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 EFX 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 organisinal 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-
Producing 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 G0 exit (22, 23). c-Myc transcription is reported to be down-regulated during differentiation (24–27) in accordance with inhibition of cell proliferation and G1 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). Refl 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 RA3T3 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. RA3T3 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% CO2, pBlERF WT and pBl-ERF M1–7 constructs were made by inserting the Xhol/XbaI fragment of the pEGFP-ERF WT or pEGFP-ERF M1–7 plasmids, respectively, into the Xhol/NheI sites of 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 × 104 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% CO2. 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/mm2 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
**MYC Regulation by ERF**

**RESULTS**

**ERF Can Inhibit Transcription from the c-MYC Promoter**—ERF can arrest cells in the G1 phase in an RB-dependent manner, can suppress ras- and ets-induced tumorigenicity, and is required for choriocarcinoma 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.

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 −1100/+340 p19Luc c-Myc promoter construct was used. C, same as in A, but cells were grown in complete media (exo) or serum-deprived for 14 h (starved). The graphs represent the average of a minimum of five independent experiments. The bars indicate standard error.

ERF can arrest cells in the G1 phase in an RB-dependent manner, can suppress ras- and ets-induced tumorigenicity, and is required for choriocarcinoma 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 may account for most of the ERF-associated phenotypes.

5-bromo-4-chloro-3-indolyl phosphate as substrates, and photographed.

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-nitrophényl]-5-phenyltetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as substrates, and photographed.

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MYC Regulation by ERF

![Graph showing MYC mRNA levels](image)

**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.

Inducible MEF3T3 cell lines that could express WTERF or ERFm1–7 in the absence of doxycycline (supplemental Fig. S1A) 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 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. 3A).

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. 3B). As expected c-Myc mRNA levels decreased during growth factor withdrawal and rapidly increased during serum stimulation (Fig. 3C) 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. 4A). 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. 1B), 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. 4B). 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. 4C, panels A–F). This was also true for the parental and the WT ERF-expressing MCF7 cells during low serum growth (Fig. 4C, panels G–J). In contrast, cells expressing the ERFm1–7 mutation failed to form soft agar colonies under low serum conditions (Fig. 4C, 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-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 spongiosotrophoblast 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 spongiosotrophoblast 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.
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. 6C). 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 KO MEFs. Despite the poor proliferation rate of c-Myc KO MEFs compared with WT MEFs, their proliferation was unaffected by the overexpression of nuclear ERF (Fig. 7A) 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 Erfs is RB-dependent. Thus, we examined c-Myc expression in RB KO MEFs in the presence or absence of serum. The level of c-Myc mRNA in RB KO MEFs was 3 orders of magnitude lower that WT MEFs and was not further reduced after serum withdrawal (Fig. 7B), suggesting that RB KO MEF growth is c-Myc-independent. This would be consistent with the inability of nuclear ERF to inhibit proliferation of RB KO MEFs. Interestingly, ERF could not be detected on the c-Myc promoter in RB KO cells independent of growth conditions, suggesting that c-Myc expression can be also repressed by an ERF-independent mechanism (Fig. 7C). 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 E2F4/5 KO and E2F5 KO MEFs. In exponentially growing cells ERF exhibits comparable
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. 7D). 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 G1 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. 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 region 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 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

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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 in c-Myc transcriptional repression. 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. 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 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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A., Maggioni, M., Polak, J. M., and Coggi, G. (1994) *Placenta* 15, 399–409

65. Martinoli, M. G., Lambert, R. D., Pothier, F., and Pelletier, G. (1992) *Mol. Reprod. Dev.* 31, 1–8

66. Hirning-Folz, U., Winking, H., and Hameister, H. (1992) *Cytogenet. Cell Genet.* 61, 289–294

67. Dakour, J., Li, H., Chen, H., and Morrish, D. W. (1999) *Placenta* 20, 119–126

68. Qiu, Q., Yang, M., Tsang, B. K., and Gruslin, A. (2004) *Mol. Hum. Reprod.* 10, 677–684

69. Coller, H. A., Grandori, C., Tamayo, P., Colbert, T., Lander, E. S., Eisenman, R. N., and Golub, T. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3260–3265

70. Santoni-Rugiu, E., Falck, J., Mailand, N., Bartek, J., and Lukas, J. (2000) *Mol. Cell. Biol.* 20, 3497–3509