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Identification and characterization of two novel alternatively spliced E2F1 transcripts in the rat CNS

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Abstract

E2F1 is a transcription factor classically known to regulate G₀/G₁ to S phase progression in 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 common in cells of the CNS and is necessary to generate diverse functional modifications to RNA or protein products from genes. Heretofore, 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: E2F1b, an E2F1 transcript retaining intron 5, and E2F1c, an E2F1 transcript excluding exon 6. These alternatively spliced transcripts are observed in the brain and neural cell types including neurons, astrocytes, and undifferentiated oligodendrocytes. The expression of these E2F1 transcripts is distinct during maturation of primary hippocampal neuroglial cells. Pharmacologically-induced global translation inhibition with cycloheximide, anisomycin or thapsigargin lead to significantly reduced expression of E2F1a, E2F1b and E2F1c. Conversely, increasing neuronal activity by elevating the concentration of potassium chloride selectively increased the expression of E2F1b. Furthermore, experiments expressing these variants *in vitro* show the transcripts can be translated to generate a protein product. Taken together, our data suggest that the alternatively spliced E2F1 transcript behave differently than the E2F1a transcript, and our results provide a foundation for future investigation of the function of E2F1 splice variants in the CNS.

Keywords

E2F1; alternative splicing; CNS

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Introduction

Alternative splicing is an evolutionarily conserved mechanism allowing cells to generate diverse functional RNA transcripts through combinatorial pairing of different splice sites (Nilsen and Graveley, 2010). Through the coordinated expression of various splicing regulatory factors, different cell types can generate a 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 splicing of RNA transcripts is 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 and Kim, 2013). Disruption of the tight regulation of RNA splicing can induce developmental defects in the CNS as evidenced by deletion of Nova2, a regulator of splicing, which leads to aberrantly spliced RNA transcripts of disabled-1 protein that results in defects in neuronal migration (Yano et al., 2010). Additionally, alternative splicing leading to the introduction of a premature stop codon in the mRNA can be coupled with the nonsense-mediated mRNA decay (NMD) pathway to temporally regulate the expression of classically generated protein products during brain development. For example, *DLG4*, encoding postsynaptic protein 95, which is crucial in synapse maturation, is alternatively spliced into a transcript containing a premature stop codon that is degraded via NMD during embryonic development. However, loss of the splicing regulators polypyrimidine tract binding protein (PTBP) 1 and 2 during brain maturation results in aberrant expression of the full length PSD-95 transcript and thus, initiates synapse formation earlier than in healthy brains (Zheng et al., 2012). Indeed, disruptions in the splicing machinery can also result in neurodegeneration, as seen in Amyotrophic Lateral Sclerosis with the dysregulation of TAR DNA-binding protein (TDP)-43 (Polymenidou et al., 2011).

E2F1 classically functions as a transcription factor regulating the G₀/G₁ to S phase transition in cycling cells, yet it also has an important role in regulating cell death (Blais and Dynlacht, 2004; Giacinti and Giordano, 2006; Iaquinta and Lees, 2007). E2F1 can induce apoptosis via indirect up-regulation of p53 expression or by direct up-regulation of pro-apoptotic target genes, including APAF1, PUMA, NOXA, Smac/Diablo, and caspases (Hershko and Ginsberg, 2004; Moroni et al., 2001; Nahle et al., 2002; Xie et al., 2006). Given this capacity to regulate cell death, the role of E2F1 in neuronal death is well characterized in a number of different *in vitro* models of toxicity (Giovanni et al., 2000; 1999; O'Hare et al., 2000; Park et al., 2000). Aberrant E2F1 expression has been reported in a number of different neurodegenerative diseases, including Alzheimer's, Huntington's, Parkinson's, and amyotrophic lateral sclerosis (Hoglinger et al., 2007; Jordan-Sciutto et al., 2001; 2002; Ranganathan et al., 2001; Lopez-Sanchez et al. 2017). However, despite the well-characterized anomalies in these diseases, little is known about E2F1 function or regulation in the CNS. We have previously reported data showing 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 explored alternative splicing as a potential mechanism for E2F1 regulation and identified two novel

E2F1 splice variants prominently observed in the brain. We did not detect these alternatively spliced transcripts in other tissues, organs, or fibroblast cell lines despite ubiquitous expression of the E2F1a transcript. Furthermore, we characterized the expression pattern of the E2F1 splice variants: during maturation of neuroglial cells, in response to pharmacologically-induced NMD inhibition, and increased neuronal activity. Taken together, our results show that E2F1 is indeed alternatively spliced into two novel transcript variants in the CNS, which are distinct from the originally identified E2F1a 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 onto poly-L-lysine coated plates and maintained in neurobasal media containing B27 supplement. Pure neuronal cultures were generated by treating neuroglial cultures with 10 μ m Ara-C, 48 hours after plating. Purity of cultures was confirmed by the absence of staining for the glial marker, glial fibrillary acidic protein (GFAP). Pure astrocyte cultures were generated by first culturing neuroglial cells in DMEM supplemented with 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 in another flask, this process was repeated twice to generate pure cultures for RNA collection (Akay et al., 2014). Pure oligodendrocyte cultures were prepared as previously described (Reid et al., 2012). Briefly, neuroglial cells were isolated from neonatal rat brain and cultured in neurobasal media 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 μ g/ml transferrin, 5 μ g/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 μ g/ml insulin, 0.4 μ g/ml T4, 0.3% glucose, 2 mM glutamine, and 10 ng/ml biotin (R&D Systems). HEK293T and Rat2 cells were maintained in DMEM supplemented with fetal bovine serum (FBS). PC-12 cells were maintained in RPMI-1640 media supplemented with horse serum and FBS. All cells were cultured at 37°C with 5% CO₂. Rat2 fibroblasts were arrested in S phase via 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 for transfection experiments in HEK293T and PC12 according to manufacturer protocols (Life Technologies). Transfection mixture was added to the cells for 2 hours and subsequently replaced with the growth media.

RNA extraction and PCR:

Tissues harvested from Sprague Dawley rats were snap frozen in liquid nitrogen and subsequently stored at –80°C or used immediately after freezing for RNA extraction. Total RNA was extracted from tissues and cells using TRIzol reagent (Life Technologies) and subsequently purified using the RNeasy plus mini kit, according to manufacturer recommendations (Qiagen). Total RNA was treated with DNase prior to cDNA synthesis using TURBO DNase (Thermo Fisher). cDNA was synthesized from 1 μ g of total RNA (as

quantified by Nanodrop) using superscript IV reverse transcriptase and oligo dT primers (Invitrogen), according to the manufacturers recommendations. Following the cDNA synthesis reaction, all samples were digested with RNase H, according to the superscript IV manufacturers recommendations. PCR reactions were performed using 2 μ L of cDNA with the AccuPrime GC-rich DNA polymerase kit and specified primers (Table 1 and Supplemental Figure 1) (Invitrogen), according to the manufacturers recommendations. To confirm PCR results with the AccuPrime GC reagents, most PCR reactions were also performed using OneTaq DNA polymerase, with the GC-rich buffer supplied in the kit (New England BioLabs). For all E2F1 PCR reactions, annealing temperatures were optimized to the specific primer sets. All E2F1 PCR reactions were performed with the following conditions: an initial melt of 95°C for 2 minutes, then by cycling through 95°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30–90 seconds (depending on product size) for 40 cycles, followed by a final extension of 10 minutes. PCR products were visualized by separation on 0.8% agarose gel containing ethidium bromide. Gels were imaged using a ChemiDoc MP system (Bio-Rad Laboratories, Inc.). For DNA sequencing of PCR products, DNA was gel extracted using the Qiagen DNA gel extraction kit (Qiagen) according to the manufacturers recommendations. DNA was submitted for sequencing (with primers specific to E2F1) by the Penn Genomics Analysis Core, which employs an ABI 3730 for Sanger sequencing. For sequencing alignments and primer design, rat E2F1 sequences were downloaded from the UCSC genome browser, rn6 assembly (Kent et al., 2002). Nicastrin- 3 isoform mRNA expression was quantified as in Confaloni et al. 2005.

Quantitative real-time PCR:

Equal amounts of cDNA were loaded in technical triplicate for quantitative PCR (qPCR) using the Fast SYBR green master mix and 7500 Fast real time PCR systems according to the manufacturers protocol (Applied Biosystems). Primers used in these experiments are listed in Table 1 and shown in Supplemental Figure S1. All qPCR primers were designed to similar amplification efficiency. Primers targeting specific E2F1 splice variants were also analyzed to ensure specificity to that variant. To determine E2F1 splice variant expression levels, qPCR results were calculated using the 2^{-CT} method, whereby the mean cycle threshold was first normalized to the endogenous control, Tata-box binding protein (TBP), and fold-change was subsequently calculated relative to the proper experimental control. All qPCR experiments were replicated biologically a minimum of four times and independently three times.

Antibodies and Reagents:

The following antibodies were purchased from the indicated vendors: E2F1 KH95 (sc-251) (Santa Cruz); FLAG tag (#2368) (Cell Signaling). 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 (Thermos), 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, 0.4 mM 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 was analyzed using Prism 6.0 (GraphPad Software) and expressed as mean $\pm$ standard error of the mean (SEM) with values of $p < 0.05$ considered significant. Unless otherwise noted, all significance was determined by one-way ANOVA with the appropriate post-hoc test.

Ethics Statement:

All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). For the studies reported here, tissues were harvested from Sprague Dawley rats post-mortem. Rats were euthanized with 20% CO₂/minute using a fixed-flow meter in a CO₂ chamber, in compliance with our approved protocol and recommendations of University Laboratory Animal Resources at Penn and our IACUC.

Results

To determine if rat E2F1 mRNA is alternatively spliced, we amplified E2F1 transcripts with primers targeting exon 1 and exon 7 of E2F1 using cDNA generated from DNase treated RNA purified from rat cortex. After separating the PCR reactions on an agarose gel we consistently noted two distinct bands, with a faint third band present above the expected PCR product for the E2F1 transcript (Figure 1A). Based on the sizes of the fragments, we hypothesized that the lower band was a deletion of one or more exons and the upper band (running larger than the expected size for the previously identified E2F1 cDNA) was due to the retention of an intron. Given that A) introns 1–4 were too large to be consistent with the size of the upper band (hypothesized intron retention) and B) the size of the lower band was not consistent with a deletion of exon 2, we next performed PCR reactions to amplify E2F1 using primers targeting exon 4 and exon 7. When visualized on an agarose gel, we noted three distinct bands that were consistent with the sizes of the three bands from the exon 1/7 PCR reaction (Figure 1B) (when accounting for the difference in amplicon size given use of the exon 4 rather than exon 1 primer). When analyzing the results of this E2F1 exon 4/7 PCR, we did notice some variability between samples in the relative abundance of the E2F1 splice variant mRNA using non-quantitative PCR (see Figure 1B), though the significance of this variability is unclear. Thus, we hypothesized these E2F1 alternative splice variants were the result of changes in the 3' half of the E2F1 mRNA. To identify the sequences of these three products, we extracted the PCR products from the gel and analyzed the DNA with Sanger sequencing. The sequencing results indicated the upper band was due to the retention of intron 5 (E2F1b) and the lower band was due to a deletion of exon 6 (E2F1c) (Figure 2). To further confirm these results, we performed PCR reactions with primers specifically

targeting the E2F1b or E2F1c variants. These reactions resulted in a single clear band at the expected sizes for the E2F1b (Figure 1C and 1D) and E2F1c variants (Figure 1E).

The data from the experiments described above, show that E2F1 mRNA can be found in three forms in the rat cortex: E2F1a, E2F1b, and E2F1c (Figure 3). The E2F1a transcript is 1,299 bp in size (excluding the 5' and 3' UTRs) and includes exons 1–7. The E2F1b variant is 1,392 bp in size and includes exons 1–7 as well as intron 5. The E2F1c variant is 1,082 bp in size and contains exons 1–5 and exon 7. It is noteworthy that for both the E2F1b and E2F1c variants, the splicing changes generate a stop codon shortly after exon 5; this stop codon is found in intron 5 in the E2F1b variant and in exon 7 in the E2F1c variant. This change is predicted to result in a truncation of the E2F1 protein for both the E2F1b and E2F1c variants. Additionally, the predicted protein produced from the E2F1b variant would have five amino acids prior to the inserted stop codon that are unique to the E2F1b protein, while the predicted protein produced from the E2F1c variant would have six amino acids prior to the inserted stop codon that are unique to the E2F1c protein (Figure 3). Thus, these truncations of E2F1 result in the loss of a portion of the E2F1 marked box region, the entire E2F1 transactivation domain, and the entire E2F1 pRb-binding domain.

Given our findings that two E2F1 splice variants are present in the rat cortex, we next collected RNA from different tissues to determine whether or not the splice variants were detectable in those tissues. PCR reactions targeting E2F1 exon 1 and exon 7 did not detect the splice variants in the liver, kidney, lung, heart, skeletal muscle, or stomach (Figure 4A), despite expression of the E2F1a mRNA. Similar results were seen in PCR reactions specifically targeted to amplify the E2F1b variant (Figure 4B), and in PCR reactions amplifying all three variants with primers targeting exon 4 and exon 7 (Figure 4C). It should be noted that because the E2F1 splice variants were only detectable in cortex tissue, we did not pursue efforts to make any quantitative comparisons of the splice variants between tissues. To determine if the E2F1 splice variants were present throughout the CNS, we evaluated the expression of E2F1 splice variants among brain regions. We detected all three E2F1 transcripts in the cortex, striatum, hippocampus, olfactory bulb, and the cerebellum (data not shown).

Based on our results showing alternatively spliced E2F1 transcripts are expressed throughout the brain, we next characterized their expression in neuronal and glial cell populations cultured *in vitro*. We amplified E2F1 using exon 4 and exon 7 primers (common to all three variants) and cDNA generated from astrocytes, neurons, oligodendrocyte precursor cells, oligodendrocytes, and mixed neuroglial cultures. We detected the presence of the E2F1c variant in pure neurons and mixed neuroglial cultures, with only modest levels present in astrocytes (Figure 5A). Given that the PCR product for the E2F1b variant migrates at a fairly similar size as the E2F1a variant product, we also performed PCR reactions with primers specific for the E2F1b variant and detected E2F1b in pure astrocytes, pure neurons, and mixed neuroglial cultures. Interestingly, the expression of both E2F1b and E2F1c was more robust in cultures containing neurons.

Previous studies have shown that E2F1 expression varies with cell cycle phase in dividing cells (Degregori and Johnson, 2006; Iaquinta and Lees, 2007). Given that mature neurons are

post-mitotic and tightly maintained in a quiescent phase (Herrup and Yang, 2007), we hypothesized that alternative splicing of E2F1 may be restricted to a specific phase of the cell cycle in non-neuronal cells. To examine this hypothesis, we employed Rat2 fibroblasts to determine if the E2F1 variants, which were not detectable in asynchronous cells, became expressed when cells were arrested in various phases of the cell cycle. We found that regardless of the cell cycle stage in which fibroblasts were arrested, E2F1b and E2F1c were not detectable, despite clear E2F1a expression (Figure 5B). As a positive control, we demonstrated that E2F1b and E2F1c were readily detectable in cortical neurons using the same reaction conditions. Furthermore, PCR reactions using genomic DNA as a template yielded a distinct pattern for the non-spliced E2F1 gene on the agarose gel, confirming that the presence of E2F1b and E2F1c were not due to amplification of genomic DNA; this was also demonstrated by the DNA sequencing discussed above (Figure 2). Taken together, these findings are consistent with our previous observations that alternative splicing of E2F1 is prominent in the brain, but not in other non-CNS tissue types.

Alternative splicing depends on the presence of splicing regulatory proteins, the expression of which is context dependent, for example, during cell differentiation or tissue development (Zheng and Black, 2013). We have already shown that all three E2F1 variants are present as cultured neuroglial cells mature *in vitro* (Figure 5A). However, we were unable to quantitatively describe the expression of the different E2F1 transcripts in these experiments with traditional PCR. Therefore, we designed and experimentally validated primers to be used for real time quantitative PCR to specifically amplify individual E2F1 transcripts (see Table 1 and Supplemental Figure S1). We subsequently analyzed the expression of each E2F1 transcript in primary cortical and hippocampal neuroglial cells as they matured *in vitro*. In cortical neuroglial cultures, all three E2F1 transcripts follow similar trends, whereby transcript expression increases modestly following the second week in culture but declines significantly after the third week in culture (Figure 6A). In contrast, the expression profile of each E2F1 variant was more distinct in the hippocampal neuroglial cultures (Figure 6B). E2F1a exhibited a modest gradual decline (not significant) in expression with time in culture, whereas the E2F1b variant exhibited a significant and dramatic decline in expression during the second and third week in culture. Furthermore, the expression of the E2F1a and E2F1b variants is lower in mature cells compared with cells collected at DIV 0. In contrast, the expression of E2F1c is significantly increased after the first week in culture and then steadily declines as the hippocampal cells age *in vitro*. Taken together, the expression profile of each E2F1 transcript is slightly different, depending on the region from which neuroglial cultures are generated. Specifically in hippocampal neuroglial cells, each E2F1 transcript displays a slightly different expression pattern, suggesting their expression may be regulated differently in these cell types.

Given the data showing distinct expression patterns of the alternatively spliced E2F1 transcripts as hippocampal cells mature *in vitro*, we next investigated potential mechanisms by which these transcripts are regulated. If translated, both of the alternatively spliced E2F1 transcripts would introduce premature termination codons. Therefore, the proteins predicted from the direct nucleotide sequence of E2F1b and E2F1c are similarly sized E2F1 truncations missing the C-terminal domains. Since a number of mRNA transcripts containing premature termination codons are targeted for destruction by non-sense mediated

decay (NMD), we evaluated whether the different E2F1 transcripts are elevated following translation inhibition-induced NMD inhibition (Bidou et al., 2012; Kervestin and Jacobson, 2012). Global protein translation inhibitors have previously been used to indirectly inhibit the NMD pathway *in vitro*, which leads to the accumulation of NMD targeted transcripts (Dang et al., 2009; Noensie and Dietz, 2001). Therefore, we treated mature primary hippocampal cells with either cycloheximide or anisomycin and analyzed the levels of E2F1 transcripts via qPCR. The expression of all three E2F1 transcripts significantly decreased in response to cycloheximide treatment (Figure 7A). Similarly, the expression of all three E2F1 transcripts significantly decreased after anisomycin induced NMD inhibition. To confirm our treatments were effective in NMD inhibition, we quantified the levels of the Nicastrin 3-isoform (exon 3 deletion), which has previously been identified as a target for NMD (Confaloni et al. 2005). Importantly, the expression of the endogenous control used for the qPCR experiments, TBP, was not disrupted by translation inhibition, thus indicating that the changes in the expression of the E2F1 variants are not global to all mRNAs.

We next sought to induce global translation inhibition through a cellular stress pathway to evaluate E2F1 variant changes. Prolonged exposure to thapsigargin, an inhibitor of endoplasmic reticulum (ER) calcium level regulation, is known to induce activation of ER stress, leading to global suppression of protein translation (Booth and Koch, 1989; Wek and Cavener, 2007). Therefore, we treated hippocampal neuroglial cell cultures with thapsigargin and evaluated the expression of different E2F1 transcripts. Similar to our previous observations following inhibition of translation with cycloheximide and anisomycin, the expression of all three E2F1 transcripts was significantly reduced after 8 and 24 hours of exposure to thapsigargin (Figure 7B). The lack of changes in E2F1 splice variant expression following short treatments with thapsigargin, suggests the changes observed following longer treatment times are not a result of acute calcium dysregulation, rather they are due to global translational inhibition following prolonged ER stress. In short, the results suggest that the E2F1 splice variants are not targeted by NMD, given that inhibition of the NMD pathway via suppression of protein translation leads to a significant reduction in all three E2F1 transcripts.

It has been reported that RNA 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 and 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 (An and Grabowski, 2007; Lee et al., 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 in expression in response to KCl-induced neuronal activity. Thus, we analyzed the expression of the E2F1 splice variants following KCl treatment in primary rat cortical cells. Neither E2F1a nor the E2F1c variant exhibited statistically significant changes following a 4 hour exposure to elevated extracellular potassium levels (Figure 7C), although E2F1a showed a non-significant trend toward an increase. However, the expression of E2F1b was significantly increased, by roughly two-fold, following a 4 hour treatment with KCl. These data suggest that the

expression of the intron 5 retaining E2F1b transcript is selectively responsive to neuronal activity, which is consistent with the observation that it is absent in other tissues.

Given the findings thus far, we next resolved to express the E2F1 splice variants in cells from a plasmid. Following expression for 48 hours, we generated cDNA and PCR amplified E2F1 transcripts with primers targeting all three variants (exon 4 forward, exon 7 reverse). In PC-12 cells that were not transfected or were transfected with the empty vector, the E2F1a, E2F1b, and E2F1c variants were all detected (Figure 8A). When E2F1a was transfected into the cells, there appeared to be less E2F1b and E2F1c present. Similar results were seen when E2F1b was expressed, with apparent decreases in E2F1a and E2F1c expression, and when E2F1c was expressed, with apparent decreases in E2F1a and E2F1b. Next, we analyzed the protein expression of these transcripts via western blot. As previously discussed, the predicted proteins translated from the E2F1b and E2F1c splice variants are hypothesized to be C-terminal deletions. Thus, they should not be detectable using many of the conventional C-terminal E2F1 antibodies. As such, when these constructs were engineered, a FLAG tag sequence was included on the N-terminus of the E2F1 CDS to distinguish between endogenous E2F1 in the cell line and enable easier detection of the truncated E2F1 variants. As a control for the experiments, a vector containing a FLAG tagged Mitogen-activated protein kinase (MAPK) sequence was included in the transfections. Western blots for the FLAG sequence indicate robust expression of the MAPK construct as well as the E2F1a protein (Figure 8B). While expression was not as robust for the E2F1b and E2F1c variants, the blots do show clear expression of a FLAG immunoreactive protein at the expected size of the truncated proteins (Figure 8B). As expected, immunoblots for E2F1 using the KH-95 C-terminal antibody showed endogenous expression of E2F1 in all experimental conditions, with an increase in E2F1 protein levels in cells transfected with the plasmid expressing E2F1a. While further experiments would be necessary to more fully characterize the primary amino acid sequence of the proteins produced from the E2F1b and E2F1c variants (i.e. mass-spec protein sequencing), these data provide evidence that, at least in this experimental paradigm, the E2F1 variants can be translated into a protein and detected via immunoblot.

Discussion

In the present study, we have identified two novel E2F1 transcripts: E2F1b which retains intron 5, and E2F1c which excludes exon 6. Similar to the E2F1a transcript, which consists of 7 exons, the alternatively spliced E2F1 transcripts appear to be expressed ubiquitously throughout the brain. On the other hand, E2F1b and E2F1c were not detected in other organs, despite the expression of E2F1a. This finding suggests a functional significance for the E2F1 splice variants within the CNS. Indeed, these E2F1 transcripts are present in a number of different CNS cell types. However, the alternatively spliced E2F1 transcripts are unlikely to be indirect byproducts of a quiescent cell cycle machinery in these terminally differentiated cell types, given their absence in Rat2 fibroblasts regardless of cell cycle stage. As primary cortical neuroglial cells mature *in vitro*, all three E2F1 transcripts exhibit decreased expression after the third week of culture. In contrast, the E2F1c variant exhibits a different pattern of expression during the maturation of hippocampal cells. Interestingly, pharmacologic manipulations leading to the suppression of protein translation and NMD

inhibition significantly reduced the levels of all three E2F1 transcripts. Lastly, elevation of neuronal activity by increasing the extracellular concentration of potassium chloride selectively increased the expression of E2F1b in the cortical cells, further suggesting that the alternatively spliced E2F1 transcripts may have distinct functional roles in the brain.

It is noteworthy that the PC-12 cell line is derived from the rat adrenal medulla which originates from the neural crest and thus contains neuroblastic cells. As such, our observation of the variants in PC-12 cells remains in keeping with our data suggesting that the variants may be specific to the CNS. When E2F1a was transfected into cells, there appeared to be less E2F1b and E2F1c present. Similar results were seen when E2F1b was expressed, with apparent decreases in E2F1a and E2F1c expression, and when E2F1c was expressed, with apparent decreases in E2F1a and E2F1b. Next, we analyzed the protein expression of these transcripts via western blot. As previously discussed, the predicted proteins translated from the E2F1b and E2F1c splice variants are hypothesized to be C-terminal deletions.

E2F1 has classically been described as a transcription factor that regulates cell cycle progression and apoptosis (Biswas and Johnson, 2012; Degregori and Johnson, 2006). However, E2F1 was recently shown to possess a novel function in the CNS, as mice lacking functional E2F1 were shown to exhibit age-dependent behavioral deficits and synaptic disruptions (Ting et al., 2014). Furthermore, E2F1 exhibits altered sub-cellular distribution in neurons where it is found predominantly in the cytoplasm, as opposed to nuclear localization in cycling cells (Strachan et al., 2005; Ting et al., 2014; Wang et al., 2010). The unique localization of E2F1 in neurons may be due, in part, to the presence of the alternatively spliced transcripts. For instance, targeted distribution of some mRNAs to the synapse by RNA-binding proteins (RBPs) is necessary for immediate local de novo protein synthesis at the synapse during neuronal stimulation (Kiebler et al., 2013). Targeted binding to mRNA by RBPs is often triggered by unique cis-acting motifs within the alternatively spliced transcripts (Buckley et al., 2014). The presence of alternatively spliced E2F1 transcripts may harbor a signal for targeted distribution of these mRNA to specific compartments to be locally translated, particularly the synapses. Differentially spliced transcripts can also lead to the translation of different protein products with modified functions. For example, context-dependent splicing of potassium calcium-activated channel subfamily M alpha 1 (*KCNMA1*), can yield similar ion channel receptor proteins with functional and physiologic differences (Navaratnam et al., 1997; Rosenblatt et al., 1997). Likewise, alternative splicing of E2F1 in the brain may create diversity in protein function and sub-cellular localization.

The intron 5 retaining E2F1 transcript identified in this study may contribute to determining E2F1 protein localization. Recent evidence has shown that intron retention serves as a critical post-transcriptional regulatory step that contributes to the targeting of RNA transcripts to specific sub-cellular compartments to subsequently undergo further RNA processing within that compartment (Buckley et al., 2014). Intron retaining transcripts have been reported to be abundantly enriched in dendritic processes of 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 brain cytoplasmic (BC1) RNA. The BC1 RNA is found predominantly in the neuronal cytoplasm, particularly in neuronal processes. Further, the structure of the BC1 5' domain is necessary and sufficient for RNA dendritic targeting (Muslimov et al., 2006; 1997). Many CIRTs present in hippocampal dendrites carry the repetitive ID element, a class of SINE that shares high homology to the 5' domain of the BC1 RNA. 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 identified an ID element embedded in intron 1 of the E2F1 gene, in addition to a number of different sequences with greater than 67% sequence similarity to previously identified SINEs (several such sequences were also identified in E2F1 intron 4), though we were unable to detect the presence of any E2F1 transcript retaining intron 1 or intron 4. Nevertheless, the presence of an ID element in intron 1 may alter the splicing of the E2F1 gene and thereby lead to expression of the E2F1b and E2F1c variants. Alternatively, it remains possible that the ID element retaining E2F1 transcript is only abundant in the hippocampal dendrites and therefore below the limit of detection in whole cell RNA isolations used in our experimental paradigm. Indeed, our identification of an E2F1 intron-retaining transcript may provide a potential mechanism for 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 E2F1b variant may similarly carry a sequence targeting this mRNA to the synapse where it is subsequently translated, thus explaining our previous observations of E2F1 protein present in 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 transcript. For example, knockdown of intron retaining high conductance potassium channel 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 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 E2F1b may contain regulatory elements that further influence the distribution of E2F1 protein or the splicing of the E2F1c variant, though further experiments would be necessary to understand this process. With respect to the sub-cellular localization of these alternatively spliced E2F1 transcripts, efforts are currently underway to determine whether these E2F1 transcripts are enriched in neuronal processes.

Aside from modifying RNA sub-cellular localization, alternative splicing in neurons can also have a direct impact on protein function through production of similar proteins with slight modifications (Zheng and Black, 2013). For example, cell cycle related protein telomere repeat-binding factor 2 (TRF2) is alternatively spliced, such that a portion of exon 7 is deleted from the mRNA. This splice variant is only present in fully differentiated neurons, indicating the splicing event is under developmental regulation. Furthermore, this exon skipping transcript is translated into a truncated TRF2 protein, which has a different sub-cellular localization and interacting partner than the full length TRF2 protein (Zhang et al., 2011). Similarly, the mRNA sequence for the E2F1b and E2F1c variants are predicted to

produce truncated E2F1 proteins that are functionally distinct from E2F1a as a result of an inserted stop codon. Our data showing a significant decrease in the expression of E2F1 transcripts following NMD inhibition suggests the E2F1b and E2F1c transcripts containing premature stop codons are not simply degraded as nonsense mRNA. Consistent with this interpretation, expression of vectors containing the E2F1b and E2F1c transcripts produced similar sized truncated E2F1 proteins lacking the C-terminal domains.

In conclusion, we have identified two novel CNS-specific E2F1 transcripts present in neurons and astrocytes. Experiments using translation inhibition to disrupt nonsense-mediated decay in neuroglial cell culture showed a significant decrease in the expression of all three E2F1 transcripts. The results show the E2F1 transcript variants display distinct expression profiles as primary neuroglial cells mature and in response to neuronal activity *in vitro*. Furthermore, our data suggest these splice variants are functionally significant and may contribute to the non-classical function and sub-cellular localization that has been well-described for E2F1 in the CNS. Future studies will investigate the potential role for these variants in neuronal development and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
qPCR	quantitative real-time PCR
TBP	tata-box binding protein
HRP	horseradish peroxidase

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Highlights

- CNS-specific E2F1 splice variants were identified in rat tissue
- E2F1b is an intron 5 retaining transcript variant
- E2F1c is a variant that lacks exon 6
- E2F1b and E2F1c proteins are c-terminal truncations due to pre-mature stop codons

Figure 1.

PCR amplification of E2F1 alternative splice variants from rat cortex. **A.** Amplification of E2F1 variants using primers against exons 1 and 7, note three different product sizes (arrows) corresponding to E2F1a (middle), E2F1b (top) and E2F1c (bottom). Low (left) and high (right) contrasts of the same gel are shown. DNA from all three bands was gel extracted and confirmed by DNA sequencing. **B.** Amplification of E2F1 variants in rat cortex with primers against exons 4 and 7, showing three different size products: E2F1a (middle), E2F1b (top) and E2F1c (bottom). Gels from two separate experiments are shown to convey variation in relative E2F1 variant abundance. DNA from all three products was gel extracted and verified by sequencing. **C.** PCR targeting E2F1b only, using a forward primer specific to intron 5 and a common reverse primer. **D.** PCR specifically targeting E2F1b, using a common forward primer and a primer specific for intron 5. All gels are representative of PCRs from at least $n = 4$ biologically different samples. **E.** PCR with a forward primer spanning the exon 5/6 junction and the exon 7 reverse primer, amplifying E2F1a (lane 1), or with a forward primer spanning the exon 5/7 junction (only present when exon 6 deleted) and the exon 7 reverse primer, amplifying E2F1c (lane 2).

Figure 2.

DNA sequencing of PCR products from figure 1A/B (**A and C**) and figure 1C (**B**), aligned to (UCSC genome browser) reference sequences for E2F1a, E2F1b, and E2F1c variants. Accurately aligned sequencing is represented by the red-filled bar. Each sequencing alignment represents a biological sample ($n = 3$ total) and is a PCR product that was visualized on agarose gel and corresponded in size to the indicated products. All sequencing (each individual bar) reads were obtained from a single unbroken sequencing reaction with a single primer and a single DNA sample. **A.** Sequencing of gel extracted PCR products from reactions with primers against E2F1 exons 1 and 7. Gaps in sequencing of a particular product are represented by open space on the bar (outlined with blue box), indicating absence of that sequence in the sequencing reaction. **B.** Sequencing of gel extracted products (as in **A**) from PCR reactions with primers against E2F1 exons 4 and 7. Product size was consistent with that for E2F1b. Note that the product was sequenced in both the forward (upper) and reverse (lower) directions. Alignments are to an E2F1 reference containing intron 5. Note the single continuous sequencing run through exon-exon junctions and the intron-exon junctions. **C.** Sequencing of gel extracted products (same PCR reactions as in **A**) aligned to the E2F1c reference sequence. The inverted red triangle (upper three bars, outlined with blue box) indicates the presence of a segment of DNA in the sequenced product, which is not present in the E2F1 reference sequence to which the reads are aligned.

In this case, the segment not present in the E2F1c reference sequence (yet present in the PCR product) is identical to E2F1 exon 6, as expected for E2F1a (top three bars of sequencing).

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Figure 3.

Visual depiction of E2F1 protein mRNA for three rat E2F1 variants: E2F1a, 1299 bp including exons 1–7, E2F1b (intron 5 retention), 1392 bp including exons 1–7 plus intron 5, and E2F1c (exon 6 deletion), 1082 bp including exons 1–5 and exon 7. Splice variant exon/intron (E2F1b) and exon/exon (E2F1c) junctions are in yellow. Above each mRNA is the predicted protein product. The pRb-binding domain is indicated by the navy colored box within the transactivation domain. E2F1a protein is the full 432 residues in length. However, the E2F1b variant is 283 residues in length due to a premature stop codon inserted by the intron retention. Retention of intron 5 results in a five amino acid sequence (VRLQT-stop) following exon 5, which is unique to this variant. The E2F1c variant is 284 residues in length, with a premature stop codon (due to a frameshift caused by loss of exon 6) present in exon 7. The E2F1c variant contains a six amino acid sequence (KLYCLE-stop) following exon 5, which is unique to this variant.

Figure 4.

Detection of E2F1 variants in tissue **A.** PCR amplification of E2F1 variants in indicated tissues, using exon 1 and 7 primers. Products visualized on agarose gel at low (top) and high (bottom) contrast. Note the presence of three separate products in the cortex sample corresponding to E2F1a, E2F1b, and E2F1c variants. Only E2F1a is present in the other tissues. **B.** PCR targeting only the E2F1b variant (exon 1 and intron 5), only detected in cortex. **C.** PCR for E2F1 variants as in **A**, using exon 4 and 7 primers. Note three bands present in the cortex sample, corresponding to E2F1a, E2F1b, and E2F1c variants. Only E2F1a was detected in all other tissues. All gels are representative of PCRs from at least $n = 4$ biological samples. PCRs performed on cDNA generated from DNase digested RNA. All PCR products for E2F1b and E2F1c variants were gel extracted and validated by DNA sequencing. Equal loading of RNA for cDNA synthesis and subsequent PCR reactions was confirmed by a PCR reaction for GAPDH (data not shown).

Figure 5.

E2F1 variants among neuronal cell types. **A.** E2F1 variants amplified with common primers (exons 4 and 7) or primers specific to E2F1b (intron 5, exon 7). **B.** Only E2F1a was detected in Rat2 fibroblasts when cells were asynchronous or arrested in G₀/G₁ phase, S phase, and M phase. Full length genomic E2F1 sequence amplified via PCR using genomic DNA; banding pattern was distinct from the three transcripts present in cortical neurons. PCR for GAPDH (bottom) indicates equal loading between samples. Images are representative of n = 3 separate experiments.

Figure 6.

qPCR characterization of E2F1 variant expression in culture. **A.** E2F1 variant fold-change (relative to DIV 7) in cortical neuroglial cultures over time. E2F1 expression normalized to TBP expression. Significance indicated by: * $p < 0.05$ compared to DIV 7, # $p < 0.05$ compared to DIV 14 **B.** E2F1 variant fold-change (relative to DIV 0) in hippocampal neuroglial cultures over time. E2F1 expression normalized to TBP. * $p < 0.01$ compared to DIV 0, # $p < 0.01$ compared to DIV 7. (**A, B**) $n = 4$ for all experiments, all data are mean $\pm$ SEM. Significance analyzed by ordinary one-way ANOVA with Tukeys multiple comparisons test.

Figure 7. qPCR analysis of E2F1 variant expression in DIV 14 rat neuroglial cultures following (A) protein translation inhibition, (B) ER stress, or (C) KCl treatment. **A.** E2F1 variant expression in hippocampal cultures following treatment with 5 μ M cycloheximide or 25 μ M anisomycin. **B.** E2F1 variant expression in hippocampal cultures after treatment with 1 μ M thapsigargin. **C.** E2F1 variant expression in cortical cultures following treatment with 20 mM KCl. (A, B, C) $n = 4$ for all treatments, all data are mean $\pm$ SEM. Variant expression normalized to TBP. Significance relative to vehicle indicated by * $p < 0.05$, ** $p < 0.01$. Significance analyzed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons.

Figure 8.

Expression of E2F1 variants in PC12 cells and HEK293T cells. **A.** Expression of E2F1 variants after transfection with indicated constructs in PC 12 cells. E2F1 amplified via PCR reactions with exon 4 and 7 primers. Transfection with E2F1 variants (indicated above lane) led to increased expression of the transfected variant. **B.** Western blot of E2F1 variant protein expression in HEK293T transfected with indicated FLAG-tagged constructs.

Table 1.

PCR and qPCR primers used in this study are listed, with information regarding region targeted by the primer.

Primer Name	Gene(s) Amplified	Sequence 5' – 3'	Target	Use	Fig. SI color
E2F1 Exon 1 Forward	All E2F1	CTCGAGATCCTGACGTGCTGCT	Exon 1	RT-PCR	Black
E2F1 Exon 4 Forward	All E2F1	GATCGGTACAGCGGCTTGAAGGC	Exon 4	RT-PCR	Black
E2F1 Exon 7 Reverse	All E2F1	GCCTCATGGGGTGGGAAAGGC	Exon 7	RT-PCR	Blue
E2F1a Forward	E2F1a	ATTCTGCAGAGACATTCAGAT	Spans Exon 5/6	RT-PCR	Orange
E2F1b Forward	E2F1b	TTCAGACCTGAAGCCTGACCAGG	Intron 5	RT-PCR	Orange
E2F1b Reverse	E2F1b	CTCTGTCCTGGTCAGGCTTCAGG	Intron 5	RT-PCR	Orange
E2F1c Forward	E2F1c	ATTCTGCAGAGAAGCTGTATTG	Spans Exons 5/7	RT-PCR	Orange
E2F1 Common Forward	All E2F1	TGTGACCTGCCAAGATCTCC	Exon 5	qRT-PCR	Red
E2F1a qPCR Reverse	E2F1a	GATCTGAAATGTCTCTGCAG	Spans Exon 5/6	qRT-PCR	Green
E2F1b qPCR Reverse	E2F1b	CTCTGTCCTGGTCAGGCTTC	Intron 5	qRT-PCR	Green
E2F1c qPCR Reverse	E2F1c	GCAATACAGTTCTCTGCAG	Span Exon 5/7	qRT-PCR	Green
TBP Forward	TATA-Box binding protein	GCTCGTTTTGTCCCCACATC		qRT-PCR	not shown
TBP Reverse	TATA-Box binding protein	TTCACATCACAGCTCCCCAC		qRT-PCR	not shown
GAPDH forward	GAPDH	GGCTCATGACCACAGTCCAT		RT-PCR	not shown
GAPDH reverse	GAPDH	CCCCTCCTGTTGTATGGGG		RT-PCR	not shown