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Transcriptional regulation of E2F-1 and eIF-2 genes by α-Pal: a potential mechanism for coordinated regulation of protein synthesis, growth, and the cell cycle

Bassey J.S. Efiok *, Brian Safer

Laboratory of Molecular Hematology, Section on Protein and RNA Biosynthesis, NHLBI, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

α-Pal regulates the basal transcription of the α and β subunits of eukaryotic initiation factor two (eIF-2), a rate-limiting enzyme for the initiation of protein biosynthesis. We recently showed that its global function may be to modulate the expression of key metabolic genes in response to cellular proliferation. In this paper, we examined a potential molecular mechanism by which α-Pal may achieve this function. When overexpressed, α-Pal upregulated protein synthesis and growth, but downregulated the cell cycle. The mechanism for the increased protein synthesis and growth appeared to be a transcriptional upregulation of the eIF-2α and eIF-2β genes. The mechanism for the cell cycle downregulation appeared to be a transcriptional downregulation of E2F-1, a transcription factor that regulates genes required for cell cycle progression beyond the G1/S interphase. Specifically, an apparently modified species of α-Pal bound to the eIF-2 promoters and induced transcriptional upregulation, whereas, an apparently unmodified species of the α-Pal bound to the E2F-1 promoter and induced transcriptional downregulation. By this mechanism, α-Pal may participate in coordinating the regulation of global protein synthesis, growth and the cell cycle; a regulation that is essential to cellular differentiation.

Keywords: α-Pal; E2F-1; Apoptosis; Cell-cycle

1. Introduction

In a normal course of life, cells grow, divide, differentiate, senesce and die. Of these, cellular growth and division require a significant increase in global protein synthesis. To distinguish cell growth from cell division cycle, we define cellular growth based on the ideas of Swann [1], as the increased synthesis of macromolecules that lead to increased cell size, but not necessarily cell division. We define the cell division cycle (or cell cycle) as the cyclical pathway in which complex interactions between cyclins, cyclin-

Abbreviations: nα-Pal, native α-Pal; rα-Pal, recombinant α-Pal; brα-Pal, bacterially produced rα-Pal; ivtα-Pal, in vitro translated α-Pal; Mα-Pal, modified α-Pal; UMα-Pall, unmodified α-Pal; BES, 2-[bis, 2-hydroxyethyl)amino]ethanesulfonic acid; RRL, rabbit reticulocyte lysate; SC50, TF-1 clone stably overexpressing α-Pal; ASC2, TF-1 clone stably underexpressing α-Pal; vTF1, vector-transfected TF-1 control clone

* Corresponding author. Fax: +1-301-496-9985; E-mail: efiokb@gwgate.nhlbi.nih.gov

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dependent kinases and other specific proteins drive a cell to duplicate its genome and divide. Normally, the relationship between protein synthesis, growth and the cell cycle is such that activation of cellular growth drives global protein synthesis, which in turn drives growth, and growth drives cell division cycle or proliferation [2–4]. Growth is so dependent on protein synthesis that it is often measured by the amount of protein synthesized. Similarly, cell division cycle is so dependent on growth that it normally does not occur without the attainment of a critical rate of growth [4,5]. For example, if the growth of yeast cells is limited, the cells arrest their division cycle [2]. Indeed, all eukaryotic cells wait at the start or restriction point until they attain the required critical rate of growth before initiating entry into S-phase. This coordinated control ensures that cellular protein content doubles before the cell commits itself to genomic duplication and mitosis. Sometimes, the relationship between protein synthesis and the cell cycle is not direct. For example, during cellular differentiation and senescence, cells withdraw from cycle, but do not abort global protein synthesis. Instead, cells adjust the rate either downward, upward or not at all, depending on the species and tissue type [4,6,7]. Yeast and *Drosophila* cells blocked in cell cycle progression continue to grow, indicating that they maintain high rates of global protein synthesis [2,8,9]. How global protein synthesis, growth and cell cycle are coordinately regulated in all these instances is unknown, even though much is now known about translation and cell cycle controls.

The eukaryotic cell cycle is primarily controlled at the restriction point or checkpoint 1 (C-point1) [4,10,11]. Upon receiving positive growth signals such as growth factors, competent quiescent cells begin to grow. They wait at C-point1 until their growth rate exceeds the required minimum and then commit to replicate DNA and divide. C-point1 is apparently maintained by the upregulated expression of the cyclin-dependent kinase inhibitors (CDKIs), p21, p27, p57 and the p16 family. CDKIs inactivate G1 cyclin-CDK activities [12–15] and, thereby, prevent the hyperphosphorylation of the retinoblastoma family proteins (pRB, p107, and p130). The pRBs thus cannot release bound E2Fs, a class of five transcription factors (E2F-1 through E2F-5) that regulate the expression of genes required for cell cycle progression beyond G1 [16–19]. Prior to commitment, the E2Fs are bound by hypophosphorylated pRBs. The resulting complexes engage E2F binding sites on target gene promoters and repress transcription, thereby preventing cells from entering S-phase. After commitment, a decline in the activities of CDKIs frees CDKs to hyperphosphorylate pRBs and release E2Fs to transactivate the expression of genes that drive cell cycle progression beyond G1. E2F-1 is the best-studied member of these transcription factors. Its overexpression in quiescent cells is sufficient to induce S-phase entry, proliferation and apoptosis. It is the only member that possesses apoptosis as well as cell cycle regulatory activities [18,20,21]. Furthermore, its apoptosis and transcription activities appear to be required for the regulation of cell proliferation and tumor formation. For example, E2F1$^{-/-}$ mice exhibit testicular atrophy, apparently due to a lack of cell proliferation, but develop aggressive lung tumors apparently due to a failure to eliminate aberrant cells by apoptosis [22–24]. E2F-1's prominent roles in cell cycle control, makes it a potential candidate to participate in the coordinated regulation of protein synthesis, growth and the cell cycle.

In eukaryotes, the translation pathway is catalyzed by a set of enzymes called eukaryotic translation initiation factors (eIF). Prominent among them is eIF-2, a heterotrimer consisting of α, β and γ subunits. eIF-2 catalyzes a rate-limiting initiation step of translation; i.e. the binding of initiator met-tRNA to the 40S ribosomal subunit [25,26]. For this reason, the overall rate of translation in most cells is dependent on eIF-2. Its α subunit is a target for post-translational modifications that lead to the regulation of protein synthesis in response to growth arrest, differentiation, viral infection, and metabolic changes [27–30]. Furthermore, quiescent T-cells and human fibroblasts responding to growth activation upregulate eIF-2α mRNA expression [31,32]. Thus, cells appear to adjust global protein synthesis in response to growth activation by regulating the expression of eIF-2α. We searched for a transcriptional regulatory link between global protein synthesis and the cell cycle by examining eIF-2α transcription factors. α-Pal, a transcription factor for both eIF-2α and eIF-2β appeared poised to mediate growth responses because of the following [33]. (1) Potential regulatory targets for α-Pal are genes that are involved in cel-

lular proliferation or the growth-responsive metabolic pathways: energy transduction, translation and DNA replication. (2) Both the *Drosophila* and sea urchin homologs of α-Pal, P3A2 and ewg, are growth and developmental transcription factors [34,35].

To determine if α-Pal can mediate a coordinated regulation of protein synthesis, growth and the cell cycle, we studied the effects of its overexpression on cellular translation, growth and division cycle activities. We report here that overexpression of α-Pal increased both protein synthesis and growth, but retarded cell cycle progression. The molecular bases for these effects are: (1) overexpression of α-Pal upregulated the transcription of eIF-2α and eIF-2β genes leading to the upregulation of cellular protein content and growth; and (2) overexpression of α-Pal led to an increase in a putative unmodified α-Pal species that repressed E2F-1 gene transcription leading to a retarded cell proliferation.

2. Materials and methods

2.1. Cell culture and antibodies

TF-1 is an erythroleukemic cell line that is dependent on IL-3 for growth and proliferation. It was purchased from ATCC. The cells were grown at a density of $0.1–0.7\times10^6$ CFU/ml in RPMI 1640 containing 10% FBS, 5 ng/ml human IL-3 and the antimicrobials; penicillin, streptomycin and amphotericin B. This medium was designated complete RPMI growth medium. Incubation conditions were 37°C and 5% CO_2. K562, like TF-1, is an erythroleukemic cell line. It was grown as previously described [36]. The cell line, 293, was purchased from ATCC, and were grown in DMEM supplemented with 10% FBS, penicillin, streptomycin and amphotericin B.

Polyclonal antibodies against α-Pal were produced using the peptides; α-PPEP3, ^{242}-NVRSDVRTEEQKQRVSWTQA^{261} and α-PPEP4; ^{35}SMLSADEDSPSSPEDTSYDDSDILNST^{63}. Analysis of the α-Pal polypeptide by PCGENE identified the strongest immunogenic epitopes within these peptides. The antibodies were produced by Advanced ChemTech (Louisville, KY) and purified by ammonium sulfate precipitation (45%) and protein A agarose affinity columns.

2.2. Recombinant DNA constructs

The α-Pal expression vectors LS51 and LS12, which overexpressed α-Pal sense and α-Pal antisense mRNA, respectively, were constructed by inserting the α-Pal cDNA into the inducible expression vector, POPRSVCAT(LacSwitch^{O} Kit, Stratagene, La Jolla, CA), using Stratagene's procedure. In LS51, the α-Pal cDNA is oriented in the sense direction, whereas, in LS12, it is oriented in the antisense direction. POPRSVCAT promoter carries the lactose operon operator switch; co-expressed repressor protein binds to it and inhibits transcription, and added IPTG counteracts the binding and induces transcription of the α-Pal cDNA. The α-Pal overexpression vector, pCIPal27, used in transient overexpression experiments, was constructed using pCIneo vector (Promega, Madison, WI).

The wild-type E2F1 promoter construct, wE2F1Luc, was kindly provided by Dr. J. Nevin of The Department of Genetics, Duke University Medical Center, Durham, NC (E2F1Luc-728, [37]). The construct consists of an 800-bp fragment of the human E2F-1 promoter inserted into pGL2 vector (Promega, Madison, WI) to drive luciferase expression. The mutant, mE2F1Luc, was generated by mutating the wild-type at seven bases that are critical to α-Pal binding using Quick Change mutagenesis kit (Stratagene, La Jolla, CA) and the following primer pair: $^{-215}$ CCGGAC**AAAGCCTtaGgaCtgCaC**GCCCCGCCATTG^{-179} and ^{179}CAATGGCGGGGC**GtGcaGtcCtaAGGCTTT**GTCCGG^{215}. The wild-type bases are shown in Fig. 5. The mutant bases are indicated above in lower case letters and have been shown to be less preferred in these positions [33]. The wild-type eIF-2α construct, weIF2αLuc, consists of a 700-bp fragment of the human eIF-2α promoter inserted into pGL2 vector to drive luciferase expression, and the mutant version, meIF2αLuc, was generated using the above Quick Change mutagenesis kit. The eIF-2β promoter constructs, weIF2βLuc (wild-type) and meIF2βLuc (mutant) were generated as described previously [38].

2.3. Stable overexpression clones

To generate stable clones overexpressing α-Pal, 3.0×10^6 TF-1 cells were cotransfected with 11.5 μg each of LS51 and P3′-SS (an expression vector for the lactose repressor protein), using the calcium phosphate-BES precipitation method [39]. To generate stable clones overexpressing α-Pal antisense RNA, 3.0×10^6 TF-1 cells were similarly cotransfected with 11.5 μg each of LS12 and P3′-SS. Vector-transfected control cells were similarly generated using 11.5 μg each of POPRSVCAT and P3′-SS. Seventy two hours after transfection, double transfectants were selected by growing in the presence of 300 μg/ml of Hygromycin and 400 μg/ml of G418 for 14 days. This pool of cells was diluted to one cell/200 μl with conditioned RPMI growth medium containing 200 μg/ml of G418, and used to seed 96-well plates at one cell/well. The plates were incubated under standard growth conditions for 2 weeks with frequent replacement of medium. G418-resistant clones were transferred to larger plates, expanded further and evaluated for α-Pal expression using Western blot, Northern blot and EMSA assays. The clones selected for this study were: SC50, which overexpressed α-Pal mRNA and protein; ASC2, which overexpressed α-Pal antisense RNA, but underexpressed α-Pal protein; and vTF1 which was a vector-transfected control.

2.4. Transient expression assays

Transfection of 293 cells was performed using Superfect transfection reagent (Qiagen, Santa Clarita, CA). The manufacturer's instructions were followed except that culture dishes were seeded with 15 000 CFU/cm^2 and transfection complexes were formed using 0.07 μg DNA per cm^2 of plate and a Superfect/DNA ratio of 4. Cells were incubated in the DNA/Superfect complexes for 6 h before they were rinsed and grown in DMEM growth medium for 12–48 h. Afterwards, the cells were harvested and processed for cell extract and luciferase expression using Promega's (Madison, WI) Luciferase assay kit. Humanized green fluorescent protein was used to assess transfection efficiencies which averaged 70%.

2.5. Preparation of α-Pal

To prepare bacterially-produced recombinant α-Pal (brα-Pal), the full-length α-Pal cDNA was cloned into the bacterial expression vector, pRSET A (Invitrogen, La Jolla, CA). The cDNA was cloned in frame with an upstream sequence that encoded a metal-binding histidine tag fused to the N-terminus of the rα-Pal. Ni^{2+} affinity column purification of brα-Pal was done as recommended. Native α-Pal (nα-Pal) was prepared as described [33]. In vitro-translated α-Pal (ivtα-Pal) was prepared by in vitro transcription of the α-Pal cDNA followed by translation in rabbit reticulocyte lysate (RRL) using Promega's TNT kit.

2.6. Preparation of nuclear extracts/protein assays

Nuclear extracts were prepared as reported by Dignan et al. [40], with the following exceptions. Lysis buffer (buffer A) contained 10 mM HEPES (pH 7.9), 10 mM KCl, 0.8 mM spermidine, 0.1 mM EDTA, 1.0 mM DTT, 1.0 mg/ml PEFABLOCK (Boehringer Mannheim, Indianapolis, IN). Nuclear extraction buffer (buffer C) contained 20 mM HEPES (pH 7.9), 470 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 2 mM DTT and 1.0 mg/ml PEFABLOCK. Protein concentrations were determined by the BCA Protein Assay method (Pierce, Rockford, IL).

2.7. Western and Northern blotting assays

These were done using standard methods [39]. For Western blots, electrophoresed proteins were transferred onto 0.2-μm PVDF membrane (Bio-Rad, Hercules, CA) using semi-dry transfer equipment (Bio-Rad), and probed with the appropriate antibodies. Blots were developed with Amersham's (Arlington Height, IL) ECL reagents. For Northern blots, 20 μg of total RNA were diluted with 2×RNA sample loading buffer consisting of 2×MOPS buffer, 13% Ficol, 7 M urea, 0.03% each of Bromphenol blue and xylene xylenol ff. The samples were electrophoresed in formaldehyde agarose gels, transferred onto 0.45-μm Magnacharge membranes (Micron Separation, Westboro, MA), and probed with ^{32}P-labeled

specific probes. Bands were quantified by volume integration using Phosphoimaging equipments (Molecular Dynamics, Sunnyvale CA).

2.8. Growth profile

Cycling cells were washed twice in growth medium. Aliquots were treated with Trypan blue dye and viable cells were counted with a hemocytometer. Triplicate T-25 flasks were seeded with 1.0×10^6 CFU of either vTF1, SC50 or ASC2 cells in 10 ml of complete RPMI medium containing 1.7 mM IPTG. The flasks were incubated at standard growth conditions for 72 h during which viable cells were counted. Viable cells as a percentage of the initial seed were plotted against time to generate a growth curve.

2.9. Apoptosis profile

SC50, ASC2 and vTF1 cells were grown in the presence of 1.7 mM IPTG for 72 h to induce maximal expression of α-Pal RNA. The cells were washed twice in RPMI growth medium containing 1.7 mM IPTG, 7.5% FBS and no IL-3, and re-suspended at a concentration of 1.0×10^6 CFU/ml. For each clone, T-25 flasks were seeded in triplicate with 1.0×10^6 CFU suspended in 10 ml of the wash medium. The flasks were incubated at standard conditions for 72 h during which viable cells were counted. Viable cells as a percentage of the initial seed were plotted against time to generate a death curve.

2.10. Electrophoretic mobility shift assays

This was performed as reported before [33].

2.11. Modification/dephosphorylation of α-Pal

To modify brα-Pal, 10 μg of brα-Pal was mixed with 30 μl of RRL and 1× buffer M, in a total volume of 60 μl. The mixture was incubated at 30°C for 30 min and then stored at −80°C until used. Buffer M (1×) contained 20 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT.

Dephosphorylation reactions were performed in a 40-μl reaction volume containing 7 ng of purified native α-Pal (or 10 μg of brα-Pal), 1.0 mg/ml BSA, 20 mM $MnCl_2$, protein phosphatase 1γ (Life Technologies, Gaithersberg, MD) or $2A_1$ (Calbiochem, San Diego, CA) and 1× phosphatase buffer (20 mM HEPES, 5.0 mM $MgCl_2$, 1.0 mM DTT, 0.1 mM EGTA). The reaction was incubated at 30°C then stored at −80°C until use.

2.12. In vitro transcription assay

brα-Pal was incubated at 25°C for 20 min in a reaction mixture containing 1× transcription buffer, 16% glycerol, 600 ng linearized template and 40 U of RNasin (Promega, Madison, WI) in a total volume of 25 μl. This allowed brα-Pal to bind to its E2F1-Pal1 site. During the incubation, a cocktail containing the following ingredients in a total volume of 25 μl was prepared: 1× transcription buffer; 20 μM $ZnCl_2$; 500 μM each of ATP, GTP and CTP; TF-1 nuclear extract (80 μg protein), H_2O, and 50 μM of α-^{32}P-labeled UTP. This cocktail was added to the first reaction mixture after the 20-min incubation. The resulting 50 μl reaction mixture was incubated at 30°C for 45 min and extracted by adding 150 μl of 10 mM EDTA, 200 μl of Trizol (Life Technologies, Gaithersburg, MD) and 100 μl of chloroform, followed by vigorous vortexing and a 10-min centrifugation in a refrigerated microfuge. The upper aqueous phase was carefully transferred into a sterile tube, precipitated with 400 μl of isopropanol and 15 μl of sterile 5.0 M ammonium acetate, and the pellet washed with 70% ETOH. The pellet was solubilized 50 μl of RNA loading buffer and electrophoresed in a 4.5% denaturing acrylamide gel for 8 h. After fixing and rinsing, the gel was dried and transcripts viewed by standard autoradiography. 1× transcription buffer contained 20 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT. Labeled UTP contained 50 μM cold UTP and 1 μl of [α-^{32}P]UTP (3000 Ci/mmol). All reagents and supplies were sterile and RNAse-free.

3. Results

3.1. Stable overexpression/underexpression of α-Pal in TF-1 cells

Our previous studies indicated that α-Pal, a tran-

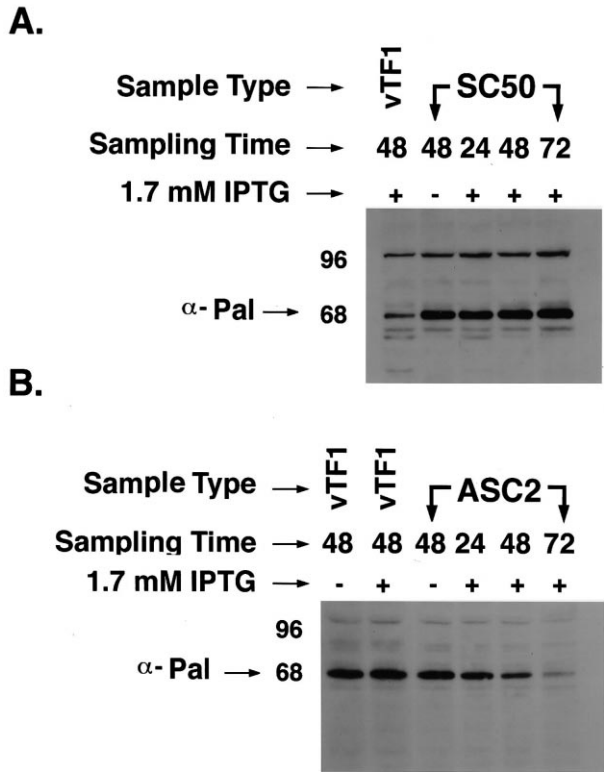

Fig. 1. Stable clones of TF-1 cells inducibly express α-Pal. SC50, ASC2 and v-TF1 (control) cells were grown in the presence of 1.7 mM IPTG, either to induce maximal expression of α-Pal, or to ensure that they all received identical treatment. Nuclear extracts were prepared at the indicated times and analyzed for α-Pal by Western immunoblotting. (A) Inducible overexpression of α-Pal in SC50 cells. Each lane contained 40 μg of proteins. (B) Inducible underexpression of α-Pal in ASC2 cells. Each lane contained 100 μg of proteins. The numbers to the left indicate molecular mass.

scription factor for eIF-2α and eIF-2β, may link the transcriptional regulation of key metabolic genes to cellular growth and development. In this study, we examined a specific metabolic pathway, protein biosynthesis, to see if and how it is linked by α-Pal to the cell division cycle. We did this by overexpressing or underexpressing α-Pal in the human cell lines, TF-1 and 293, and assessing the effects on pertinent cellular parameters. The α-Pal cDNA was inserted into the vector POPRSVCAT as described in Section 2 to generate constructs which expressed either the sense or antisense mRNA when transfected into cells. The sense construct was transferred into TF-1 cells to generate the stable clone, SC50, which overexpressed both the α-Pal mRNA and protein. The antisense construct was transferred into TF-1 cells to generate the stable clone, ASC2, which overexpressed the α-Pal antisense mRNA and, hence, underexpressed the protein. The empty POPRSVCAT vector was similarly transferred to generate the vector-transfected control cells, v-TF1.

SC50, ASC2 and v-TF1 cells were treated with 1.7 mM IPTG either to induce optimal expression of α-Pal, or to ensure that the cells received similar treatments. Cells were harvested at various times and processed for total RNA and nuclear extracts. Western blot analysis of the nuclear extracts (Fig. 1A) showed that at 72 h after IPTG treatment, SC50 overexpressed the recombinant α-Pal (rα-Pal) protein by about 5-fold relative to v-TF1. However, the overexpression was barely inducible because the lactose operator switch that was used to control the expression was leaky. Northern blot analysis confirmed a correlating increase in α-Pal mRNA (data not shown), indicating that the increased expression of α-Pal was transcriptionally driven. In contrast, ASC2 inducibly underexpressed the native protein (nα-Pal) by about 6-fold compared to the v-TF1 control, indicating that ASC2 overexpressed α-Pal antisense RNA inducibly. Northern blot analysis also confirmed a correlating increase in the antisense RNA (data not shown). The two clones, SC50 and ASC2, were subsequently used to evaluate the effects of stable overexpression of α-Pal in TF-1 cells.

3.2. *Overexpression of α-Pal results in increased eIF-2 transcription and changes in cellular protein synthesis*

α-Pal regulates the transcription of eIF-2α and 2β genes and thus regulates the expression of functional eIF-2, a rate-limiting initiation factor for protein synthesis [36,38]. Overexpressed α-Pal, therefore, can modulate the transcription of the eIF-2α and 2β genes and affect cellular global protein synthesis. To assess these effects, SC50, ASC2 and v-TF1 were treated with IPTG to induce maximal α-Pal RNA overexpression, and the treated cells were harvested at various times and used to prepare total RNA. Aliquots of the 72-h cultures were also used to prepare whole cell extracts for measuring cellular protein content. Results of Northern blot analysis showed that, after 48 h of induction, eIF-2α and eIF-2β transcripts increased by about 3-fold in

SC50 as compared to v-TF1 (Fig. 2A). Thus, the overexpressed α-Pal upregulated the transcription of these genes as expected. In contrast, eIF-2α and eIF-2β transcripts in ASC2 were unchanged or decreased by less than 10%. This indicated that α-Pal underexpression did not significantly repress the basal transcription of the eIF-2α and eIF-2β genes as would be expected. Our unpublished data indicate that residual α-Pal in the ASC2 cells was still sufficient to maintain basal transcription rate.

The observed effects on the transcription of eIF-2α and eIF-2β genes were reflected at the level of translation. Assessment of total protein in the cells showed that SC50 contained 219 pg of protein per CFU in comparison to 180 pg/CFU for v-TF1 and 173 pg/CFU for ASC2 (Fig. 2C). Similar results were obtained for other clones: SC29 and SC40 that stably overexpressed α-Pal, contained more proteins, and ASC1 and ASC3 that stably underexpressed α-Pal, contained slightly less proteins than v-TF1. Together, these results indicate that overexpression of α-Pal induced the upregulation of eIF-2 subunit genes leading to a 20% increase in cellular global protein synthesis. In contrast, underexpression of α-Pal did not decrease the transcription of eIF-2 subunit genes, and consequently, did not significantly decrease protein synthesis from the basal level.

3.3. *Overexpression of α-Pal retards cell proliferation, whereas underexpression stimulates proliferation*

The rate at which a cell grows is a function of the rate of global protein biosynthesis [3,5]. Normally, growth is coupled to the cell division cycle such that when a growing cell exceeds a critical size, cell division occurs, thereby maintaining a uniform cell size. Under normal conditions, therefore, increased growth results in increased cell size, increased cell

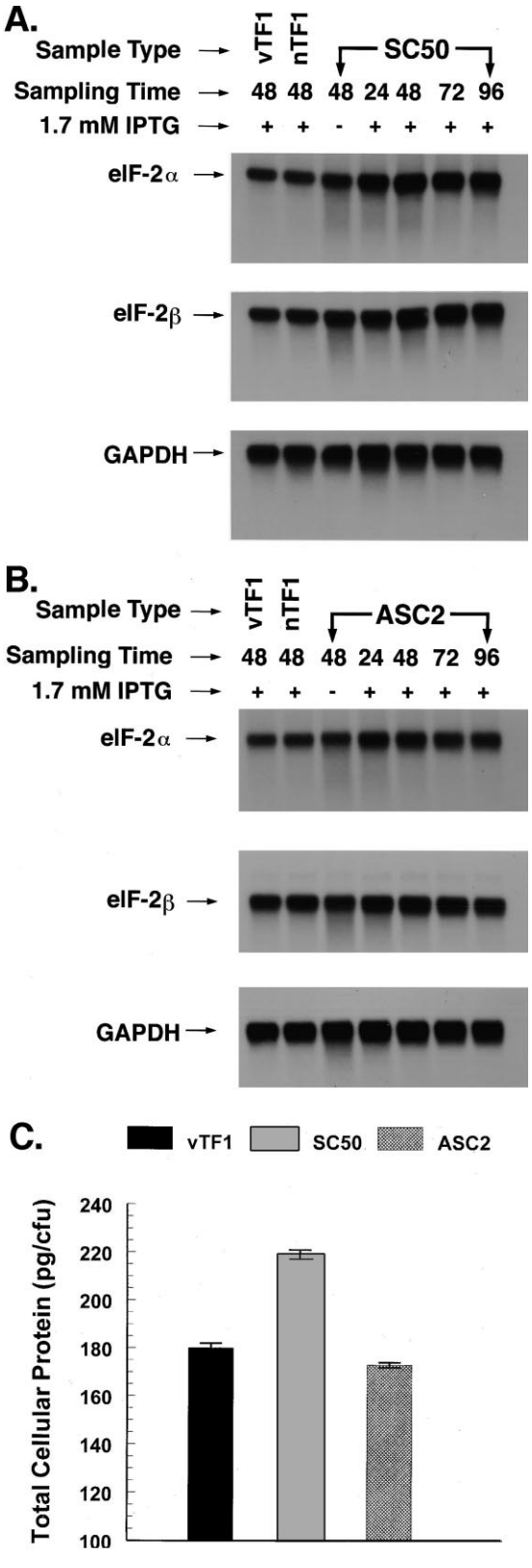

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Fig. 2. Overexpressed α-Pal upregulated the transcription of eIF-2α and eIF-2β genes and cellular protein content. Total RNA was prepared from cells grown as described for Fig. 1, and was analyzed for eIF-2α and eIF-2β transcripts by Northern blotting. Each lane contained 20 μg of total RNA. (A) Expression profile of eIF-2α and eIF-2β in SC50. (B) Expression profile of eIF-2α and eIF-2β in ASC2. (C) Cellular protein contents at the 72nd hour. The data represent averages of six experiments.

division and increased cell number. Conversely, decreased growth leads to decreases in these parameters. Since overexpression of α-Pal in TF-1 cells led to increased protein synthesis and underexpression led to near-normal synthesis, we determined how these changes affected cellular growth and cell division. Growth and cell division were assessed by determining changes in cell number and size in response to the induction of α-Pal overexpression. The results show that SC29 and SC50 cells that overexpressed α-Pal, were retarded in cell proliferation as compared to v-TF1 (Fig. 3A). The cells, however, increased in size (Fig. 3B). Similar growth and proliferation profiles were also obtained for another overexpressing clone, SC40, and G418-resistant pools. These results indicate that cells of this clone grew at an increased rate, but the growth was somewhat uncoupled from cell division cycle since it led to increased cell size and not increased proliferation. In agreement, ASC2 and ASC3 that underexpressed α-Pal, showed increased proliferation and decreased size as compared to v-TF1 (Fig. 3). Similar growth and proliferation profiles were also obtained for another underexpressing clone, ASC1, and G418-resistant pools. Transiently transfected 293 cells also showed retarded proliferation in response to α-Pal overexpression, indicating that the above effect on growth is not limited to TF-1 cells. These results suggest that α-Pal positively regulates cellular growth, but negatively regulates the cell cycle.

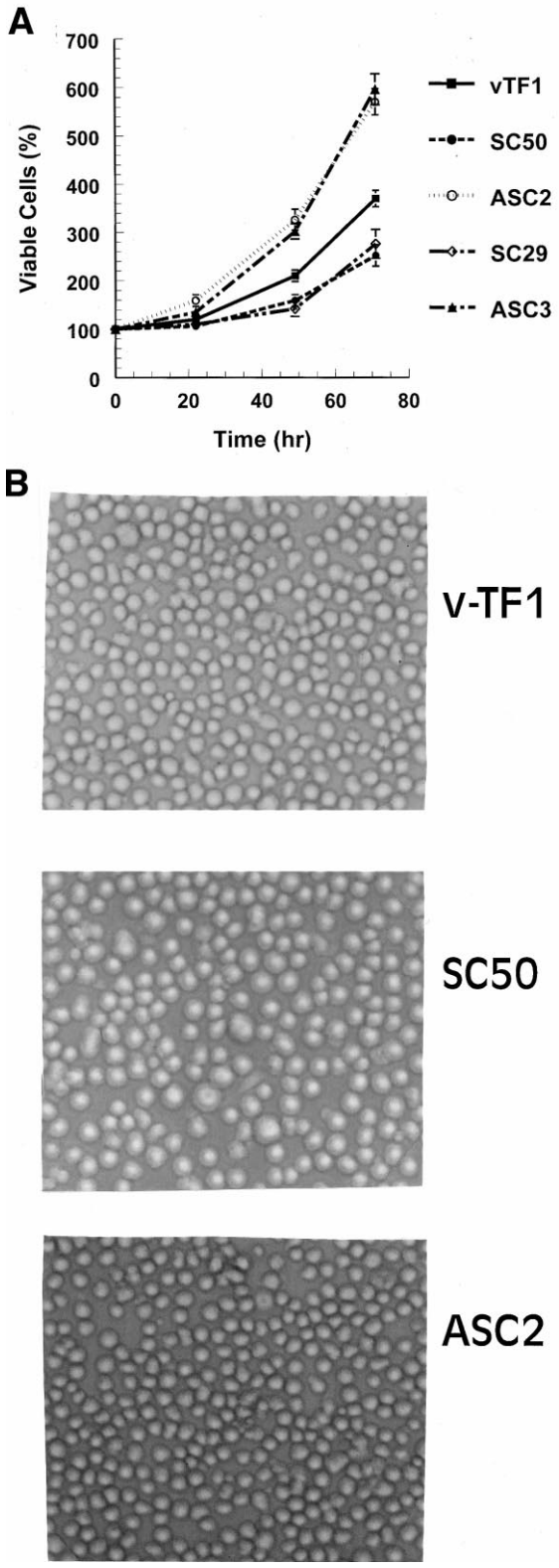

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Fig. 3. (A) Cell proliferation was retarded by overexpression, but stimulated by underexpression of α-Pal. SC29, SC50, ASC2, ASC3 and v-TF1 cells were grown as described in Section 2, and aliquots were analyzed at the indicated times to generate the growth curve (see Section 2). Each data point represents the average of six experiments. (B) Cell size was increased by overexpression, but decreased by underexpression of α-pal. v-TF1, SC50 and ASC2 cells were grown for 72 h as described in Section 2 for Fig. 3A. The cells were photographed directly in the incubation flask without any processing, using a Zeiss light microscope set at ×20 magnification. Each image was printed at ×66 magnification (Reduced to ×43 for publication).

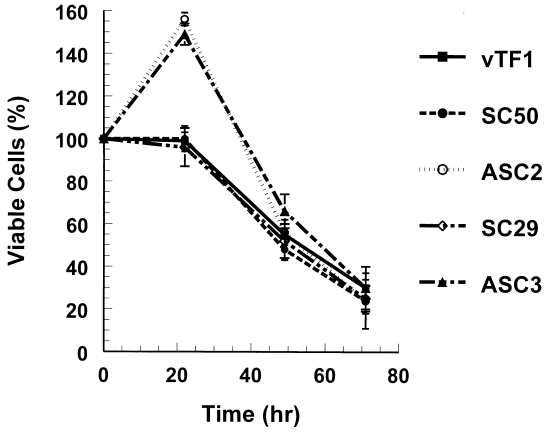

Fig. 4. The increased proliferation was accompanied by increased apoptosis. Cells grown for 72 h in the presence of 1.7 mM IPTG, were processed and analyzed for apoptosis (see Section 2). The data represents averages for six experiments. Cells underexpressing α-Pal failed to exit the cell cycle in the absence of IL-3, and apoptosed faster than control cells.

3.4. The increased proliferation was accompanied by increased apoptosis

Since cell cycle perturbation is often accompanied by changes in the rate of apoptosis, we evaluated the rate of apoptosis of SC29, SC50, ASC2, ASC3 and v-TF1 cells in response to α-Pal overexpression. These cells, like the parental TF-1, are normally dependent on IL-3 for survival and proliferation. Following IL-3 withdrawal, the cells withdraw from cycle but remain viable for about 24 h. Afterwards, a majority of them apoptose within the following 48 h [41]. Fig. 4 shows that SC29 and SC50 that overexpressed α-Pal, behaved similarly to v-TF-1 in that they withdrew from cycle within the first 24 h and apoptosed at about the same rate thereafter. G418-resistant pools and SC40 that also overexpressed α-Pal, gave similar results (data not shown). In contrast, ASC2 and ASC3 that underexpressed α-Pal, consistently failed to withdraw from cycle within 24 h of IL-3 withdrawal. Most notably, they continued to proliferate during the first 24 h and thereafter apoptosed at a faster rate than v-TF1. G418-resistant pools and ASC1 that also underexpressed α-Pal, gave similar results (data not shown). Thus, underexpression of α-Pal apparently led to: (1) fast cell cycling; (2) failure to withdraw from cell cycle in response to the absence of growth signal; and (3) increased rate of apoptosis. These effects are very similar to those observed when E2F-1 is overexpressed in cells. When overexpressed, E2F-1 drove quiescent cells into cycle, and increased the rate of apoptosis [20,21,42–46]. The premature entry of the quiescent cells into cycle indicates a failure to withdraw from cell cycle in the absence of growth signal. E2F-1 mediated apoptosis is often dependent on certain experimental conditions, such as low serum. Similarly, the increased apoptosis of the α-Pal underexpressing clones, ASC2 and ASC3, were dependent on the absence of serum. Taken together, the above results suggest that underexpressing α-Pal leads to the upregulation of E2F-1 and a consequent increase in cell proliferation when the growth signal, IL-3, was present, or a consequent increase in apoptosis when the growth signal was absent.

3.5. α-Pal interacts differentially on the eIF-2α and E2F-1 gene promoters

The above results suggest that α-Pal negatively regulates E2F-1 expression. In agreement, SC50 cells that stably overexpressed α-Pal, moderately downregulated E2F-1 mRNA and ASC2 that underexpressed α-Pal, moderately upregulated the mRNA (data not shown). To confirm this and to elucidate the molecular mechanism involved, a series of assays were performed. We started out by searching the human E2F-1 promoter for α-Pal DNA-recognition sites. Three sites were identified within the proximal region. One of these, E2F1-Pal1, is embedded in a region known to have strong influence on E2F-1 transcription, and has previously been shown to mediate transcriptional repression of the E2F-1 promoter [47]. Using EMSA, the α-Pal binding characteristics of this site were compared to those of the high affinity site on eIF-2α promoter (eIF-2PalH). α-Pal preparations used in the binding studies were: native α-Pal (nα-Pal) that was purified from K562 nuclear extract; in vitro translated α-Pal (ivtα-Pal) that was histidine-tagged rα-Pal translated in vitro in rabbit reticulocyte lysate (RRL); and affinity purified bacterially produced rα-Pal (brα-Pal) that was histidine-tagged homodimeric rα-Pal produced in *Escherichia coli*. The results (Fig. 5B) show that neither the nα-Pal, nor the ivtα-Pal, bound the E2F1-Pal1 site even though they bound eIF-2PalH with high affinity. In contrast, brα-Pal bound both sites with sim-

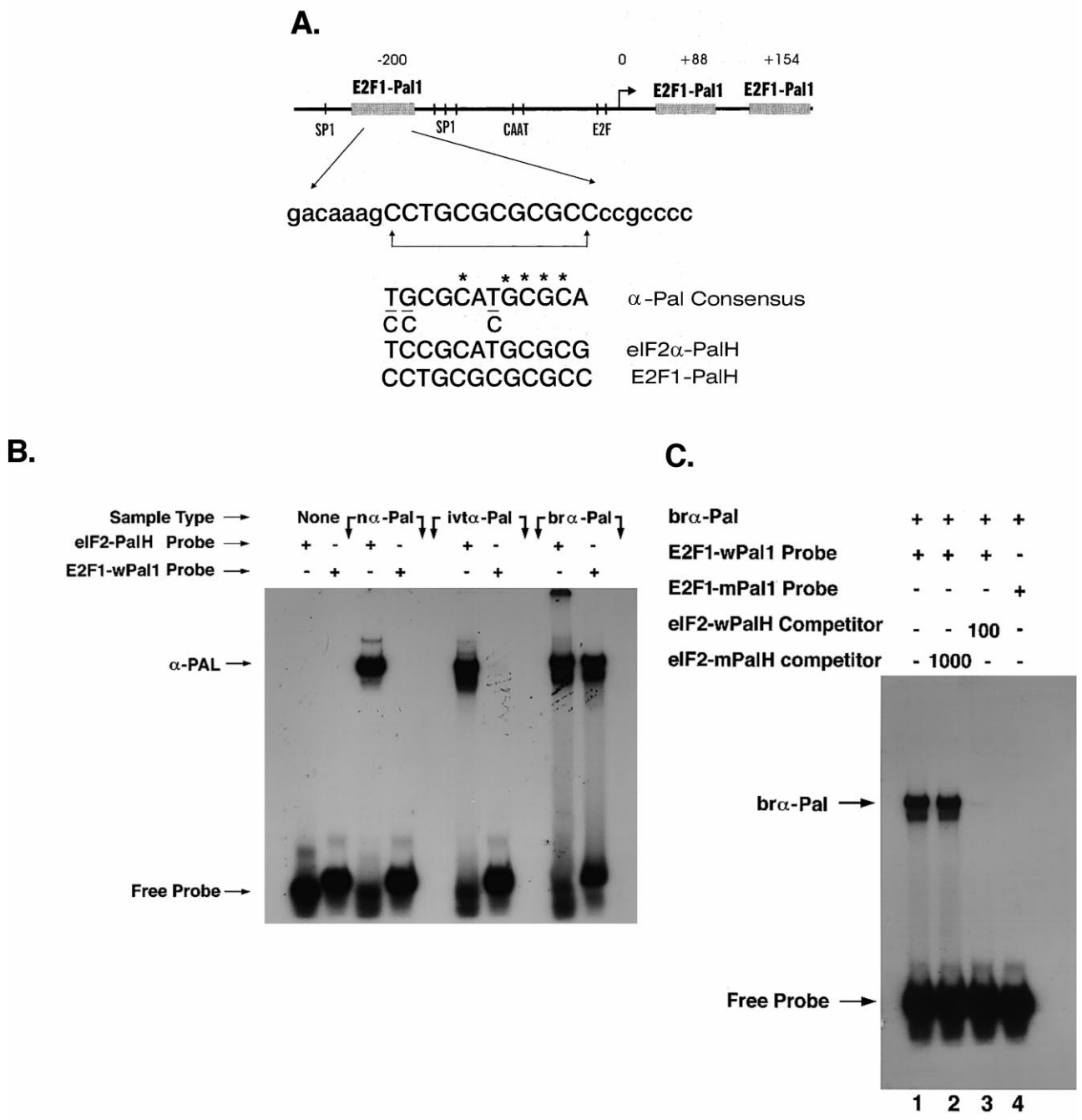

Fig. 5. α-Pal binds to its sites on eIF-2α and E2F-1 promoters with different affinities. (A) Location and sequence of the upstream α-Pal binding site on human E2F-1 promoter. The top schematic illustrates the locations of three α-Pal binding sites E2F1-Pal1, E2F1-Pal2 and E2F1-Pal3. The sequence of E2F1-Pal1 is shown and is then compared to those of the α-Pal consensus and the high-affinity site on the eIF-2α promoter (eIF2-PalH). *, indicates bases critical to binding. (B) Autoradiograph of EMSA comparing the affinities of eIF2-PalH and E2F1-Pal1 for nα-Pal, ivtα-Pal and brα-Pal. Both nα-Pal and ivtα-Pal bound eIF2-PalH with high affinity (K_d = 111 pM), but failed to bind E2F1-Pal1. In contrast, brα-Pal bound both sites. (C) EMSA demonstrating the specificity of binding of brα-Pal to E2F1-Pal1. The binding was not disrupted by 1000-fold excess cold mutant eIF2-PalH (lane 2), but was disrupted by either 100-fold excess cold wild-type eIF2-PalH (lane 3) or mutant E2F1-Pal1 (lane 4).

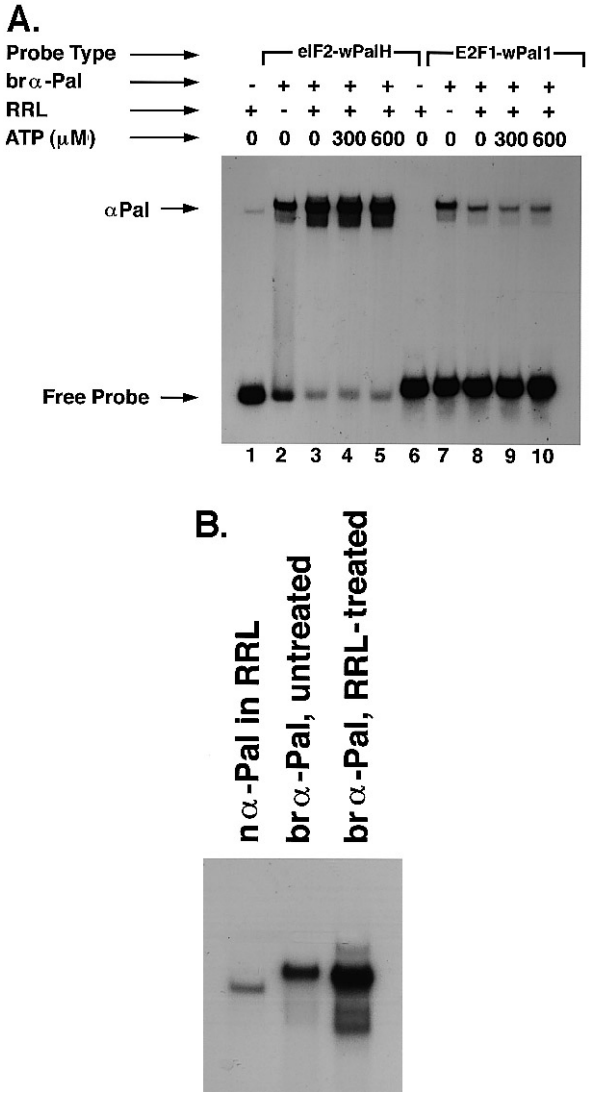

Fig. 6. Modified brα-Pal exhibited differential binding affinities towards eIF2-PalH and E2F1-Pal1. brα-Pal was treated with RRL as described in Section 2. (A) Autoradiograph of EMSA showing binding activities of the modified brα-Pal towards eIF2-PalH and E2F1-Pal1. The modified brα-Pal showed increased binding affinity for eIF-2PalH (lanes 2–5), but decreased affinity for E2F1-Pal1 (lanes 7–10). The indicated ATP values were added to the modification reactions to determine if the mechanism was kinase dependent. (B) Autoradiograph of EMSA comparing the mobilities of nα-Pal, brα-Pal and modified brα-Pal. The samples were electrophoresed for twice as long as in A. The modified brα-Pal has a mobility that appears to be intermediate to nα-Pal and brα-Pal.

ilar affinities. In competition assays, 1000-fold excess cold mutant eIF-2PalH failed to disrupt binding of the brα-Pal to E2F1-Pal1 (Fig. 5C, lane 2), whereas 100-fold excess cold wild-type eIF-2PalH probe abolished the binding (lane 3). Also, when the E2F1-Pal1 site was mutated at bases which were previously shown to be critical to α-Pal binding, the binding was completely abolished (lane 4). These results confirm the specificity of the binding of brα-Pal to E2F1-Pal1.

As the cDNA templates for the ivtα-Pal and brα-Pal were identical, but ivtα-Pal acquired the binding characteristics of nα-Pal, and brα-Pal did not, then either ivtα-Pal had been modified by something in the RRL in which it was translated, or brα-Pal was modified in bacteria so that it behaved differently. Both possibilities were tested. First, phosphorylation is the most common modification that cells use to alter transcription factor activity, and brα-Pal may have been inappropriately phosphorylated in bacteria. Therefore, brα-Pal was dephosphorylated with protein phosphatase 1 (PP1), protein phosphatase II (PP2) or lambda protein phosphatase (λPP), and then retested for binding by EMSA. The treated and the untreated proteins behaved similarly with regard to binding affinities towards E2F1-Pal1 and eIF-2PalH probes (data not shown). Therefore, the ability of brα-Pal to bind to both probes in Fig. 5B was not due to inappropriate phosphorylation in bacteria. Secondly, brα-Pal was treated with catalytic amounts of RRL to see if it could be modified to acquire the binding characteristics of the native protein. The results (Fig. 6A) showed that brα-Pal acquired the binding characteristics of nα-Pal in that it decreased affinity for the E2F1-Pal1 probe, but significantly increased affinity for eIF-2PalH. These results indicate that the switch in binding affinities was caused by a modification of the brα-Pal homodimer in RRL. The modification was not phosphorylation because neither additional ATP (Fig. 6A, lanes 4, 5, 9, 10) nor other common nucleotide triphosphates (data not shown) had any effect on the extent of the α-Pal modification. The modified rα-Pal acquired an apparent electrophoretic mobility that was intermediate to nα-Pal and brα-Pal (Fig. 6A,B), indicating that it could be a heterodimer. Together, the results in this section show that, α-Pal interacts with its binding site on E2F-1 promoter differently than it interacts with those on the eIF-2α promoter. They also imply that nα-Pal could be a heterodimer that binds eIF-2PalH site with high affinity but have very low affinity for the E2F1-Pal1 site, and that only

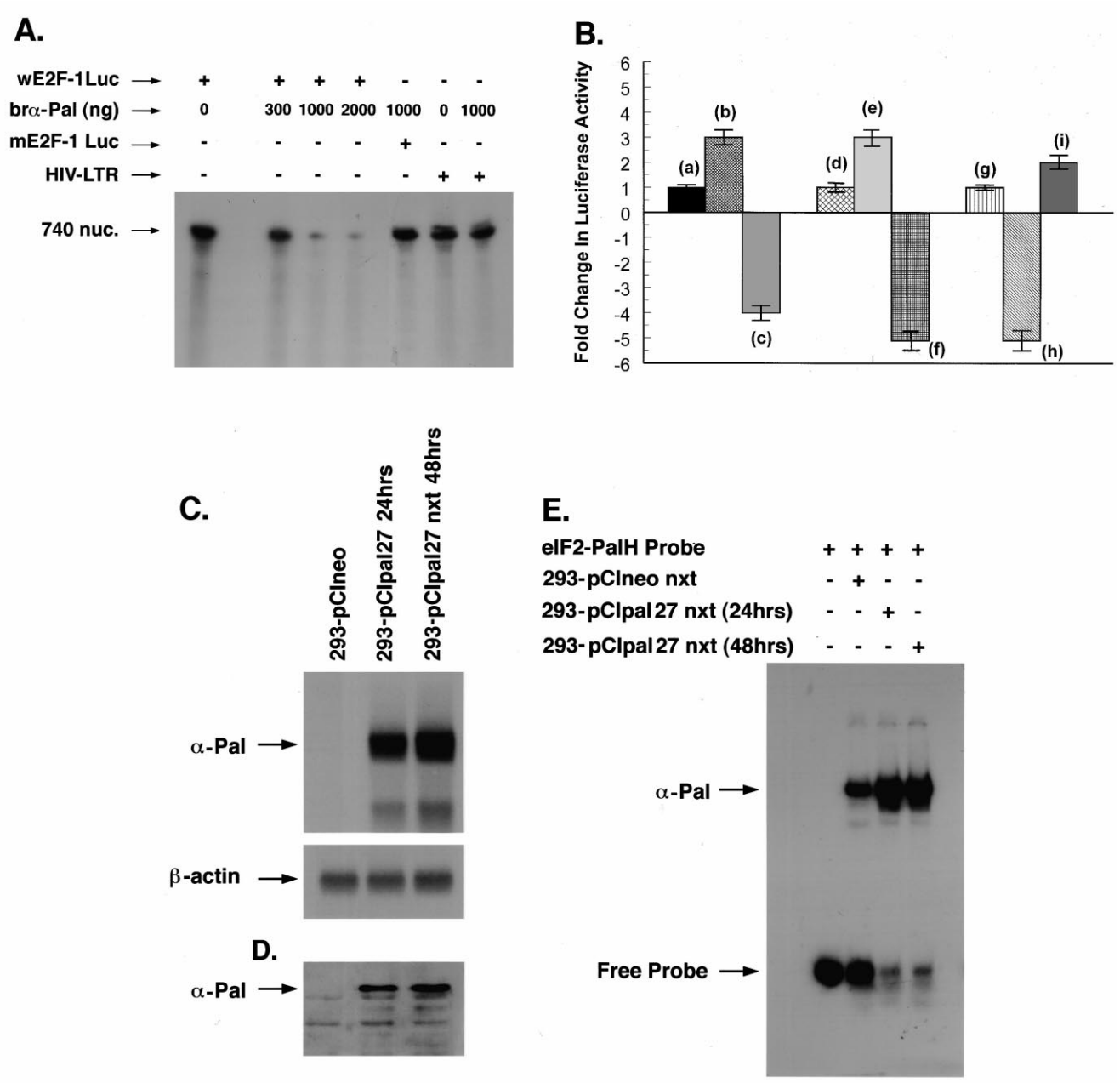

Fig. 7. Binding of UMα-Pal to E2F-1Pal1 site repressed transcription from E2F-1 promoter. (A) Autoradiograph of ^{32}P-labeled transcripts generated by in vitro transcription from E2F-1 promoter. Linearized wE2F1Luc vector was transcribed in vitro in the presence of various amounts of brα-Pal as described in Section 2. As controls, linearized mE2F1Luc and HIV-LTR vectors were also transcribed. As shown, nanogram quantities of brα-Pal repressed E2F-1 driven transcription. (B) Plots of luciferase activities assessing the effects of rα-Pal overexpression on transcription driven by E2F-1, eIF-2α, and eIF-2β promoters. The cell line, 293, was co-transfected with the following pairs of promoter/expression vectors: weIF2αLuc/pCIneo (a), weIF2αLuc/pCIPal27 (b), meIF2αLuc/pCIPal27 (c), weIF2βLuc/pCIneo (d), weIF2βLuc/pCIPal27 (e), meIF2βLuc/pCIPal27 (f), wE2F1Luc/pCIneo (g), wE2F1Luc/pCIPal27 (h), mE2-F1Luc/pCIPal27 (i); which are described in Section 2. After 48 h, the cells were processed and assayed for transient luciferase activity. In response to rα-Pal overexpression, weIF2αLuc and weIF2βLuc upregulated transcription 3-fold, but wE2F1Luc downregulated transcription 5-fold. (C–E) Extent of rα-Pal overexpression in 293 cells. Autoradiographs of Northern blot (C), Western blot (D) and EMSA (E) are shown. Phosphoimager analysis of the Northern blot indicated a 20-fold overexpression of rα-Pal.

α-Pal homodimer binds to the E2F1-Pal1 site with high affinity.

3.6. The differential interactions of α-Pal on eIF-2α and E2F-1 promoters mediate opposite transcriptional effects

The observed differential binding affinities of α-Pal can potentially be responsible for the differential expression of the eIF-2 and E2F-1 genes and the corresponding differential effects on cell growth and proliferation rates. If so, binding of α-Pal to the eIF-2α promoter would upregulate transcription, but binding to the E2F-1 promoter would repress transcription. To examine this, we first examined the effect of rα-Pal on the in vitro and in vivo transcription from the E2F-1 promoter. For the in vitro transcription, linearized wE2F1Luc, a luciferase reporter construct driven by the human E2F-1 promoter, was transcribed using TF-1 nuclear extract. The results (Fig. 7A) show that nanogram quantities of brα-Pal repressed transcription from the E2F-1 promoter in a concentration-dependent manner; with 300 ng repressing by 40% and 2000 ng by 95%. The repression was alleviated when we used mE2F1Luc, a mutant construct that could no longer bind brα-Pal with high affinity, indicating that the repression was mediated via binding of brα-Pal to the E2F1-Pal1 site. Additionally, transcription from HIV-1 LTR that lacks authentic α-Pal sites, was not affected by 1000 ng of the brα-Pal. In vivo transcription was assessed by transient transfection assays. When the α-Pal-overexpressing vector, pCI-Pal27, and E2F1Luc promoter constructs were cotransfected into 293 cells, the results (Fig. 7B) show that cells cotransfected with wE2F1Luc and pCI-Pal27 repressed E2F1 transcription by about 5-fold compared to control cells that were cotransfected with wE2F1Luc and the empty expression vector, pCI-neo. When mE2F1Luc and pCI-Pal27 were cotransfected, the repression was relieved and transcription increased moderately by two-fold, indicating that the repression was mediated by the E2F1-Pal1 site.

Co-transfection of α-Pal expression vector and luciferase constructs of eIF2α and eIF-2β promoters into 293 cells showed that transcription from the wild-type promoters was stimulated by about 3-fold. Conversely, transcription from the mutant promoters that could no longer bind α-Pal with high affinity, were inhibited by about 4–5-fold. Taken together, the results of transcription assays confirm that binding of α-Pal to E2F1-Pal1 repressed the transcription of E2F-1 gene. Also, in agreement with earlier work [36,38], binding of α-Pal to the eIF-2α and eIF-2β promoters stimulated transcription. Fig. 7C–E shows that the rα-Pal was significantly overexpressed in the 293 cells as assessed by Northern blotting (C), Western blotting (D) and EMSA (E). Phosphoimager analysis of the Northern blot gave a 20-fold overexpression.

3.7. Unmodified nα-Pal binding activity is increased in α-Pal overexpressing cells

The results of the above binding studies and unpublished data suggest that there are two forms of nα-Pal dimers in cells: modified α-Pal (Mα-Pal) and unmodified α-Pal (UMα-Pal). According to Figs. 5B and 6 Mα-Pal appears to be a heterodimer and binds preferentially to eIF-PalH, and UMα-Pal appears to be a homodimer like brα-Pal, and may be the only form that binds to the E2F1-Pal1 site with high affinity. The data also suggests that UMα-Pal is present at only trace level in cells, and that the steady state concentration of Mα-Pal far exceeds that of UMα-Pal. By overexpressing α-Pal, the level of UMα-Pal may have increased beyond normal, leading to a downregulation of E2F-1 gene transcription (Fig. 7). To determine the level of UMα-Pal binding activity in cells and whether this activity was increased in response to rα-Pal overexpression, its binding activity to E2F1-Pal1 probe was assessed by EMSA using nuclear extracts from 293 cells transiently overexpressing α-Pal. The results (Fig. 8A) show firstly that UMα-Pal binding activity in 293 cells was generally at trace level compared to Mα-Pal (Lanes 6 vs. 1). Secondly, the binding activity of UMα-Pal was significantly increased in response to α-Pal overexpression (lanes 6 vs. 7). Lanes 8–10 show that even a 1000-fold excess of cold mutant eIF-PalH failed to disrupt binding of UMα-Pal to E2F1-Pal1 probe, even though 100-fold excess of the cold wild-type eIF2-PalH probe abolished the binding. Thus the demonstrated binding was α-Pal specific. Fig. 8B shows that UM-α-Pal binding activity was at trace level in TF-1 cells also, and increased

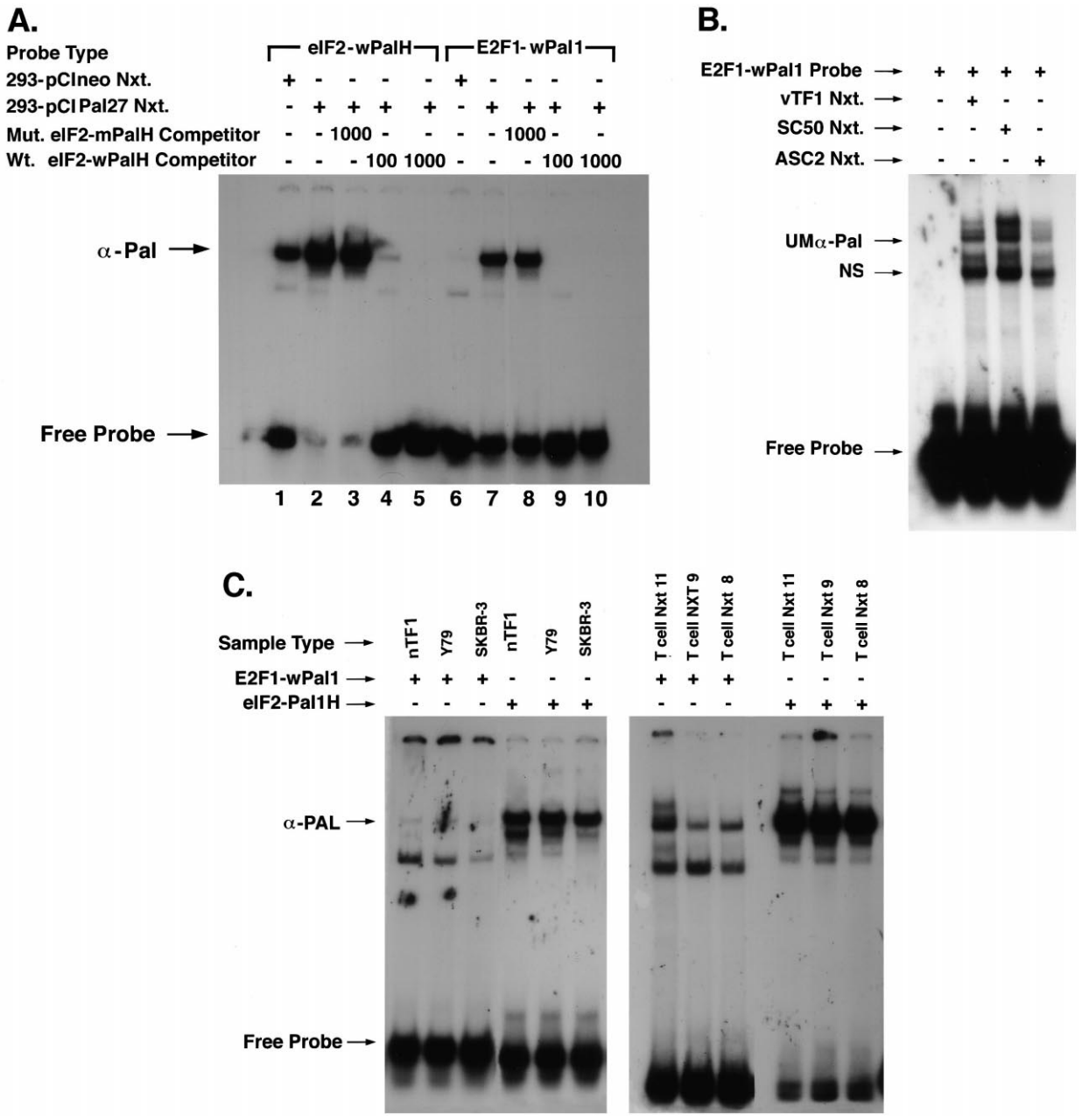

Fig. 8. UMα-Pal is significantly upregulated in response to rα-Pal overexpression. (A) Autoradiograph of EMSA illustrating binding activities of both Mα-Pal and UMα-Pal in response to rα-Pal overexpression in 293 cells. Each lane contained 5 μg of nuclear extract proteins. Lanes 1 and 2 indicate a significant overexpression of both Mα-Pal and UMα-Pal as detected by their eIF2-wPalH binding activities. Lanes 3–5 confirm the specificity of the binding since binding was unaffected by 1000-fold excess of cold eIF2-mPalH, but was abolished by 100-fold excess of cold E2F1-wPal1. Lanes 6 and 7 indicate a significant increase in UMα-Pal as detected by its E2F1-wPal1 binding activity. Lanes 8–10 confirm the specificity of the binding since binding was unaffected by 1000-fold excess of cold eIF2-mPalH, but was abolished by 100-fold excess of cold eIF2-wPalH probe. (B)Autoradiograph of EMSA illustrating increased E2F-1Pal1 binding activity of UMα-Pal in SC50 cells (lane 3). In agreement, ASC2 showed a decreased UMα-Pal (lane 4). Each lane contained 10 μg of nuclear extract proteins. (C) Autoradiograph of EMSA assessing the E2F-1Pal1 binding activities of UMα-Pal in the cancer cell lines; NTF-1, Y79 and SKBR-3 (lanes 1–3). For comparison, the corresponding eIF-2PalH binding activities of Mα-Pal are shown in lanes 4–6. Lanes 7–9 show UMα-Pal activities in T cell nuclear extracts and lanes 10–12 show the corresponding Mα-Pal binding activities in the same samples. Each lane contained 5 μg of protein.

in SC50 cells that stably overexpressed Pα-Pal. In tandem, the activity decreased in ASC2 cells that stably underexpressed α-Pal. Thus UMα-Pal in TF-1 cells also increased in response to α-Pal overexpression. The presence of only trace UMα-Pal in TF-1 and 293 cells most likely explains why relatively small changes in cellular levels of α-Pal was able to induce observable changes in the proliferation rates of the cells.

Assay of two other cancer cell lines, (Y79 and SKBR-3) showed similar trace levels of UMα-Pal (Fig. 8C). Normal activated human T-lymphocytes also contained relatively trace levels of UMα-Pal except that, on average, the levels were 3–5-fold greater than those of cancer cells. In all cases, though, the level of UMα-Pal was proportional to that of Mα-Pal (Fig. 8C). This indicates that the two forms of the protein are at steady state with one another in vivo. The lower, more-abundant, binding activity marked UN in Fig. 8A is considered non-specific since it was not competed away by cold probe.

4. Discussion

The transcription factor, α-Pal, regulates the expression of eIF-2α and eIF-2β, and is implicated in coordinating the regulation of key metabolic genes in response to changes in the growth status of the cell [33]. We have studied how its overexpression affects the regulation of key genes in a specific metabolic pathway, protein biosynthesis, and how the effects correlate with changes in cell growth and division cycle. When overexpressed, α-Pal differentially regulated cellular protein synthesis and proliferation activities: It upregulated protein synthesis but downregulated proliferation. The molecular basis for the increased protein synthesis was probably the transcriptional upregulation of the α and β subunits of eIF-2 genes (Figs. 2 and 7, and [38]). The molecular basis for the downregulation of proliferation appears to be the transcriptional inhibition of E2F-1 gene. This negative regulation appears to be mediated by a functional α-Pal binding site located 210 bp upstream of the first transcription start site on human E2F-1 promoter. Although this site should bind α-Pal with high affinity based on predictions from previous kinetic studies [33], it surprisingly showed negligible binding to Mα-Pal. In contrast, it bound UMα-Pal readily (Fig. 5B). Moreover, this binding mediated a transcriptional downregulation (Fig. 7) that contrasts with the transcriptional upregulation observed for the binding of Mα-Pal to sites on the eIF-2 promoter (Fig. 7, and [38]).

How α-Pal discriminates between the two types of sites and exerts opposite transcriptional effects is unknown. It is likely that heterodimerization with another protein and the unique architecture of the eIF-2 and E2F-1 sites play important roles. Nevertheless, these opposite transcriptional effects may explain the opposite phenotypic changes reported earlier. Overexpression of α-Pal in TF-1 cells likely resulted in elevated Mα-Pal and UMα-Pal (Figs. 1A, 7C–D and 8A). These bound to and upregulated the transcription of eIF-2 subunit genes, leading to their increased expression and increased protein synthesis. Because UMα-Pal also bound to its E2F1-Pal1 site and downregulated the expression of E2F-1 gene, cell cycle progression was retarded. Thus, increased protein synthesis failed to drive increased cell proliferation. Underexpression of α-Pal predictably resulted in the decreased levels of both forms of α-Pal (Figs. 1B and 8B). In the experiments reported here, the decrease in Mα-Pal was insufficient to decrease the transcription of eIF-2 subunit genes below basal level. Consequently, neither protein synthesis nor growth was significantly repressed. However, the decrease in UMα-Pal was sufficient to relieve whatever repression the normally trace level of UMα-Pal exerts on the expression of E2F-1 gene. This likely led to the upregulation of E2F-1 expression with an attendant increase in proliferation. The associated increase in the rate of cell division in the face of a normal rate of growth most likely resulted in the smaller sizes of ASC2 cells that underexpressed α-Pal.

Our model lacks direct evidence that the regulation of E2F-1 by α-Pal is solely responsible for the observed effects of α-Pal overexpression. As such, we cannot rule out the possibility that other mechanisms are responsible. An appropriate experiment is to overexpress α-Pal in E2F-1 deficient background to see if the effects are abrogated. We plan to address this question in future experiments using transgenic and knockout models, since human E2F-1 deficient cell lines are not available.

The mechanism described above may participate in coordinating cellular global protein synthesis, growth and division cycle activities during differentiation and after cells have terminally differentiated. Differentiating vertebrate and invertebrates cells must first exit their division cycle during these stages [4,48]. Permanent, terminally differentiated cells such as neurons and myocytes, maintain high metabolic activity, renew their components and can increase their size in response to increased growth or functional demands [4]. These activities require high levels of translational activity and, as such. They can mount up high levels of global protein synthesis even though they are non-mitotic. We note that skeletal muscle tissue, which is populated with terminally differentiated myotubes, overexpress α-Pal mRNA. Furthermore, SC50 clones upregulated the expression of $p27^{kip}$ that effects cell cycle exit in differentiating, terminally differentiated and senescent cell (figures not shown). Another situation in which the above mechanism can apply is the coordinated regulation of protein synthesis, growth and cell proliferation in the imaginary disks of the developing *Drosophila*. When Weigmann et al. [9] blocked cell division in the developing *Drosophila* wing imaginary disk, cell growth continued, resulting in fewer larger cells, but a normal disk size. These cells apparently continued with high rates of global protein synthesis resulting in their larger sizes. Whether it is differentiation or developmental patterning, α-Pal likely participates in the transcriptional repression of E2F-1 gene and couples this to the transcriptional modulation of eIF-2 genes. By this mechanism, α-Pal may coordinate the regulation of cellular global protein synthesis and cell cycle exit during these processes. The mechanism may explain the previously reported roles of α-Pal homologs in *Drosophila* and sea urchin embryogenesis [34,35]. E2F1 transcription has also been shown to be partially repressed by pRB-E2F complexes that bind to autoregulatory E2F sites on the E2F1 promoter [49–53].

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