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The RAS-dependent ERF Control of Cell Proliferation and Differentiation Is Mediated by c-*Myc* Repression*[S]

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The ERF transcriptional repressor is a downstream effector of the RAS/ERK pathway that interacts with and is directly phosphorylated by ERKs *in vivo* and *in vitro*. This phosphorylation results in its cytoplasmic export and inactivation, although lack of ERK activity allows its immediate nuclear accumulation and repressor function. Nuclear ERFs arrest cell cycle progression in G_1 and can suppress *ras*-dependent tumorigenicity. Here we provide evidence that ERF function is mediated by its ability to repress transcription of c-*Myc*. Promoter reporter assays indicate a DNA binding-dependent and repressor domain-dependent *Myc* transcriptional repression. Chromatin immunoprecipitations in primary cells suggest that ERF specifically binds on the c-*Myc* promoter in an E2F4/5-dependent manner and only under conditions that the physiological c-*Myc* transcription is stopped. Cellular systems overexpressing nuclear ERF exhibit reduced c-*Myc* mRNA and tumorigenic potential. Elimination of *Erf* in animal models results in increased c-*Myc* expression, whereas $Erf^{-/-}$ primary fibroblasts fail to down-regulate *Myc* in response to growth factor withdrawal. Finally, elimination of c-*Myc* in primary mouse embryo fibroblasts negates the ability of nuclear ERF to suppress proliferation. Thus *Erf* provides a direct link between the RAS/ERK signaling and the transcriptional regulation of c-*Myc* and suggests that RAS/ERK attenuation actively regulates cell fate.

ETS2-repressor factor (ERF)[3] is a ubiquitously expressed transcriptional regulator of the ETS family of transcription factors, with tumor suppressor activity, that is regulated by the RAS/ERK signaling pathway. ERF is shown to be bound and phosphorylated both *in vivo* and *in vitro* by ERKs (1, 2). It interacts specifically with active and inactive ERKs via two distinct F*X*F motifs and can effectively block ERK-substrate interaction (3). In the absence of growth factors, ERF is dephosphorylated and located in the nucleus, whereas upon mitogenic stimulation and in exponentially growing cells, it is actively transported into the cytoplasm through a CRM-dependent mechanism (4). Phosphorylation-deficient ERF mutants are able to reverse RAS-induced tumorigenicity and arrest fibroblasts in the G_0/G_1 phase of the cell cycle, determining ERF as a *bona fide* ERK substrate and an effector of the RAS/ERK pathway (2–4). ERF-mediated cell cycle arrest can be abolished by the overexpression of cyclins D and E or the inactivation of the retinoblastoma protein, providing a strong link with cell cycle regulation (2, 4). Homozygous deletion of *Erf* leads to a block of chorionic trophoblast differentiation, the absence of chorioallantoic fusion, persisting chorion layer, the absence of labyrinth formation, expansion of the giant cell layer, diminishing of the spongiotrophoblast layer, and eventual embryo death by 10.5 dpc (5). Trophoblast stem cell lines derived by $Erf^{-/-}$ embryos exhibit delayed differentiation kinetics and decreased expression of spongiotrophoblast terminal differentiation markers suggesting that the ERF is required for extraembryonic ectoderm and trophoblast stem cell differentiation. Thus, there is emerging evidence for ERF contribution in cell cycle inhibition and promotion of differentiation. However, relevant downstream ERF targets have not yet been identified, rendering unclear its mechanism of action.

c-MYC is a ubiquitously expressed transcription factor that in physiological levels binds about 10% of the human promoters (6) and regulates crucial cell functions such as proliferation, differentiation, apoptosis, metabolism, and cell growth (for review see Ref. 7). c-MYC induces the activity of cyclin-cyclin-dependent kinase complexes or affects directly the expression of cell cycle regulators (8–15), suggestive of its role in cell cycle progression and consistent with the severely retarded proliferation of the c-$Myc^{-/-}$ primary mouse fibroblasts because of G_1 and G_2 phase lengthening (16, 17). Heterozygous c-*Myc* fibroblasts show slower growth rates (18), whereas c-MYC controls mammalian and fly body size in a dose-dependent manner (19–21), indicating that subtle perturbations in c-MYC levels lead to profound defects in cell and organismal physiology.

Tight regulation of c-*Myc* expression is achieved in transcriptional, post-transcriptional, translational, and post-translational levels. c-*Myc* mRNA expression is driven mainly by two promoters, P1 and P2, with the latter being responsible for pro-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[3] The abbreviations used are: ERF, ETS2-repressor factor; WT, wild type; dpc, days post-coitum; RT-qPCR, reverse transcription-quantitative PCR; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MEF, mouse embryo fibroblast; KO, knock out; RB, retinoblastoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase.

ducing the majority of c-*Myc* transcripts under physiological conditions. Early studies have shown that c-*Myc* transcripts are present in low levels in growing cells, drop even more in the absence of growth factors, and re-emerge upon induction by mitogens and subsequent G_0 exit (22, 23). c-*Myc* transcription is reported to be down-regulated during differentiation (24–27) in accordance with inhibition of cell proliferation and G_1 cell cycle arrest (28–31). No single growth factor or signaling pathway appears to be responsible for c-*Myc* promoter full activity. Rather, the c-*Myc* promoter is regulated by different pathways that converge to a large panel of transcription factors acting at different time points, cell types, and developmental stages, ensuring proper and accurate c-*Myc* transcription (32).

The RAS pathway, which is involved in cell cycle regulation in response to mitogens, tumorigenesis, and differentiation, has been established that can affect c-MYC levels primarily at a post-translational level. Upon serum stimulation, the RAS/ERK pathway phosphorylates c-MYC at serine 62, whereas at the same time the RAS/phosphatidylinositol 3-kinase/Akt pathway inhibits GSK3 activity preventing the destabilizing phosphorylation at threonine 58 (33, 34). There is also indirect evidence that the RAS/ERK pathway can affect c-*Myc* mRNA levels. ETS1/2 transcription factors have been shown to bind the c-*Myc* promoter *in vitro* and to regulate c-*Myc* transcription (35, 36). Overexpression of an activated or a dominant negative mutation of Raf1 led to c-*Myc* mRNA up-regulation or down-regulation, respectively (37). Serine 10 phosphorylation of histone H3 at the c-*Myc* coding region is observed in mouse fibroblasts upon stimulation of ERK activity (38). Finally, transcriptome analysis after ERK activation revealed c-*Myc* mRNA up-regulation (39). However, direct molecular links between the RAS/ERK pathway and the c-*Myc* transcriptional regulation are missing.

In this study, we use biochemical and genetic approaches to provide evidence that ERF when nuclear represses c-*Myc* transcription and that this repression mediates ERF function. Promoter reporter assays indicate that ERF can repress c-*Myc* promoter-driven transcription in an ERF localization-, DNA binding domain-, and repressor domain-dependent manner. Chromatin immunoprecipitation assays indicate that endogenous ERF binds the P1-P2 promoter region in serum-deprived primary MEFs consistent with c-*Myc* transcriptional repression. Overexpression of nuclear ERF represses c-*Myc* mRNA levels in normal and RAS-transformed fibroblasts and in MCF7 adenocarcinoma cells, and suppresses transformation in RAS-transformed fibroblasts and MCF7 cells. Elimination of *Erf* increases c-*Myc* expression in the mouse embryo and placenta and attenuates its repression upon growth factor withdrawal in MEFs. Finally, the cell cycle arrest induced by nuclear ERF is abrogated in the absence of c-*Myc*. Thus, c-*Myc* is identified as the first cellular target of the ERF transcriptional repressor, providing insight into the mechanism of *Erf* function and the first direct linking between the RAS/ERK signaling pathway and c-*Myc* transcriptional regulation.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Plasmids—Primary *Erf* KO and WT mouse embryos at embryonic day 10 or 13.5 were dissected and plated in high glucose DMEM supplemented with 10% FBS, penicillin/streptomycin, and 50 μM β-mercaptoethanol (Invitrogen). Ref1 and MEF3T3 cells (Clontech) were grown in high glucose DMEM supplemented with 10% FBS and penicillin/streptomycin (Invitrogen), whereas for the MCF-7 cell lines low glucose DMEM was used. Finally, the RAS3T3 cell lines were maintained in low glucose medium supplemented with 8% bovine serum (Invitrogen) and penicillin/streptomycin. Transfections were performed with the calcium phosphate method. Stable MCF7 cell lines were selected with 1000 μg/ml G418 for 3 weeks, and individual clones were tested for ERF expression by Western blot. MEF3T3 Tet-Off cell lines were selected with 400 μg/ml hygromycin for 2 weeks, and individual clones were tested by Northern blot and GFP expression. MEF3T3 Tet-Off cells were maintained in 100 ng/ml doxycycline. To induce ERF expression, cells were extensively washed with phosphate-buffered saline, and fresh media, without doxycycline, were added. Cells were harvested 3 days after doxycycline removal. RAS 3T3 ERF WT and M1–7 cell lines and RB KO MEFs were described previously (2, 4). All cell lines were maintained in a humidified 37 °C incubator with 5% CO_2. pBI-ERF WT and pBI-ERF M1–7 constructs were made by inserting the XhoI/XbaI fragment of the pEGFP-ERF WT or pEGFP-ERF M1–7 plasmids, respectively, into the XhoI/NheI sites of a modified pBI vector (40). pSG5-ERF WT, pSG5-ERF M1–7, and pSG5-ERF ΔDBD were described previously (1). pSG5-ERF ΔRD was created by deleting the SmaI/BglII fragment from the respective pSG5-ERF WT construct. The −1100/+580 and −140/+340 p19Luc c-*Myc* promoter plasmids were described previously (41).

Soft Agar Assay—Tissue culture medium and agarose were mixed with 5×10^4 cells to a final agarose concentration of 0.35%. The samples were immediately plated in 6-well plates coated with 0.5% agarose in medium and cultured in a humidified 37 °C incubator with 5% CO_2. Cells were fed every 3 days with 0.35% agarose in medium, and colony formation was observed after 3 weeks. Photographs were taken with a Leica DFC 300 camera on an inverted microscope.

BrdUrd Incorporation Assay—WT and c-*Myc* knock-out primary MEFs were transfected either with empty vector or ERF M1–7, and BrdUrd incorporation assay was performed as described previously (2).

Luciferase Assay—Ref1 cells were plated in 35-mm plates at a density of 200 cells/mm^2 and were transfected overnight by calcium phosphate with 0.1 μg of the indicated reporter plasmid, 2 μg of the effector plasmid, and 1 μg of the RSV-GAL plasmid to normalize transfection efficiency. Cells were lysed with lysis buffer (Promega), and luciferase activity was measured in an FB12 luminometer.

RNA Extraction and RT-qPCR—Cells were harvested and lysed with 1 ml of TRIzol reagent (Invitrogen). RNA was extracted and DNase I-treated, and 5 μg of total RNA were reverse-transcribed with the Amersham Biosciences first-strand cDNA synthesis kit. Quantitative PCR was performed with RNA-specific primers in an ABI Prism 7000 real time PCR machine, using the Brilliant SYBR Green QPCR Master mix (Stratagene). Results were normalized and quantified by the manufacturer's software and further analyzed with Microsoft

Office Excel software. The primer sets used were the following: mouse c-*Myc* mRNA, 5′-mouse *Erf* mRNA, 5′-CACCGAGATTCCTGAGAGC-3′ and 5′-AATCCTGTACCTCGTCCGAT-3′ and 5′-TCTTCTCCACAGACACCACA-3′; AGAGACTAAAGAGAGCTGTCC-3′; mouse *Cph* mRNA, 5′-AGACCAGCAAGAAGATCACC-3′ and 5′-GGAAAATATGGAACCCAAAG-3′; human c-*MYC* mRNA, 5′-CTCAACGACAGCAGCTCG-3′ and 5′-CAGAAGGTGATCCAGACTC-3′; human *GAPDH* mRNA, 5′-TCGACAGTCAGCCGCATCTT-3′ and 5′-CGCCCAATACGACCAAATCC-3′.

Chromatin Immunoprecipitation and qPCR—ChIP assays were performed as described previously (42) with a few modifications. Briefly, 5–10 × 10^6 primary MEFs grown under the indicated conditions in 150-mm plates were fixed with 1% formaldehyde in phosphate-buffered saline for 10 min at room temperature or at 37 °C. Cells were scraped off the plate and incubated in a hypotonic buffer containing 25 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, and the nuclei were isolated after cell disruption in a Dounce homogenizer with a glass pestle B. Nuclear extracts were sonicated in sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS) and were pre-cleared with protein A beads. The precleared extracts were incubated overnight with the indicated antibodies, and protein A beads were added for 1 h. The immunoprecipitated material was washed twice with each of the following buffers: sonication buffer, wash buffer A (sonication buffer with 500 mM NaCl), wash buffer B (20 mM Tris, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% deoxycholate), and TE and eluted twice with 50 mM Tris, pH 8.0, 1 mM EDTA, 1% SDS at 65 °C. The extracts were de-cross-linked in elution buffer supplemented with 250 mM NaCl at 65 °C overnight and treated with RNase and proteinase K, and DNA was phenol/chloroform-extracted. Radioactive PCRs were done by adding 0.1 μCi of radiolabeled ATP and CTP, and the products were electrophoresed in 6% polyacrylamide gel in TBE buffer. qPCR was performed as described with the following primer sets: 5′-distal, 5′-GAATACTACGCTGTGCATTC-3′ and 5′-AGGATAAGCAAATCCCGAGG-3′; 5′-proximal, 5′-ACGCAGGGCAAGAACACAG-3′ and 5′-GCGCTATTACTGTTTACACC-3′; P1-P2, 5′-TTGGAAGAGCCGTGTGTGC-3′ and 5′-AGTGAGAAGTGTCTGCCCG-3′; Intron1, 5′-CCTGAGCTGTTTGGAGAAGG-3′ and 5′-TCCCAGGCTGTCAGAAATGC-3′; and mouse GAPDH, 5′-CCAGTATGACTCCACTCACG-3′ and 5′-GACTCCACGACATACTCAGC-3′.

In Situ Hybridization—In situ hybridizations of frozen placenta sections were performed as described previously (5). Briefly, paraformaldehyde-fixed cryosections were hybridized overnight at 55 °C in 50% formamide, 5× SSC, 5× Denhardt's, 0.25 mg/ml yeast RNA, 0.5 mg/ml herring sperm DNA, and 1 ng/μl of each c-*Myc* and *Erf* riboprobes labeled with digoxigenin-11-UTP or fluorescein-12-UTP (Roche Applied Science), respectively. After hybridization, the sections were washed, treated with RNase, blocked with 10% fetal calf serum, incubated with the appropriate antibody, developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate or 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as substrates, and photographed.

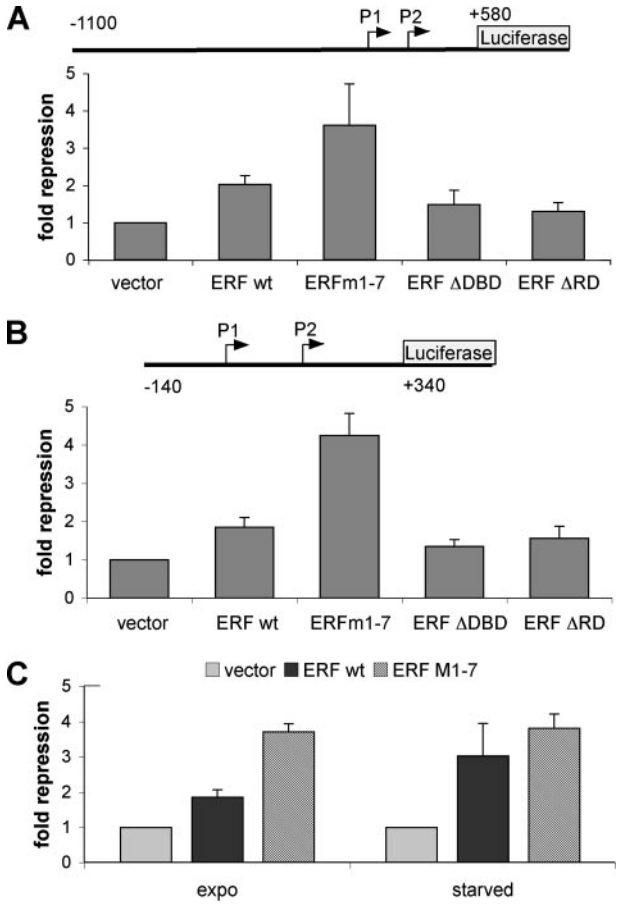

FIGURE 1. **ERF represses *c-Myc* promoter in Ref1 cells.** *A,* Ref1 cells were co-transfected with 0.1 μg of the −1100/+580 p19Luc c-*Myc* promoter construct, 2 μg of the indicated ERF constructs, and 1 μg of the RSV-GAL as transfection efficiency control. *B,* same as in *A*, but −140/+340 p19Luc c-*Myc* promoter construct was used. *C,* same as in *A*, but cells were grown in complete media (*expo*) or serum-deprived for 14 h (*starved*). The *graphs* represent the average of a minimum of five independent experiments. The *bars* indicate standard error.

RESULTS

ERF Can Inhibit Transcription from the c-MYC Promoter—ERF can arrest cells in the G_1 phase in an RB-dependent manner, can suppress *ras*- and *ets*-induced tumorigenicity, and is required for chorion cell differentiation (1, 2, 4, 5), but little is known about its downstream targets that may mediate the ERF effects. The major immediate-early promoter of the human cytomegalovirus (43, 44) and the utrophin-A promoter (45) that have been identified as ERF targets could not account for ERF function. c-*Myc*, a ubiquitously expressed gene that contains functional ets-binding sites, has been shown to accelerate cell proliferation and block differentiation, and its expression pattern mirrors ERF nuclear localization and could be a valid ERF target and could account for most of the ERF-associated phenotypes.

To test this hypothesis, we performed promoter reporter assays in Ref1 cells using plasmids containing a 1680-bp region of the murine c-*Myc* gene flanking the major P1-P2 promoters (41) (Fig. 1*A*). WT ERF could repress transcription from the c-*Myc* promoter by 2-fold, whereas the phosphorylation-defi-

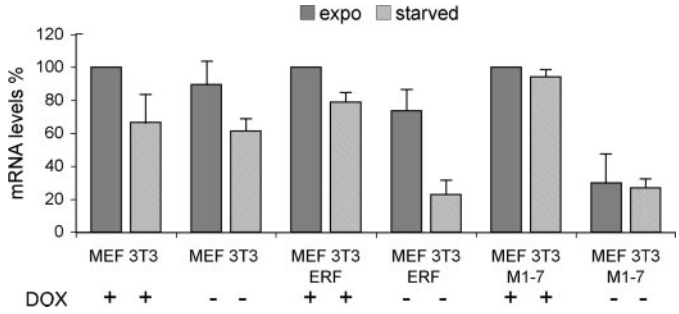

FIGURE 2. **Nuclear ERF decreases c-*Myc* mRNA levels.** WT ERF protein and ERFm1–7 constitutive nuclear mutant protein were expressed from an inducible promoter (Tet-Off) in the respective stably transfected MEF3T3 cell lines, and *c-Myc* mRNA levels were quantified by RT-qPCR during exponential growth and after 4 h of serum starvation. Normalization was performed against *CPH* mRNA levels. 100% represents the c-*Myc* mRNA level for each cell line under growth conditions in the presence of doxycycline (*DOX*). The values shown are the average of three independent experiments.

cient nuclear ERFm1–7 mutant decreased transcription by 4-fold (Fig. 1*A*). Direct DNA binding onto the c-*Myc* promoter is required for ERF repression as indicated by the ERFΔDBD mutation that eliminates the ETS DNA binding domain (Fig. 1*A*, *ERF ΔDBD*). The ERF repression domain is also required for the c-*Myc* promoter repression (Fig. 1*A*, *ERF ΔRD*), suggesting that the observed repression is not because of the displacement of endogenous activating factors but to active and direct repression by ERF. Identical results were observed when a minimal c-*Myc* promoter fragment from +140 to −340 bp was used (Fig. 1*B*) indicating that the interaction occurs within the previously identified *ets*-responsive region. To examine if the observed difference between the WT ERF and ERFm1–7 was because of differences in nuclear accumulation, transfected cells were grown in the absence of serum for 14 h. Under these conditions WT ERF could repress c-*Myc* promoter-driven transcription to the same extent as the constitutively nuclear mutant ERFm1–7 (Fig. 2*C*), suggesting that the WT ERF can repress c-*Myc* transcription when nuclear in a physiologically relevant setting.

Inducible MEF3T3 cell lines that could express WTERF or ERFm1–7 in the absence of doxycycline (supplemental Fig. S1*A*) were generated to test the effect of ERF expression on endogenous c-*Myc*. Under growth conditions induction of WTERF expression had negligible effect on c-*Myc* mRNA levels compared with the uninduced or the induced parental cells. In contrast, expression of ERFm1–7 decreased c-*Myc* expression to 30%. Serum withdrawal had a modest effect on parental or uninduced cells but resulted in 75% reduction of c-*Myc* mRNA levels in both WT and ERFm1–7-expressing induced cells (Fig. 2). These data suggest that nuclear ERF can repress the endogenous c-*Myc* expression under normal growth conditions and that increased ERF levels enhance c-*Myc* repression during growth factor withdrawal.

ERF Interacts with c-MYC Promoter in Vivo—To establish that indeed ERF could regulate transcription from the c-*Myc* promoter, we investigated the association of the endogenous ERF protein with the c-*Myc* promoter. WT primary MEFs were utilized for ChIP experiments. MEFs were grown from 13.5 dpc embryos to passage 3, and at this point were either serum-starved for 4 h or continued to grow in 10% serum. ChIP assays

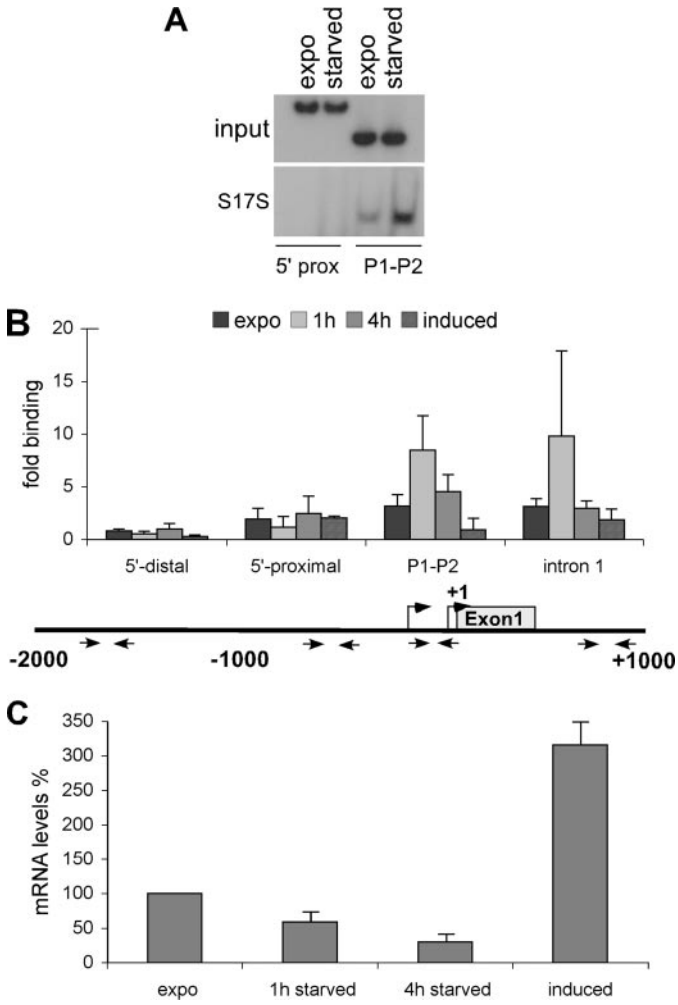

FIGURE 3. **ERF binds the c-*Myc* P1-P2 promoter region.** *A,* ChIP assay was performed in exponentially growing (*expo*) or 4-h serum-deprived (*starved*) primary MEF with 15 μl of S17S anti-ERF antibody, followed by ^{32}P-labeled PCR using primers spanning the +48 to +236 (*P1-P2*) and the −512- to −259-bp (*5′ prox*) region of the c-*Myc* promoter, and nondenaturing PAGE. Labeled products were detected by autoradiography. *B,* ChIP assay was performed as in *A* in primary MEF growing in complete media (*expo*) cells or in the absence of serum for 1 (*1 h*) or 4 h (*4 h*), or serum-deprived for 4 h and then induced for 15 min with 20% of fetal bovine serum (*induced*), and quantified by qPCR. Normalization of the ERF binding on the *c-Myc* promoter region was done relative to the ERF binding on a nonspecific region (*Gapdh* coding region). The values shown are the average of three independent experiments. A schematic representation of the murine c-*Myc* 5′ upstream region is shown *below* the graph. *Arrows* represent the positions of primer sets used and correspond to the graph above. *C,* MEFs were treated as in *B*, and total RNA was extracted. RT-qPCR with *c-Myc* RNA-specific primers was performed to quantify *c-Myc* mRNA levels in comparison to *CPH* mRNA levels.

were performed using the ERF-specific S17S rabbit polyclonal antibody, and the precipitated material was PCR-amplified using primers either from the P1-P2 region of the c-*Myc* promoter or a proximal 5′ primer set. An increased specific interaction of ERF with the P1-P2 promoter region of the c-*Myc* gene could be observed only during serum withdrawal, whereas ERF was nuclear and the c-*Myc* transcription decreased (Fig. 3*A*).

We further analyzed the specificity and the kinetics of the ERF binding on c-*Myc* promoter region in conjunction with c-*Myc* expression. MEFs were serum-starved for 1 and 4 h or starved for 4 h and then induced with 20% serum, and primer

sets spanning the c-*Myc* genomic locus were used to map the ERF occupancy and ERF binding on the c-*Myc* locus, and c-*Myc* mRNA levels were quantified by real time PCRs. Background ERF binding could be seen at the 5′ far upstream region of c-MYC at all times. In contrast, ERF protein could be detected on the c-*Myc* P1-P2 promoter region even in exponentially growing cells. Consistent with the kinetics of ERF nuclear accumulation, strong induction of ERF binding in the P1-P2 region was observed 1 h after serum withdrawal, whereas ERF levels dropped after 4 h of serum starvation. ERF binding on the c-*Myc* promoter after serum induction was decreased below the basal level observed during exponential growth (Fig. 3*B*). As expected c-*Myc* mRNA levels decreased during growth factor withdrawal and rapidly increased during serum stimulation (Fig. 3*C*) mirroring the binding of ERF onto the c-*Myc* promoter. The direct ERF effect on c-*Myc* transcription is also supported by the RNA polymerase II occupancy of the c-*Myc* promoter (supplemental Fig. S2). These data strongly suggest that under physiological conditions ERF may bind in the P1-P2 promoter region of c-*Myc* and repress its expression.

Erf Can Suppress Transformation through c-MYC Repression—We have shown that the tumorigenic potential of *ras*-transformed NIH3T3 cells can be suppressed by ERF mutations with nuclear localization (2). Cells expressing the nuclear ERFm1–7 mutant exhibited dramatically decreased tumorigenicity and had a 70% decrease in c-*Myc* mRNA levels compared with the parental RAS-3T3. In contrast, cells expressing WT ERF were comparable with the parental RAS-3T3 tumorigenicity and comparable with c-*Myc* transcription (Fig. 4*A*). These data suggest that the suppression of the *ras*-induced cellular transformation by ERF may be directly due to the ability of ERF to repress c-*Myc* transcription.

To further test this hypothesis we used the mammary epithelial adenocarcinoma cells MCF7 that have been shown to depend on c-*Myc* expression for their tumorigenic potential (46–48). We established MCF7 cells lines that overexpress either WT or the m1–7 nuclear mutation of ERF (supplemental Fig. 1*B*), and we tested their tumorigenic potential by soft agar assays. During normal serum growth c-*Myc* mRNA levels were unaffected by the expression of the transgenes. During low serum growth, the parental and the WTERF-transformed cells exhibited marginal decrease in c-*Myc* mRNA levels, consistent with the presence of activated ERK at this stage (not shown). In contrast, cells expressing the ERFm1–7 nuclear mutation exhibited a 4-fold decrease in c-*Myc* mRNA (Fig. 4*B*). Significantly, c-*Myc* mRNA levels were consistent with anchorage-independent growth ability of the cells. In complete serum media colony formation was comparable for all cell lines (Fig. 4*C*, *panels A–F*). This was also true for the parental and the WT ERF-expressing MCF7 cells during low serum growth (Fig. 4*C*, *panels G–J*). In contrast, cells expressing the ERFm1–7 mutation failed to form soft agar colonies under low serum conditions (Fig. 4*C*, *panels K* and *L*). These data indicate that repression of c-*Myc* expression may indeed be the reason for the suppression of oncogenic transformation by *ERF*.

ERF Regulates c-MYC Expression in Vivo—Elimination of *Erf* leads to failure of terminal trophoblast stem cell differentiation and chorioallantoic attachment in the mouse placenta (5). Mis-

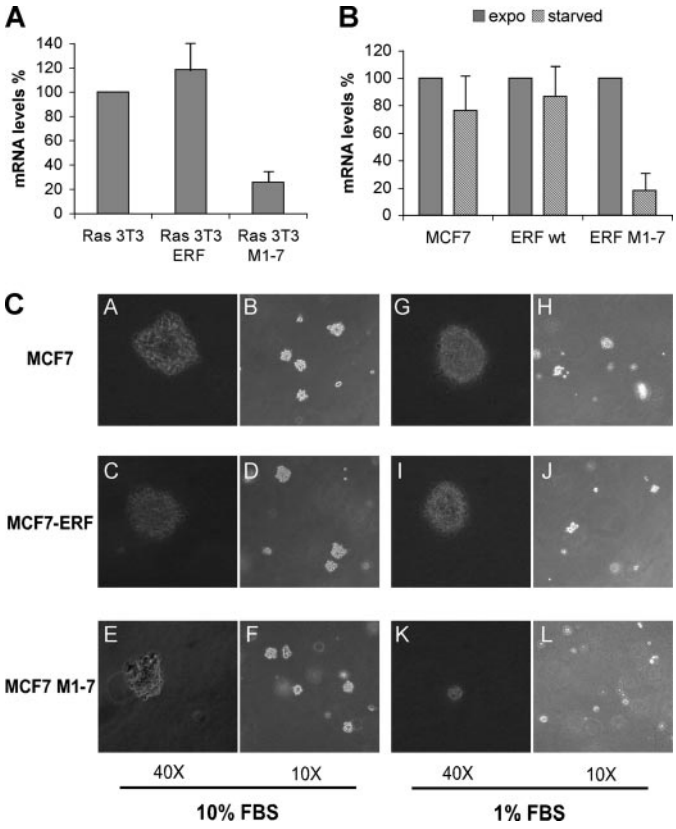

FIGURE 4. **The suppression activity of *Erf* follows *c-Myc* mRNA levels.** *A,* we have shown that that nuclear ERF (*ERFm1–7*) can suppress *ras*-induced transformation in fibroblasts. Total RNA was extracted from exponentially growing parental, WTERF-, or ERFm1–7-expressing *ras*-transformed NIH3T3 cells. *c-Myc* mRNA was quantified by RT-qPCR in comparison with the *Cph* mRNA levels. The values shown are the average of three independent experiments. *B,* total RNA was extracted from exponentially growing (*expo*) or 4-h serum deprived (*starved*) parental, WTERF- or ERFm1–7-expressing MCF7 adenocarcinoma cells. *c-MYC* mRNA was quantified by RT-qPCR in comparison with *GAPDH* mRNA levels. The values shown are the average of three independent experiments. 100% represents the c-*MYC* mRNA level for each cell line under growth conditions. *C,* 5×10^4 parental, WTERF-, or ERFm1–7-expressing MCF7 cells were seeded in DMEM containing 0.35% agarose supplemented with either 10 or 1% FBS in 35-mm plates. Cells were grown in a humidified 37 °C incubator with 5% CO_2. The formation of colonies was observed 2 weeks later in a Leica DM IRE2 microscope, and photographs were taken by a Leica DFC300 FX CCD camera.

regulated c-*Myc* can affect differentiation in a number of systems (49) and can induce pluripotency (50). We thus examined the expression of c-*Myc* and *Erf* in WT developing mouse placentas at 9.5 and 10.5 dpc by double *in situ* hybridization. At 9.5 dpc *Erf* was expressed along the basal chorion layer, whereas c-*Myc* was expressed mostly along the inner chorion layer and the spongiotrophoblast layer, with minimal expression in cells that expressed *Erf* (Fig. 5, *a–d*). At 10.5 dpc the *Erf* expression was more restricted to chorion diploid cells, whereas c-*Myc* expression was scattered throughout the labyrinth and the spongiotrophoblast layers. At this stage an extensive exclusion of c-*Myc* expression could be observed in cells that expressed *Erf* (Fig. 5, *e–h*). *Erf* KO placentas expressed c-*Myc* throughout the chorion, including the chorion basal layer (supplemental Fig. S3). c-*Myc* mRNA levels were found almost 3-fold higher in *Erf* KO placentas as compared with their WT littermates supporting the hypothesis of *Myc* regulation by *Erf in vivo*.

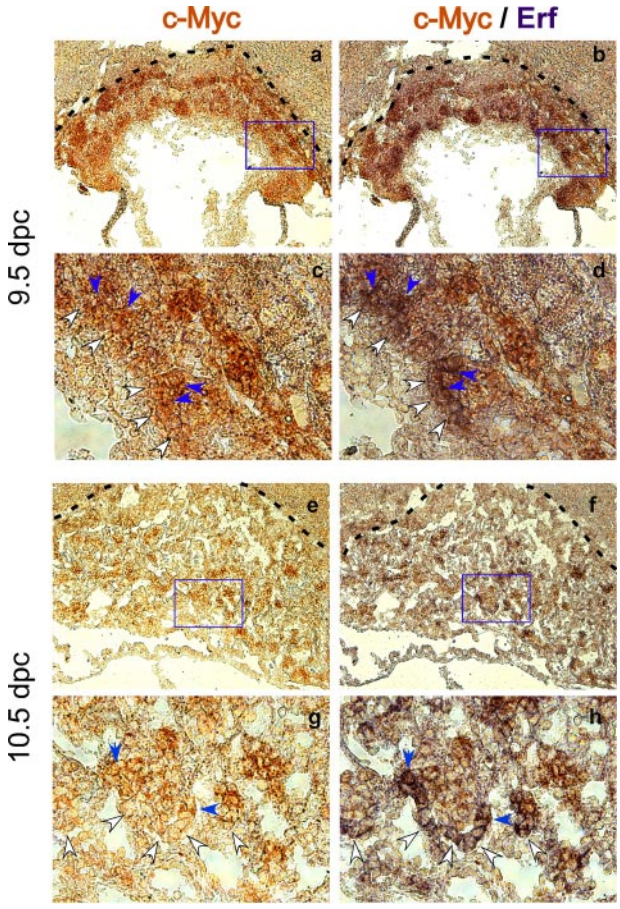

FIGURE 5. **Different cell populations express c-*Myc* and *Erf* in the mouse placenta.** Cryosections from 9.5 dpc (*a–d*) and 10.5 dpc (*e–h*) WT placentas were hybridized with digoxigenin-labeled c-*Myc* and fluo-labeled *Erf* probes and stained first with 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride for c-*Myc* (*orange*) and then 5-bromo-4-chloro-3-indolyl phosphate for *Erf* (*purple*). Photographs of the same sections were taken after each staining step with a ×10 (*a, b, e,* and *f*) or ×40 lens (*c, d, g,* and *h*). The *blue boxes* in *a, b, e,* and *f* indicate the positions of *c, d, g,* and *h*, respectively. *White arrowheads* indicate areas with *Erf* expression and minimal c-*Myc* expression. *Blue arrowheads* indicate cells that co-express *Erf* and c-*Myc*. The *dotted line* indicates the boundaries of the placentas.

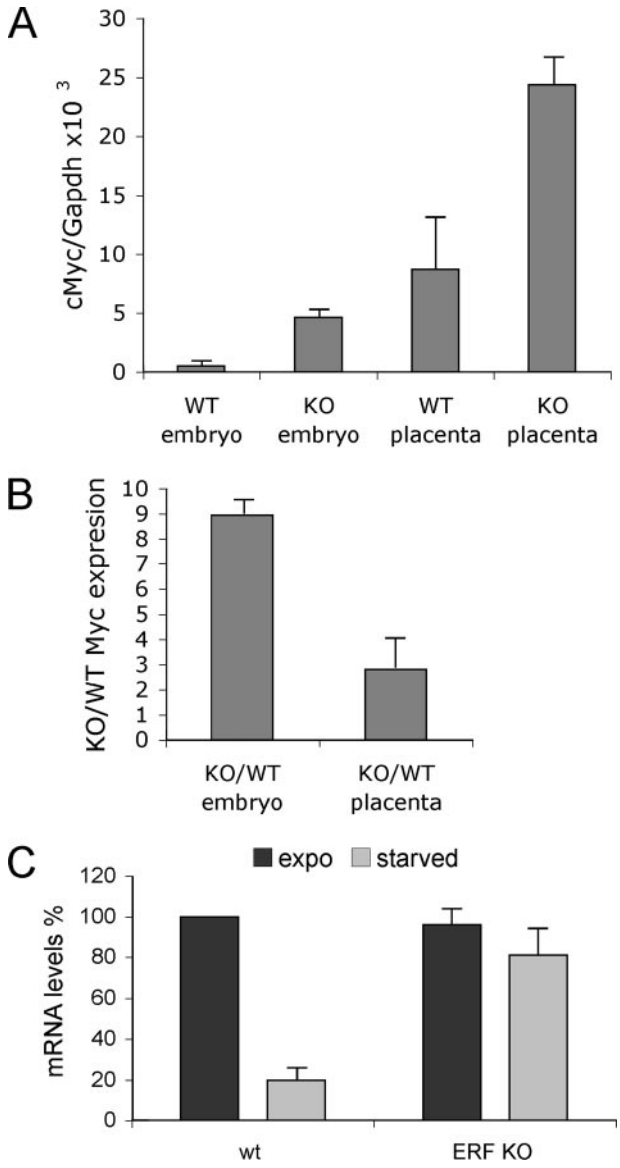

FIGURE 6. **c-*Myc* mRNA levels are increased in the absence of *Erf*.** *A,* c-*Myc* expression in 9.5 dpc WT and *Erf*-KO embryo and placenta littermates was quantified by RT-qPCR in comparison to *Gapdh* mRNA. *B,* data from *A* expressed as fold increase of c-*Myc* mRNA in the absence of *Erf*. *C,* c-*Myc* expression in WT and *Erf*–KO MEFs derived from 10 dpc littermates growing in complete or serum-deprived media for 4 h. c-*Myc* mRNA levels were quantified by RT-qPCR in comparison with the *Cph* mRNA. The *graph* represents the average of four independent experiments.

To minimize contribution of other possible developmental differences, we generated MEFs from 10 dpc *Erf* KO embryos and their WT littermates and tested c-*Myc* expression. c-*Myc* mRNA levels in complete serum media were identical in both WT and *Erf* KO MEFs. Four hours of growth factor withdrawal, however, was sufficient to decrease c-*Myc* levels by 80% in WT MEFs but only a marginal 20% in *Erf* KO MEFs (Fig. 6*C*). These data indicate that indeed nuclear ERF may be responsible for c-*Myc* transcriptional repression in the absence of RAS/ERK signaling.

We have shown previously that overexpression of nuclear ERF can inhibit proliferation of many cells, including primary MEF (2, 4). Thus, we examined the effect of nuclear ERF in the proliferation of c-$Myc^{-/-}$ MEFs. Despite the poor proliferation rate of c-$Myc^{-/-}$ MEFs compared with WT MEFs, their proliferation was unaffected by the overexpression of nuclear ERF (Fig. 7*A*) supporting the hypothesis that the ERF inhibition on cell proliferation is mediated by c-*Myc* transcriptional repression. We have also shown that proliferation inhibition by nuclear *Erf* is *Rb*-dependent. Thus, we examined c-*Myc* expression in $Rb^{-/-}$ MEFs in the presence or absence of serum. The level of c-*Myc* mRNA in $Rb^{-/-}$ MEFs was 3 orders of magnitude lower that WT MEFs and was not further reduced after serum withdrawal (Fig. 7*B*), suggesting that $Rb^{-/-}$ MEF growth is c-*Myc*-independent. This would be consistent with the inability of nuclear ERF to inhibit proliferation of $Rb^{-/-}$ MEFs. Interestingly, ERF could not be detected on the c-*Myc* promoter in $Rb^{-/-}$ cells independent of growth conditions, suggesting that c-*Myc* expression can be also repressed by an ERF-independent mechanism (Fig. 7*C*). Pocket binding proteins are established regulators of c-*Myc* transcription. E2F4/5 has been shown to repress c-*Myc* (8). We thus examined the binding of ERF on the c-*Myc* promoter in $E2f4^{-/-}$ (51) and $E2f5^{-/-}$ (52) MEFs. In exponentially growing cells ERF exhibits comparable

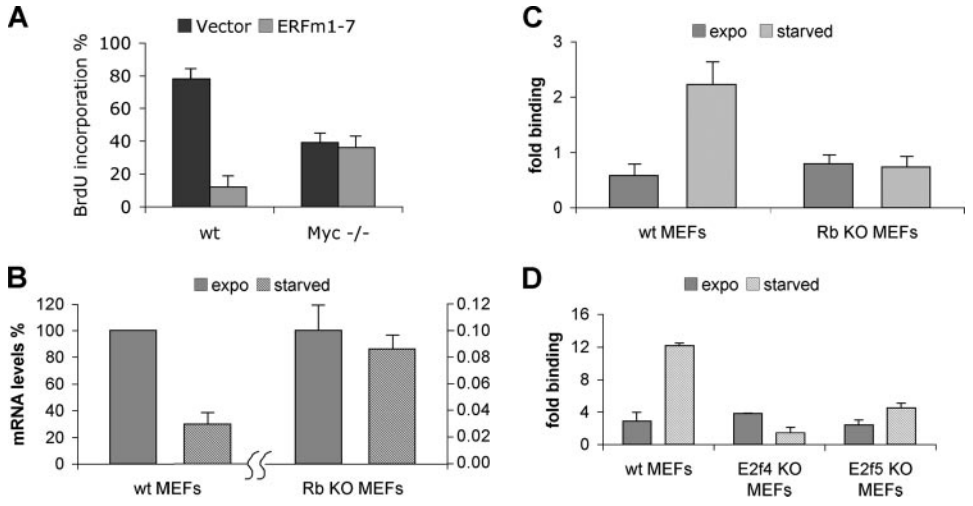

FIGURE 7. **The *ERF*-induced cell cycle arrest is c-*Myc*-dependent.** *A,* primary WT or c-*Myc*$^{-/-}$ MEFs were transfected with the nuclear ERFm1–7 mutant or an empty vector and pEGFP to identify transfected cells. Twenty four hours post-transfection the cells were treated with BrdUrd for 12 h and stained for GFP and BrdUrd. The *graph* represents percentage of BrdUrd-positive transfected cells. A minimum of 100 GFP-positive cells was scored in three independent experiments (*B*). We have shown that ERFm1–7 fails to inhibit proliferation of *Rb*$^{-/-}$ primary MEFs. c-*Myc* expression in WT and *Rb*$^{-/-}$ MEF growing in complete (*expo*) or serum-deprived (*starved*) media for 4 h was quantified by RT-qPCR in comparison with *Cph* mRNA. The *graph* represents the average of two independent experiments. The *right y* axis refers to the c-*Myc* mRNA levels in RB KO cells and is 3 orders of magnitude lower than the *left y* axis that refers to the WT MEFs. *C,* same cells as in *B* but with 1-h serum deprivation were analyzed for ERF binding in the P1-P2 c-*Myc* promoter region by ChIP. The *graph* represents the average of two independent experiments. *D,* binding of ERF onto the c-*Myc* promoter was analyzed by ChIP in E2F4 KO and E2F5 KO primary MEFs as in *C*. The *graph* represents the average of two independent experiments.

binding in WT, *E2f4*$^{-/-}$, and *E2f5*$^{-/-}$ cells. However, in the absence of serum ERF fails to bind c-*Myc* promoter in *E2f4*$^{-/-}$ MEFs and has a modest increase in *E2f5*$^{-/-}$ MEFs (Fig. 7*D*). These data strongly suggest that c-*Myc* expression is a prerequisite for the *Erf*-mediated inhibition of cell proliferation and that ERF-mediated repression of c-*Myc* involves pocket binding proteins.

DISCUSSION

We have shown that the ubiquitously expressed ERF transcriptional repressor is a downstream effector of the RAS/ERK pathway (1). ERF interacts with and is phosphorylated by ERKs *in vivo* and *in vitro*, in response to mitogenic stimulation and cell cycle progression (2, 3), and serves as a sensor of ERK activity via nucleo-cytoplasmic shuttling (4). Nonphosphorylated ERF can reverse *Ras*-induced tumorigenicity and arrest cells in the G_1 phase of the cell cycle. Loss of ERF during mouse embryonic development blocks chorionic trophoblast cell differentiation at a time point that ERK activity is attenuated in these cells (5). ERF function suggests that, in addition to well established functions of RAS/ERK activation, lack of RAS/ERK signaling actively affects cellular processes also. To that extent ERF targets provide new insight in a major signaling pathway.

In this study we provide biochemical and genetic evidence that c-*Myc* is a target of ERF. Promoter reporter and chromatin immunoprecipitation assays indicate that ERF binding on the c-*Myc* promoter reduces c-*Myc* transcription. Cellular and *in vivo* systems overexpressing or eliminating *Erf* correlate nuclear ERF presence and function with the reduction or elevation of c-*Myc* mRNA. Finally, elimination of c-*Myc* in cellular systems eliminates the *Erf* effect. Thus *Erf* provides a direct transcriptional link between a major mitogenic pathway and a global transcriptional effector of cell proliferation, growth, differentiation, and apoptosis (6, 53–55).

Our promoter assays indicate that in order for ERF to repress *Myc*, promoter-driven transcription must be nuclear and capable of interacting with DNA, suggesting a direct interaction with the minimal c-*Myc* promoter. Two other ETS proteins ETS2 and METS/PE2 have also been suggested to interact with c-*Myc* promoter and activate or repress transcription (24, 36). In all cases the exact binding motif on the c-*Myc* promoter remains poorly defined. *In vitro* binding assays indicated that ERF binds upstream of the P2 promoter, consistent with the reporter and ChIP assays, but the strength and specificity of this interaction are weak.[4] This is a characteristic of many transcription factors, and it is postulated that the unique c-*Myc* promoter structure allows proper binding only by multifactorial assemblies ensuring the precise spatial and temporal regulation of c-*Myc* transcription (for review see Ref. 32). However, direct interaction of ERF with the P1-P2 c-*Myc* promoter can be detected in a considerably more physiological setting via ChIP after serum withdrawal and faithfully reflects both ERF localization and *Myc* transcription. This interaction appears to be dependent on E2F4 and E2F5, supporting the multifactorial interaction hypothesis. It is of interest that ERF binding on the c-*Myc* promoter decreases after prolonged growth factor withdrawal. It is conceivable that after the initial repression, ERF is replaced by other factors known to repress c-*Myc* transcription like Ids (56) and p53 (29) and the redistribution of the RB family and pocket binding proteins that are critical for c-*Myc* transcriptional regulation (57–59).

The mechanism of transcriptional repression by ERF, however, remains elusive. The 70-amino acid C-terminal ERF repressor domain is necessary for *Myc* repression, indicating an active repression mechanism rather than a simple displacement of ETS activators or a scaffolding function. In this study, we observed that under conditions that ERF occupies the c-*Myc* promoter region, the RNA polymerase II complex is not present (supplemental Fig. S2). Our unpublished data[4] indicate that Gal4-ERF hybrids can repress transcription from Gal4-driven promoter in a trichostatin-independent manner and without affecting histone acetylation as determined by ChIP. These data indicate that ERF may act by interfering with the assembly of an active preinitiation complex at the c-*Myc* promoter and would

[4] M. Verykokakis, C. Papadaki, E. Vorgia, L. Le Gallic, and G. Mavrothalassitis, unpublished data.

be consistent with the immediate and transient binding of ERF. Although perturbations in chromatin and/or alterations in histone modifications cannot be excluded, it is more likely that other repressing factors that are known to mediate chromatin acetylation and remodeling, such as RB-E2F-DP complexes, may be involved in a later stage of *Myc* transcription repression (26, 57, 60). Thus our biochemical analysis suggests that ERF directly interacts with the c-*Myc* promoter and represses its transcription as an immediate response to ERK activity loss. Given the role of ERK kinase in MYC activation (33) and the autoregulatory function of MYC (61), it would appear that ERK activity is both a positive and negative regulator of MYC expression.

The ability of ERF to regulate *Myc* transcription is also supported by our genetic experiments. In all the cell lines tested, expression of an exclusively nuclear form of ERF resulted in inhibition of cellular c-*Myc* expression. This inhibition was growth factor-independent in NIH3T3 fibroblasts but serum-dependent in MCF7 epithelial cells. It is conceivable that this difference is because of different levels of ERK activity among the cell lines, as MCF7 cells exhibit a higher ERK activity. It is also plausible that different factor combination is responsible for *Myc* activation in the two cell types. However, in both cell types expression of nuclear ERF and decreased *Myc* transcription resulted in suppression of cellular transformation. The transformed phenotype of MCF7 cells has been shown to be *MYC*-dependent (46–48). However, in NIH3T3 cells *ras* is sufficient to induce transformation (62), but it would appear that this is because of an already elevated c-*Myc* given the need for *myc* overexpression for primary embryo fibroblast transformation (63). Thus, our data suggest that the ability of nuclear ERF to suppress cellular transformation is because of its ability to repress c-*Myc* transcription.

Our loss of function experiments further support the role of ERF as c-*Myc* transcriptional repressor. The delayed and limited c-*Myc* down-regulation in response to serum withdrawal in primary $Erf^{-/-}$ MEFs strongly suggest the role of ERF in this process. The manifestation of the effect only after growth factor removal minimizes the possibility of cell type differences because of *Erf* inactivation, whereas the sort duration of the experiment minimizes the possibility that persisting c-*Myc* transcription is an indirect effect of *Erf* loss. Indeed whole genome analysis from four biological replicates from WT and $Erf^{-/-}$ MEFs identified a very limited number of genes that failed to down-regulate as a result of serum withdrawal in $Erf^{-/-}$ MEFs compared with their WT littermates.[4] One of these genes was c-*Myc,* confirming our qPCR data, whereas none of the other genes could account for *Myc* regulation, further supporting the specific and direct effect of ERF on *c-Myc* transcriptional regulation.

The expression pattern of *Erf* and c-*Myc* in the developing placenta is also consistent with the role of ERF as a c-*Myc* repressor. *Myc* is expressed during early placentation, and its expression is decreased and finally eliminated at later stages (64, 65) similar to *Erf* (5). Although their expression is not strictly mutually exclusive, it appears that *Myc* expression is minimal and decreased in cells expressing *Erf,* consistent with its repression function. Despite the plethora of *Myc* transgenic

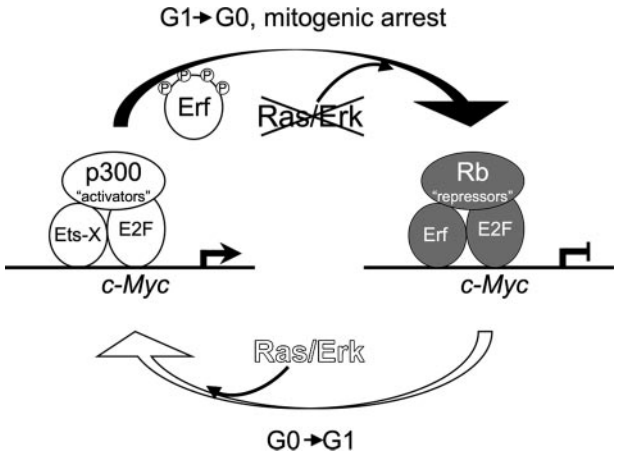

FIGURE 8. **A simplified model for the role of ERF in c-*Myc* regulation.** In the absence of RAS/ERK activity ERF is nonphosphorylated, and in the nucleus, where in association with pRB family proteins and E2Fs, it represses c-*Myc* transcription. In the presence of nuclear ERK activity, ERF is exported in the cytoplasm allowing activating E2Fs and probably other Ets proteins to induce c-*Myc* transcription.

animals, their tissue-specific expression limits our knowledge on the effect of MYC overexpression in placenta development. However, the aberrant *Myc* expression in the murine trisomy 15 syndrome correlates with placenta defects (66). In addition *Myc* has been shown to affect trophoblast differentiation (67) and migration (68) in response to RAS/ERK signaling supporting the hypothesis of c-*Myc* as and *Erf* effector. The role of c-*Myc* in mediating *Erf* function, however, is more apparent in $Myc^{-/-}$ and $Rb^{-/-}$ MEFs that are not inhibited by nuclear ERF as their WT counterparts. In both cell lines the expression of c-*Myc* in absent or negligible indicating the need for an active *Myc* for ERF function. This is further supported by our previous finding that overexpression of the c-MYC-regulated cyclins D and E (11–13, 69, 70) can reverse ERF-mediated suppression (4). Overall, our data indicate reciprocally that *Myc* repression is required for *Erf* function and that *Erf* is necessary for *Myc* repression and place *Erf* upstream of *Myc*.

An emerging model based on our findings and known *Myc* regulatory mechanisms would suggest that ERF signals the immediate attenuation of c-*Myc* transcription in response to RAS/ERK signaling loss. In contrast, in the presence of RAS/ERK signaling, ERF translocates to the cytoplasm releasing the promoter to activating factors (Fig. 8). This would suggest that *Ras* may not activate *Myc* but rather inactivates it in its absence. It would be interesting to investigate the range of *Ras* and/or *Myc* phenotypes that might be recapitulated by *Erf* loss or suppressed by nuclear ERF. In most somatic cells RAS and MYC activity is absent, although ERF is present in the nucleus. In contrast, during oncogenic transformation RAS and/or MYC activation plays a fundamental role in many tumor types. Our data thus far indicate that ERF mediates *Myc* down-regulation in response to RAS signaling attenuation and to that extent could be valuable in regulating this process.

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