blocked at room temperature and incubated with primary antibodies overnight at 4°C and appropriate secondary 30 minutes at room temperature. Tyramide Signal Amplification system [Perkin Elmer] was used according to manufacturer instructions (Wang et al. 2010).

Image acquisition and analysis: Images from samples were captured at 600x on a laser confocal microscope with Biorad Radiance 2100 (Biorad) or 200x on a standard epifluorescent microscope (Nikon E400). Total E2F1, PSD-95 pixel intensity and MAP2 area in an image were quantified using Metamorph 6.0 (Universal Imaging). Quantitative analysis of neuritic arborization of Td-tomato (Clontech) transfected neurons was performed using NIH ImageJ Analysis Plugin (Ghosh Lab, UCSD) (Dijkhuizen & Ghosh 2005). The parameters of the analysis are: starting radius=25µm, ending radius=350µm, radius size step=15µm, radius span=1.5µm.

Statistical analysis. All data were analyzed by Prism 5.0 software (GraphPad Software) and expressed as mean ± SEM with values of $p < 0.05$ considered significant. Unless otherwise noted, all asterisks denote statistical significance by comparing the means to that of the control group using one-way ANOVA and Newman-Keuls post-hoc test.

Results

1) E2F1 expression increases as neurons mature *in vitro*

E2F1 expression has been previously demonstrated to increase *in vivo* during brain development and maturation from embryogenesis through adulthood (Kusek et al. 2001, Ting et al. 2014). However, the contributions of this increase in E2F1 expression from individual neural cell types have not been documented. Here, we examined specifically the expression of E2F1 in neurons as they mature in culture. Primary cortical neurons from different ages in culture were immunostained for E2F1 and neuronal dendritic marker microtubule-associated protein 2 (MAP2). We observed an increase in neuronal E2F1 expression as the cortical neurons mature from 7 days *in vitro* (DIV) to 21DIV (Fig 1A). Consistent with previously published report, E2F1 expression mainly co-localized with MAP2 as opposed to DAPI-positive nuclei, confirming that E2F1 expression in differentiated neurons is predominantly cytoplasmic (Strachan et al. 2005b, Wang et al. 2010). Next, we examined whether increased neuronal E2F1 expression was brain region dependent. Primary hippocampal neurons at various days grown *in vitro* were immunostained for E2F1 and neuronal marker growth associated protein 43 (GAP43). GAP43 was chosen as a neuronal marker because the staining of GAP-43 included axonal processes in addition to the dendritic processes labeled by neuronal marker MAP2 that was previously used. As expected, neuronal exhibited an age-dependent increase in expression from DIV1 to DIV14 (Fig 1B). Notably, we observed significant E2F1 expression in the growth cones of the primary axons at DIV 1 and 2 that

were not previously documented. The maturation of these neurons was evident by the increasing elaboration of neurites marked either by MAP2 or GAP43 immunostain. Taken together, our data demonstrate that E2F1 expression is predominantly cytoplasmic and increases as neurons mature *in vitro*.

2) PSD-95 expression is reduced in the primary neurons derived from the E2f1^{tm1} animals

Previously, we reported a significant reduction in the level of PSD-95 in the mouse brains lacking functional E2F1 (Ting et al. 2014). In addition, the loss of PSD-95 manifested early during embryonic development, suggesting that normal PSD-95 expression in synapse formation and/or maturation depends upon E2F1 expression. Therefore, we sought to determine whether PSD-95 expression was dependent on E2F1 by examining PSD-95 expression in primary neurons cultured from E2f1^{tm1} mice *in vitro*. Consistent with previous *in vivo* findings, we observed significantly reduced expression of PSD-95 in neurons derived from E2f1^{tm1} mice at DIV17 compared with wildtype mice (Fig 6A). Further analysis at DIV21-28, demonstrated recovery of PSD-95 expression in E2f1^{tm1} neuronal cultures to wildtype levels. Interestingly, despite the reduction in PSD-95, the expressions of NMDA receptor subunit 1 (NR1) and AMPA receptor subunit 2 (GluR2) were not disrupted regardless of the age of culture, suggesting that other glutamatergic receptors in the synapses were not perturbed in neurons lacking functional E2F1. To corroborate our findings, we also examined the levels of PSD-95 expression in these cultures by immunocytochemistry. As shown in figure 6B, total PSD-95 levels were significantly reduced at 17 DIV in neurons derived from E2f1^{tm1} mice. To control

for cell density, we measured total MAP2 area and found no significant change at the time when reduced PSD-95 levels were observed (Fig 6Biii.). Together, these data suggest that E2F1 may regulate PSD-95 expression as primary neurons mature *in vitro*.

3) Reducing E2F1 expression by shRNA knockdown leads deficits in neuritic arborization

Since experiments utilizing neurons derived from E2f1^{tm1} mice lack direct temporal control in E2F1 expression, we next sought to reduce E2F1 expression by RNA interference to determine the effect of E2F1 knockdown on neuronal physiology *in vitro*. We designed and developed three separate shRNA in pSuper-vectors targeted at various regions of the E2F1 mRNA: 987, 1018, and 1027 (Brummelkamp et al. 2002). When these shRNA vectors and E2F1 were co-expressed in HEK293T cells, all three vectors were able to reduce the transfected E2F1 expression (Fig 3A). Since E2F1 has been shown to negatively regulate BiP expression, we used endogenous expression of BiP to verify the efficiency of E2F1 knockdown. As predicted, we indeed observed an increase in BiP expression with a corresponding decrease in E2F1 expression as a result of the knockdown. In addition, we also verified the knockdown efficiency of endogenous E2F1 by overexpressing these vectors in Rat2 fibroblast cells. As a result, we observed that while pSuper-1018 and pSuper-1027 were efficient in reducing endogenous E2F1 expression, pSuper-984 or pSuper-scrambled were unable to reduce endogenous E2F1 expression (Fig 3B). Furthermore, we also examined whether increased amount of knockdown vectors would lead to greater suppression of E2F1 expression and found that indeed pSuper-1018 and pSuper-1027 were able to reduce E2F1 expression in a plasmid

dosage-dependent manner (Fig 3C).

We next examined the effect of E2F1 knockdown on PSD-95 expression in neurons that were cultured for either one week or three weeks. When we examined the morphologies of the primary hippocampal neurons transfected with the E2F1 silencing vectors by Scholl's analysis, we observed a significant repression of dendritic arborization in the 2-weeks old neurons (Fig 3E). Interestingly, reducing E2F1 expression in 1-week old neurons did not result in a similar phenotype as that of 2-week old neurons, suggesting that E2F1 expression and activity are critical during the second week of differentiation *in vitro* for normal neuronal morphology.

Discussion

Although E2F1 has been characterized extensively in the context of neurodegeneration in the presence of neurotoxins, its activity and function in healthy neurons have been largely unexplored. In the context of cell cycle, E2F1 is critical in suppressing the aberrant re-activation of the cell cycle machinery that leads to death of post-mitotic neurons (Wang et al. 2007a). The suppression of cell cycle in neurons also requires CDK5, which complexes with E2F1 in the brain to inhibit the ability of E2F1 to initiate cell cycle re-entry through transcriptional regulation (Zhang et al. 2010). Interestingly, a pro-survival role of E2F1 in post-mitotic neurons has been observed specifically in rat cerebellar granular neurons as overexpression of E2F1 in these cells during potassium deprivation resulted in reduced cell death (Yuan et al. 2011). In the

present study however, we directly examined the role of E2F1 in healthy neurons by initially characterizing E2F1 expression as neurons mature in culture and subsequently examining the effect of E2F1 depletion on neuronal physiology.

Consistent with the previous observations, we report a predominant cytoplasmic localization of E2F1 in primary neurons that are cultured *in vitro* (Wang et al. 2010, Ting et al. 2014). Moreover, E2F1 in this compartment increases as neurons increase in age, suggesting that increased E2F1 activity may be necessary during the neuronal maturation process. One of several critical processes involved in neuronal maturation *in vitro* is the establishment of synaptic connectivity with neighboring neurons (Waites *et al.* 2005). While the rate of synaptogenesis is relatively low during the early period of neuronal culturing (DIV 7), synaptogenesis subsequently increases exponentially at later time points, starting around DIV14 and onwards (Romijn *et al.* 1981, Ichikawa *et al.* 1993). Correlative to the timeline of synapse formation, cytoplasmic E2F1 also increases from DIV 7 to DIV14 in both primary cortical and hippocampal neuron, underscoring the possibility that E2F1 activity may be involved in synaptogenesis. Given that E2F1 is observed close to or at the synapses, it would be important to characterize E2F1 expression specifically in the synaptosomes as neuronal cultures increase in age in future studies to determine if indeed E2F1 is recruited to the synapse as part of neuronal development (Ting et al. 2014).

Since E2F1 activity has been linked to PSD-95 expression *in vivo*, we hypothesized that the expression of PSD-95 in neurons derived from the E2f1^{tm1} mice would be similarly reduced (Ting et al. 2014). In support of our hypothesis, we observed

an age-dependent reduction of PSD-95 expression in the absence of E2F1 activity, specifically at DIV17. In contrast however, we did not observe any changes in expression of other synaptic protein such as glutamate receptor subunits NR1 or GluR2, suggesting that E2F1 does not regulate all processes associate with synapse formation. On the other hand, since we were unable to determine whether the recruitment of these receptor proteins to the synapse is impaired in the absence of E2F1 activity, it remains possible that the levels of these receptor proteins are only disrupted locally at the synapse. Future studies are necessary in order to further explore this possibility.

Given that E2F1 expression is relatively low during the first week of *in vitro* culturing, it is not surprising that we did not observe a difference in PSD-95 levels at DIV 7. Interestingly however, we did not detect the reduction of PSD-95 levels in the older cultures at DIV 21, 24, or 28 even though synaptogenesis persist at this time. We speculate that, similar to the age-dependent PSD-95 expression profile in the E2f1^{tm1} mice, the loss of PSD-95 levels is only apparent initially, when the numbers of synapses are not yet saturated. However, when the numbers of synapses reach saturation in the older cultures, the effect of E2F1 on PSD-95 observed in the earlier cultures is masked by the overabundance of synaptic proteins as a result of the exponential rate of synaptogenesis. Alternatively, it is also plausible that E2F1 activity is only necessary initially, during the early stage of synapse formation, but no longer required once the synapses have matured. Consistent with this interpretation, our preliminary data indicates that E2F1 depletion by RNAi in neurons at first week led to a trend of PSD-95 reduction while no change at the third week (data not shown). The change in PSD-95 expression may not have reached statistical significance due to the low knockdown

efficiency. Studies are currently underway to increase the efficiency of E2F1 depletion by transfection of validated siRNA in primary neurons.

Despite the inefficiency in E2F1 depletion through shRNA, we did observe a robust effect of E2F1 knockdown on neuronal morphology. Specifically, neuritic arborization of neurons whose E2F1 expression has been reduced by shRNA vectors was impaired. Importantly, neuritic arborization is only affected when E2F1 is reduced during the second week of culturing but not the first week, suggesting that the phenotype is unlikely due to the transfection of the shRNA plasmids. Rather, since E2F1 expression is elevated at the second of week of culture, E2F1 depletion would be more effective at this time. The impairment in neuritic arborization following E2F1 knockdown may also be relevant in the context of the role of E2F1 in PSD-95 expression during synaptogenesis as proper dendritic arborization is crucial for the proper development of synaptic circuitry (Cline 2001). It is possible that loss of E2F1 leads to reduced neuritic extension leading to fewer formations of synapses. Future studies would be necessary in delineating the effect of E2F1 on dendritic arborization as well as PSD-95 expression. Taken together, our studies present evidence for the first time that E2F1 is crucial in neuronal physiologic development outside the context of cell cycle or death, particularly during synapse formation and neuritic arborization. Disruptions to this E2F1 role may further contribute to neuronal dysfunction in the presence of neurotoxins during neurodegeneration.

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Abbreviations PSD-95, postsynaptic density protein 95; shRNA, small-hairpin RNA; MAP-2, microtubule associated protein -2; GAP-43, growth associated protein 43, DIV, days *in vitro*; NR1, N-methyl-D-aspartate receptor subunit 1; GluR2, α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit 2.

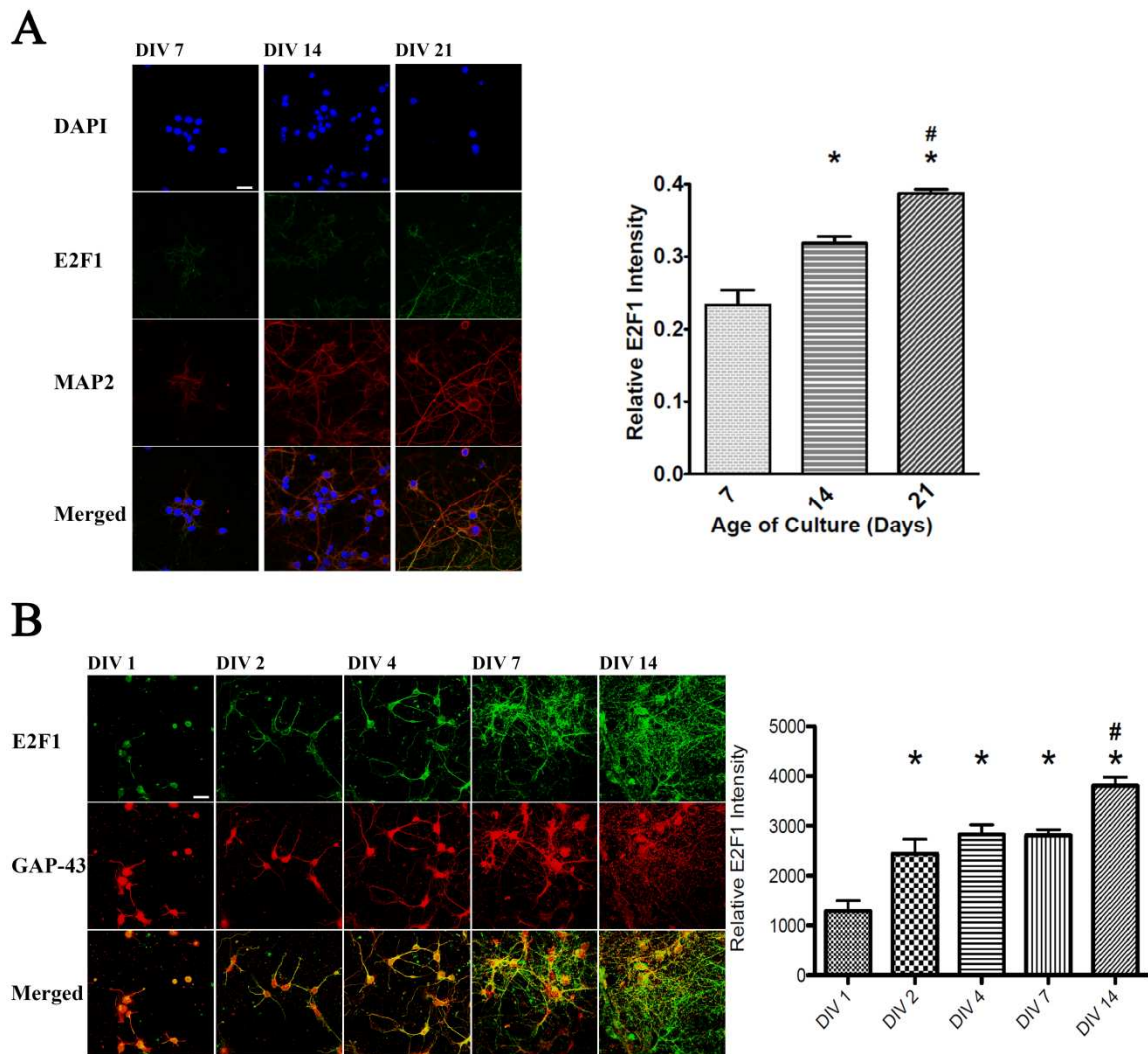


Figure 1. E2F1 expression increases as neurons mature *in vitro*. A) Primary cortical neuroglial cells from DIV 7, 14, and 21 were immunostained with E2F1 (green), MAP2 (red), and labeled with nuclear marker DAPI (blue). E2F1 predominantly co-localized with MAP2 marker but not nuclear marker DAPI. Quantification of the E2F1 intensity (arbitrary units) which co-localized with MAP2 and subsequently normalized to MAP2 area is presented in the right panel. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to 7DIV, # denotes $p < .05$ compared to 14 DIV). B) Primary

hippocampal neuroglial cells from DIV 1, 2, 4, 7, and 14 were immunostained with E2F1 (green) and GAP-43 (red). Quantification of the E2F1 intensity (arbitrary units) which co-localized with GAP-43 and normalized to GAP-43 area is presented in the right panel. Data are represented as mean \pm SEM (* denotes $p < .05$ compared to DIV1, # denotes $p < .05$ compared to DIV7). Data were analyzed using one-way ANOVA and Newman-Keuls post-hoc test. Scale bar=30 μ m.

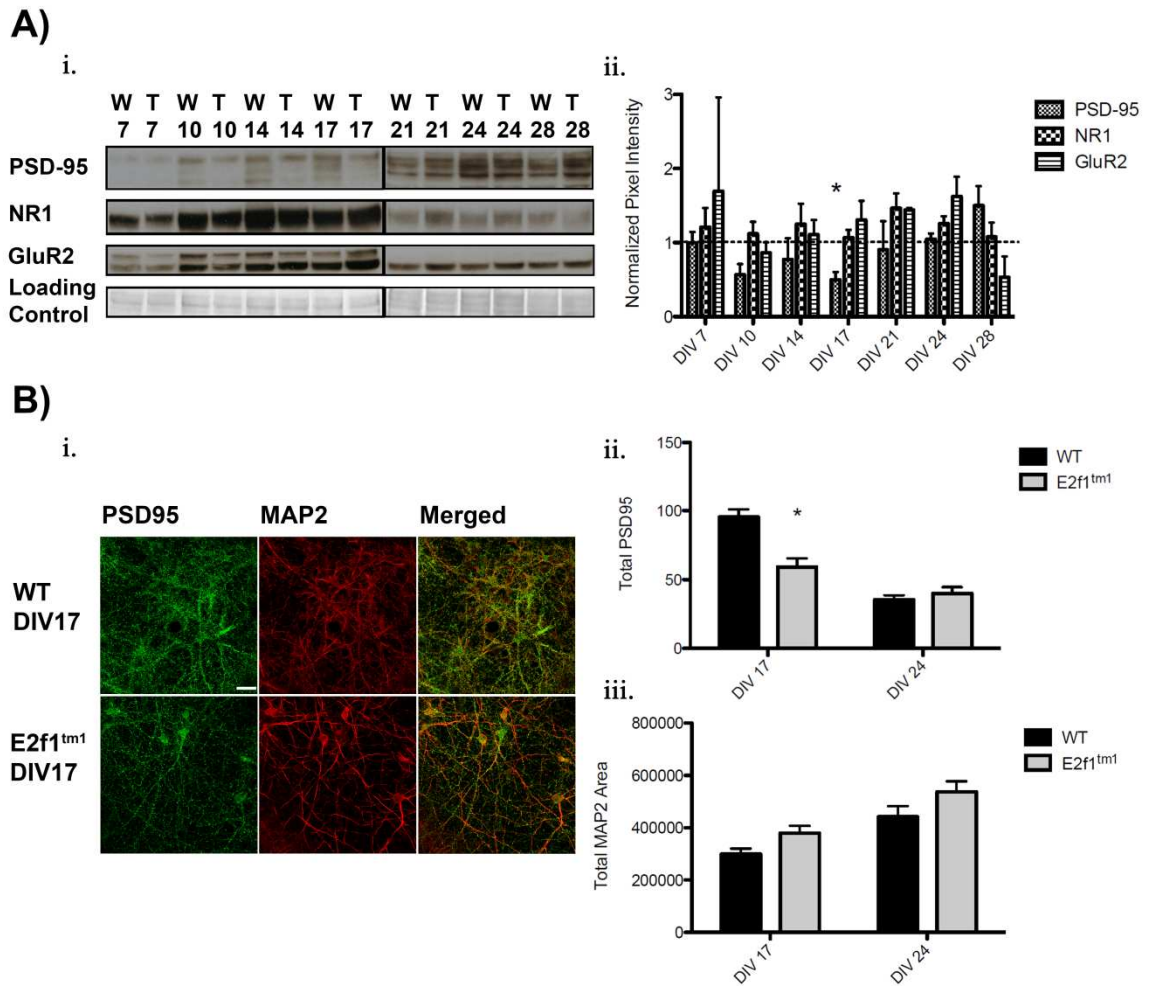


Figure 2. PSD-95 expression is reduced in the neurons derived from E2f1^{tm1} mice. A) Lysates were collected from primary neuroglial cells derived from wildtype or E2f1^{tm1} mice at DIV 7, 10, 14, 17, 21, 24, and 28 and immunoblotted for PSD-95, NR1, and GluR2. The representative immunoblot of three independent experiments is shown on the left (i.) while the densitometry analysis of each protein band of interest in the E2f1^{tm1} mice normalized to that of wildtype is shown on the left (ii.). Data are represented as mean \pm SEM (* denotes $p < .05$ compared to wildtype, student's t-test). B) Cultured from wildtype or E2f1^{tm1} mice at DIV 17 and 24 were immunostained for PSD-95 (green) and MAP2 (red). Immunostaining images from confocal microscopy of PSD-95 and MAP2 at DIV 17 is shown on left (i.) while the quantification of total PSD-95 intensity

(arbitrary units) and MAP-2 area (arbitrary units) shown on the right (ii. and iii.) Data are represented as mean \pm SEM (* denotes $p < .05$ compared to wildtype, student's t-test).

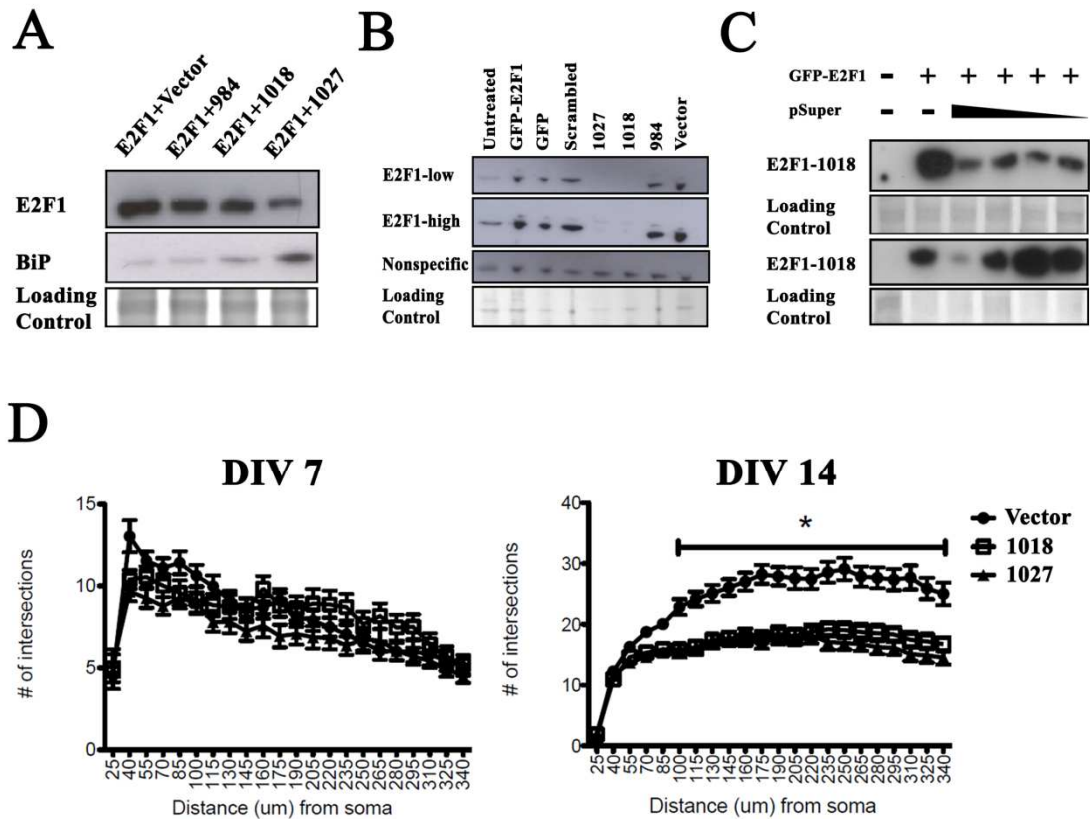


Figure 3. Reducing E2F1 expression leads to defects in neuritic arborization. A) Lysates were collected from HEK293T cells co-transfected with rat E2F1 fused to GFP (GFP-E2F1) and either pSuper-vector only, or E2F1 shRNAs: pSuper-984, pSuper-1018, or pSuper-1027, and immunoblotted for GFP-E2F1 and BiP. B) Lysates were collected from Rat2 cells transfected with GFP-E2F1, GFP, pSuper-Scrambled, pSuper-1027, pSuper-1018, pSuper-984, and pSuper-vector only and immunoblotted for E2F1. C) Lysates were collected from HEK293T cells co-transfected with 1 μ g of GFP-E2F1 DNA and either 3 μ g of pSuper-vector, 3 μ g of pSuper-1018/ 1027, 1 μ g pSuper-1018/1027, 0.6 μ g pSuper-1018/1027, and 0.3 μ g pSuper-1018/1027 and immunoblotted for GFP-E2F1. E) Primary hippocampal neurons were transfected with Td-tomato and either pSuper-vector, 1018, or 1027 at DIV 3 and fixed at DIV 10 (left) or transfected at DIV 10 and fixed at DIV 14 (right). Analysis by Sholl's method is shown with the data represented as mean \pm SEM (* denotes $p < .05$ compared to pSuper-vector, two-ways ANOVA, Bonferroni post-hoc test)

**CHAPTER 5: Identification and characterization of two novel
alternatively spliced E2F1 transcripts in the brain**

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Abstract

E2F1 is a transcription factor classically known to regulate G₀/G₁ to S phase progression during the cell cycle. In addition, E2F1 also regulates a wide range of apoptotic genes and thus has been well studied in the context of neuronal death and neurodegenerative diseases. However, its function and regulation in the mature central nervous system are not well understood. Alternative splicing is a well conserved post-transcriptional mechanism that is common in cells of the CNS and is necessary to generate diverse functional modifications to RNA or protein products from the same gene. Despite this, physiologically significant alternatively spliced E2F1 transcripts have not been reported. In the present study, we report the identification of two novel alternatively spliced E2F1 transcripts: an E2F1 transcript retaining intron 5 and an E2F1 transcript excluding exon 6. These alternatively spliced transcripts are specific to the brain and neural cell types including the neurons, astrocytes, and oligodendrocytes. Additionally, the expression of the E2F1 transcripts are distinct during the maturation of primary hippocampal neuroglial cells. Pharmacologically induced global translation inhibition with cycloheximide or thapsigargin lead to significantly reduced expression of classic E2F1 and E2F1 transcript excluding exon 6. On the other hand, although anisomycin induced translation inhibition lead to reduced expression of these two transcripts, it had no effect on the expression of E2F1 transcript retaining intron 5. Increasing neuronal activity by elevating concentration of potassium chloride selectively increased the expression of E2F1 transcript retaining intron 5 but not the other transcripts. Taken together, our data suggest

that the alternatively spliced E2F1 transcripts behave differently than the classic E2F1 transcript and provide a foundation for future investigation to study their functions in the brain.

Introduction

Alternative splicing is an evolutionarily conserved mechanism that allows the cells to generate diverse functional RNA transcripts through combinatorial pairing of different splice sites (Nilsen & Graveley 2010). Through the coordinated expression of different splicing regulatory factors, different cell types can generate vast number of different proteins and functional modifications necessary for their own physiologic development from an identical genome. Tissue specific alternatively spliced RNA transcripts are particularly common during the development of the central nervous system (CNS) as neural cells enter their fully differentiated state (Grabowski 2011). A recent example of this developmentally controlled spliced RNA transcripts is seen in the gene encoding the neuronal per-arnt-sim domain protein 3 (NPAS3) such that the splice variant is only expressed in the ventricular zone during brain development (Shin & Kim 2013). Disruption of tightly regulated switch of spliced RNA transcripts can induce developmental defects in the CNS as seen when deletion of Nova2, a regulator of splicing, leads to aberrantly spliced RNA transcript of disabled-1 protein leading to neuronal migration defects (Yano *et al.* 2010). In addition, alternative splicing that introduces a premature stop signal in the mRNA can be coupled with the nonsense-mediated mRNA decay (NMD) pathway to temporally control the expression of the classically generated protein product during brain development. For example, *DLG4*, the gene that encodes postsynaptic protein 95 that is crucial in synapse maturation, is alternatively spliced into a transcript containing a premature stop codon that is degraded by NMD during embryonic

development. However, loss of splicing regulatory proteins polypyrimidine tract binding proteins (PTBP) 1 and 2 during brain maturation leads to the transcription of full length postsynaptic density-95 (PSD-95) transcript and proteins necessary for synapse formations (Zheng *et al.* 2012). Thus, tight regulation of the splicing machinery is critical for CNS physiology and its disruption can contribute to neuronal degeneration as seen in the disruption of TAR DNA-binding protein (TDP)-43 (Polymenidou *et al.* 2011) .

E2F1 is classically described as a transcription factor regulating G₀/G₁ to S phase in cycling cells but also has an important role in determining cell deaths (Blais & Dynlacht 2004, Giacinti & Giordano 2006, Iaquina & Lees 2007). E2F1 is able to induce apoptosis by indirectly upregulating the level of p53, the master regulator of apoptosis and cell cycle arrest, or by directly upregulating pro-apoptotic targets such as APAF1, PUMA, NOXA, Smac/Diablo, and caspases through gene transcription (Moroni *et al.* 2001, Nahle *et al.* 2002, Hershko & Ginsberg 2004, Xie *et al.* 2006) . Because of this capacity, the role of E2F1 in neuronal death has been well characterized in various models of toxicity *in vitro* (Giovanni *et al.* 1999, Giovanni *et al.* 2000, O'Hare *et al.* 2000, Park *et al.* 2000a). In fact, aberrant E2F1 expression is common in various neurodegenerative diseases including Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (Ranganathan *et al.* 2001, Jordan-Sciutto *et al.* 2001, Jordan-Sciutto *et al.* 2002a, Hoglinger *et al.* 2007). However, despite its well-characterized implications in these diseases, little is known about E2F1 function or regulation in the CNS. We have previously provided evidence that E2F1 has a physiologic role in the CNS as transgenic mice lacking functional E2F1 exhibit age-dependent synaptic disruption and behavioral deficits (Ting *et al.* 2014). In the present study, we explore alternative splicing as a

potential mechanism for E2F1 regulation and identified two novel splice variants of E2F1 transcripts more prominently observed in the brain. In addition, we did not detect these alternatively spliced transcripts in other tissues, organs or fibroblast cell lines despite ubiquitous expression of the classic full length E2F1 transcript. Furthermore, we characterized their expression pattern as neuroglial cells mature, their responses during pharmacologic-induced NMD inhibition, and during elevated neuronal activity. Taken together, E2F1 is alternatively spliced into brain-specific transcripts that are distinct from the classic E2F1 mRNA.

Materials and Methods

Cell culture and transfection: Primary cortical or hippocampal neuroglial cultures were isolated from the brains of embryonic day 17 Sprague Dawley rats as previously described (Wilcox et al, 1994). Dissociated cells were seeded on poly-L-lysine coated plates and the cultures are maintained in neurobasal media with B27 supplement. Pure neuronal cultures were generated by treating neuroglial cultures with 10 μ m of Ara-C 48 hours after plating and confirmed by the absence of glial marker glial fibrillary acidic protein. Pure astrocytic cultures were generated by first culturing neuroglial cells in DMEM supplemented with fetal bovine serum (FBS) for 7-10 days at which point glial cells constitute approximately 90% of the cells. The cells were manually shaken off and sub-cultured to another culturing flask which is repeated for twice for astrocytic purity prior to RNA collection (Akay *et al.* 2014). Pure oligodendrocyte cultures were prepared as described (Reid *et al.* 2012). Briefly, neuroglial cells were isolated from neonatal rat

brain and cultured in neurobasal supplemented with B27, 10 ng/ml basic fibroblast growth factor, 2 ng/ml platelet-derived growth factor (R&D Systems) and 1 ng/ml neurotrophin-3 (Peprotech). To differentiate cells into oligodendrocytes, cultures were subsequently maintained in differentiation media containing 50% DMEM, 50% Ham's F12 with 50 µg/ml transferrin, 5 µg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 µg/ml insulin, 0.4 µg/ml T4, 0.3% glucose, 2 mM glutamine, 10 ng/ml and biotin (R&D Systems). HEK293T and Rat2 cells were maintained in DMEM supplemented with fetal bovine serum (FBS) while PC-12 cells were in RPMI-1640 supplemented with horse serum and FBS. All cells were cultured at 37°C with 5% CO₂. Rat2 fibroblasts were arrested in S-phase by double thymidine block, M phase by thymidine-nocodazole block, and G₀/G₁ phase by serum starvation as previously described (Whitfield *et al.* 2002). Lipofectamine 2000 was used in transfection experiments in HEK293T or PC12 according to manufacturer protocol [Life Technologies]. Transfection mixture was added to the cells for 2 hours and subsequently replaced with the growth media.

RNA processing: Harvest tissues were incubated in RNAlater reagent [Life Technologies] overnight. Total RNA was extracted from tissues and cells using TRIzol reagent [Life Technologies] and purified using the RNeasy plus mini kit according to manufacturer instructions [Qiagen]. Equal amounts of RNA was reverse transcribed to cDNA using SuperScript II reverse transcriptase with Oligo(dT) primers [Life Technologies]. DNA products were amplified with different target specific primers (Table 1) using the OneTaq DNA polymerase [New England BioLabs] and processed by electrophoresis on a 1% agarose gel.

Quantitative real-time PCR: Equal amounts of cDNA were loaded in triplicates for quantitative real-time PCR (qPCR) using the Fast SYBR green master mix and 7500 Fast real time PCR systems according to manufacturer protocol [Applied Biosystems]. Primers used in these experiments are listed in table 1. The expression levels of different E2F1 transcripts were determined using the delta delta Ct method in which the mean cycle threshold were first normalized to the endogenous control, Tata-box binding protein (TBP) and subsequently to the vehicle within the experiment. All Q-PCR experiments were replicated biologically and independently at minimum of four times.

Antibodies and Reagents: The following antibodies were purchased from the indicated vendors: E2F1 E2F1 KH95 (sc-251) [Santa Cruz]; FLAG tag (#2368) [Cell Signaling]; MAT tag (M6693) [Sigma]. The following chemical reagents were used from the indicated vendors: Protein assay dye (500-0005), PVDF membrane [BioRad], protease inhibitor cocktail [Sigma], PAGERuler plus protein ladder [Thermo], Luminata Forte Western HRP substrate [Millipore], agarose powder [Life Technologies], RNasin RNase inhibitor [Promega], Fast SYBR Green Master Mix [Applied Biosystems]. All HRP conjugated secondary antibodies were obtained from Pierce.

Immunoblotting: Cultured cells were homogenized in ice cold, whole cell lysis buffer containing 50 mM Tris, 120 mM NaCl, 0.5% NP-40, 0.4mM sodium orthovanadate and protease inhibitor cocktail. Protein concentrations were determined using the Bradford method. Equal amounts of proteins (2-10 ug) were loaded for immunoblotting and confirmed by staining the gel with Coomassie. Autographs were scanned and cropped using Adobe Photoshop (Adobe Systems).

Statistical analysis. All data were analyzed by Prism 5.0 software (GraphPad Software) and expressed as mean \pm SEM with values of $p < 0.05$ considered significant. Unless otherwise noted, all asterisks denote statistical significance by comparing the means to that of the vehicle group using one-way ANOVA and Newman-Keuls post-hoc test.

Results

1) Identification of two novel E2F1 mRNA transcripts

To determine if the E2F1 gene is alternatively spliced, we amplified E2F1 mRNA transcripts with primers targeting exon 1 and exon 7 of the E2F1 gene from a cDNA library generated from primary rat cortical cells. As shown in figure 1A, we consistently observed three E2F1 DNA products using this primer set, indicating that E2F1 is indeed spliced into at least three different E2F1 mRNA transcripts in primary rat cortical cells. To identify how the three E2F1 transcripts are spliced, we cloned these DNA products into an expression vector and sequenced each spliced variants. As expected, one of the DNA products was the classic, full length E2F1 transcript carrying exon 1 through 7 with the size of 1299 bp (Figure 1B). Interestingly, the largest DNA product was an E2F1 transcript carrying exon 1 through 7 and retaining the full intron normally found between exons 5 and 6 (intron 5) with the size of 1392 bp. On the other hand, the smallest DNA product was an E2F1 transcript carrying all the exons excluding the full exon 6 with the size of 1082 bp. It is important to note that the three DNA bands shown in figure 1A do not reflect the full sizes of the cloned E2F1 transcripts because the primer pairs used

precluded portions of exon 1 and exon 7 of the E2F1 gene.

To further verify the presence of all three E2F1 transcripts in our primary rat cortical cells, we designed primer sets that would amplify all three transcripts in the same reaction or specifically amplify one transcript, but not the others. Using primers pairs that target exon 4 and exon 7, which are common in all three transcripts, we were able to amplify three DNA bands corresponding to each of the E2F1 transcripts as shown in lane 1 of figure 1C. Each of the three DNA bands were extracted and sequenced to ensure that the amplified products were indeed the three alternative spliced E2F1 transcripts. Using primers that target intron 5 and exon 7, we were able to only amplify the E2F1 transcript retaining intron 5 in lane 2 as indicated by the arrow. The presence of an upper band is inconsistent in PCR reactions and does not reflect any specific gene when the DNA product was extracted and sequenced. Using a primer that spans exon 5&6 and a reverse primer targeting exon 7, we predictably only observed a single band that corresponds to the classic E2F1 transcript (Figure 1D, Lane 2). Using a primer that spans exon 5&7 and a reverse primer targeting exon 7, we observed a single band that corresponds to the E2F1 transcript missing exon 6 (Figure 1D, Lane 3). Together, we demonstrate that E2F1 is spliced into two novel transcripts that either retains intron 5 or excludes exon 6 in primary rat cortical cells.

2) Alternative splicing of E2F1 occurs predominantly in the brain

Given that our cDNA libraries were generated using RNA collected from cultured primary cortical cells, it remains possible that the observed splicing of E2F1 is an inessential artifact of cells cultured *in vitro*. Therefore, we amplified all E2F1 transcripts

using cDNA generated from various tissues as template and with the common primer pairs. As expected, we found that all three E2F1 transcripts are expressed in the adult brain (Figure 2A). More importantly, despite ubiquitous expression of classic E2F1 (E2F1-Classic) across all tissues tested, the E2F1 transcript retaining intron 5 (E2F1+Int5) and the E2F1 transcript missing exon 6 (E2F1 Δ Exon6) are only expressed appreciably in the brain. Although alternatively spliced E2F1 transcripts are expressed abundantly in the brain, E2F1 Δ Exon6 is faintly detectable in the spleen (Fig 2A, lane 5) indicating that alternative E2F1 splicing may not be completely exclusive in the brain. Furthermore, although we can readily detect E2F1+Int5 in embryonic brain (Fig 2A, lane 3), both E2F1+Int5 and E2F1 Δ Exon6 are more readily detectable in adult brain (Fig 2A, lane 1), suggesting that the expression of the alternatively spliced E2F1 transcripts may increase as the brain matures *in vivo*.

Since E2F1+Int5 and E2F1 Δ Exon6 are expressed abundantly in the whole brain, we next determined if the alternative splicing of E2F1 occurs in any specific regions of the brain. When we amplified E2F1 transcripts in the brain using cDNA generated from various brain regions with the common primer pairs, we found that all three E2F1 transcripts were expressed throughout all tested brain regions (Figure 2B). Because the expression of E2F1+Int5 was low in brain regions such as striatum, olfactory bulbs, spinal cord, and cerebellum, we verified their presence using primers specifically amplifying E2F1+Int5 and again found that the E2F1+Int5 is expressed ubiquitously in all tested regions. Taken together, the expression of alternatively spliced E2F1 products shows specificity for the brain compared with other organs. Further, the expression of alternatively spliced E2F1 varies across different brain regions and brain development.

3) The alternative spliced E2F1 transcripts are expressed in neuroglial cells but not fibroblasts

Since the alternatively spliced E2F1 transcripts are highly expressed throughout the brain, we characterized their expression in the neural and glial populations cultured *in vitro*. When we amplified all E2F1 transcripts using cDNA generated from astrocytes, neurons, oligodendrocyte precursor cells, oligodendrocytes, and mixed neuroglial cultures with the common primer pair, we were able to detect the presence of E2F1 Δ Exon6 in all tested neuroglial cell types (Figure 3A). Because E2F1+Int5 migrates at a similar size as E2F1-Classic, we also used primer pair specific for E2F1+Int5 and were able to detect presence of this variant in all neuroglial cell types. Interestingly, the expression of both E2F1+Int5 and E2F1 Δ Exon6 was more robust in the cultures that contained neurons despite similar E2F1-Classic expression suggesting that the alternative splicing of E2F1 may be more prominent in neurons.

E2F1 expression is highly regulated depending on the stage of the cell cycle progression in dividing cells (DeGregori & Johnson 2006, Iaquinta & Lees 2007). However, since post-mitotic neurons are tightly maintained in a quiescent phase, we hypothesized that the alternative splicing of E2F1 may be restricted to the quiescent phase of the cell cycle and greatly limited in other phases (Herrup & Yang 2007). Because we could not directly manipulate cell cycle in post-mitotic neurons, we utilized Rat2 fibroblast cell lines and arrested these cells in various stages of the cell cycle and determined the expression of the alternatively spliced E2F1 transcripts. Contrary to what we predicted, we observed that regardless of the cell cycle stage that the fibroblasts were

arrested in, E2F1+Int5 and E2F1ΔExon6 were not detectable, despite clear E2F1-Classic expression (Figure 3B). As a control, we demonstrate that E2F1+Int5 and E2F1ΔExon6 were readily detectable in the cortical neurons using the same reaction parameters. Furthermore, PCR reactions using genomic DNA as template yielded a distinct migratory pattern of unspliced E2F1 gene confirming that the presence of E2F1+Int5 and E2F1ΔExon6 were not a result of amplification of genomic DNA. Taken together, these findings are consistent with our previous observation that alternative splicing of E2F1 is prominent in neuroglial cells residing in the brain, but not in the fibroblasts.

4) Characterization of alternatively spliced E2F1 transcripts as neuroglial cells mature *in vitro*

Alternative splicing is dependent upon the presence of splicing regulatory proteins whose expression is context dependent, such as through cell differentiation or tissue development (Zheng & Black 2013). Previously, we have shown that all three E2F1 transcripts are present as cultured neuroglial cells mature *in vitro*. However, we were unable to quantitatively describe the levels of different E2F1 transcript expression in these experiments with traditional RT-PCR. Therefore, we designed and verified additional primers for real time qPCR aimed at specifically amplifying each E2F1 transcript (Supplemental Figure 1). We subsequently analyzed the expression of each E2F1 transcript in primary cortical and hippocampal neuroglial cells as they mature *in vitro*. In the cortical neuroglial cells, all three E2F1 transcripts follow similar trends, such that each transcript expression increases modestly during the second week in culture but declines significantly during the third week in culture (Figure 4A). In contrast, the

profile of each E2F1 transcripts expression was more distinct in the hippocampal neuroglial cells (Figure 4B). E2F1-Classic exhibited a trend of gradual modest decline in expression, whereas E2F1+Int5 exhibited a more significant and dramatic decline in expression during the second and third week in culture. Furthermore, the expression of E2F1 transcripts is lower in mature cells compared to cells collected at DIV 0. In contrast, the expression of E2F1 Δ Exon6 is significantly elevated in mature cells and then steadily declines as the hippocampal cells age *in vitro*. Taken together, the expression profile of each E2F1 transcripts are slightly different depending on the source of neuroglial neurons. Specifically in hippocampal neuroglial cells, each E2F1 transcripts display a slightly different expression pattern suggesting that they may be regulated differently in these cell types.

5) Alternatively spliced E2F1 transcripts exhibit distinct expression patterns in response to different protein translational inhibitors

Because of the distinct expression pattern of the alternatively spliced E2F1 transcripts as the hippocampal cells mature *in vitro*, we next investigated their potential regulatory mechanism. If translated, both of alternatively spliced E2F1 transcripts would introduce premature termination codons; and therefore, the proteins predicted from the direct nucleotide sequence of E2F1+Int5 and E2F1 Δ Exon6 are similarly sized truncated E2F1 missing its C-terminal domains. Since many mRNA transcripts that contain premature termination codons are targeted for destruction by NMD, we determined whether the different E2F1 transcripts are elevated following translational inhibition-induced NMD inhibition (Bidou *et al.* 2012, Kervestin & Jacobson 2012).

Global protein translation inhibitors have been previously used to indirectly inhibit NMD pathway *in vitro* (Noensie & Dietz 2001, Dang *et al.* 2009). Therefore, we treated mature primary hippocampal cells with either cycloheximide or anisomycin and examined the levels of each alternatively spliced transcript by qPCR. Interestingly, the expressions of all E2F1 transcripts dramatically reduced over time in response to cycloheximide induced NMD inhibition (Figure 2). Similarly, the expression of E2F1-Classic and E2F1 Δ Exon6 also reduce over time in response to anisomycin induced NMD inhibition (Figure 2A, C). In contrast however, E2F1+Int5 expression is distinctively unchanged in response to anisomycin (Figure 2B). Importantly, the expression of the endogenous control, TBP, is not disrupted by the translation inhibition indicating that the changes in expression of E2F1 transcripts are not global to all mRNAs. Taken together, the alternatively spliced E2F1 transcripts respond differently to two distinct translation inhibitors suggesting that their regulation may be distinct from each other. Further, because E2F1 transcripts for E2F1-classic and E2F1 Δ Exon6 are degraded following NMD inhibition, these findings suggest that these transcripts are not subject to NMD degradation.

6) All E2F1 transcripts are reduced in response to prolonged ER-stress induced translation inhibition

Due to the distinctive response of the E2F1+Int5 and E2F1 Δ Exon6 following anisomycin, we sought to induce global translation inhibition through a more physiologic mechanism. Prolonged exposure to thapsigargin, an inhibitor of endoplasmic reticulum (ER) calcium level regulation, has been previously shown to potently induce the activation of canonical ER stress pathway leading to global protein translation inhibition

(Booth & Koch 1989, Wek & Cavener 2007). Therefore, we treated the hippocampal cells with thapsigargin and examined the expression of different E2F1 transcripts. Similar to what we observed previously when protein translation was inhibited by cycloheximide, the expressions of all three E2F1 transcripts were reduced, specifically after 8 hours and 24 hours of exposure to thapsigargin (Figure 6). Interestingly, in contrast to anisomycin induced translation inhibition, the expression of E2F1+Int5 was also reduced in response to prolonged thapsigargin treatment indicating that E2F1+Int5 was indeed reduce in expression following translation inhibition. Lastly, the lack of change in the expressions of the E2F1 transcripts at the early timepoints of thapsigargin treatment suggest that the changes observed at the later time points did not result from acute calcium dysregulation, but rather global translational inhibition following prolonged ER stress induction. Taken together, our results indicate that the alternatively spliced E2F1 transcripts are not targeted by the NMD surveillance as the inhibition of the NMD pathway from protein translational suppression leads to significant reduction of all three E2F1 transcripts.

7) E2F1 transcript retaining intron 5 is selectively upregulated with increased neuronal activity

Recently, it has been shown that the splicing machinery and the splicing of target mRNA is highly responsive to intracellular calcium levels, particularly in neurons whose electrical activity is critical for their functions (Li *et al.* 2007, Sharma & Lou 2011) . For example, transcripts containing exon 21 of the NMDAR1 are selectively repressed in response to KCl-induced neuronal activity in both differentiated P19 cells and primary

cortical cells (Lee *et al.* 2007, An & Grabowski 2007). Given that the alternatively spliced E2F1 transcripts are predominantly expressed in the brain, particularly in highly excitable neurons, we hypothesized that these transcripts would similarly change expression in response to KCl-induced neuronal activity. Therefore we examined the expression of the three E2F1 transcripts following KCl treatment in the primary rat cortical cells. Neither E2F1 Δ Exon6 nor E2F1-classic exhibited statistically significant changes during a 4 hours exposure to elevated extracellular potassium level (Figure 7C), although E2F1 classic showed a non-statically significant trend toward an increase. Interestingly however, the expression of E2F1+Int5 is significantly upregulated after 4 hours of treatment with KCl by approximately two fold. These data suggests that the expression of the intron retaining E2F1 transcript is selectively responsive to neuronal activity, which is consistent with the observation that it is absent in other tissues.

Discussions

Tissue-specific or cell-type specific alternative splicing of a specific gene is a common mechanism in evolution which yields similar proteins with diverse functions from a very limited gene pool (Nilsen & Graveley 2010). In the present study, we have identified two novel E2F1 transcripts: E2F1+Int5 which retains intron 5, as well as the E2F1 Δ Exon6, which excludes exon 6. Similar to the classic E2F1 transcript which is joined by seven exons, the alternatively spliced E2F1 transcripts are expressed ubiquitously throughout the brain. On the other hand, neither E2F1+Int5 and E2F1 Δ Exon6 are undetectable in other organs despite the expression of E2F1-Classic

suggesting that they may be functionally significant in the brain. Moreover, these E2F1 transcripts are present in various CNS cell types, specifically the neurons, astrocytes and oligodendrocytes. However, the presence of alternative spliced E2F1 transcripts are unlikely to be indirect byproducts of a quiescent cell cycle machinery in these terminally differentiated cell types since they are absent in the Rat2 fibroblast cell lines regardless of their stage of the cell cycle. As primary cortical neuroglial cells mature *in vitro*, all three E2F1 transcripts exhibit decreased expression during the third week of culture. In contrast, the three E2F1 transcripts exhibit diverse expression pattern during the maturation process of the hippocampal cells. Interestingly, pharmacologic manipulations that lead to protein translation suppression and NMD inhibition significantly reduced the levels of all three E2F1 transcripts similarly; however, treatment of hippocampal cells with anisomycin did not affect expression of E2F1+Int5, despite downregulation of, E2F1-Classic and E2F1 Δ Exon6. Lastly, elevation of neuronal activity by increasing the extracellular concentration of potassium chloride selectively increased the expression of E2F1+Int5 in the cortical cells, further suggesting that the alternatively spliced E2F1 transcripts may have distinct functional roles in the brain.

E2F1 has been classically described as a transcription factor that regulating cell cycle progression and apoptosis (DeGregori & Johnson 2006, Biswas & Johnson 2012). However, E2F1 was recently shown to possess novel protein functions in the CNS as mice lacking functional E2F1, which exhibit age-dependent behavioral deficits and synaptic disruptions (Ting et al. 2014). Further, E2F1 exhibits different subcellular distribution in neurons in contrast to its expected nuclear localization as a transcriptional regulator in the cycling cells (Wang et al. 2010, Strachan et al. 2005b, Ting et al. 2014).

The unique E2F1 localization in neuron may be in part due to its alternative spliced transcripts. For instance, targeted distribution of some mRNAs to the synapse by RNA binding proteins (RBPs) is necessary for the immediate local de novo protein synthesis at the synapses during neuronal stimulation (Kiebler *et al.* 2013). These targeted binding by the RBPs are often triggered by the unique cis-acting element within the alternatively spliced transcripts (Buckley *et al.* 2013). The presence of alternatively spliced E2F1 transcript may harbor a signal for targeted distribution of these mRNA to specific compartments to be locally translated, particularly the synapses. In regards to the effect of alternative splicing on protein function, differentially spliced transcripts can also lead to the translation of different protein products with modified functions. For example, context-dependent splicing of the *KCNMA1* can yield the similar ion channel receptors proteins with functional and physiologic differences (Navaratnam *et al.* 1997, Rosenblatt *et al.* 1997). Likewise, alternative splicing of the E2F1 gene in the brain may create diversity in terms of its protein functions as well as the subcellular localization of its proteins.

The intron-retaining E2F1 transcript identified in this study may contribute in determining E2F1 protein localization. Recent emerging evidence has shown that intron retention serves as a critical post-transcriptional regulatory step that contributes to the targeting of RNA transcripts to specific subcellular compartment which subsequently undergo further RNA processing within that compartment (Buckley *et al.* 2013). Intron retaining transcripts are found to be abundantly enriched in dendritic processes of the hippocampal and cortical neurons *in vitro* (Khaladkar *et al.* 2013). One common feature of these cytoplasmic intron retaining transcripts (CIRTs) is that they often carry short

interspersed repetitive elements (SINE) derived from the BC1 RNA. The BC1 RNA is found predominantly in the neuronal cytoplasm, particularly in the neuronal processes. Further, the structure of the BC1 5' domain is necessary and sufficient for RNA dendritic targeting (Muslimov *et al.* 1997, Muslimov *et al.* 2006). Many CIRTs present in the hippocampal dendrites carry the repetitive ID element, a class of SINE, that shares high homology to the 5' domain of the BC1 RNA. Furthermore, over-expression of exogenous transcripts fused with the ID element disrupts the endogenous distribution of CIRT as well as the proteins they encode, suggesting that the ID element within the retained introns are functionally significant in targeting of their RNA transcripts and subsequent translated proteins (Buckley *et al.* 2011). Interestingly, we also identified an ID element embedded in intron 1 of the E2F1 gene, though we were unable to detect the presence of any E2F1 transcript retaining intron 1 (data not shown). However, the presence of ID element in intron 1 may alter the splicing of the E2F1 gene and therefore lead to the expression of the E2F1+Int5 and E2F1 Δ Exon6. Alternatively, it remains possible that the ID element retaining E2F1 transcript is only abundant in the hippocampal dendrites and therefore below limit of detection in the whole cell RNA isolations used in our current studies. Nevertheless, our identification of an E2F1 intron retaining transcript may provide a potential mechanism for its protein distribution and the observed effects on synaptic integrity and behavior in the transgenic mice with dysfunctional E2F1 (Ting *et al.* 2014). Intron 5 of the E2F1+Int5 may similarly carry a unique code such that the cells target this RNA to the synapses where it is subsequently translated, thus explaining localization of E2F1 protein in the synaptic fractions (Ting *et al.* 2014).

Intron retention in alternatively spliced transcripts can also affect the expression and distribution of the protein that is classically produced from the gene. For example, knockdown of the intron retaining high conductance potassium channels transcripts reduced dendritic spine localization of the channels and altered the excitability of these cells (Bell *et al.* 2008). Furthermore, intron retention is highly associated with the other alternatively spliced transcripts of these channels suggesting that these splicing events may be co-regulated (Bell *et al.* 2010). Therefore, the intron retained in E2F1+Int5 may contain regulatory element that may further influence the distribution of E2F1 proteins or the splicing of the E2F1 Δ Exon6, though future knockdown experiments will be necessary to confirm these possibilities. In regards to the subcellular localization of these alternatively spliced transcripts, efforts are currently underway in determining whether these E2F1 transcripts are enriched in the neuronal processes.

Aside from modifying RNA subcellular localization, alternative splicing in neurons can also directly impact protein functions by producing similar proteins with slight modifications from the same gene (Zheng & Black 2013). For example, cell cycle related protein telomere repeat-binding factor 2 (TRF2) is alternatively spliced such that part of its exon 7 is deleted from its mRNA message. This switch of splicing from its normal length transcript is only present in fully differentiated neurons indicating that the splicing event is under strict developmental regulation. Furthermore, this exon skipping transcript is translated into a truncated TRF2 protein that has a different subcellular localization and interacting partner than the classic full length TRF2 protein (Zhang *et al.* 2011). In a similar fashion, E2F1+Int5 and E2F1 Δ Exon6 may produce truncated E2F1 proteins that are functionally distinct from full length E2F1 as a result of shifting stop

codons. Our data that show dramatic reduction in the expression of E2F1 transcripts following NMD inhibition indicates that the transcripts with premature stop codons as in E2F1+Int5 and E2F1 Δ Exon6 are not simply degraded as nonsense mRNA. Consistent with this interpretation, overexpression of vectors containing E2F1+Int5 and E2F1 Δ Exon6 transcripts produced similar sized truncated E2F1 proteins missing its C-terminal domains (Supplementary Figure 2). In conclusion, we have identified two novel brain-specific E2F1 transcripts that are present in neurons, astrocytes, and oligodendrocytes. As evidenced by their distinct expression pattern as primary neuroglial cells mature *in vitro*, and in response to neuronal activity or anisomycin, they are physiologically and functionally significant and may contribute to the non-classic role and subcellular localization of E2F1 in the brain.

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Abbreviations

CNS, central nervous system; NPAS3, neuronal per-arnt-sim domain protein 3; NMD, nonsense-mediated mRNA decay; PTBP, polypyrimidine tract binding proteins; PSD-95, postsynaptic density-95; TDP-43, TAR DNA-binding protein-43; FBS, fetal bovine serum; qPCR, quantitative real-time PCR; TBP, tata-box binding protein; HRP, horseradish peroxidase; RBP, RNA-binding proteins

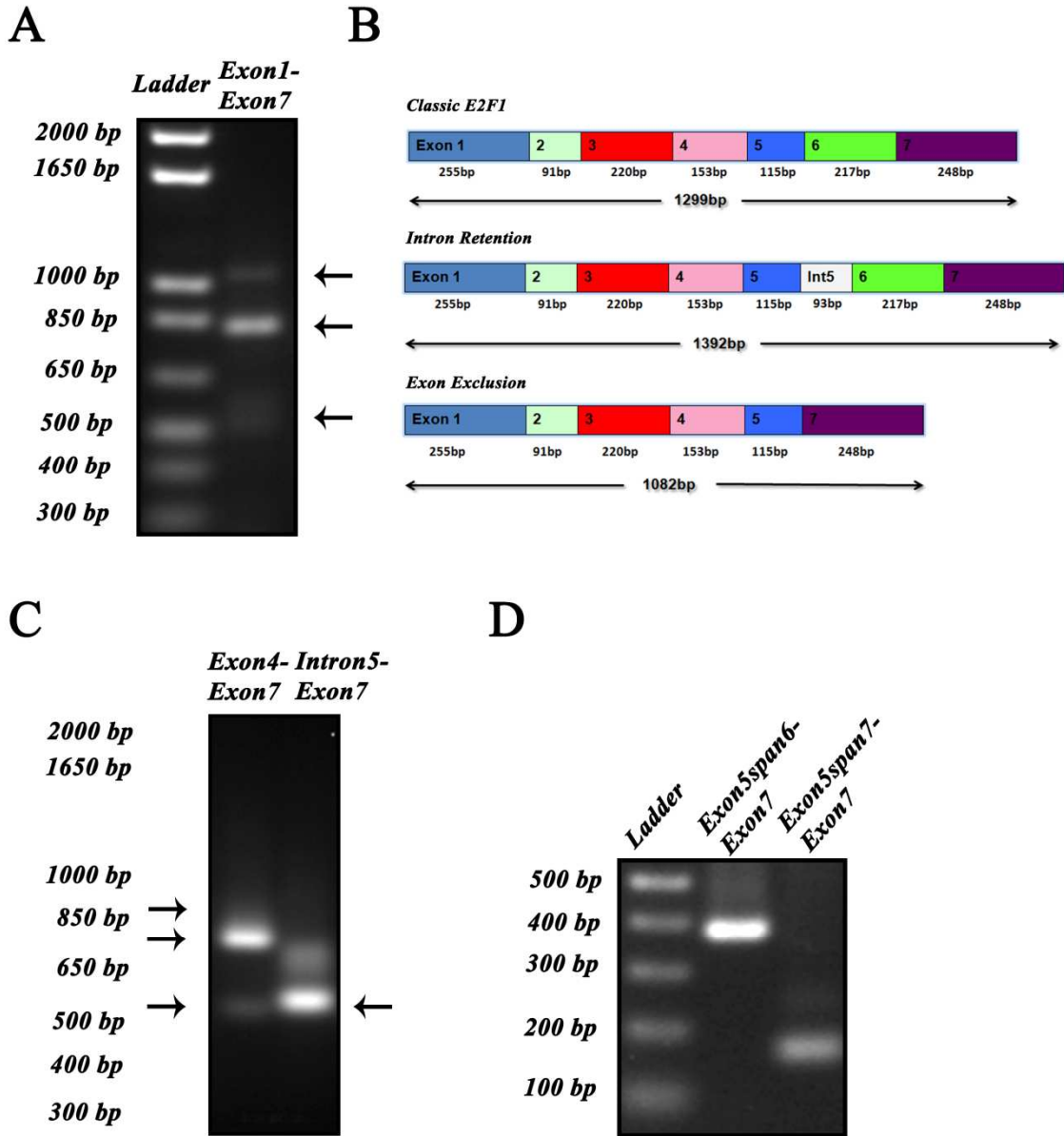


Figure 1. Identification of the alternatively spliced E2F1 transcripts in primary rat neuroglial cultures. A) Three distinct DNA products were amplified using primers targeted towards the exon 1 and exon 7 of the E2F1 gene from cDNA generated from neuroglial cells. B) Schematic of the three E2F1 mRNA transcripts identified in the neuroglial cells with their exact sizes. C) Three DNA products representing the three E2F1 transcripts amplified using primers common to all three E2F1 transcripts in lane 1.

The bright band indicated by the single arrow reflects the E2F1+Int5 transcript amplified using a primer targeted specifically inside intron 5 (lane 2). D) PCR using primers spanning the exon 5:exon 6 border amplified E2F1+Classic in lane 2 while primers spanning the exon 5: exon 7 border amplified E2F1 Δ Exon6 in lane 3.

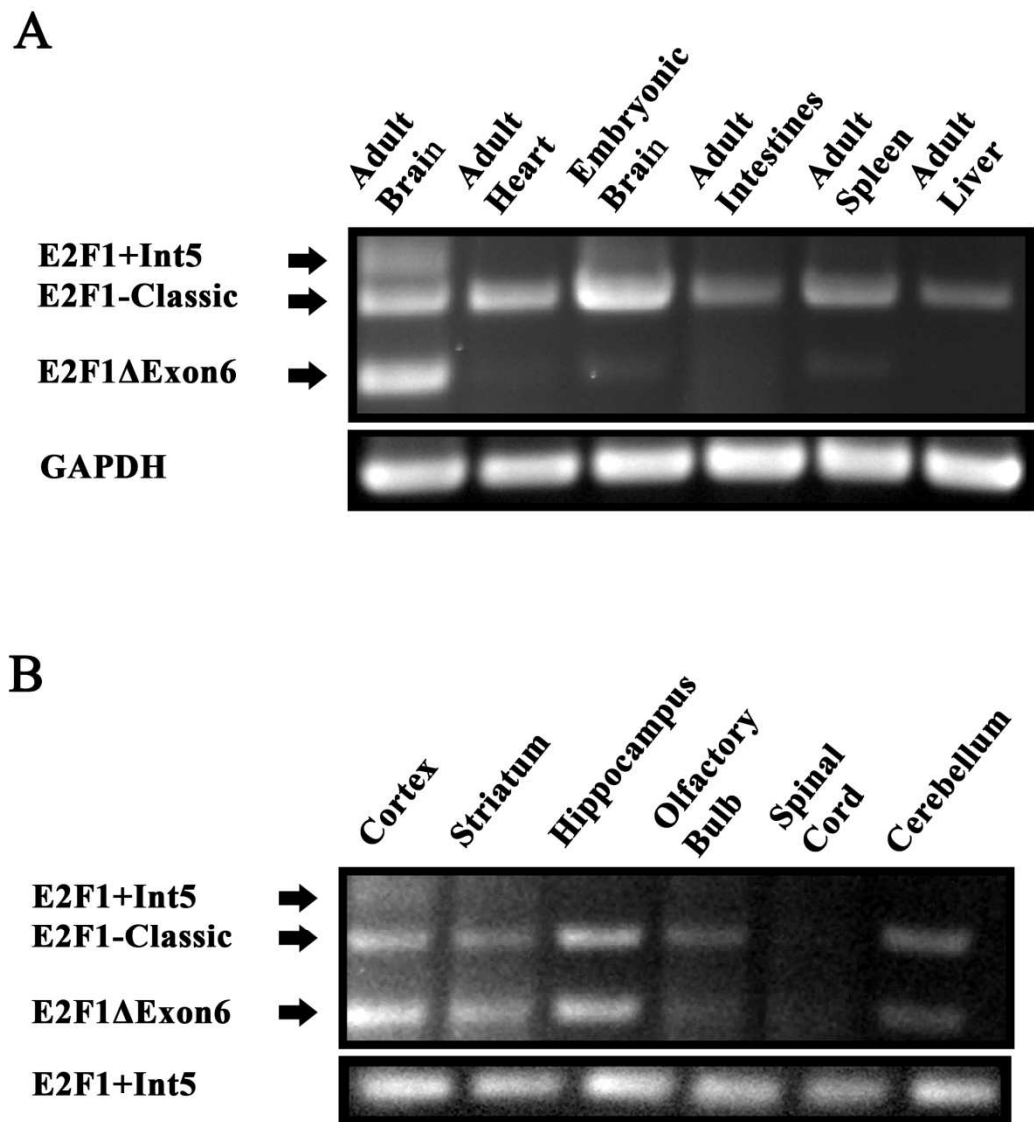
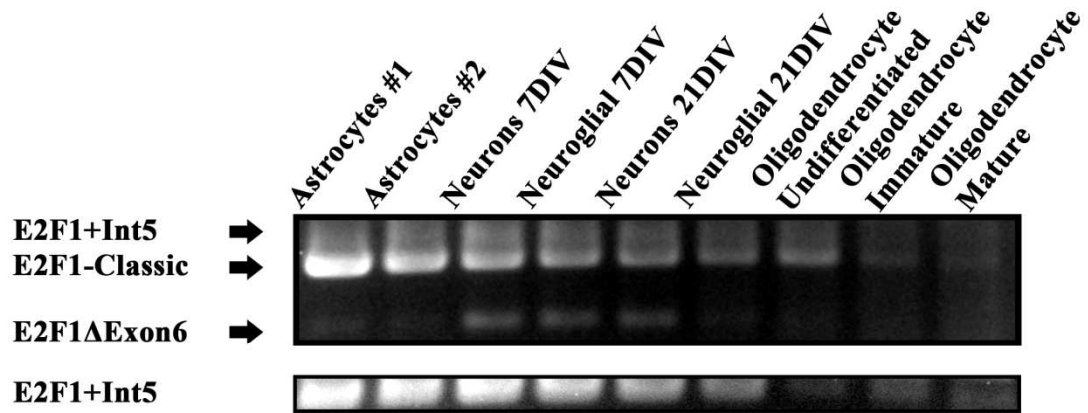


Figure 2. Alternatively spliced E2F1 transcripts are expressed predominantly in the brain. A) Three distinct E2F1 DNA products were amplified using common primers in the adult and embryonic brain, lane 1 and 3, respectively, while only E2F1-Classic was detected in the other organs. DNA product of GAPDH gene was shown as a quality

loading control. B) Alternatively spliced E2F1 transcripts were detectable in all brain regions tested including cortex, striatum, hippocampus, olfactory bulb, spinal cord, and cerebellum. Primers specifically targeting E2F1+Int5 were used to verify the presence of this transcript in all brain regions.

A



B

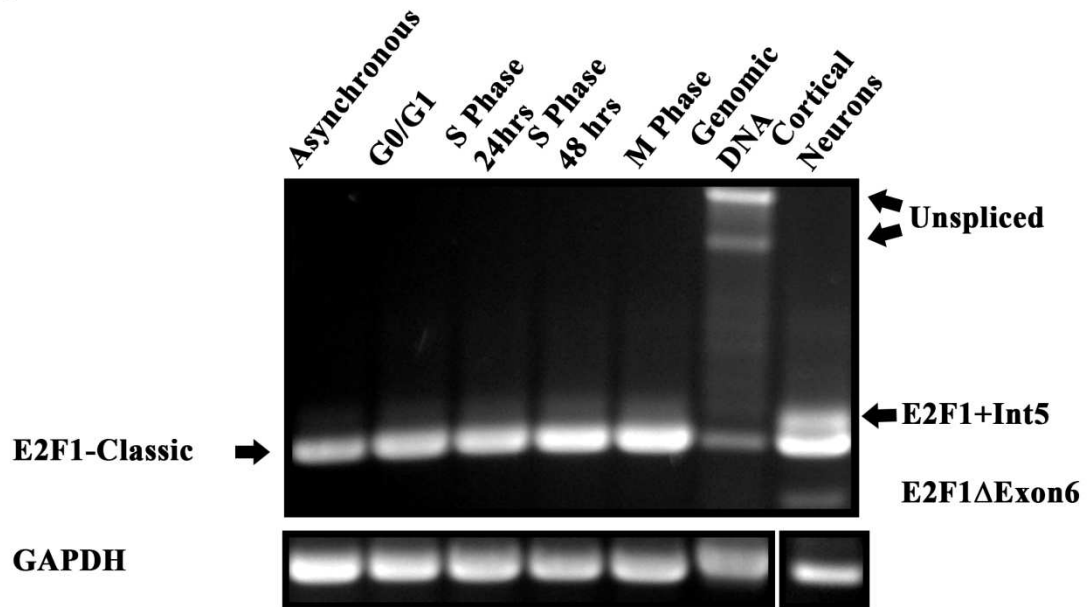


Figure 3. Alternatively spliced E2F1 transcripts are detectable in neural cells but not in fibroblasts. A) Three E2F1 DNA products were amplified using common primers in pure astrocytes, pure neurons, mixed neural cells, precursor, immature, and mature oligodendrocytes. Primers specifically targeting E2F1+Int5 were used to verify the presence of this transcript in all tested neural cell types. B) Only E2F1-Classic was

detectable when Rat2 fibroblast cells were arrested in G₀/G₁ phase, S phase, and M phase. Unspliced E2F1 DNA products from PCR reaction using genomic DNA displayed distinct migratory pattern than the three amplified E2F1 transcript expressed in the cortical neurons.

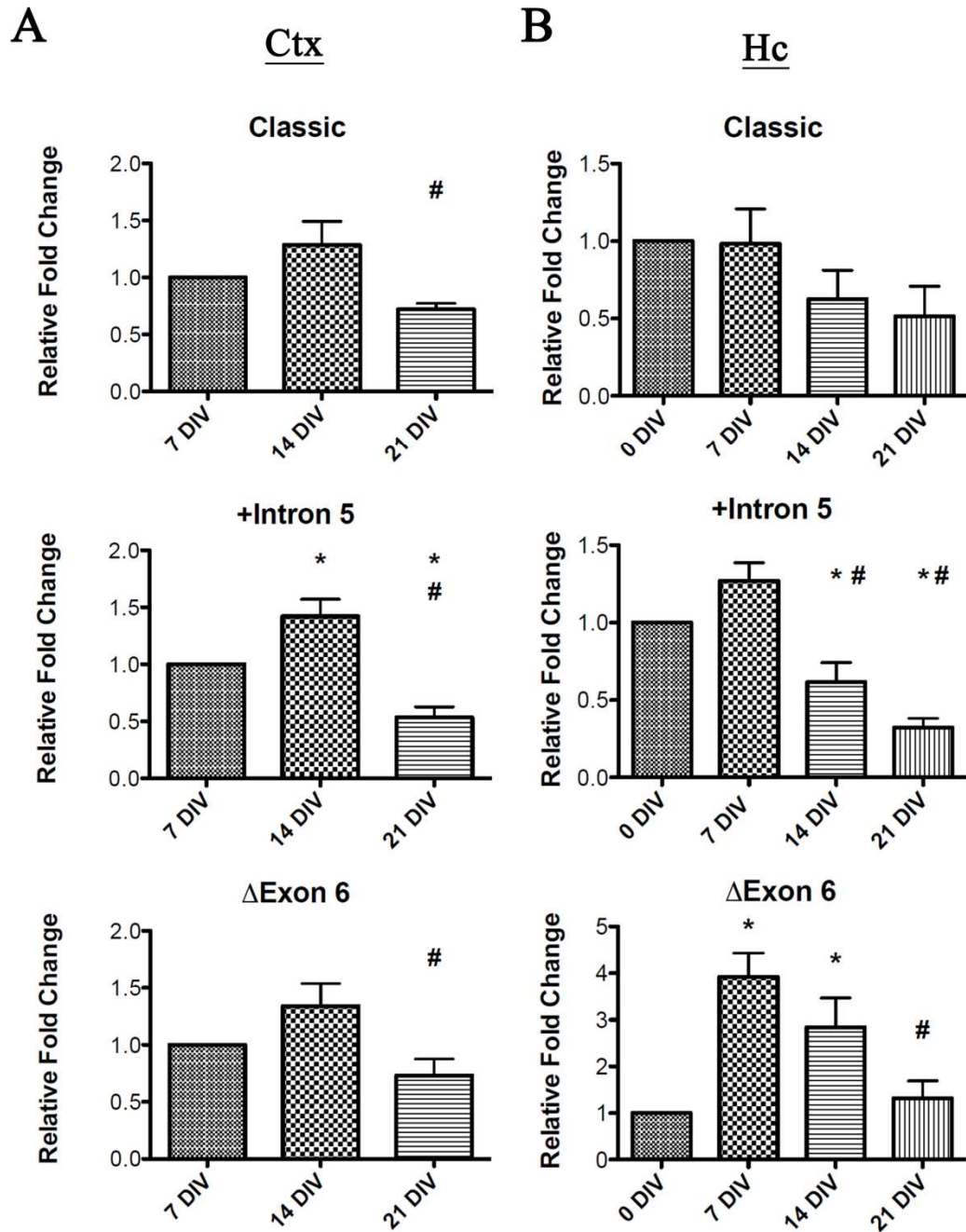


Figure 4. Characterization of the expression profiles of the three E2F1 transcripts as primary neuroglial cells mature *in vitro*. A) Expression profiles of E2F1-Classic, E2F1+Int5, and E2F1ΔExon6 in primary cortical neuroglial cells. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to 7DIV, # denotes $p < .05$ compared to 14 DIV). B) Expression profiles of E2F1-Classic, E2F1+Int5, and

E2F1 Δ Exon6 in primary hippocampal neuroglial cells. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to 0 DIV, # denotes $p < .05$ compared to 7 DIV).

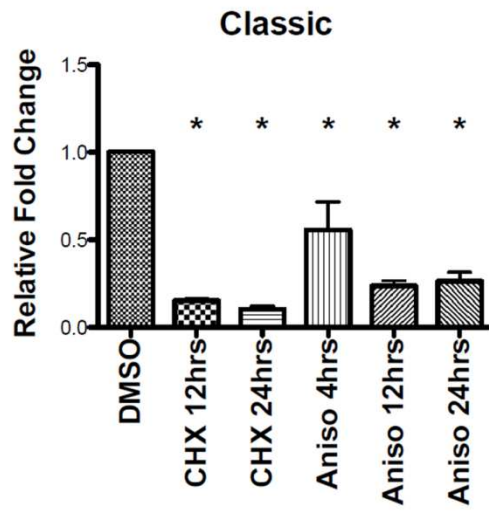
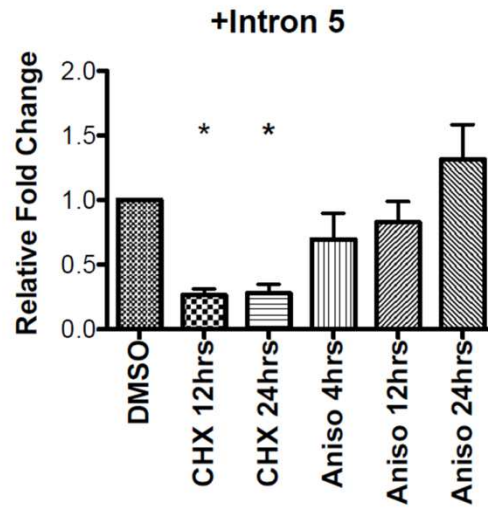
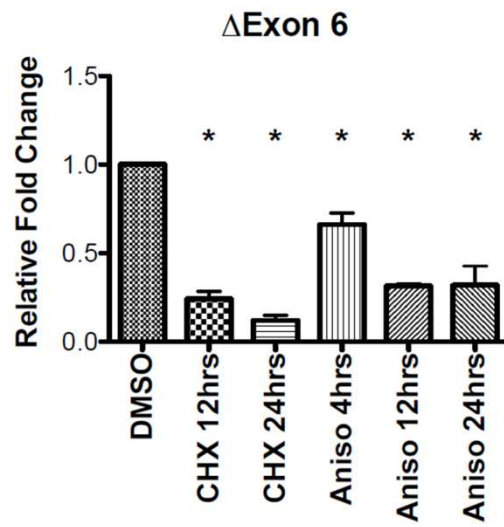
A**B****C**

Figure 5. Expression of alternatively spliced E2F1 transcripts following global protein translation inhibition. Expression of E2F1-Classic (A), E2F1+Int5 (B), and E2F1 Δ Exon6 (C) in 14 DIV hippocampal neuroglial cultures following 5 μ M cycloheximide or 25 μ M anisomycin treatment. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to DMSO).

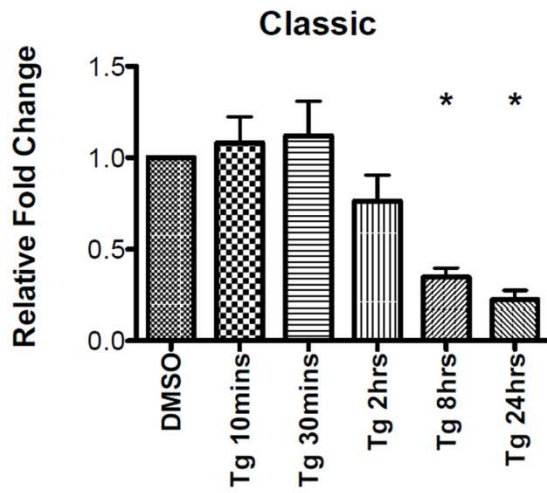
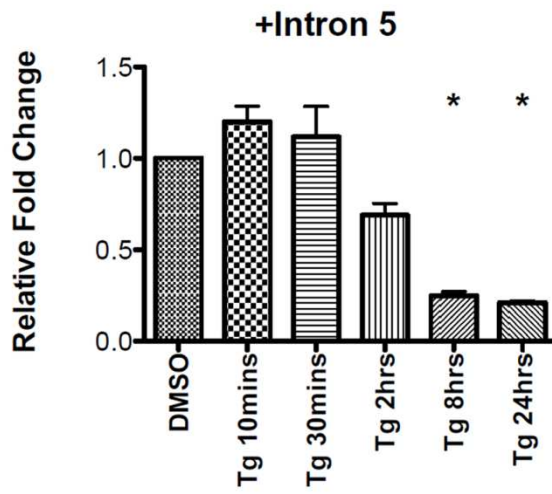
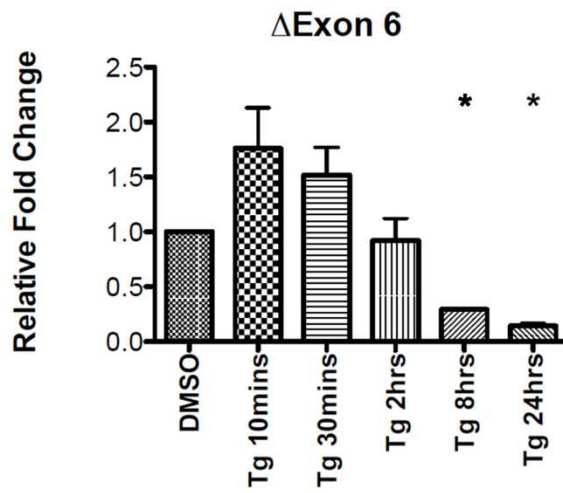
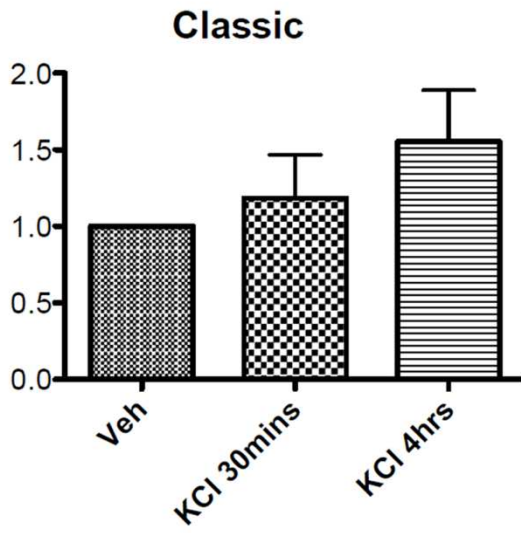
A**B****C**

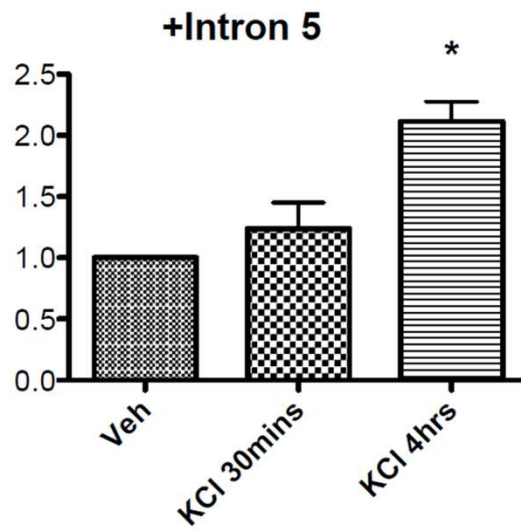
Figure 6. Expression of alternatively spliced E2F1 transcripts in response to thapsigargin induced ER stress. Expression of E2F1-Classic (A), E2F1+Int5 (B), and E2F1 Δ Exon6 (C) in 14 DIV hippocampal neuroglial cultures following 1 μ M thapsigargin treatment. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to DMSO).

A

Relative Fold Change

**B**

Relative Fold Change

**C**

Relative Fold Change

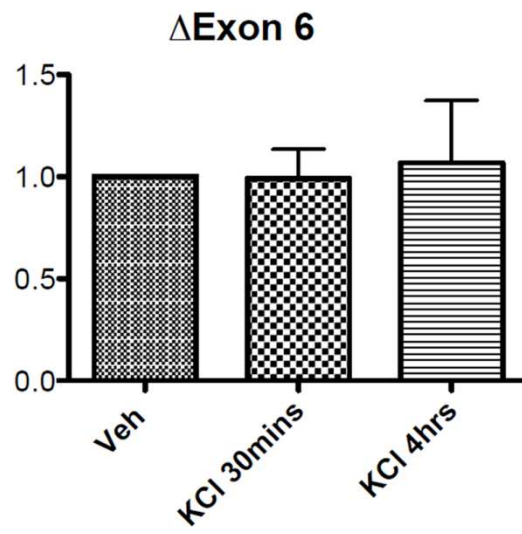


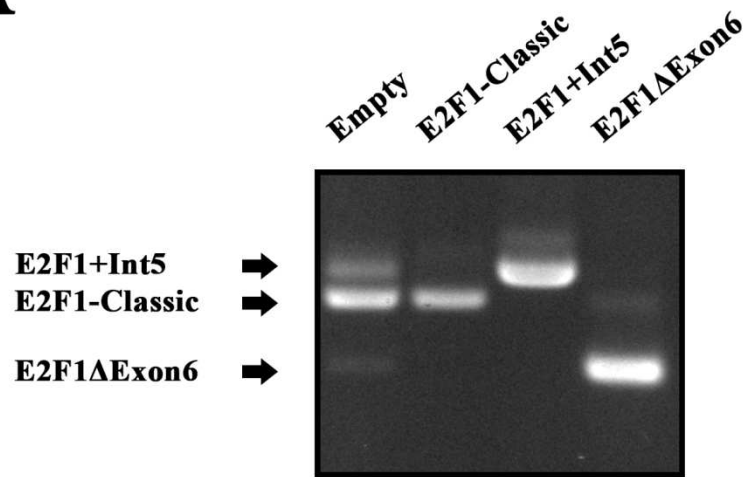
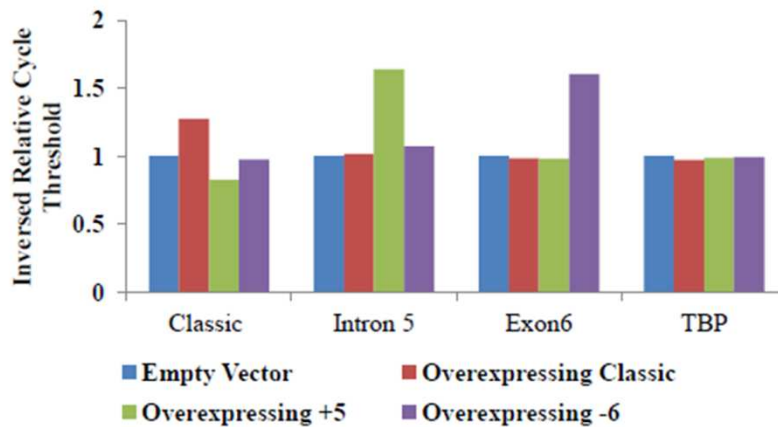
Figure 7. Expression of E2F1+Int5 is selectively upregulated in response to KCl induced neuronal activity. Expression of E2F1-Classic (A), E2F1+Int5 (B), and E2F1 Δ Exon6 (C) in 14 DIV cortical neuroglial cultures following 20mM KCl treatment. All data are represented as mean \pm SEM (* denotes $p < .05$ compared to Veh).

Table 1. List of Primers Used

Primer	Sequence 5' – 3'	Target	Region
GAPDH forward	GGCTCATGACCACAGTCCAT	Glyceraldehyde 3-phosphate dehydrogenase	
GAPDH reverse	CCCCTCCTGTTGTTATGGGG	Glyceraldehyde 3-phosphate dehydrogenase	
E2F1 Exon 1 F	CTCGAGATCCTGACGTGCTGCT	All E2F1	Exon 1
E2F1 Exon 4 F	GATCGGTCAGCGGCTTGAAGGC	All E2F1	Exon 4
E2F1 Exon 7 R	GCCTCATGGGGTGGGGAAAGGC	All E2F1	Exon 7
E2F1-classic F	ATTCTGCAGAGACATTCAGAT	E2F1 Classic	Span Exon 5&6
E2F1-intron5 F	CTGGGCTGGTGGTCACCTAGGC	E2F1+Int5	Intron 5
E2F1-Intron5 R	GCCTTCAAGCCGCTGACCGATC	E2F1+Int5	Intron 5
E2F1-exon6 F	ATTCTGCAGAGAAGCTGTATTG	E2F1 Δ Exon6	Span Exon 5&7
TBP forward	GCTCGTTTTGTCCCCACATC	TATA-Box binding protein	
TBP reverse	TTCACATCACAGCTCCCCAC	TATA-Box binding protein	
E2F1 Common	TGTGACCTGCCAAGATCTCC	All E2F1	Exon 5

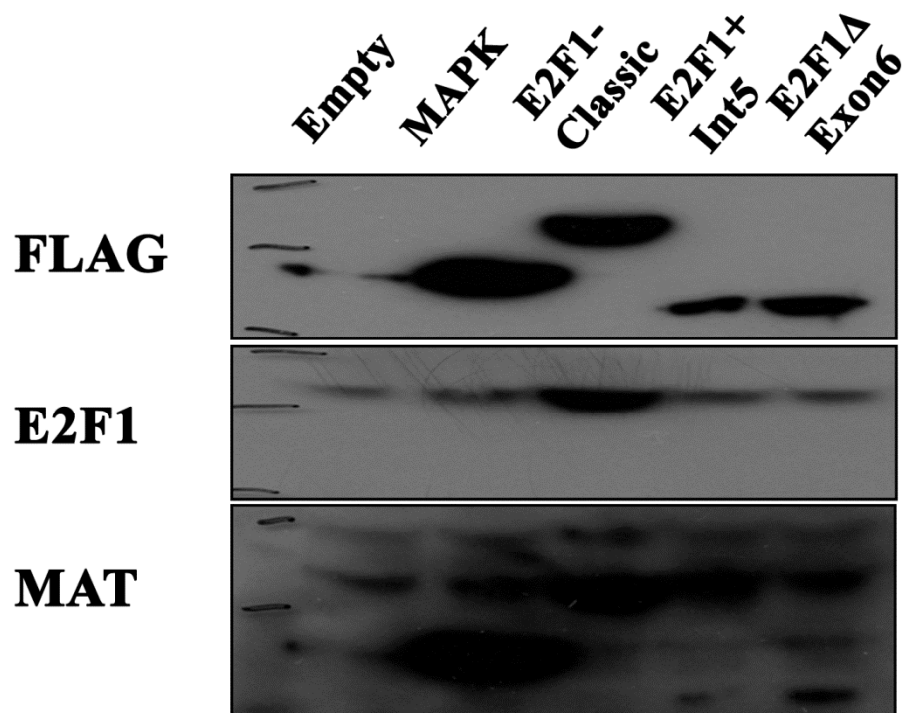
E2F1-classic R2	GATCTGAAATGTCTCTGCAG	E2F1 Classic	Span Exon 5&6
E2F1-int5 R2	CTCTGTCCTGGTCAGGCTTC	E2F1+Int5	Intron 5
E2F1-exon6 R2	GCAATACAGCTTCTCTGCAG	E2F1 Δ Exon6	Span Exon 5&7

Table 1. Table of primers used in this study, their corresponding target transcript, and the region in which they were designed to anneal.

A**B**

Supplemental Figure 1. Verification of the qPCR primers. A) PC12 cells were transfected with either an empty vector, expression vector containing E2F1-Classic, E2F1+Int5, or E2F1ΔExon6. All three E2F1 transcripts were amplified using the common primers. In conditions where the cells were overexpressing vector containing a given E2F1 transcript, the signal for that transcript is selectively intensified

demonstrating that each vector indeed contained that specific E2F1 transcript. B) Expressions of each E2F1 transcript were assessed using their respective qPCR primers. In condition where cells were overexpressing a given E2F1 transcript vector, the cycle threshold for that transcript is selectively reduced demonstrating that the qPCR primers used indeed only anneal to their designated targets.



Supplemental Figure 2. Immunoblot of protein products of vectors containing E2F1-Classic, E2F1+Int5, or E2F1ΔExon6. HEK293T cells were transfected with vectors containing E2F1-Classic, E2F1+Int5, or E2F1ΔExon6 transcripts, and MAPK as positive control. Immunoblots using N-terminal tag FLAG antibody, E2F1 C-terminal epitope antibody, and C-terminal tag MAT antibody reveal that alternatively spliced E2F1 transcript produce similarly sized truncated E2F1 protein missing its C-terminus. Endogenous E2F1 is also detected when E2F1 antibodies were used. Interestingly, despite MAT tag cloned downstream of the epitope recognized by the E2F1 antibody, it is still expressed by the truncated E2F1.

CHAPTER 6: Persistent expression of residual E2F1 mRNA and immunoreactive protein in the E2F1 knockout mouse model

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Abstract

E2F1 is a transcription factor initially characterized as a transcriptional regulator of G1 to S phase progression in dividing cells. In an E2F1 knockout model, the E2F1 gene was disrupted by replacing exons 3 and 4 with a neomycin resistance gene in the reverse orientation resulting in a transgenic mouse lacking E2F1 permitting the study of E2F1 function *in vivo*. Studies utilizing this mouse model demonstrate that E2F1 activity is essential for apoptosis, development, and cell differentiation. While knockout mouse models have been commonly used in elucidating the role of the knocked out gene *in vivo*, studies have shown that residual mRNA species persist, and in some cases, are subsequently translated into mutant protein. In the present study, we provide evidence to suggest that the E2f1^{tm1} mice that have been previously used as a knockout model for E2F1 indeed possess residual mRNA messages and mutant proteins detectable by various E2F1 antibodies.

Keywords: E2F, mouse model, knockout, residual mRNA, mutation

Introduction

E2F1 is a highly conserved cell-cycle related transcription factor regulating genes that are required for the transition from G1 to S phase in dividing cells. It is one of 8 identified E2F family members critical for development of various tissues (McClellan & Slack 2007). Although E2Fs have highly homologous consensus DNA binding sequence, their functions and interacting partners are not entirely overlapping (Blais & Dynlacht 2004). Specifically, only E2F1 has the capacity to regulate cell death via induction of the p53-dependent and p53-independent apoptosis as well as transcription-dependent and, transcription-independent mechanisms (DeGregori & Johnson 2006). Furthermore, E2F1 can regulate other transcriptional target such as microRNAs and the perturbation of this activity has been implicated in cancer development (Petrocca et al. 2008).

Two transgenic mice designed to eliminate E2F1 gene expression were reported in 1996. Both models were designed to delete the genomic region that codes for the E2F1 DNA binding domain. Investigation of the phenotype observed in these animals revealed that E2F1 possesses strong apoptotic inducing activity, which had been previously undocumented (Yamasaki et al. 1996, Field et al. 1996). Subsequent utilization of the E2F1 mouse model was further instrumental in identifying other unknown role of E2F1 *in vivo*. A short but non-exhaustive list of E2F1 functions identified by studying the E2F1 knockout include epidermal wound healing, adipocyte differentiation, β cell function in the pancreas, oxidative metabolism, mammary gland development, and neuronal death (D'Souza *et al.* 2002, Fajas et al. 2002, Fajas et al. 2004, Blanchet et al. 2011, Andrechek *et al.* 2008, O'Hare et al. 2000, Giovanni et al. 2000).

Many of these reported functions are dependent on E2F1 transcriptional activity. Therefore, it is appropriate that the E2F1 knockout mouse model used in these studies lacked the sequences coding for the DNA binding domain.

Though rarely reported, there have been cases in which mRNA message of the gene that is knocked out in a mouse model is maintained and subsequently translated into mutant proteins (Muller 1999). These types of leaky mutations often result from the aberrant mRNA splicing or read-through transcription and translation of the inserted selection marker. In the present study, we provide evidence to suggest that the E2F1 knockout mouse model is also leaky as the E2f1^{tm1} mice possess residual mRNA messages and mutant proteins that are detectable by various E2F1 antibodies.

Materials and Methods

Animals: E2f1^{tm1} (B6;129S4-E2F1^{tm1M^{eg}}/J; strain # 002785) and Wildtype F2 hybrids (B6129SF2/J; strain # 101045) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were described in Field et al. (1996), and are a hybrid of C57BL/6 and SV129 strains (Field et al. 1996). The same strain mixture was used for wildtype controls in each successive generation of breeding. All mice were housed at the University of Pennsylvania animal facilities on a 12 h light/dark cycle and water were provided *ad libitum* in conventional style cages.

Immunoblotting: Harvested tissues were homogenized in ice-cold tissue lysis buffer, including protease and phosphatase inhibitors. Tissues were harvested from single

animal and not pooled, and homogenates were rotated at 4°C for 1-2 hrs to increase protein extraction. Protein concentrations were determined using Bradford method. Equal amounts of proteins were loaded for immunoblotting. Equal protein loading across gel lanes was confirmed by staining the gel with Coomassie and membrane with fast green. Broad range of the immunoblots is shown to demonstrate the specificity of the antibodies as well as the approximate size of the immunoreactive protein bands. For densitometric analysis, autographs were scanned and cropped using Adobe Photoshop (Adobe Systems).

RNA processing and RT-PCR: Harvested tissues were incubated in RNAlater reagent [Life Technology] overnight and extracted for RNA using the RNeasy Mini Plus kit according to manufacturer protocol [Qiagen]. RNA is reverse transcribed to cDNA using the SuperScript First-Strand Synthesis for RT-PCR kit using oligoDT according to manufacturer protocol [Life Technology]. The cDNA products served as templates for downstream PCR reaction using the AccuPrime GC-Rich DNA polymerase according to manufacturer protocol [Life Technology]. Products were run on a 1-2% agarose gel and stained with ethidium bromide.

Reagents and Antibodies: Lysis buffers were prepared as the following: 25 Tris (hydroxymethyl) aminomethane (pH 7.5), 250 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1x protease inhibitor cocktail [Sigma]. The following reagents were used and purchased from indicated vendors: Coomassie (161-0786); Protein assay dye (500-0005), PVDF membrane [BioRad], Fast Green FCF [Sigma], 1 Kb plus ladder [Invitrogen], HRP-conjugated secondary antibodies [Pierce]. The following antibodies

were used and purchased: E2F1 (#3742) [Cell Signaling]. E2F1 KH95 (ab4070) [Abcam]. E2F1 KH95 (sc-251), 658c, (sc-81257), C20 (sc-193), KH20 (sc-56662), 1G10 (sc-56658), H137 (sc-22820), E2F2 C20 (sc-633), E2F3 C18 (sc-878), E2F4 C108 (sc-512), E2F4 monoclonal D3 (sc-6851) [Santa Cruz].

Primers: *3UTR R-* CCCATTTTGGTCTGCTCAAT; *COMMON -*
CTAAATCTGACCACCAAACGC; *WT -* GGATATGATTCTTGGACTTCTTGG;
MUTANT - CAAGTGCCAGCGGGGCTGCTAAAG; *PGKSEQ1 F -*
ACTTGGCGCTACACAAGTGG; *NEOSEQ R -* TTCCATTTGTCACGTCCTGCA;
EXON 3-5F - ATATCCAGTGGCTCCTGGCCTAT; *EXON 3-5F2 -*
CATATCCAGTGGCTGACCTTCGC; *EXON 5-6F -*
GCTGTGGATTCTTCAGAGACATTTC; *EXON 7R -*
CTCAATGTGGCAGCAACCAAACC

Results

1) E2f1^{tm1} mice express a protein that is detectable by various E2F1 antibodies

During our initial characterization of the biochemical changes in the E2f1^{tm1} mouse brains, we observed that protein lysates from the brains of E2f1^{tm1} mice contained a protein corresponding in size to E2F1 and detectable by widely used E2F1 antibodies. Due to this unexpected result, we began a systematic analysis to determine whether the E2f1^{tm1} mice may indeed be expressing a mutant protein that is similar to wildtype E2F1. Lysates collected from the olfactory epithelium (OE) from 12 wildtype animals displayed

a strong E2F1 immunoreactive band at approximately 60 kDa. As a positive control, lysates collected from HEK293T cells overexpressing human E2F1 were loaded to ensure the ability for the antibody to detect E2F1. Consistent with our previous observation, OE lysates from 10 E2f1^{tm1} mice all displayed an E2F1 antibody immunoreactive, similarly sized protein band (Figure 1A). To rule out the possibility that the unexpected bands may be due to inadvertent mixture of the E2f1^{tm1} mice and wildtypes during the breeding procedure within our institution, we collected additional lysates from the olfactory bulbs (OB), cortex (CTX), and OE from mice directly purchased from Jackson Laboratory and immunoblotted for E2F1. In addition, we used 7 different commercially available E2F1 antibodies targeting various epitopes of the E2F1 protein to verify that the band we are detecting corresponds to E2F1 (for mapping of the antibodies, refer to Figure 4A). Consistent with our previous results, OB, OE, and CTX lysates from 4 additional E2f1^{tm1} mice all displayed a similar sized protein band detectable by all 7 E2F1 antibodies (Figure 1B).

We next asked whether the E2F1 antibodies immunoreactive protein band in the E2f1^{tm1} mice is expressed ubiquitously throughout various brain regions as is E2F1 in wildtype mice. Lysates collected from OB, CTX, hippocampus (HC), and OE from both wildtype and E2f1^{tm1} mice all displayed similar sized protein band recognized by KH20 and KH95 E2F1 antibodies targeting the N-terminus and the C-terminus, respectively (Figure 1C). To determine whether the E2F1 antibodies immunoreactive band is present exclusively in the brain, we immunoblotted lysates collected from the brain, heart, and kidney of the wildtype and E2f1^{tm1} mice and found that though E2F1 expression level varies across brain, heart, and kidney, mice of both genotypes express a similar sized

protein that is immunoreactive to 3 different E2F1 antibodies (Figure 1D). Taken together, our results indicates that E2f1^{tm1} mice ubiquitously express a mutant protein that shares multiple domain homology as the wildtype E2F1 since the mutant protein is recognized by various E2F1 antibodies targeting different epitopes.

2) The expression profile of the E2F1 antibodies immunoreactive protein in the E2f1^{tm1} mice corresponds to that of wildtype E2F1

Given that the immunoreactive protein band in the E2f1^{tm1} mice displays comparable size and distribution pattern throughout various regions of the brain as that of normal E2F1 protein in the wildtype mice, we hypothesized that its age dependent expression would similarly correlate with that of wildtype E2F1. To test this, we collected cortical lysates from the wildtype and E2f1^{tm1} mice at 40, 90, 270, 365, and 465 days postnatal and assessed protein levels by immunblotting using 2 E2F1 antibodies to distinct domains of E2F1. Consistent with our hypothesis, we observed that the expression of E2F1 in the wildtypes and the E2F1 antibody-immunoreactive protein in the E2f1^{tm1} mice increase as these animals age *in vivo*. To ensure antibody specificity, we also immunoblotted the same lysates using the KH95 antibody and obtained similar results. Furthermore, as a negative control, we used preadsorbed KH95 antibody and were able to eliminate the signal from both wildtype E2F1 and the E2F1 antibody-immunoreactive protein in the E2f1^{tm1} mice confirming that these antibodies do indeed recognize its target antigen in the E2F1 protein (Figure 2B).

To determine whether this corresponding age-dependent increase in expression of E2F1 and E2F1 antibody-immunoreactive protein are limited in the cortex, we also collected OE lysates from the wildtype and E2f1^{tm1} mice at 40, 90, 180, 270, and 365

days postnatal and immunoblotted for E2F1 expression. Consistent with the previous results, the expressions of wildtype E2F1 and the E2F1 antibody-immunoreactive protein in the E2f1^{tm1} mice increase in an age-dependent fashion in the OE (Figure 2C). Taken together, our results indicates that the E2F1 immunoreactive protein found in the E2f1^{tm1} mice follow similar age-dependent expression pattern as the wildtype E2F1 protein throughout the brain.

3) Mutated E2F1 mRNA messages persist in the E2f1^{tm1} mice

Because the remarkable resemblance of the E2F1 antibody-immunoreactive protein in the E2f1^{tm1} mice to the normal E2F1 protein found in the wildtype, we hypothesized that there are residual E2F1 mRNA messages in the E2f1^{tm1} mice. First, we genotyped the E2f1^{tm1} mice obtained directly from Jackson Laboratory using standard genotyping primers WT, Mutant, and Common primers (for mapping of all primers used, refer to Figure 4B). As expected, we observed a single band of 227 base pairs (bp) for all E2f1^{tm1} mice and a single band of 172 bp for WT and no amplification when no template was added (Figure 3A). After confirming the genotype of our animals, we collected mRNA and genomic DNA from E2f1^{tm1} and WT mice. When we amplified the reverse transcribed mRNA using primers targeting the mutated region of the E2F1 gene and the 3' untranslated region of the E2F1 mRNA, we observed a single band of the predicted size in the E2f1^{tm1} mice but not in the WT (Figure 3B). Importantly, the amplified product using genomic DNA from the E2f1^{tm1} mice as reaction template migrated at a significantly larger size as a result of intervening introns indicating that the amplified product from our reverse transcribed mRNA is not a product of contaminating genomic DNA. Furthermore, exon 7, which is downstream of the mutated region, is predictably

retained in the residual mRNA message in the E2f1^{tm1} mice (Figure 3C).

According to the original design of the generation of the E2f1^{tm1} mice, the mutation vector is constructed such that the neomycin cassette is inserted within the exon 3 of the E2F1 gene while simultaneously deleting exon 4. This strategy would then presumably disrupt the downstream translation of the E2F1 protein (Field et al. 1996). To test whether the neomycin cassette is retained in the residual mRNA found in the E2f1^{tm1} mice, we designed primers targeting the neomycin cassette and found that the residual mRNA indeed carry the neomycin cassette while no such product was visible in the wildtype mice (Figure 3D&E). Given that the bands amplified from the genomic DNA of the E2f1^{tm1} mice using the same primer pairs migrated at a significantly larger size, the amplified products in the RT-PCR reactions are residual mRNA that have undergone intronic splicing.

Finally, we systematically designed exon border spanning primers to determine whether the splicing of the E2F1 gene downstream of the inserted mutation in the E2f1^{tm1} mice is similar to that of wildtype E2F1 mRNA message. In these experiments, PCR reaction using genomic DNA never yielded amplified product since genomic DNA possess intervening introns and the primers are designed to span exon borders. Interestingly, while the border junction of exon 5, 6, and 7 of the residual mRNA in the E2f1^{tm1} mice are spliced normally as that of wildtype E2F1 mRNA, exon 3 is not spliced into exon 5 as what was predicted in the residual mRNA (Figure 3F&G). We subsequently sequenced the mutant residual mRNA and found that the initial 22 bp of the exon 5 sequence is absent in the mRNA message. When we redesigned the exon 3 and 5 border spanning primer by taking into account of the skipping of the initial 22 bp of exon

5, we were indeed able to observe an amplified product in the E2f1^{tm1} mice (Figure 3H). Taken together, our results indicate that despite targeted genomic mutation, residual mutated E2F1 mRNA persists in the E2f1^{tm1} mice. Furthermore, this residual mRNA retains the neomycin cassette and a disrupted splicing of the exon 3 and exon 5 border.

Discussion

Although targeted gene disruption knockout models are especially useful in elucidating the role of the disrupted gene *in vivo*, the incomplete removal of the gene may lead to residual mRNA and subsequent production of a mutant protein (Hall *et al.* 2009). In the present study, we present evidence indicating that although the E2f1^{tm1} mice were designed and generated as an E2F1 knockout mouse model, these mice maintain significant levels of residual mRNA. Through our sequencing analysis, we report here that the residual mRNA message in the E2f1^{tm1} mice retain exon 1, 2, an interrupted exon 3 with embedded neomycin resistance gene cassette, part of exon 5, and full length exon 6 and 7 (Figure 4A). Though the presence of residual mRNA message that retain part or the entire inserted positive selection marker in knockout animal models is not unique to E2f1^{tm1} mice, it is unusual that such strategy would also lead to the exclusion of part of the immediate downstream exon (Muller 1999). The exclusion of 22 bps in exon 5 do not appear to be part of the original design of the E2f1^{tm1} mice since the alterations to the homologous sequence necessary for recombination in the gene targeting vector appears to end in the intronic sequence upstream of exon 5. The exclusion of the 22 bps of exon 5 sequence may lead a shifting of the open reading frame such that translation of mutant

protein in the E2f1^{tm1} mice is now permissive. It is also possible that the insertion of the neomycin cassette in the mouse genome perturbed the normal splicing of the E2F1 gene resulting in the aberrant splicing evident in the residual mRNA.

It is possible that these residual mRNA messages are translated into a mutant protein that shares several domain homologies as wildtype E2F1. With a wide range of commercially available E2F1 antibodies targeting different epitopes throughout the E2F1 protein, we systemically mapped the domains of the mutant protein found in E2f1^{tm1} mice (Figure 4B). Unexpectedly, almost all of the major domains of the wildtype E2F1 protein are conserved in mutant protein in the E2f1^{tm1} mice including the transactivation domain, pRB binding site, part of the “marked box”, and part of the N terminal cyclin A binding domain. The integrity of the DNA binding domain or the heptad repeat of E2F1 was not tested by antibody mapping in this study since antibodies raised against this region would lack E2F1 specificity as this region is highly conserved with other E2F family members (Zheng et al. 1999). Both of these domains are necessary for E2F1 heterodimerization with DP1 and binding to DNA and thus crucial for E2F1 transcriptional regulatory function (Cress & Nevins 1994). However, given that the DNA binding domain and heptad repeat correspond to the sequences of exon 3 and exon 4 of the E2F1 gene, it is nearly impossible for the mutant protein to retain a functional DNA binding domain or the heptad repeat as exon 3 is intersected by the neomycin cassette while the exon 4 completely deleted (Cao *et al.* 2011).

It is not unusual that mouse models with a gene knocked out would carry low levels of that residual mRNA that are not degraded by the nonsense mRNA degradation pathway. For example, in PSD-93 knockout mice, low levels of its mRNA messages are

maintained in the brains of these homozygous null animals despite lack of evident translation of these messages into proteins (McGee *et al.* 2001). Another example of persisting residual mRNA is in cystic fibrosis mouse model which also maintains low levels of Cfr mRNA likely as a result of aberrant splicing and exon skipping (Dorin *et al.* 1994). Alternatively, the residual mRNA from the “knocked out” gene can be translated into mutant proteins and examples of these leaky mutations include knockout mouse models targeting the N-myc, β APP, adhesion molecule L1, CD18, and DNA methyltransferase (Moens *et al.* 1992, Muller *et al.* 1994, Dahme *et al.* 1997, Wilson *et al.* 1993, Li *et al.* 1992). The leaky mutations may be a result of usage of alternative promoter, simply read-through of transcription through the positive selection marker, aberrant splicing of the mutated exons.

However, in many of these cases, the resulting mutant proteins are often expressed at lower levels and display a different molecular weight since at least part of the encoded protein sequences are disrupted. It is therefore surprising that the mutant protein in the E2f1^{tm1} mice is expressed at comparable levels, runs at similar size, exhibit similar age-dependent expression levels, and shares multiple protein domain homologies as the wildtype E2F1 protein. We speculate that the residual mRNA is a result of the usage of alternative cryptic splicing site as suggested by the exclusion of 22 bps of exon 5. The fact that the regulatory 3' untranslated region of the E2F1 mRNA is conserved in the E2f1^{tm1} mice may explain why the age-dependent expression profile of E2F1 in wildtype and mutant protein in E2f1^{tm1} mice is remarkably similar. Translation of the residual mRNA into the mutant protein in the E2f1^{tm1} mice results from the read-through transcription of only part of the inserted selection marker and subsequent downstream

exons. In addition, because E2F1 expression increases dramatically in adult, it is possible that these mutant proteins are not detectable in the ES cells or primary cultures generated from embryonic tissues earlier.

In the present study, we present data indicating that significant levels of residual aberrantly spliced E2F1 mRNA message persist in the E2f1^{tm1} mice. These messages are likely translated into a mutant E2F1 protein that is recognized by various E2F1 antibodies raised against various antigen epitopes. The mutant protein in the E2f1^{tm1} mice is expressed at comparable levels, runs at similar size, and exhibits similar age-dependent expression profile as wildtype E2F1. Given that the gene is disrupted at the critical region that codes for the DNA binding domain of E2F1 and that the residual mRNA do indeed carry such mutations, E2f1^{tm1} mice resembles more closely to a functional knockout of the E2F1 DNA transcriptional regulation than a complete knockout. However, cautions are warranted during the interpretation of the results from these E2f1^{tm1} mice since it is possible these mutant proteins may also have unpredicted gain of functions. Future characterization of the mutant protein using mass spectrometry protein sequencing would help illustrate additional potential mutant gain of function of these proteins.

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Abbreviations: bp, base pairs; CTX, cortex; HC, hippocampus; OB, olfactory bulbs; OE,

olfactory epithelium.

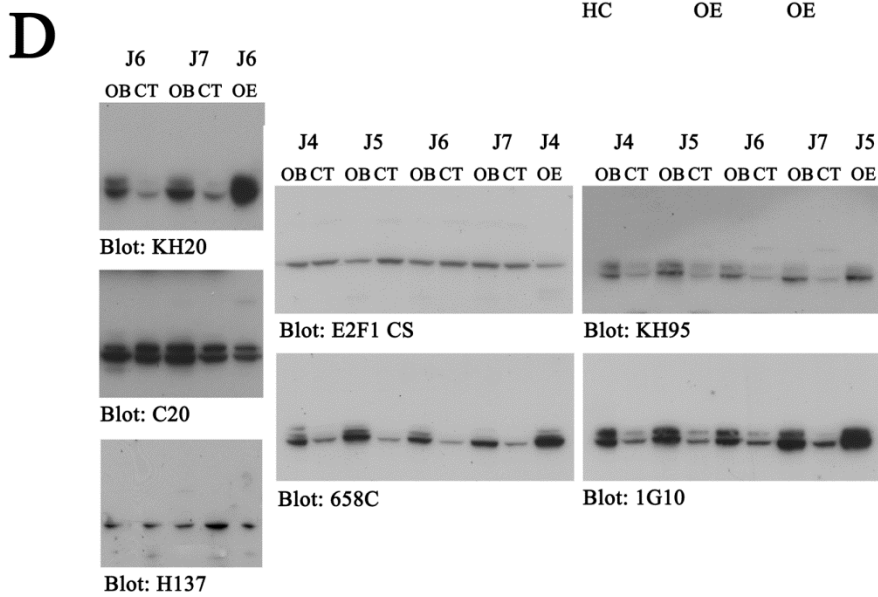
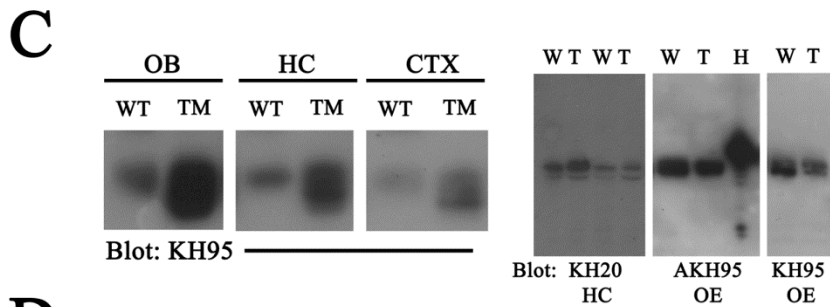
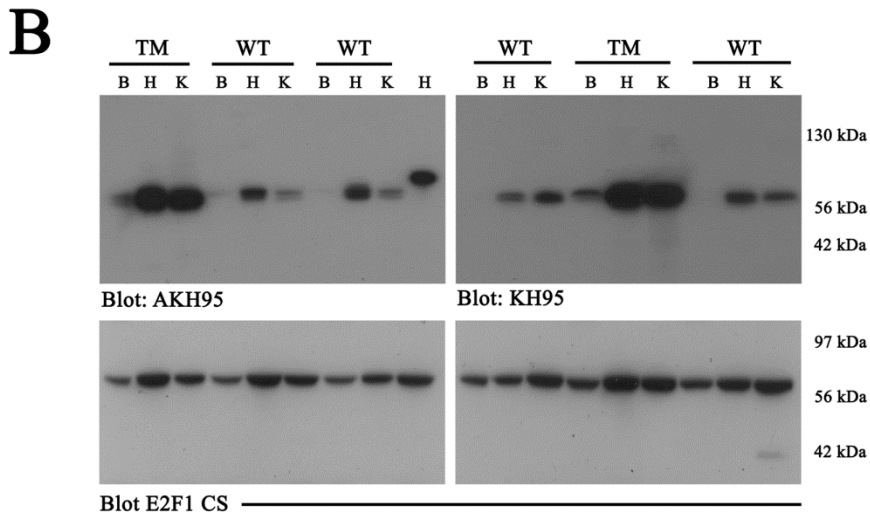
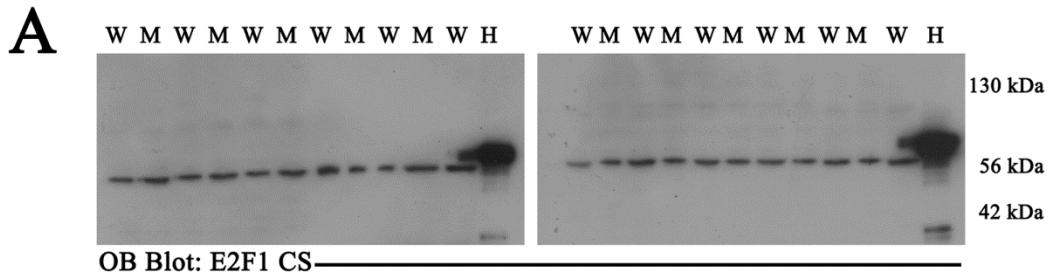


Figure 1. E2f1^{tm1} mice express a protein that is detectable by various E2F1 antibodies. A) Immunoblots of OB lysates from adult wildtype (W, n=12) and E2f1^{tm1} mice (T, n=10) probed with E2F1 antibodies from Cell Signaling. Lysates from HEK293 cells overexpressing human E2F1 (H) are included as positive control for antibody detection. B) Immunoblots of OB, CTX, and OE lysates from E2f1^{tm1} mice directly obtained from Jackson Laboratory probed by the following E2F1 antibodies: KH20, C20, H137, CS, KH95, 658C, and 1G10. C) Immunoblots of OB, HC, CTX lysates from P90 wildtype (W) and E2f1^{tm1} mice (T) probed with indicated antibodies. KH95 antibodies from two different companies (Santa Cruz - KH95; Abcam - AKH95) were used to demonstrate reproducibility of E2F1 detection. Positive control (H) from overexpressed E2F1 lysates is included. D) Immunoblots of brain (B), heart (H), and kidney (K) lysates from WT and E2f1^{tm1} mice probed with indicated E2F1 antibodies. Positive control (H) from overexpressed E2F1 lysates is included.

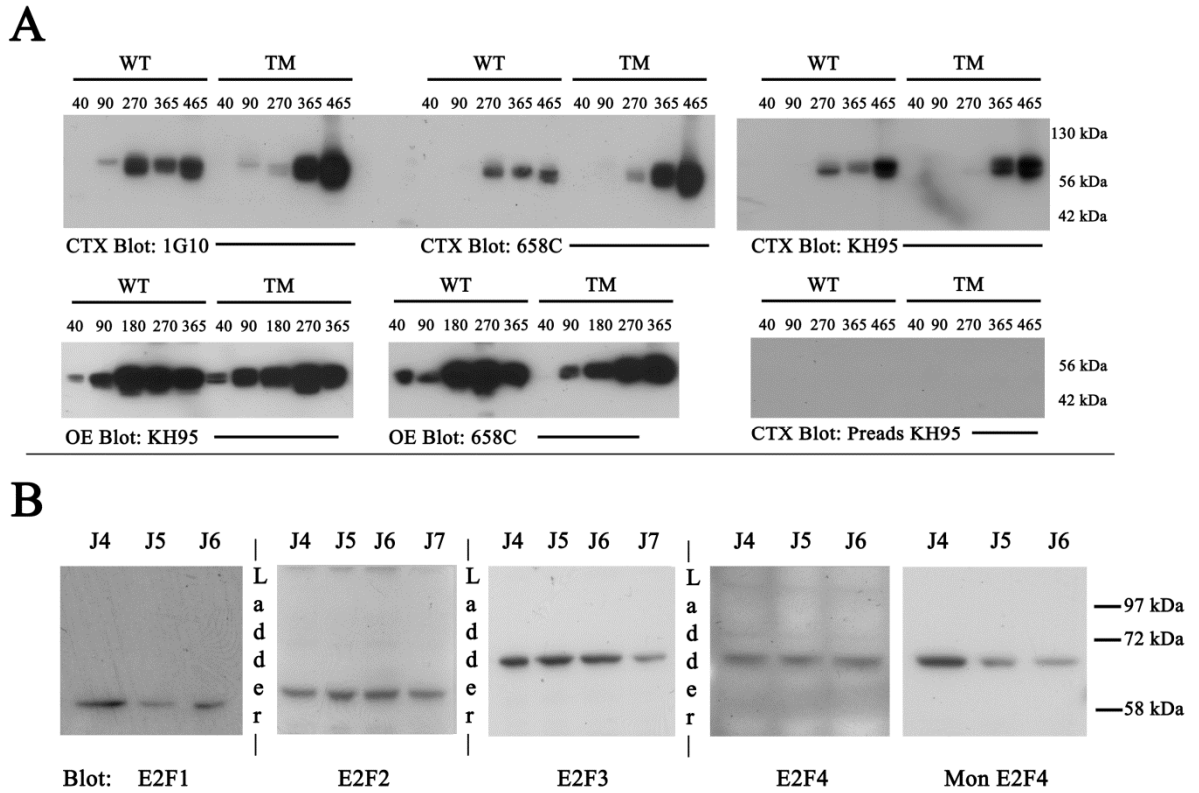


Figure 2. The expression profile of the mutant protein in $E2f1^{tm1}$ mice corresponds to that of wildtype E2F1. A) Immunoblot of CTX lysates from WT and $E2f1^{tm1}$ mice (TM) animals at indicated postnatal ages probed with indicated E2F1 antibodies. (n=5 per age group, per genotype) B) Immunoblot of CTX lysates from WT and $E2f1^{tm1}$ mice animals at indicated postnatal ages probed with KH95 antibodies. Immunoblot of cortical lysates probed with KH95 antibodies preadsorbed with a blocking peptide is included as negative control demonstrating specificity of this antibody for E2F1. C) Immunoblot of OE lysates from WT and $E2f1^{tm1}$ mice animals at indicated postnatal ages probed with indicated E2F1 antibodies.

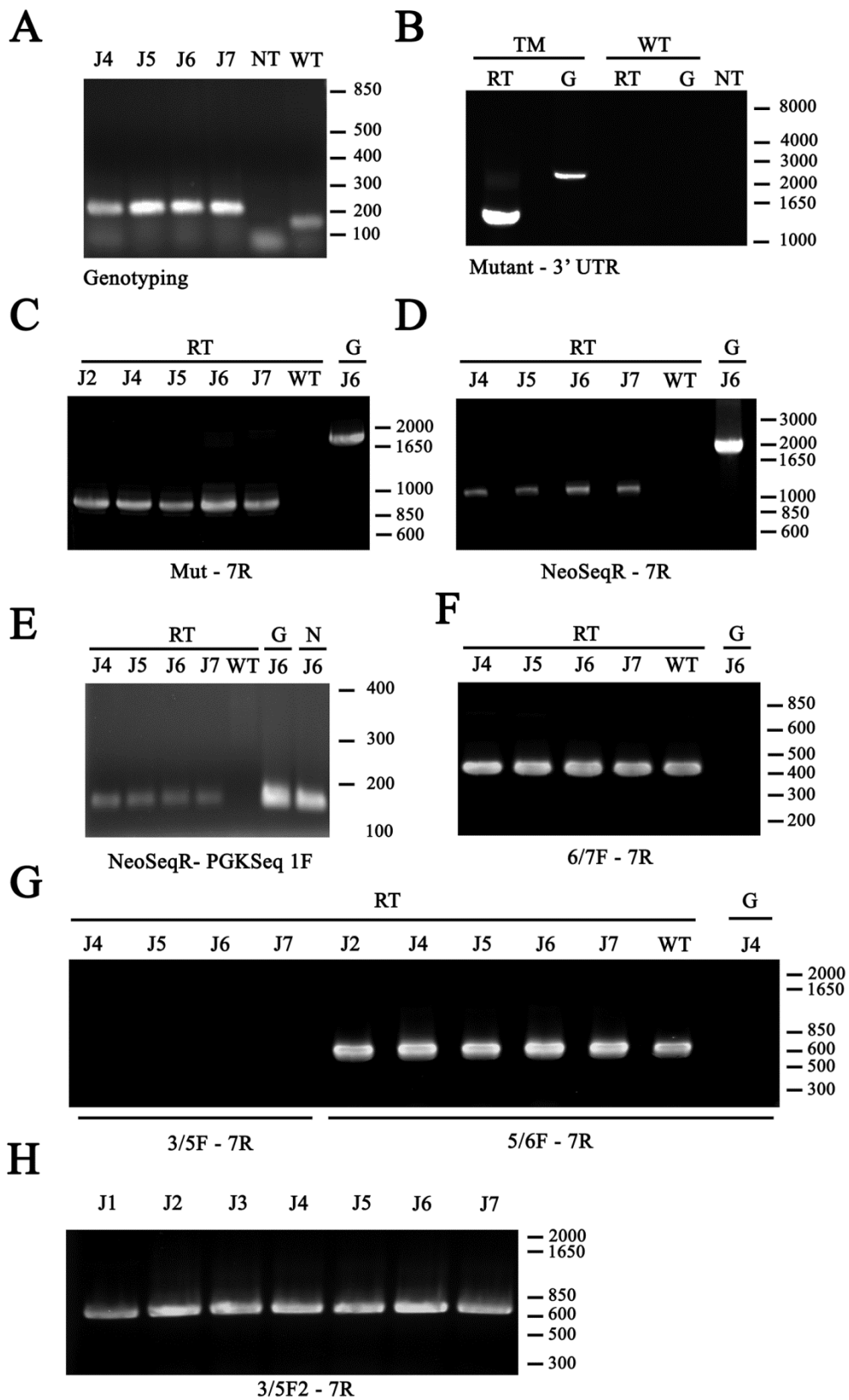


Figure 3. Mutated E2F1 mRNA messages persist in the E2f1^{tm1} mice. A) Standard genotyping PCR reaction using three primers (wildtype, mutant, common) with genomic DNA isolated from WT and E2f1^{tm1} mice obtained directly from Jackson Laboratories (J). Amplified products from genomic DNA isolated from E2f1^{tm1} mice display a band at 226 bp while WT at 172 bp. Reaction without any added template (NT) is included as a negative control. B) PCR using reverse transcribed polyA⁺ RNA (RT) or genomic DNA (G) isolated from WT and E2f1^{tm1} mice using mutant forward and 3' UTR reverse primers. No products are amplified using templates from WT with this set of primer pairs. C) PCR using mutant forward and exon 7 reverse primer (7R). D) PCR using forward primer targeting the neomycin cassette and 7R. E) PCR using forward primer and reverse primer both targeting the neomycin cassette. F) PCR using forward primer spanning the border between exon 6 and exon 7 and 7R. No product was amplified when genomic DNA was used as a template because of intervening introns. G) PCR using forward primer spanning the border between exon 3 and exon 5 and 7R or spanning the border between exon 5 and 6 and 7R. H) PCR using redesigned forward primer that span the border between exon 3 and exon 5 but excluding the first 22 bps of exon 5.

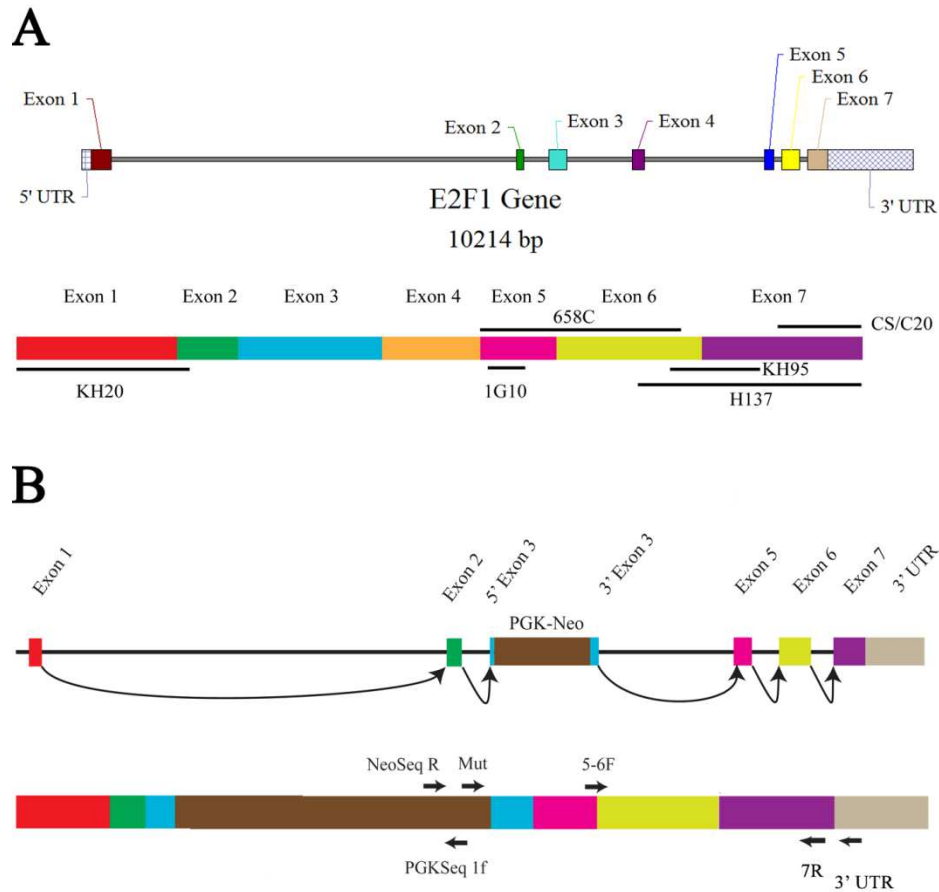


Figure 4. Schematics of E2F1 gene. A) Splicing of the E2F1 gene in the E2f1^{tm1} mice (Top). Note that exon 3 is aberrantly spliced into exon 5 by excluding the first 22 bps of exon 5. Schematic of the residual mRNA present in the E2f1^{tm1} mapped with the primers used in the study (Bottom). B) E2F1 mRNA messages mapped with the regions that various E2F1 antibodies used in this study were raised (Top). Map of the predicted E2F1 protein domains corresponding to its mRNA message (Bottom).

CHAPTER 7: Discussion and Future Directions

Since the inception of cART as a therapy to suppress HIV viral replication in HIV-infected patients, the incidence of HAD has dramatically decreased; whereas the prevalence of the minor cognitive impairments associated with HIV infection collectively known as HAND has steadily increased (Childs et al. 1999, Sacktor et al. 2001). Although cART treatments are effective in suppressing HIV viral load and in certain patient cohorts lead to the improvement of clinical symptoms associated with HAND, the drugs have been shown to induce neurotoxicity (Sacktor *et al.* 2000, Sacktor *et al.* 2003, Sacktor *et al.* 2006, Sacktor *et al.* 2009). For example, administration of cART drugs in simian model of HIV infection *in vivo* and primary cortical neurons *in vitro* both led to significant neurodegeneration (Akay et al. 2014). Furthermore, cART treatment has been shown to be associated with worsened neurocognitive status in one cohort, whereas the interruption of cART treatment led to an improved neurocognitive status in another (Marra *et al.* 2009, Robertson *et al.* 2010). Therefore, despite the tremendous advantages of cART in increasing the lifespan of HIV-infected patients, other adjunct therapy specifically addressing the neurological symptoms of HAND is necessary to effectively treat this disease.

Aberrant activation of the cell cycle machinery is a common pathological feature of many neurodegenerative diseases which ultimately lead to neuronal dysfunction and death (Busser et al. 1998, Jordan-Sciutto et al. 2002b, Jordan-Sciutto et al. 2002a, Mosch et al. 2007, Varvel et al. 2008, Bhaskar et al. 2009, Judge et al. 2011, Esteras *et al.* 2012,

Bhaskar et al. 2014). Given that the transcription factor E2F1 is the gatekeeper of the irreversible entry into cell cycle, it receives significant interest in the context of neuronal death and neurodegenerative diseases (Jordan-Sciutto et al. 2001, Hoglinger et al. 2007, Wu *et al.* 2012). Furthermore, these studies often emphasize the potential of inhibiting E2F1 function as novel therapeutic target in post-mitotic neurons (Giovanni et al. 2000, O'Hare et al. 2000). However, the physiologic function of E2F1 in the context of the healthy brain is still largely unknown, and thus the therapeutic strategies targeting the inhibition of E2F1 function may unintentionally impede and disrupt the physiologic E2F1 functions that may be necessary for optimal brain function. Therefore, a greater understanding of E2F1 physiologic function in the brain is imperative in developing future therapeutic interventions targeting E2F1, particularly in HAND.

In the present studies, we aim to elucidate the physiologic function of E2F1 in the context of the healthy brain. Specifically, in chapter 2, we provided evidence that E2F1 does not mediate neuronal death through the transcriptional regulation of its classic apoptotic and cell cycle-related targets in an *in vitro* model of HIV-associated neurotoxicity (Wang et al. 2010). In support of this finding, the mRNA expression of classic apoptotic targets of E2F1 in the presence of HIV-associated neurotoxicity were unchanged; while only the expression of the DHFR protein was significantly elevated in patients with HAND among the classic cell cycle targets. Given that the levels of DHFR mRNA did not change, it is likely that its change in protein expression is independent of E2F1 regulation. However, despite the absence of changes in E2F1 classic targets, E2F1 may still contribute to neuronal death through the regulation of the non-classic neuronal-specific targets such as neuropilin-1 (Jiang et al. 2007). A comprehensive identification

of novel targets through the microarray analysis of E2F1-bound targets in neurons in the future would be necessary to further characterize the transcription-dependent mechanism of E2F1-related neuronal death.

Alternatively, E2F1 may mediate neuronal death in a transcription independent mechanism since E2F1 expression is predominantly cytoplasmic in the neurons of healthy brains, which is consistent with previous studies (Jordan-Sciutto *et al.* 2002b, Strachan *et al.* 2005b). Akin to the ability p53 to form complexes with other proteins in the mitochondria to induce apoptosis in cycling cells, E2F1 may also induce neuronal death through its interactions with other proteins despite localizing outside of the nucleus (Marchenko *et al.* 2000, Mihara *et al.* 2003, Endo *et al.* 2006). One potential transcriptional independent mechanism to induce neuronal death is through the E2F1 destabilizing effect on the tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) proteins (Phillips *et al.* 1999). Destabilization of TRAF2 leads to the suppression of NF-kappa B pro-survival pathway, which is disrupted in E2F1- dependent Fas ligand-induced neuronal death (Hou *et al.* 2002). Another potential transcription independent E2F1 target is calpain, as over-expression of DNA-binding incompetent E2F1 led to increased calpain activity and calpain-dependent death. (Strachan *et al.* 2005a). Activation of calpain and the cleavage of downstream targets such as p35 into p25 can directly lead to CDK5 over-activity, which has been implicated in neurodegeneration (Patrick *et al.* 1999, Lee *et al.* 2000). Therefore, the cytoplasmic E2F1 may mediate neuronal death through a transcription-independent mechanism. In addition, its localization in healthy brain further suggests that under physiologic condition, E2F1 may have alternative function in post-mitotic neuron outside the context of

neurodegeneration.

In chapter 3, we investigated the role for E2F1 in the brain and reported that the targeted mutation to E2F1 gene in mice lead to age-dependent behavioral and synaptic perturbations. Furthermore, consistent with previous findings, we reported that E2f1^{tm1} mice exhibited deficits in neurogenesis in the olfactory bulbs and hippocampus of adult and aged mice (Cooperkuhn et al. 2002). Since deficits in neurogenesis have been shown to disrupt behaviors, the persistent reduction in neurogenesis in the E2f1^{tm1} mice may partially account for the age-dependent olfactory and memory impairments (Gheusi et al. 2000, Rochefort et al. 2002, Saxe et al. 2006, Winocur et al. 2006, Jessberger et al. 2009, Adlard *et al.* 2010, Pan *et al.* 2012). Although the loss of E2F1 function in the neural progenitors likely impedes their ability to proliferate in the E2f1^{tm1} mice, we were unable to rule out whether the loss of E2F1 in other cell types leads to changes in the extracellular environment and thereby contributing to the deficits in adult neurogenesis. Since the soluble trophic factors in the extracellular environment released by the astrocytes also affect the rate of neurogenesis and the fate of newly generated cells, loss of E2F1 function in the astrocytes in the E2f1^{tm1} mice may account for the persistent deficits in neurogenesis (Song *et al.* 2002, Mudo *et al.* 2009). In support of this possibility, E2F1 has been demonstrated to regulate the key aspects of the signaling pathway of several neurotrophic factors such as FGF and IGF via their transcriptional regulation (Tashiro et al. 2003, Schayek et al. 2010). In addition, activation of the TGF and VEGF pathway can lead to changes in the E2F1 expression and activity in the context of neurogenesis (Zhu et al. 2003, Spender & Inman 2009). Therefore, the loss of E2F1 function in the brain may lead to non-cell autonomous changes leading to the

impairment in the adult neurogenesis we observed. Future studies targeting region- and/or cell type- specific depletion of E2F1 using conditional transgenic mice are necessary to further explore these possibilities.

A potential pitfall in our selection of behavioral paradigm in characterizing memory impairments presented in chapter 4 is using novel object recognition as a measure of hippocampal dependent learning and memory. The involvement of the hippocampus in this task is still heavily debated since novel object recognition does not require hippocampus proper but requires the accessory areas of the hippocampal formation, where the role for neurogenesis has not been firmly established (Broadbent *et al.* 2010, Gaskin *et al.* 2003, Broadbent *et al.* 2004, Barker & Warburton 2011). Therefore, additional classic hippocampal-dependent memory tasks such as the Morris water maze, radial maze, and novel spatial-cued recognition will be necessary in the future to thoroughly link the memory impairments in the E2f1^{tm1} mutant animals to deficits in adult neurogenesis in the hippocampus. Furthermore, to establish that the E2F1-mediated deficits in adult neurogenesis mechanistically lead to changes in behaviors, future experiments may aim to re-introduce E2F1 protein function in the brain by viral transduction and examine whether the behavioral phenotypes are restored. Alternatively, if the behavioral phenotypes are indeed through E2F1-mediated loss of neurogenesis, then chemical or surgical ablation of neurogenesis in the DG or the SVZ should not lead to additional deficits. Additionally, experiments investigating the synaptic changes in other areas of the brain without neurogenesis would also be important to delineate E2F1 function in neurogenesis and at the synapse. These experiments would further clarify the mechanistic link between E2F1-mediated loss of

neurogenesis and behavioral phenotypes.

The ages in which the behavioral phenotypes in the E2f1^{tm1} mice were observed closely correlate to those of the synaptic disruptions, suggesting that these phenotypes may be mechanistically linked. In support of this interpretation, changes in PSD-95 expression have been associated with behavioral impairments (Soule et al. 2008, Sun et al. 2009, Wakade et al. 2010). Mechanistically however, how the loss of E2F1 function leads to the synaptic disruptions is unclear. One potential explanation is that the loss of E2F1 function leads to reduced number of adult-born neurons resulting in reduction in the number of newly formed synapses in the circuitry. However, since the adult-born neurons incorporated into the olfactory bulbs are typically inhibitory interneurons, it is unlikely that the deficits in adult neurogenesis account for the changes in the expression of predominantly excitatory synaptic proteins such as PSD-95, NMDAR, and synGAP (Betarbet *et al.* 1996, Petreanu & Alvarez-Buylla 2002, Carleton et al. 2003). Instead, we hypothesized and demonstrated in Chapter 4 that E2F1, independent of its role in neurogenesis, may also directly contribute to the regulation of PSD-95 expression and neuronal physiology. Since fully differentiated, post-mitotic neurons, once dissociated from embryonic cortex, cannot replicate even in the presence of neurogenic trophic factors, the reduced PSD-95 expression in the primary neurons cultured from the E2f1^{tm1} mice is independent of the effects of E2F1 in neurogenesis (Jin *et al.* 2002a, Jin *et al.* 2002b, Sun *et al.* 2006). Furthermore, the impairment of neuritic arborization following reduced E2F1 expression in hippocampal neurons also supports the idea that E2F1 has a direct role in neuronal physiology.

Because the loss of E2F1 function led to a reduction of various synaptic molecules in mice, the electrical signaling properties of the neurons depleted of E2F1 may be altered (Cull-Candy & Leszkiewicz 2004). Alternatively, the changes in the expression of different glutamate receptor subunits as a result of E2F1 depletion may also lead to changes in susceptibility to excitotoxicity or HIV-associated neurotoxicity (Liu *et al.* 2007, O'Donnell *et al.* 2006). Furthermore, loss of PSD-95 in the absence of E2F1 may alter the surface expression of different glutamate receptors, further altering the electrical properties of these neurons (Tu *et al.* 1999, Petralia *et al.* 2005). Future investigation of the electrical, biophysical, and pharmacological properties of the NMDA receptors expressed in neurons lacking E2F1 for would be instrumental to determine how these changes may affect the susceptibility of neurodegeneration in the presence of HIV-associated neurotoxicity. In addition, assessment of the ability of the E2f1^{tm1} mice to form long-term potentiation or depression would strengthen the relationship between the synaptic perturbations and the behavioral deficits.

Consistent to our central hypothesis that E2F1 has a synaptic role in the CNS, we further demonstrated that the cytoplasmic E2F1 in the neurons localizes to the synapse in chapter 3. Curiously, E2F1 is not the first cell cycle related protein enriched in the synapses. Similar to E2F1, core subunits of the origin recognition complex (ORC), which normally initiates DNA replication machinery in cycling cells, localize to the synapse and the depletion of ORC3 leads to impairment in dendritic branching (Huang *et al.* 2005). CDK5 and its co-activator p35 can also be found in the synapses in adult brain and their functions have been implicated in neuronal migration, synapse formation, neurotransmission, and neurodegeneration (Patrick *et al.* 1999, Humbert *et al.* 2000,

Smith & Tsai 2002). Interestingly, E2F1 has been shown to complex with either ORC or CDK5 in cycling cells, supporting the possibility that E2F1 may also directly interact with these proteins in the synapse (Mendoza-Maldonado *et al.* 2010, Zhang *et al.* 2010). Another novel E2F1 interacting protein partner at the synapse is NPDC-1 since NPDC-1 is found in the synapse and the interaction of NPDC-1 and E2F1 have been documented (Sansal *et al.* 2000, Evrard & Rouget 2005). Similarly, E2F1 can also interact with GSK-3 β , whose activity has been implicated in various aspect of neuronal physiology including neuronal morphology, synaptic plasticity, and neurotransmission, at the synapse since they also interact in neural cells (Jiang *et al.* 2005, Hooper *et al.* 2007, Peineau *et al.* 2007, Ahmad-Annur *et al.* 2006, Zhou *et al.* 2008). With a large number of potential E2F1 interacting partners implicated in synaptic functions, it would be crucial for future studies to identify these true interactions amongst these candidates by immunoprecipitating E2F1 complexes from the synapse. It may be through the loss of these protein interactions in the synapses underlying the synaptic disruptions in the E2f1^{tm1} animals. Interestingly, we have indeed identified a putative Src homology 3 (SH3) recognition motif in E2F1 protein that may be necessary for its localization and interaction with synaptic molecules in the synapse (McPherson 1999). To test this possibility, site directed-mutation to this region in the future may be utilized to examine whether E2F1 synaptic localization and/or interaction with candidate proteins in the synapse are disrupted.

However, what is unusual about our findings is that given E2F1 proteins are located predominantly in the presynaptic fractions of the synapses; the majority of the synaptic or morphological changes in these neurons are predominantly postsynaptic.

Furthermore, we did not detect any changes in the expression of synaptophysin across different ages in the E2f1tm. It is possible that the presynaptic changes in the absence of E2F1 function is more subtle and cannot be determined through synaptic protein expressions. Other presynaptic changes that should be examined in the future include the rate of synaptic vesicle release, calcium influx, and neurotransmitter uptake or packaging. However, if the changes in the E2f1^{tm1} mutants are indeed exclusively predominantly post-synaptic, then several potential mechanisms could explain these discrepancies. First, similar to CREB, E2F1 as a transcription factor, upon synaptic stimulation, can translocate into the nucleus from the presynaptic terminal and regulate gene expression related to postsynaptic changes (Riccio et al. 1997, Lai et al. 2008, Ch'ng *et al.* 2012). Second, E2F1 may interact with other synaptic molecules trans-synaptically or in the synapses to mediate its changes post-synaptically (McClelland *et al.* 2010). Third, although rare, E2F1 as a transcription factor can be secreted extracellular to mediate post-synaptic changes (Raghavan *et al.* 2008). Future experiments addressing these possibilities would be instrumental in understanding the mechanism in which the loss of E2F1 function lead to changes in the synapse.

Our findings in chapter 5 illustrate that unlike in other tissue, the E2F1 gene is alternatively spliced into two novel mRNA transcripts in the brain, particularly in the neurons. The presence of alternatively spliced products indicates that E2F1 has an additional level of regulation that is absent in other tissues, which is consistent with our hypothesis that E2F1 has alternate function in the brain. Interestingly, the two alternatively spliced mRNA products E2F1+Int5 and E2F1 Δ Exon6, when translated into proteins without further RNA editing, produce similarly sized truncated version of E2F1

lacking the transactivation domain. These modified protein products would therefore only be present in the brain and may thus restrict the ability for E2F1 to transcriptionally induce apoptotic or cell cycle-related targets in post-mitotic neurons. The precedence for brain-specific protein isoforms whose functions in neural cells are altered is exemplified in TRF2 and ETS domain protein Elk-1. Neuronal specific splice variant TRF2-S sequesters master neuronal gene-silencer repressor element-1 silencing transcription factor (REST) in the cytoplasm to maintain differentiation; while sElk-1 induces Elk-1 cytoplasmic localization and PC-12 differentiation (Zhang et al. 2011, Vanhoutte *et al.* 2001). To determine whether E2F1+Int5 and E2F1 Δ Exon6 are translated into endogenous protein isoforms, antibodies that detect the N-terminal region of the E2F1 proteins will be needed. The detection of these brain-specific isoforms will underscore the importance of E2F1 brain-specific functions.

The presence of alternatively spliced E2F1 transcripts may also have potential implications as to how the E2F1 proteins are localized to the neuronal processes and synapses. Cytoplasmic intron-retaining transcripts in neurons often contain ID element that confers their cytoplasmic localization that is subsequently edited prior to local translation (Buckley et al. 2011). The E2F1 gene contains such ID element in its intron 1, though an alternatively spliced transcript retaining this element has yet to be identified. Nevertheless, we predict that E2F1+Int5 transcript identified in our studies harbors a signal that affects the subcellular distribution of the E2F1 proteins. To address this possibility in the future, we would deplete of the alternatively spliced E2F1 transcripts via RNA interference and examine the localization of endogenous localization of E2F1 mRNA transcripts and E2F1 proteins. Furthermore, to test if alternatively splicing affects

E2F1 function, we would also examine the effect of RNAi on neuritic arborization and/or PSD-95 expression.

Finally in Chapter 6, we presented evidence that the E2F1 transgenic mouse model originally intended as a total depletion of the E2F1 protein is incomplete since residual mutated E2F1 mRNA and proteins can be detected. Considering the unexpected splicing of the E2F1 gene identified in chapter 5, it is not surprising that the original strategy to completely remove E2F1 proteins leads to the detection of residual mutant E2F1 mRNA in the brain. However, our data does advocate for more careful interpretation of studies utilizing these animals since the mutant E2F1 proteins, though lacking transcriptional regulatory functions, may serve as a dominant negative repressor to compete away endogenous protein interaction (Liu & Greene 2001). Therefore, it would be prudent in the future to determine whether the protein interacting partners of the mutant E2F1 in the E2f1^{tm1} mice overlap that of wildtype E2F1.

In conclusion, our present studies provide a myriad of supporting evidence that E2F1 has alternate functions in the brain relating to the neuronal physiology that are distinct from its classic cell-cycle and apoptosis regulatory role observed in cycling cells. Specifically, E2F1 has a synaptic role in neurons, modulating the expression of various synaptic molecules. Although we cannot currently pinpoint the mechanism in which E2F1 exert its effects in neurons, we speculate that it may be related to its post-transcriptional regulation and subcellular localization. Recent studies indicate that long-term synaptic plasticity relies on the local translation of transcription factors that are rapidly transported into the nucleus to modulate gene expression (Wang *et al.* 2009,

Ch'ng et al. 2012). Potentially, E2F1 may serve as this signaling molecule in neurons given that its proteins have been observed to shuttle between the nucleus and the cytoplasm (Strachan et al. 2005b). On the other hand, E2F1 may also function directly at the synapse through its potential interaction with other synaptic signaling molecules such as CDK5 and GSK-3 β (Humbert et al. 2000, Hooper et al. 2007). Precedence for unique functions for transcription factors outside of the nucleus in neurons have been observed in Elk-1 (Barrett *et al.* 2006b, Barrett et al. 2006a, Sharma *et al.* 2010). Here, we also offer some future direction earlier aimed to explore these possibilities that would provide additional insight to the uncharacteristically unique E2F1 functions in the brain. Furthermore, these studies would help to design more effective and efficient adjunctive therapeutic strategies targeting the unique functions of repurposed cell cycle related proteins such as E2F1 in HAND and other neurodegenerative diseases.

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