Cloning and Characterization of Mouse E2F8, a Novel Mammalian E2F Family Member Capable of Blocking Cellular Proliferation.

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Running Title: E2F8 overexpression blocks cellular proliferation

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The E2F transcription factor family plays a crucial and well-established role in cell cycle progression. Deregulation of E2F activities in vivo leads to developmental defects and cancer. Based on current evidence in the field, mammalian E2Fs can be functionally categorized into either transcriptional activators (E2F1, E2F2 and E2F3a) or repressors (E2F3b, E2F4, E2F5, E2F6 and E2F7). We have identified a novel E2F family member, E2F8, which is conserved in mice and humans and has its counterpart in Arabidopsis thaliana (E2Ls). Interestingly, E2F7 and E2F8 share unique structural features that distinguish them from other mammalian E2F repressor members, including the presence of two distinct DNA-binding domains and the absence of DP-dimerization, Retinoblastoma-binding, and transcriptional activation domains. Like E2F7, over-expression of E2F8 significantly slows down the proliferation of primary mouse embryonic fibroblasts. These observations, together with the fact that E2F7 and E2F8 can homodimerize and are expressed in the same adult tissues, suggest that they may have overlapping and perhaps synergistic roles in the control of cellular proliferation.

The E2F transcription factor family plays a crucial and well-established role in the regulation of cellular proliferation differentiation and apoptosis. While E2Fs can act as transcriptional activators, they can also repress gene expression when bound to the Retinoblastoma (Rb1) tumor suppressor protein or related pocket proteins (p130 and p107). Rb1 negatively regulates the E2F transcriptional activity by binding to and masking the transactivation domain of E2F (1, 2). When bound to E2F, Rb1 can also directly repress E2F target genes by recruiting chromatin remodeling complexes and histone modifying activities to the promoter (3-9). During the G1-S transition of the cell cycle, the cyclin dependent kinases (cdks) phosphorylate Rb1 resulting in the release of E2F from Rb1 containing complexes and the expression of E2F target genes. Many studies indicate E2F as a key mediator of Rb1 function in cellular proliferation, differentiation and apoptosis (10-12). E2Fs are also regulated at the level of transcription, post-translational modifications, subcellular localization, association of co-factors and degradation (11, 12).

Since the identification of the founder E2F family member, E2F1, six additional family members have been identified. Structurally, E2F1-6 share a highly conserved DNA-binding and dimerization domain. E2F1-6 bind DNA target sequences as heterodimers with DP1 or DP2. Although heterodimerization is required for high affinity sequence specific DNA binding, the specificity of the dimer is determined by the E2F component (10, 12). The most recently identified E2F family member, E2F7, is unique in having a duplicated conserved E2F-like DNA-binding domain and in lacking a DP-dimerization domain. E2F1-5 possess conserved transactivation and pocket protein-binding domains at the C-terminus.
which are absent in E2F6 and E2F7 (10, 14-16). Based on structural and functional considerations, E2Fs have been classified as either ‘activator’ (E2F1, E2F2, E2F3a) or ‘repressor’ E2Fs (E2F3b, E2F4, E2F5, E2F6, E2F7). Activator E2Fs are maximally expressed late in G1 and can be found in association with E2F regulated promoters during the G1-S transition, coinciding with the activation of many E2F-target genes. Mouse embryonic fibroblasts ablated for all the three activator E2Fs are severely compromised in E2F-target gene expression as well as the capacity to proliferate, underscoring the importance of the activator subclass in cell cycle progression (28). In contrast to the activator E2Fs, E2F3b, E2F4 and E2F5 are expressed in quiescent cells and can be found associated with E2F-binding elements on E2F-target promoters during G0 (12, 17-19). Among the repressor subclass, E2F6 mediates repression via recruitment of the polycomb group of proteins or complexes containing Mga and Max proteins (20, 21). While the most recently identified E2F7 protein can function as a repressor independent of DP interaction, its exact mechanism of mediating repression is not understood (14-16).

E2Fs regulate the transcription of a multitude of genes involved in DNA replication, cell cycle regulation, chromatin assembly and condensation, chromosome segregation, DNA repair, and checkpoint control (10-13). Consistent with its role in various important cellular processes, E2Fs are evolutionarily conserved among plants and animals with the exception of yeast. *Drosophila melanogaster* has two E2F genes namely dE2F1, which acts as an ‘activator’ E2F, and dE2F2, which represents a ‘repressor’ E2F. Each of these E2Fs heterodimerize with a single DP protein found in *Drosophila* called dDP (22). E2F like activities have also been found in *Xenopus levis* and *Caenorhabditis elegans* (23, 24). In the plant, *Arabidopsis thaliana*, six E2Fs (AtE2Fa-f) and two DPs (AtDPa-b) have been described. Of these, AtE2Fa-c are reminiscent of the mammalian activator E2F1-3a subclass and E2Fd-f resemble the recently discovered E2F7 in that they possess a duplicated DNA-binding domain and exhibit repressor function. Besides human and mouse, *Arabidopsis* is the only other species where E2Fs with duplicated DNA binding domain have been described (25, 26).

Here we report the identification and characterization of yet another mouse E2F family member that we call E2F8, which closely resembles E2F7. E2F8 is expressed in a cell-cycle-regulated fashion, has duplicated DNA-binding domains that are essential for binding to consensus E2F binding DNA elements, represses E2F-target genes and negatively influences cellular proliferation.

Materials and Methods

Cloning of the E2F8 cDNA- The mouse testis cDNA library (Origene) was screened using the primer pairs 5’- AGTACCCCAACCTGCTGAATA and 5’- GGACTTGTCTTTGCGGCTGTTTAC; 5’- CCGGCACACCTCCACAAAAAC and 5’- TCCCCGCGTAGAGAAGAGG. Each primer pair resulted in a single sharp band in three wells that were positive out of 96 wells in the master plate. Subsequently, we screened the subplates to obtain the clone with the full length E2F8 cDNA. We subcloned a 5X Myc-tag in between the BamH1 and EcoR1 sites of the pcDNA3.1/HisB (Invitrogen) vector in frame with the 6X Histidine tag. Next we subcloned the 2583 bp open reading frame of E2F8 in frame with the His and Myc tags, thus generating a construct with full length E2F8 ORF tagged with 6X His and 5X Myc-tag at the N-terminus.

Generation of Mouse Embryonic Fibroblasts and in vitro cell culture- Primary MEFs were generated using standard techniques as described before (28). All cell lines were grown in DMEM with 15% FBS. Cells were starved in DMEM containing 0.2% FBS when they were about 70% confluent. After starving for 48-60 hours they were stimulated to grow with DMEM containing 15% FBS. Cells were harvested at different time points after serum stimulation for BrdU incorporation assay, Northern blot assay and Real-time RT-PCR.

BrdU incorporation assay- BrdU was added to the media and cells were incubated with BrdU for 2 hours before harvesting and fixing them in Methanol/Acetone 1:1. We stained cells in 35mm dishes with anti-BrdU primary antibody (NA-61 from Oncogene) and anti-mouse Rhodamine secondary antibody and counterstained with 6-
diamidino-2-phenylindole (DAPI). At least 400 cells / 35mm plate were counted.

**Northern Blot Assay**- For the cell cycle Northern Blot analysis, total RNA was isolated from MEFs using TRIzol (GibcoBRL), and mRNA was subsequently purified using PolyATtract mRNA isolation system (Promega). PolyA mRNA was separated on a 1% agarose gel containing 6% formaldehyde and transferred onto a Gene Screen membrane (NEN Life Science Products). The mouse tissue Northern Blot was purchased from Origene. The 3’ 1850bp of the E2F8 cDNA including the 3’UTR was used as a probe for both the Northern Blot analyses. The probe was radiolabeled with 50 µCi [α-32P]dCTP using Prime-It RmT (Stratagene). Hybridization was carried on overnight under high stringency conditions (5x SSPE, 50% Formamide, 5x Denhardts, 1%SDS at 42 °C) and washed several times (0.2xSSC, 0.2%SDS, at 65 °C) before autoradiography.

**Real-Time RT-PCR**- Approximately 1x10^6 cells were harvested at the indicated time point and total RNA was isolated using the Qiagen RNA miniprep column as described by the manufacturer, including a DNase treatment before elution from the column. Reverse transcription of 2ug of total RNA was performed by combining 1ul of Superscript III reverse transcriptase (Invitrogen), 4ul of 10X buffer, 0.5ul of 100mM oligo dT primer, 0.5ul of 25mM dNTPs, 1.0ul of 0.1M DTT, 1.0ul of RNase Inhibitor (Roche) and water up to a volume of 20ul. Reactions were incubated at 50° for 60 minutes and then diluted 5 fold with 80ul of water. Real-time RT-PCR was performed using the BioRad iCycler PCR machine. Each PCR reaction contained 0.5ul of cDNA template and primers at a concentration of 100nM in a final volume of 25ul of SYBR Green Reaction Mix (BioRad). Each PCR reaction generated only the expected amplicon as shown by the melting-temperature profiles of the final products and by gel electrophoresis. Standard curves were performed using cDNA to determine the linear range and PCR efficiency of each primer pair. Reactions were done in triplicate and relative amounts of cDNA were normalized to GAPDH. The sequences of the primer pair used for the E2F8 cDNA were 5’-CCGGCACAACCTCAACAAAAAC and 5’-TCCCCCGCGTAGAGAAGAGG. Primer sequences of the E2F target genes are available upon request.

**5’ RACE PCR**- Total RNA was isolated from WT primary Mouse Embryonic Fibroblasts (MEFs) and mouse thymus; and the mouse testis cDNA was purchased in the Marathon-Ready cDNA kit (Clontech). cDNA was prepared and 5’RACE PCR was performed using the BD SMART RACE cDNA Amplification Kit (BD Biosciences) following the manufacturer’s protocol. The reverse primer used for 5’ RACE PCR were TACAGCGTAAGGACTTTGCTTTTG mapping to the exon 6 of E2F8 gene and the nested primer was CGTCCCCGAGGGTTTGTGAGGT located in the exon 5 of the E2F8 gene. The products of 5’ RACE PCR were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen). At least 20 colonies from each tissue type were sequenced.

**Luciferase Reporter Assay**- A 3.5 Kb promoter fragment was isolated from the BAC clone RPCI 24-294G9 and subcloned into pBluescript vector. The primer pair used for amplifying the long promoter fragment (LP) was 5’-GAGAGAGGTACCGTCTCTCCCAACCCCTCGTT TG and 5’-AGAGAGAGCTTTGCTGAAGTTTCTCCTTG ACAC and that for amplifying the short promoter fragment (SP) was 5’-GAGAGAGGTACCAGCTCTGAAGGAGGATT GACAGG and 5’-AGAGAGAGCTTTGCTGAAGTTTCTCCTG ACAC. The amplified fragments were subcloned into the pGL2Basic vector using the HindIII and Kpn1 restriction sites.

The two E2F binding consensus sites in the E2F8 promoter fragment were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer. The mutations introduced are depicted in Fig. 4a. All constructs were confirmed by sequencing.

Subconfluent REF52 cells grown in triplicates were transfected using the Superfect reagent (Qiagen). The cells were transfected with the firefly luciferase expression vectors and thymidine kinase (TK) Renilla luciferase as internal control. Then the cells were serum starved in DMEM containing 0.2% FBS for 48 hours and stimulated to enter the cell cycle by DMEM containing 15% FBS. Cells were harvested at the
indicated time points and luciferase was detected by the Dual-Luciferase Reporter Assay System (Promega).

**Western Blot analysis**—Cell protein lysates were separated in SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Blots were first incubated in blocking buffer (10% skim milk in Tris-buffered saline-Tween) for one hour and subsequently incubated overnight in blocking buffer containing the antibody specific for myc-tag 9E10 (Santa Cruz, SC-40). The primary antibody was then detected using horseradish-peroxidase-conjugated secondary antibody and ECL reagent as described by the manufacturer (Amersham).

**Immunofluorescence**—MEFs were grown in 35mm dishes and transfected with His-myc-tagged E2F8 or control vector. The cells were fixed with 4% paraformaldehyde and Methanol/Acetone (1:1). The myc antibody 9E10 (Santa Cruz, SC-40) was the primary antibody used and rhodamine-conjugated anti-mouse IgG (Vector Laboratories) was used as the secondary. The cells were counterstained with DAPI. The procedure for the staining was same as described before (14).

**Electromobility Shift Assay for DNA binding**—The probe used for the DNA binding assay was a fragment of the Adenoviral E2 promoter containing two E2F binding sites. The complimentary strands of the probes were biotinylated on the 5’ end and were annealed together to make a double stranded probe end labeled with Biotin. The sequence of the wild-type probe was 5’-TCGAGACGTAGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTAGTCCTTAACTGCA and that of the mutated probe was 5’-TCGAGACGTAGTTTTAAGGCGCTTAAATTTGAGAAAGGGCTTGAAACTAGTCCTTAACTGCA. The binding reaction was carried out in a 20ul volume using 40 fmoles biotinylated probe and 8 pmoles of non-biotinylated WT or mutated probe as and when required. The binding conditions were the same as described before (16). The Supershift analysis was carried out as previously described using an antibody specific against myc (Santa Cruz, SC-40). Proteins were translated using the TNT Quick Coupled Transcription/Translation System (Promega). After carrying out the binding reaction at 30°C for 30 minutes, Ficoll was added to it to a final concentration of 4% and it was separated on a 4% Polyacrylamide gel. After running, it was transferred to Hybond N+ membrane (Amersham) and UV crosslinked. Then it was probed using the LightShift™ Chemiluminescent EMSA Kit (Pierce) following manufacturer’s protocol. The chemiluminescence was detected on Hyperfilm (Amersham).

**Site directed mutagenesis of the DNA binding Domains**—Site directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s protocol. We used the following primers for DBD1 5’-CGGAAGGAGAAGAGCGAATTCTTGCTATGCACAA and 5’-TGGTGGCATAGCAAGAATTCGCTCTTCTCCTTCCG; and the following for DBD2 5’-CGCAAAGACAAGTCCGAATTCGTGATGACCCAGAAG and 5’-CTTCTGCTCATCAGAATTGGACTTGGCTTCCG.

**Structural Analysis**—Sequence alignments of the E2F8 DNA binding domains by the ClustalW method were used to generate models for E2F8 DNA binding (34). Modeling requests were submitted to the SWISS-MODEL protein modeling server using the previously solved E2F4/DP2 crystal structure PDB file (1CF7) as the template.

**Co-Immunoprecipitation**—Transiently transfected 293 cells were harvested in cold PBS and cell pellets were lysed in 10 times volume of lysis buffer (0.05M sodium phosphate PH7.3, 0.3M NaCl, 0.1% NP40, 10% glycerol with protease inhibitor cocktail). Lysates were incubated with protein G Plus/ Protein A Agarose beads (Calbiochem) at 4°C for 1hour to preclear. The precleared lysates were incubated with appropriate antibody overnight; protein G Plus/ Protein A Agarose beads were added and incubated for 1 hour at 4°C. Protein binding to the beads were released and resolved by SDS-PAGE, followed by immuno blotting. Immunoprecipitation and Immunoblotting was performed using M2 monoclonal anti-Flag (Sigma), anti-HA (Roche), anti-Myc 9E10 (Santa Cruz Biotechnology) antibodies.

**Retroviral Infection**—Full-length cDNAs for His-Myc tagged E2F8 was subcloned into the pBABE
retroviral vector containing a hygromycin-resistance gene. High-titer retroviruses were produced by transient transfection of retroviral constructs into the Phoenix-Eco packaging cell line as described previously (33). MEFs were infected with the retrovirus using standard methods and selected in the presence of 200 \mu g \text{ ml}^{-1} \text{ hygromycin.}

**Proliferation Assay**- MEFs were plated at 4 x 10^4 cells per 60-mm plate and grown in DMEM with 15%FBS. Duplicate plates were counted daily using a Beckton Dickson Coulter Counter and were replated every 72 hours at the same density of the initial plating.

**RESULTS**

**Identification and Cloning of mouse E2F8**- We performed a homology search across the sequenced mouse genome (Celera and GenBank\textsuperscript{TM} Databases) using the E2F3 DNA-binding domain (DBD) amino acid sequence as the reference. The search retrieved the known E2Fs (E2F1-7) and a potentially novel E2F gene, which we named E2F8. The in silico predicted E2F8 protein (Celera and GenBank\textsuperscript{TM} Databases) possesses two E2F like DBDs but shares no homology to any other known domains conserved across the E2F family members. We also performed a BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) using the predicted E2F8 transcript as a query. The search recovered several mouse ESTs, from a variety of mouse tissues and developmental stages (UniGene Cluster Mm.240566). Using E2F8 specific primers, we screened the mouse testis cDNA library (Origene Technologies Inc.) and retrieved the full length mouse E2F8 cDNA clone.

Analysis using the UCSC Genome Browser – BLAT tool (29) revealed that the mouse E2F8 gene is located on chromosome 7 and contains 13 exons separated by 12 introns (Fig. 1A). We sequenced a BAC clone containing the E2F8 gene to determine the sequences across the splice junctions. From 5' to 3' end of the gene, the 12 splice sites have the universal consensus splice junction dinucleotides GT/AG.

The first initiation ATG, as predicted by the presence of a Kozak consensus sequence and an in-frame termination codon, is in exon 2 and the stop codon TGA is in exon 13, giving rise to a 2583 bp open reading frame, encoding 860 amino acids (Fig. 1B). E2F8 mRNA has a 300 bp long 5' untranslated region (UTR) and a 587 bp long 3' UTR. There are two consensus polyadenylation signals- AATAAA and ATTAAA, which are 327 and 238 bp upstream of the poly(A) tail respectively. The polyadenylation signal that is relevant in this particular clone is the non canonical polyadenylation signal TATAAA located 18 bases upstream of the poly(A) tail (30).

**E2F8 Protein Characteristics and Homology with the Other E2Fs** - The E2F8 protein consists of 860 amino acids with a predicted molecular mass of ~95Kd. It has two E2F like DBDs and three putative nuclear localization signals (NLS) (Fig. 1C, D). The presence of two E2F like DBDs is reminiscent of the mammalian E2F7 and Arabidopsis E2F-d-f. Alignment of the DBDs of mouse E2F1-8 and Arabidopsis E2F-d using the ClustalW program shows high homology with notable conservation of the RRXYDI DNA recognition motif (Fig. 2A). In spite of significant homology in the duplicated DBD, E2F8 is devoid of any other E2F-like domains including the pocket protein binding, transactivation and DP dimerization domains - a characteristic shared by mammalian E2F7 and Arabidopsis E2F-d-f proteins (Fig. 2C).

Phylogenetically, the DBDs of Arabidopsis E2F-d-f and mouse E2F7 and E2F8, cluster together (Fig. 2B). Significantly, when the full-length mouse E2F proteins are analyzed for evolutionary relationship on the basis of their primary structure, we also obtain a segregation pattern that is reflective of their known functional characteristics. The acquisition of additional E2Fs may stem from a developmental requirement for E2F activity in multiple different tissues as organisms evolve to be structurally and functionally more complex down the path of evolution (Fig. 2D).

**E2F8 expression pattern in tissues and over the cell cycle**- As a first step towards the understanding of E2F8 function, we investigated its tissue and cell cycle expression patterns. A 3'～2500 bp fragment of E2F8 cDNA, which lacked any significant sequence overlap with any of the other known E2Fs, was used to probe tissue-specific Northern blots. We found that E2F8 is highly expressed in the liver, skin, thymus and testis, but not in the brain, muscle and stomach (Fig. 3A). Interestingly, this pattern of expression...
is almost identical to that previously found for E2F7, giving rise to the possibility that E2F7 and E2F8 may have overlapping or complementary functions in these organs.

As discussed above, the known E2F family members fall into two distinct categories with regard to their patterns of expression during the cell cycle. Consistent with their function, expression of the activator subclass (E2F1, E2F2 and E2F3a) is maximal as cells enter the cell cycle, while the expression of the repressor subclass (E2F3b, E2F4 and E2F5) remains unchanged throughout all of the cell cycle. To determine whether the expression of E2F8 is cell cycle dependent, E2F8 mRNA levels were measured by Northern Blot and Real-time RT-PCR analysis in synchronized and cycling populations of primary mouse embryonic fibroblasts (MEFs). MEFs were synchronized in quiescence (G0) by starvation in media containing 0.2% FBS for 48 hours and then stimulated by the addition of media containing 15% FBS. RNA was isolated from cells harvested at different time points following serum stimulation. Poly-A mRNA was purified and subjected to denaturing electrophoresis as described in the methods section, transferred to membrane and hybridized to an E2F8-specific radioactive-labeled probe (Fig. 3B). Both the Northern blot and the Real-time RT-PCR analysis suggest that E2F8 expression is cell growth dependent, with maximal expression levels found during S phase (Fig. 3C). The serum-induced activation of E2F8 expression was mild when compared to the activation of Cdc6 expression, which is a well-established cell-cycle-regulated E2F target gene. This mild induction in E2F8 expression was not simply a consequence of an inability of primary MEFs to respond to serum, since E2F8 expression was also poorly induced in p53 mutant MEFs, which can be efficiently induced to enter the cell cycle in response to serum addition.  

**Regulation of the E2F8 Promoter**- In order to understand the basis of E2F8’s tissue specific and cell-growth dependent expression patterns we sought to confirm its transcriptional start site – the genuine 5’ end of the E2F8 gene. To this end we performed 5’ RACE (Rapid amplification of cDNA ends) PCR with mRNA isolated from the adult mouse thymus, testis and primary mouse embryonic fibroblasts (MEFs). Approximately 20 clones were analyzed from each tissue type, revealing the presence of three alternative first exons (exons 1a, 1b and 1c; Fig. 4A). As a reference, we labeled the first nucleotide of exon 1b as +1. All the three alternative first exons spliced precisely to the common exon 2, which contains the ATG of the full length E2F8 open reading frame. The universal splice junction dinucleotide GT/AG was present in each of the alternative first exons. Usage of either exons 1a or 1b preserved the full length open reading frame of E2F8. Usage of exon 1c introduces 2 short ORFs preceding the ATG of the full length E2F8 ORF, which could potentially produce proteins of 98 and 30 amino acids long. However, none of these ATGs fulfilled the Kozak criteria for efficient initiation of translational, and are most likely not used as initiating codons. Hence, the ATG located towards the 5’end of exon 2, which contains a perfect Kozak sequence, is predicted to represent the translational start site for the full length E2F8 ORF. Usage of the 3 alternative exons 1a, 1b or 1c gives rise to the same full length ORF.

To investigate the transcriptional regulation of E2F8 gene expression, we analyzed the putative E2F8 promoter region for the presence of consensus binding sites of different trans acting factors. Since E2Fs are known to be auto-regulated, it came hardly as a surprise to find two canonical E2F binding elements (‘a’ and ‘b’), at positions +257 and +385. We cloned a ~ 2.9-kb genomic fragment containing the −1993 to +855 region of the E2F8 gene into a firefly-luciferase transcriptional reporter plasmid, and called this construct the long promoter, or LP. A second E2F8 genomic fragment extending from +166 bp to +855 bp, which represents the most proximal sequence near exon 1c (Fig. 4A), was also cloned into the firefly-luciferase reporter plasmid, which we called the short promoter, or SP. We tested the promoter activity of these constructs in REF52 cells, using the TK promoter driven Renilla-luciferase plasmid as an internal control for transfection efficiencies.

Since both our reporter constructs contained the E2F DNA binding elements ‘a’ and ‘b’, we first sought to determine whether these constructs were responsive to the overexpression of E2F1 – an activator E2F. As expected, E2F1 overexpression led to a dose-dependent activation of luciferase activity from either the LP or SP
reporter constructs (Fig. 4B). We thus hypothesized that the observed growth dependent expression of E2F8 could be due to an E2F autoregulatory loop mediated via these putative E2F binding elements. To test this hypothesis, we analyzed the transcriptional activity of these reporter constructs in synchronized REF52 cell populations. We transfected our reporter constructs in REF52 cells and serum starved them in media containing 0.2% FBS for 60 hours in order to synchronize them in G0, and then re-stimulated the cells with media containing 15% FBS. As expected, expression from both the SP and LP constructs was significantly increased in the 18 hour re-stimulated samples indicating that the cell growth regulation of E2F8 is, at least in part, transcriptional in nature (Fig. 4C). To assess the role of the two consensus E2F binding elements in regulating the expression of E2F8, we mutated both the E2F binding elements ‘a’ and ‘b’ in the SP reporter construct (SP*ab) and reassessed their promoter activity in E2F1 overexpressing cells. As shown in Fig. 4E, mutation of the two E2F DNA recognition sites reduced but did not completely eliminate the E2F responsiveness of these reporters, indicating that additional non-consensus E2F target sites must also mediate their E2F responsiveness. To determine whether these consensus E2F sites played a role in the growth regulation of E2F8, we analyzed the activity of both the wild type (SP) and mutant SP reporter constructs (SP*b) in quiescent REF52 cells. Mutation of site b (SP*b) led to a small but reproducible two-fold increase in reporter expression, indicating that E2F8 expression in quiescent cells is likely repressed through an E2F dependent mechanism (Fig. 4D). While the expression of E2F8 is likely to be complex, the above data suggests that the E2F binding sites contribute to the positive and negative regulation of its expression during the cell cycle.

Subcellular localization and DNA binding activity of E2F8- In order to gain insight into the possible function of the E2F8 protein product, we overexpressed a Myc-tagged version of the murine E2F8 protein (Myc-E2F8) in MEFs and assessed its effect on cellular proliferation. Western blot analysis of Myc-E2F8 transfected cell lysates using Myc epitope-specific antibodies (9E10) revealed a protein product that migrated in SDS-PAGE with a mobility of approximately 115 kDa. Two additional Myc-E2F8 specific products of approximately 110 kDa and 82 kDa were also evident in these lysates, and probably represent degradation cleavage products of the full length protein (Fig. 5A). Consistent with the presence of three potential nuclear localization signals (NLS) immunofluorescence microscopy using anti-myc epitope antibodies revealed Myc-E2F8 to be completely localized to the nucleus (Fig. 5B).

All known E2F family members possess a highly conserved E2F DNA-binding domain that can mediate specific binding to consensus E2F DNA binding elements. The ability of in vitro translated Myc-E2F8 to bind the adenoviral E2 promoter fragment containing two intact E2F binding sites was tested by ElectroMobility Shift Assays (EMSA). As shown in Fig. 5C, in vitro translated Myc-E2F8 bound specifically to the E2 probe as indicated by the appearance of two distinct bands which were both supershifted with anti-Myc antibodies. In vitro translated Myc-E2F3/DP1 served as a positive control, and in vitro translated luciferase protein served as a negative control for DNA binding activity. The binding of E2F8 to the E2 probe was demonstrated to be specific since the binding could be efficiently competed with excess unlabeled E2 probe, but not with excess of a mutant E2 probe containing a 2 bp substitution within the E2F binding consensus site.

Like Arabidopsis E2Fd-f and mammalian E2F7, E2F8 contains two DBDs (referred to as DBD1 and DBD2 from N to the C terminus). This domain arrangement is identical to the recently described E2F7 protein, which binds DNA independently of DP (16). Given this similarity and the high degree of homology between E2F and DP DNA binding domains, we tested whether modeling of E2F8 would allow the DNA binding domains to adopt a structure homologous to E2F/DP binding as determined from the crystal structure of the E2F4/DP2 heterodimer (31). Sequence alignments by the ClustalW method and through the SWISS-MODEL server showed that DBD2 has a higher sequence homology to the DP DNA binding domain than DBD1. Based upon conservation of the residues involved in heterodimerization and binding of the E2F DNA consensus sequence, we were able to model E2F8 binding to DNA with DBD2 adopting the structure
of the DP binding partner. Our in silico analysis and model for DNA binding suggest that E2F8 binds DNA with DBD1 in the position of the E2F binding partner and DBD2 in the position of the DP binding partner (Fig. 5E).

To determine whether each domain directly contributes to E2F8 DNA binding activity, we have introduced point mutations in DBD1, DBD2 or both DBD1 and 2 (DBD1-2) that are predicted to disrupt DNA binding activity. The conserved leucine 118 and glycine 119 in DBD1, and leucine 266 and arginine 267 in DBD2 were replaced with glutamate and phenylalanine, respectively (Fig. 5E). These leucines contribute to the dimerization interface of the DNA binding domains and are conserved across all E2F family members as shown in Fig. 2A. In E2F1, the corresponding conserved leucine at position 132 is thought to make important heterodimerization contacts with DP, and its mutation abrogates DNA binding activity (31, 32). Disruption of these two amino acids in DBD1 or DBD2 of E2F8 completely abrogated its DNA binding capacity, indicating that the integrity of both the DBDs of E2F8 is important for its DNA binding function (Fig. 5D). These observations are consistent with our modeling of E2F8, which indicates that DNA binding is dependent upon dimerization interactions at both interfaces of DBD1 and DBD2 (Fig. 5E) (16, 26).

E2F8 forms homodimers- Previous work from LaThangue’s group demonstrated that the related E2F7 family member can form homodimers (16). To test the possibility that E2F8 could also oligomerize, we co-expressed Flag-tagged E2F8 and HA-tagged E2F8 in the 293 cells and assessed their ability to interact with each other by immunoprecipitation and immunoblotting using anti-Flag or anti-HA antibodies. To rule out any non-specific antibody interactions, singly transfected cells were also immunoprecipitated with either Flag-E2F8 or HA-E2F8 as controls (Fig. 6A). In this analysis, HA-tagged E2F8 could be detected in the anti-Flag immunoprecipitates derived from the doubly transfected cells, but not from the singly transfected HA-tagged E2F8 samples. Likewise, Flag-tagged E2F8 could be detected in the HA immunoprecipitates from doubly but not singly transfected samples. These data indicate that E2F8 can indeed form oligomers.

These findings raise the possibility that the inability of the E2F8 DBD mutants to bind to E2F consensus sites could be due to the disruption of E2F8 oligomerization. Co-immunoprecipitation assays demonstrated that the single (DBD1 or DBD2) or double (DBD1-2) DBD mutants of E2F8 still retained the capacity to oligomerize (Figure 6B, and data not shown). These results demonstrate that oligomerization of E2F8 is independent of its DNA binding activity.

E2F8 overexpression blocks cellular proliferation- E2Fs are thought to be critical players in orchestrating the control of cellular proliferation. To determine the potential role of E2F8 in the control of cellular proliferation, E2F8 was overexpressed in MEFs and proliferation was monitored over a period of seven days. To this end, primary MEFs were infected with retroviruses expressing Myc-E2F8, and transduced cells were selected for 48 hours in Hygromycin. Cells were then plated in media containing 15% FBS and viable cells were harvested and counted every 24 hours over a period of seven days. Relative to control-treated cells, MEFs over-expressing Myc-E2F8 proliferated considerably slower (Fig. 7A). Consistent with the observed growth retardation, the expression of E2F target genes in synchronized populations of Myc-E2F8 over-expressing cells was significantly reduced (Fig. 7B). Whether the inhibition of E2F target gene expression is a direct or indirect effect of E2F8 overexpression remains to be determined.

**DISCUSSION**

The E2F family members play important roles in cellular proliferation, apoptosis and differentiation in both Rb1-dependent and independent manners. E2F activities have been described in the vast majority of eukaryotes studied, ranging from plants to mammals, with the exception of yeast. Mammals have seven distinct E2F genes, with some family members encoding multiple related isoforms through differential promoter usage or alternative splicing. The identification of yet another mammalian E2F family member, E2F8, provides further complexity to the E2F family of transcription factors. Interestingly, E2F8 has the distinctive feature of possessing two tandem DBDs,
there is little amino acid sequence conservation between E2F8 and the other E2Fs.

E2F8 is an E2F member based on the presence of conserved DNA binding domains and its ability to bind to E2F consensus sites found in many E2F-regulated promoters. Structure modeling predicts that the duplicated DBDs of E2F8 can interact with each other to form a functional DNA binding unit, thus alleviating the requirement to interact with DP. Interestingly, this modeling predicts that the key conserved leucine residues, which are important for the interaction between the DBDs of the E2F and DP heterodimers, are also important for the intramolecular interactions between DBD1 and DBD2. This prediction is supported by our data demonstrating that introduction of point mutations at this conserved leucine residue in either of the E2F8 DBDs abrogates DNA binding activity. Co-immunoprecipitation assays demonstrate that E2F8, like E2F7, can form homodimers, suggesting that these two unique E2Fs could potentially form contacts with multiple consensus E2F sites at once.

E2F7 and E2F8 share a number of characteristics that could reflect their unique function. Each is expressed in a cell growth-dependent manner, with peak levels found during S phase, and are expressed in the same adult tissues of mice. Both have the ability to homodimerize, and to repress E2F-dependent gene expression. Importantly, their over-expression can lead to a pronounced decrease in the proliferative capacity of cells. These observations have led us to suggest that these two E2F family members may have overlapping and/or synergistic functions in the control of cellular proliferation. While the identification of E2F8 adds further complexity to the E2F family of transcription factors, our findings begin to place E2F members into distinct subclasses that have general structural and functional themes which might be used to differentially regulate cellular proliferation.

REFERENCES


**FOOTNOTES**

We thank Charles Bell for his advice in modeling E2F8 structure. This work was supported by grants from the National Institute of Health. F.G. was supported by T32 NIH postdoctoral fellowship and G.L. is a Leukemia and Lymphoma Society Scholar.

1 The abbreviations used are: Rb, retinoblastoma; DBD, DNA-binding domain; DHFR, dihydrofolate reductase; TK, thymidine kinase; DAPI, 4,6-diamidino-2-phenylindole; Pol α, polymerase α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame; BrdU, 5-bromo-2′-deoxyuridine.

**FIGURE LEGENDS**

Fig. 1. Structure of mouse E2F8 gene, mRNA and protein A. Schematic representation of the E2F8 genomic locus on the *Mus musculus* chromosome 7. The 13 exons are represented by boxes and the intronic regions by lines. The lengths of the introns and exons are indicated. B, Structure of the E2F8 mRNA. The open reading frame (ORF) extends from 301 - 2883 base pairs. The 5’ and 3’ untranslated regions are shaded light grey. The Poly-adenylation signals (PAS) are represented by arrows in the 3’
C, Schematic diagram of full length E2F8 protein. The nuclear localization signals (NLS) and the DNA binding domains (DBDs) as predicted by \textit{in silico} analysis are indicated and the amino acid positions are indicated in parentheses. \(D\), Amino acid sequence of full length E2F8 protein. From the N-terminus to the C-terminus the DBDs are named DBD1 and DBD2 and are indicated by bold fonts in the primary structure of full length E2F8 protein.

\textbf{Fig. 2}. Comparative analysis of E2F8 and other known E2F and DP family members \(A\), Sequence alignment of the DNA binding domains (DBD) of mouse E2F 1-8 (represented as mE2F1-8), mouse DP1-2 (represented as mDP1-2) and \textit{Arabidopsis thaliana} E2Fd (AtE2Fd) using the ClustalW program. The conserved RRXYD motif that makes DNA base contacts is indicated with a line. The amino acids LG in mE2F8 DBD1 and LR in mE2F8 DBD2 are marked with asterisks (**) indicating that these amino acids when mutated to E and F abolished DNA binding of E2F8 as shown later in Fig. 5d. Conserved amino acids are shaded yellow. \(B\), Phylogenetic relationship of the DBD amino acid sequences between E2F8, the E2F/DP family in mouse and E2Fd-f of \textit{Arabidopsis thaliana} (phenogram). The length of each horizontal line represents the evolutionary distance between branching points, while the units at the bottom of the tree indicate the number of substitution events. The dotted line on the phenogram indicates a negative branch length. \(C\), Schematic representation of the domain structure of full length E2F1-8 proteins. The domains indicated are NTD (N-terminal domain), DBD (DNA binding domain), LZ (Leucine Zipper), Marked (Marked box), Rb\(^1\) bind (Rb\(^1\) binding) and transactivation domains. \(D\), Phylogenetic relationship of the full length amino acid sequences between murine E2F1-8 (phenogram). The length of each horizontal line represents the evolutionary distance between branching points, while the units at the bottom of the tree indicate the number of substitution events. The dotted line on the phenogram indicates a negative branch length.

\textbf{Fig. 3}. Tissue specific and cell-cycle dependent expression of E2F8. \(A\), Mouse tissue blot from ORIGENE, was either hybridized with an E2F8 specific probe or a \(\beta\)-actin probe as a loading control. \(B\), Primary MEFs were brought to quiescence by serum starvation and stimulated to proliferate by the addition of fresh media with 15% serum. Cells were harvested for RNA at 0, 12, 18 and 24 hours after serum stimulation as indicated and Northern Blot analysis was performed as described in the methods section. The membrane was probed with E2F8 specific probe or with GAPDH probe as a loading control. \(C\), Primary MEFs or p53-/- MEFs were brought to quiescence by serum starvation and stimulated to proliferate by addition of 15% FBS containing media. Cells were harvested for RNA at 0, 9, 12, 15, 18, 21 and 24 hours after serum stimulation and real-time RT-PCR was performed with primers for E2F8 and cdc6 as described in the methods section. Real-time RT-PCR for GAPDH was done to standardize the amount of cDNA in each sample. Cells treated identically were harvested for BrdU incorporation. BrdU was added to the media 3 hours before harvesting. Cells were stained with anti-BrdU antibody with DAPI counterstaining and BrdU positive cells were counted as described in the methods section.

\textbf{Fig. 4}. Regulation of E2F8 promoter. \(A\), Schematic representation of the E2F8 promoter region and that of the Luciferase reporter constructs – short promoter (SP) and long promoter (LP). Alternative first exons 1a, 1b and 1c are shown and their positions relative to each other are indicated. The two E2F binding DNA elements (‘a’ and ‘b’) are shown between the exons 1b and 1c and the mutations introduced therein are indicated. \(B\), Induction of Luciferase activity of SP and LP constructs by transient transfection of 50ng and 150ng of E2F1 or the control vector in REF52 cells. Proliferating cells were transfected and brought to quiescence by serum starvation before harvesting for measuring Luciferase activity as described in the methods section. TK promoter driven Renilla Luciferase reporter construct was used as a control for transfection efficiencies. Each experiment was done in triplicates and the results were reproduced at least 3 times. \(C\), REF52 cells transiently transfected with SP or LP constructs were brought to quiescence and stimulated to grow by adding 15% FBS containing media. Cells were harvested 0 and 18 hours after serum stimulation and Luciferase activity was measured. \(D\), REF52 cells transiently transfected with SP or SP*\(b\) (SP with ‘b’ elements mutated) constructs were brought to quiescence and
Luciferase activity measured. *E*, Fold induction of Luciferase activity by 150 ng or 1500 ng of E2F1 or the control vector, was measured in lysates from REF52 cell transfected with SP or SP*ab (SP construct mutated for both the E2F binding elements ‘a’ and ‘b’).

**Fig. 5.** Subcellular localization and DNA binding activity of E2F8. *A*, Western Blot analysis of lysates from MEFs transiently transfected with myc-tagged E2F8 or the control vector. Myc-8 specific products are indicated by arrows. *B*, MEFs transiently transfected with myc-tagged E2F8 or the control vector were stained with anti-myc antibody and counterstained with DAPI, showing that myc-tagged E2F8 is nuclear localized. *C*, Electro-mobility Shift Assay (EMSA) was performed with biotin-labeled Adenoviral E2 promoter fragment and *in vitro* translated myc-tagged E2F8 protein (Myc-8, indicated by black arrows). *In vitro* translated Luciferase was used as a negative control and myc-tagged E2F3a (Myc-3a) and DP1 (indicated by white arrows) was used as a positive control. The binding resulted in two new bands (indicated by a black or white arrow) which were supershifted using the anti-myc antibody or competed out using non-biotinylated ‘cold’ competitor (cc). The ‘cold’ competitor (cc*) point mutated at the E2F binding elements was unable to compete out the binding indicating sequence specific interaction. *D*, *In vitro* translated E2F8 point mutated at DBD1 (Myc-8-DBD1) or DBD2 (Myc-8-DBD2) or DBD1 and 2 (Myc-8-DBD1-2) were unable to bind the biotin end-labeled E2 promoter fragment. *In vitro* translated Myc-tagged wild-type E2F8 (Myc-8) was used as a positive control and Luciferase as a negative control. *E*, Structural model for DNA binding by E2F8. Left: the crystal structure of the E2F4/DP2 heterodimer with E2F4 (pink) and DP2 (teal) bound to an E2F DNA consensus sequence is shown (1CF7.pdb) (31). Right: our structural model based on homology modeling with the solved structure for the E2F4/DP2 heterodimer, illustrating interactions between DBD1 (gold) and DBD2 (blue) during DNA binding by E2F8. Below: the boxed dimerization interfaces between DBDs 1 and 2 are enlarged in order to illustrate the protein regions that make conserved dimerization contacts. The amino acid sidechains that were mutated for EMSA and co-immunoprecipitation experiments are highlighted in red and blue for DBD1 and DBD2, respectively.

**Fig. 6.** E2F8 can homodimerize. *A*, Lysates from 293 cells transfected with both HA-tagged E2F8 (HA-8) and Flag-tagged E2F8 (Flag-8) were co-immunoprecipitated using anti-Flag antibody and immunoblotted with anti-HA antibody or vice versa. Singly transfected 293 cell lysates with either Flag-E2F8 or HA-E2F8 were used as negative control to rule out any non-specific antibody interactions. *B*, Myc-tagged E2F8 (Myc-8) and Flag-tagged E2F8 (Flag-8) were co-transfected in 293 cells. Lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-myc antibody. This was repeated with lysates from Myc-E2F8 and Flag-E2F8 with DNA binding domains 1 and 2 mutated (Myc-8-DBD1-2 and Flag-8-DBD1-2). Immunoprecipitation with normal mouse IgG (Calbiochem) was used as a negative control to rule out non-specific interactions.

**Fig. 7.** E2F8 overexpression inhibits cellular proliferation. *A*, Primary MEFs infected with E2F8 overexpressing or control retrovirus, were plated at a density of 4X10^4 cells per 60 mm plate. Cells were grown in 15% FBS containing media and were harvested every 24 hours for 7 days and counted. *B*, Real-time RT-PCR for the E2F target genes cyclin A2, polymerase α, cdc6, cyclin E, DHFR and E2F2 was performed on the cells infected with E2F8 overexpressing retrovirus or the control virus. Real-time RT-PCR for GAPDH was done as a control for the amount of cDNA in each sample. The values on the Y-axis represent fold induction.
Figure 1

A) **Mus musculus** chromosome 7

B) E2F8 mRNA

C) E2F8 Protein

D) E2F8 Gene

E2F8 mRNA:

ORF: 2583 bp

E2F8 Protein:

860 amino acids
**Figure 2**

A

```
... P SRHEKSLRVMSSQKLVALFLS... VSPQIVSEIAAXILIGE... mE2F8 DBD1
... N SRKDKSLRVMSSQKLVALFLS... VSPQIVSEIAAXILIGE... mE2F8 DBD2
... R P RKF DVLVYTRKFMDVLVR... SADDKPLLKNKVALKLS... mE2F6 DBD1
... R P RKF DVLVYTRKFMDVLVR... SADDKPLLKNKVALKLS... mE2F6 DBD2
... R P RKF DVLVYTRKFMDVLVR... SADDKPLLKNKVALKLS... mE2F7 DBD1
... R P RKF DVLVYTRKFMDVLVR... SADDKPLLKNKVALKLS... mE2F7 DBD2
```

B

```
... mE2F1 DBD1
... mE2F1 DBD2
... mE2F2 DBD1
... mE2F2 DBD2
... mE2F3 DBD1
... mE2F3 DBD2
... mE2F4 DBD1
... mE2F4 DBD2
... mE2F5 DBD1
... mE2F5 DBD2
... mE2F6 DBD1
... mE2F6 DBD2
```

C

```
E2F1  NTD   DBD   LZ   Marked   Rb bind
E2F2  NTD   DBD   LZ   Marked   Rb bind
E2F3  NTD   DBD   LZ   Marked   Rb bind
E2F4  NTD   DBD   LZ   Marked   Rb bind
E2F5  NTD   DBD   LZ   Marked   Rb bind
E2F6  NTD   DBD   LZ   Marked   Rb bind
E2F7  NTD   DBD   LZ   Marked   Rb bind
E2F8  NTD   DBD   LZ   Marked   Rb bind
```

D

```
... mE2F2
... mE2F3
... mE2F1
... mE2F4
... mE2F5
... mE2F6
... mE2F7
... mE2F8
```

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**Nucleotide Substitutions (x100)**

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**Transactivation**

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E2F1  NTD   DBD   LZ   Marked   Rb bind
E2F2  NTD   DBD   LZ   Marked   Rb bind
E2F3  NTD   DBD   LZ   Marked   Rb bind
E2F4  NTD   DBD   LZ   Marked   Rb bind
E2F5  NTD   DBD   LZ   Marked   Rb bind
E2F6  NTD   DBD   LZ   Marked   Rb bind
E2F7  NTD   DBD   LZ   Marked   Rb bind
E2F8  NTD   DBD   LZ   Marked   Rb bind
```
Figure 3

A

Brain Heart Kidney Liver* Lung Muscle Skin* Small Intestine* Stomach* Testis* Thymus*

6.0kb 5.0kb 4.0kb 3.0kb 2.5kb 2.0kb

6000 5000 4000 3000 2500 2000

E2F8

β-Actin

B

WT MEFs

0h 12h 18h 24h

E2F8

GAPDH

C

WT MEFs

P53-/- MEFs

Fold Induction

0hr 9hr 12hr 15hr 18hr 21hr 24hr

0hr 9hr 12hr 15hr 18hr 21hr 24hr

Fold Induction

% BrdU

% BrdU
Figure 4

A

-513 -393 +1 +191 +257(a) +560 +936 +1305 (ATG)

TTTCCcGC

+385(b)

GCgCAAA

-1993 +168 +855 +855

LP

Luc

SP

B

C

Relative luciferase activity

0 20 40 60 80 100 120

SP LP

0hr 18hr

0 2

0 2 4

0 2 4

0 2 4

0 2

E2F1 Control

Relative luciferase activity

0 5 10 15

SP SP*ab

E2F1 Control

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Figure 6

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Figure 7

A

![Graph showing the number of cells (x10⁴) over days after plating (0-7). The graph compares control and E2F8 treatments.](image)

B

- cyc A2
- polα
- cdc6
- cyc E1
- dhfr
- E2F2

![Graphs showing expression levels of various genes over hours post-stimulation (0-24)].(image)
Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation
Baidehi Maiti, Jing Li, Alain de Bruin, Faye Gordon, Cynthia Timmers, Rene Opavsky, Kaustubha Patil, John Tuttle, Whitney Cleghorn and Gustavo Leone

J. Biol. Chem. published online February 18, 2005

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