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Article

Continuous ERK Activation Downregulates Antiproliferative Genes throughout G1 Phase to Allow Cell-Cycle Progression

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Summary

Background: The ERK family of MAP kinase plays a critical role in growth factor-stimulated cell-cycle progression from G0/G1 to S phase. It has been suggested that sustained activation, but not transient activation, of ERK is necessary for inducing S phase entry. Although the essential role of ERK MAP kinase in growth factor-stimulated gene expression, especially expression of immediate-early genes, is well established, it has remained unclear how ERK activity duration affects the promotion of G1 phase progression to S phase.
Results: We have found that inhibition of ERK activation by the MEK inhibitor or dominant-negative MEK1 even immediately before the onset of S phase leads to the cessation of S phase entry. Our analyses reveal that there are ERK-dependent downregulated genes, whose expression levels return to their original levels rapidly after ERK inactivation, and that their downregulation mostly requires AP-1 activity. Remarkably, microinjection experiments demonstrate that many of the downregulated genes act as antiproliferative genes during G1 phase and that their forced expression to the levels before growth factor stimulation even in late G1 phase blocks S phase entry.
Conclusions: Thus, continuous ERK activation downregulates antiproliferative genes until the onset of S phase to allow successful G1 phase progression. This mechanism may also work as a fail-safe mechanism, which prevents inappropriate stimuli that induce transient ERK activation from causing S phase entry.

Introduction

Mammalian cultured cells proliferate in the presence of serum containing growth factors. When cultured fibroblasts are deprived of serum, they enter the quiescent state, termed G0 phase. The addition of growth factors induces the re-entry of the cell cycle: the quiescent cells leave the G0 phase, progress through G1 phase, replicate their DNA, and divide. Extracellular signal-regulated kinase (ERK), a member of the MAPK family, is one of key molecules in growth factor signaling [1–9]. ERK is phosphorylated and activated by MEK in response to growth factor stimulation, and activated ERK phosphorylates and activates nuclear targets such as Elk-1 to upregulate immediate-early genes such as *c-fos*. The expression of the immediate-early genes has been implicated to regulate subsequent induction of delayed early genes, which include cyclin D [10–12]. Cyclin D-CDK4/6 complex then initiates Rb phosphorylation, which activates the E2F family of transcription factors and induces the expression of target genes, including cyclin E. Cyclin E/CDK2 complex further phosphorylates Rb and activates the E2F family. These sequential events, including sequential induction of a number of genes, lead to the synthesis of proteins required for S phase entry [13]. Previous studies have shown that ERK activation is required for not only the induction of immediate-early genes but also the induction and maintenance of the increased expression of cyclin D1. As the expression of cyclin D1 occurs several hours after growth factor stimulation, sustained ERK activation for at least several hours, not transient activation, is required for sequential induction of gene expression and therefore for successful S phase entry [14–17]. Thus, the duration of ERK activity has been implicated as a critical factor for ensuring G1 phase progression. In fact, a number of studies have shown the correlation between sustained ERK activation and successful S phase entry in various cell lines [18–20]. Then, how sustained ERK activation regulates sequential induction of gene expression has been a problem to be solved. Recent studies have identified one of the molecular mechanisms that answer this question [21, 22]. Thus, only sustained ERK activation induces sustained phosphorylation of immediate-early gene products, which leads to their stabilization and activation, which are important for cell-cycle progression from G0/G1 to S phase. However, it has not been fully elucidated how sustained ERK activation regulates successful S phase entry.

In this study, our systematic analysis of the effect of the ERK activity duration on gene expression profiles has identified a set of genes whose expression levels are downregulated by ERK activation and return to the original levels rapidly after ERK inactivation. This has for the first time directed our attention to ERK-dependent downregulated genes and their function in cell-cycle progression. Their downregulation is shown to be dependent, at least in part, on AP-1 activity, suggesting that continuous downregulation of these genes throughout G1 phase may act as one of downstream effectors of the immediate-early gene products stabilized by sustained ERK signal. Interestingly, many of these ERK-dependent downregulated genes are identified as antiproliferative genes that have the ability to suppress S phase entry even at mid or late G1 phase. Our result also demonstrates that ERK inactivation by the MEK inhibitor or dominant-negative MEK1 any time before the onset of S phase leads to the cessation of S phase entry. Collectively, our results

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show requirement of continuous ERK activation throughout G1 phase for successful S phase entry and identify one of the underlying mechanisms: ERK activity-dependent downregulation of antiproliferative genes. This mechanism may also be used as a fail-safe mechanism that prevents inappropriate extracellular stimuli that induce transient ERK activation from promoting cell proliferation, and thus could be a G1 phase progression-monitoring system.

Results

Requirement of Continuous ERK Activation throughout G1 Phase for S Phase Entry

In quiescent NIH3T3 cells stimulated with FGF or PDGF, the ERK activation declined to about 10% of the maximal activation within 3 hr but persisted thereafter, and the cells entered S phase (Figure 1A, FGF and PDGF). In contrast, in cells stimulated with EGF or IGF1, the ERK activation dropped to nearly zero within 3 hr, and the cells did not enter S phase (Figure 1A, EGF; and see Figure S4A in the Supplemental Data available with this article online, IGF1). Thus, our data have indicated a correlation between sustained ERK activation and successful S phase entry (Figure 1A), in agreement with previous reports [16, 18, 19]. However, how long ERK activity should be sustained has not been precisely determined before. Then, to address this, we added the MEK inhibitor U0126 to FGF-stimulated cells at varying times after FGF stimulation and assayed the S phase entry at 24 hr (Figure 1B, right). In the absence of U0126, about half of the NIH3T3 cells began to enter S phase at around 15 hr after FGF stimulation, and 85% of the cells entered S phase at 24 hr (see Figure 1B, left). When U0126 was added before FGF stimulation, no ERK activation occurred, and the cells failed to enter S phase (Figure 1A, FGF + U0126). The addition of U0126 at each time point induced almost complete ERK inactivation within 10 min (Figure 1A, FGF + U at 1 hr; and data not shown). When U0126 was added at 5 hr, only 10% of the cells were able to enter S phase at 24 hr (Figure 1B, right, middle). Rather surprisingly, even when U0126 was added at 14 hr, the S phase entry was inhibited by 34.8%, and only 53.4% of cells entered S phase at 24 hr (Figure 1B, right, middle, and bottom). In other words, the ERK activation for 14 hr (= t′53.4) was required for 53.4% of cells to enter S phase. On the other hand, 53.4% of cells entered S phase at 15.7 hr (= t53.4) after FGF stimulation in the absence of U0126 (Figure 1B, left). This may imply that sustained ERK activation until 1.7 hr (= t53.4 [15.7 hr] − t′53.4 [14 hr]) before the onset of S phase is required for S phase entry. In fact, the time differences between the broken line in Figure 1B (left) and the solid line in Figure 1B (right) (e.g., t43.1 [14.3 hr] − t′43.1 [11 hr] = 3.3 hr, t25.1 [11 hr] − t′25.1 [8 hr] = 3 hr) fell within 2–3 hr at any time points after FGF stimulation. This suggests, therefore, that sustained ERK activation until about 2 or 3 hr before the onset of S phase is required for quiescent cells to enter S phase. It should be noted that ERK5 was not activated significantly in quiescent NIH3T3 cells after stimulation by FGF or PDGF (data not shown). Therefore, activation of ERK1/2 MAPK is important for cell-cycle progression of NIH3T3 cells, although U0126 is known to inhibit the MEK5/ERK5 pathway as well [23]. To confirm this, we microinjected a plasmid of S218A/S222A (SASA) MEK1, which is a dominant-negative form of MEK1 that inhibits ERK1/2 activation but not ERK5 activation, into NIH3T3 cells at varying time. Cell staining with anti-phospho ERK1/2 antibody showed that ERK1/2 was completely inactivated in the SASA MEK1-injected cells (data not shown). Considering that it takes about 2–3 hr for the injected SASA MEK1 plasmid to be expressed, injection of SASA MEK1 at varying times resulted in the same extent of inhibition of S phase entry as U0126 addition (Figure 1B, right, bottom). These results thus demonstrate that ERK1/2 activation should be sustained until late G1 for ensuring successful S phase entry.

Sustained ERK Activation Downregulates a Set of Genes throughout G1 Phase

To identify those genes whose expression levels are regulated by the ERK pathway during G0/G1 to S phase, we performed the genome-wide analysis by using Affymetrix GeneChip oligonucleotide microarrays, which contain over 39,000 transcripts and variants, including about 34,000 well-substantiated mouse genes. Our analysis showed that sequential induction of immediate-early genes, cyclin D1, and cyclin E and other E2F target genes requires ERK activation. This is consistent with previous reports [24, 25]. On the other hand, our microarray analysis revealed the existence of ERK-dependent downregulated genes. Here, we have chosen to focus on those mRNAs whose levels decreased in response to FGF. First, we identified those genes whose expression level in FGF-stimulated cells at 4, 7, or 12 hr was statistically different from the expression level in unstimulated cells and that in the U0126-treated, FGF-stimulated cells. In 173 genes out of these genes, the expression level was decreased by 2-fold or more at either time point after FGF stimulation, as compared to the expression level before stimulation, and the degree of the decrease was reduced by more than 50% by U0126 pretreatment in replicate experiments. We defined these 173 genes as ERK-dependent downregulated genes (Figure 2A and Table S2). The expression level of almost all of these genes was maintained at a lower level throughout G1 phase.

We then examined the effect of the ERK activity duration on expression profiles of these ERK-dependent downregulated genes. We first compared the gene expression profiles at 7 hr after FGF stimulation when U0126 was added before stimulation or added at 1 hr after stimulation or not added. Rather surprisingly, the expression profile at 7 hr in the total absence of ERK activation was very similar to the profile in the presence of the first 1 hr ERK activation (Figure 2A). This suggests that strong ERK activation during the initial 1 hr alone without continued presence of active ERK could not induce decreased expression of these ERK-dependent downregulated genes at 7 hr. We then examined expression profiles of the ERK-dependent downregulated genes at 12 hr after FGF stimulation when U0126 was added at 7 hr or added before stimulation or not added. The result showed that the effect of the U0126 addition at 7 hr on the expression profile at 12 hr is very similar to that of the addition before stimulation (Figure 2A),

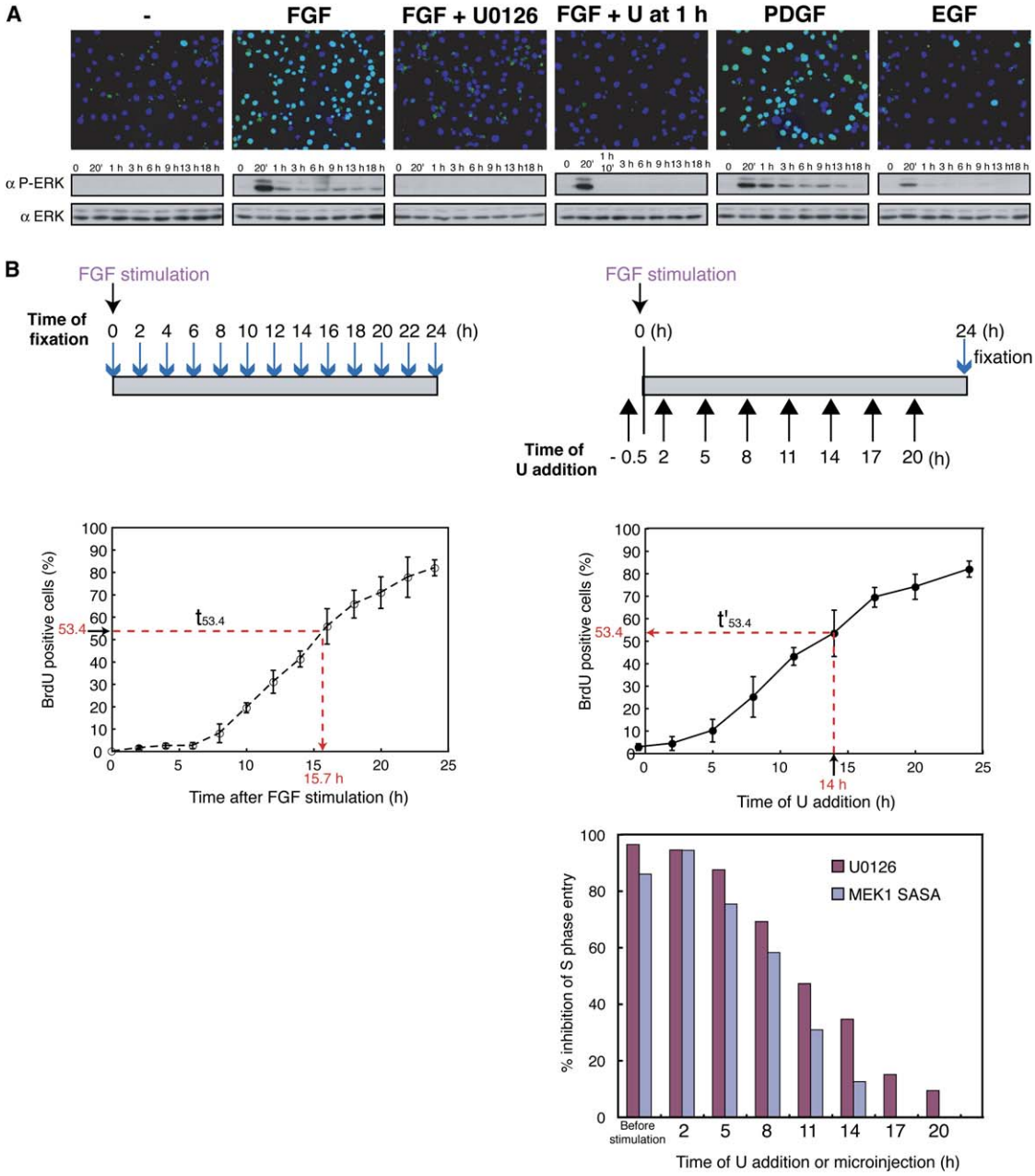

Figure 1. Sustained ERK Activation throughout G1 Phase Is Required for S Phase Entry

(A) Serum-starved NIH3T3 cells were stimulated with bFGF (50 ng/ml), PDGF (20 ng/ml), or EGF (30 ng/ml). U0126 (20 μM) was added 30 min before (FGF + U0126) or 1 hr after (FGF + U at 1 hr) stimulation. Cells were incubated for 24 hr in the presence of BrdU, fixed, and stained with anti-BrdU (green). Cell nuclei were also visualized with Hoechst (blue). At the same time, the cell extracts were subjected to immunoblot analysis with antibodies against ERK and phosphorylated ERK (p-ERK).

(B) Serum-starved NIH3T3 cells were stimulated with bFGF (50 ng/ml) and cultured in the presence of BrdU. At the indicated times after stimulation, the cells were fixed and stained with anti-BrdU antibody (left, upper), and the percentages of BrdU-positive cells were measured (left, middle). U0126 (final 20 μM) was added to the FGF-stimulated cells at the indicated times after stimulation, and the cells were fixed and stained with anti-BrdU antibody at 24 hr after FGF stimulation (right, upper). The percentages of BrdU-positive cells were measured (right, middle), and the percent inhibitions of BrdU incorporation were determined (right, bottom, U0126). More than 100 cells were counted in each sample. Values represent means ± SD from three independent experiments. An empty vector or an expression plasmid for MEK1 SASA, together with the GFP expression plasmid, was injected into nuclei of NIH3T3 cells at the same time points as U0126 addition. Cells were incubated for 24 hr after FGF stimulation in the presence of BrdU, fixed, and stained with anti-BrdU antibody. The injected cells were detected by GFP expression. The percent inhibitions of BrdU incorporation in the MEK1 SASA-injected cells compared to the empty vector-injected cells were determined (right, bottom, MEK1 SASA).

indicating that sustained ERK activation until, or immediately before, 12 hr is required for maintenance of the decreased expression levels of the ERK-dependent downregulated genes throughout G1 phase.

Identification of the ERK-Dependent Downregulated Genes as Antiproliferative Genes

We extracted 29 genes, whose decreased expression levels were almost totally dependent on ERK activity,

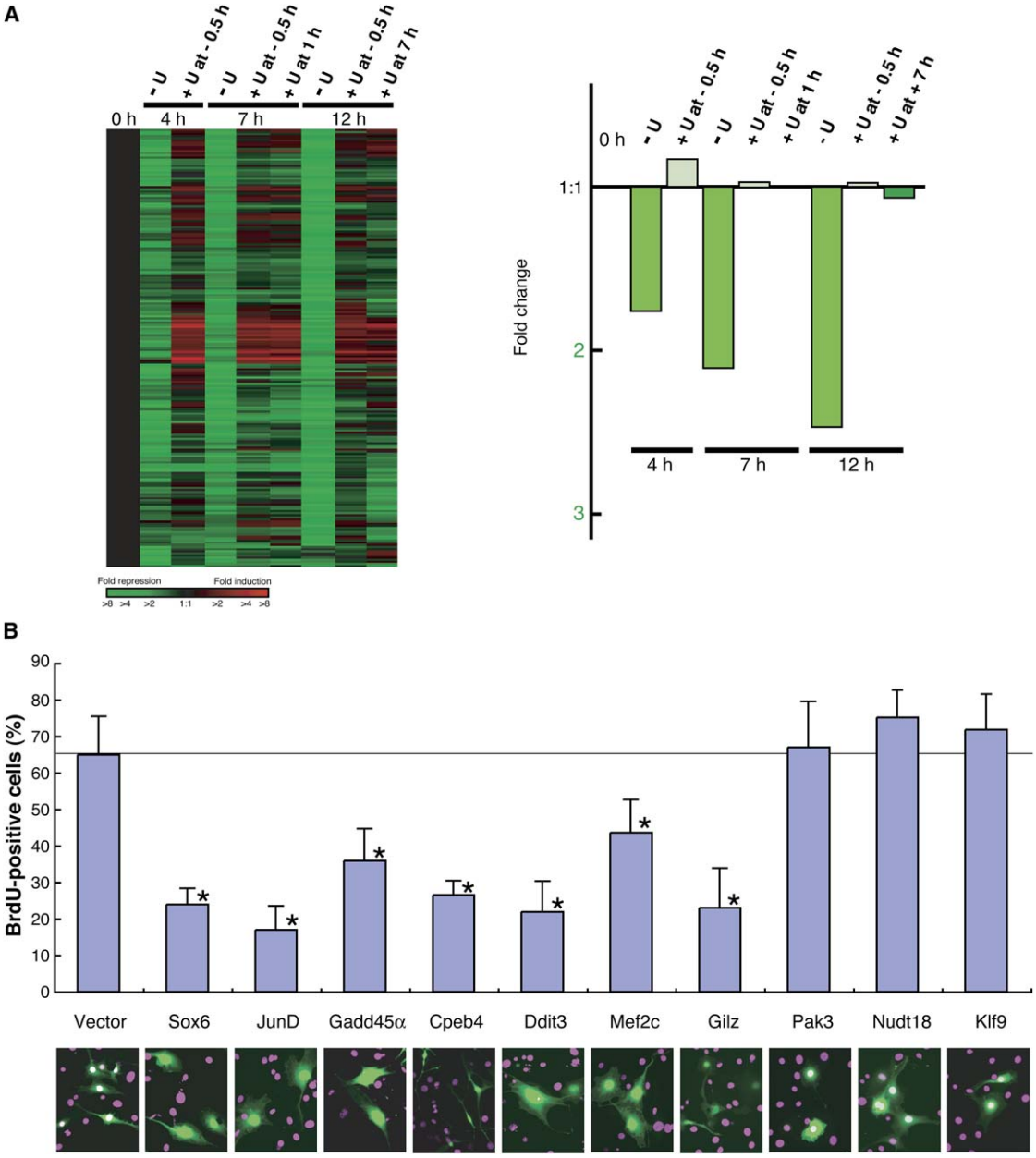

Figure 2. ERK Downregulates a Set of Antiproliferative Genes during G1 Phase Progression

(A) Expression profiles (left) and average expression profiles (right) of ERK-dependent downregulated genes (173 genes) at 4 hr, 7 hr, or 12 hr after FGF stimulation when U0126 was added before (+U at −0.5 hr), at 1 hr after (+U at 1 hr), or at 7 hr (+U at 7 hr) after stimulation, or not added (−U), are shown. Each horizontal line displays the expression data for one gene, where the ratio of the mRNA level to its level in the serum-starved (0 hr) cells is represented by color according to the color scale at the bottom. The entire data set is shown in Table S2.

(B) An empty vector or an expression plasmid for each indicated gene (100 μg/ml), together with the GFP expression plasmid, was injected into nuclei of serum-starved NIH3T3 cells. After 4 hr, cells were stimulated with bFGF (50 ng/ml) and incubated for 24 hr in the presence of BrdU. Cells were fixed and stained with anti-BrdU antibody (magenta). The graph shows the percentages of BrdU-positive cells in the injected cells. At least 30 cells were injected for each sample in a series of experiments. The values (means ± SD) were obtained from three to seven independent series of experiments. Significant differences from the control group (empty vector) are indicated with a single ($p < 0.005$, Student's t test) asterisk.

by the following four criteria (Table 1). The expression levels were downregulated at 4, 7, and 12 hr after FGF stimulation (by more than 2.5-fold change at least at one time point). The FGF-dependent decrease in the expression levels was almost completely suppressed by prior treatment with U0126. Their downregulation was not induced by the initial 1 hr ERK activation alone. Their downregulated expression levels returned to the original or higher levels at 12 hr when U0126 was added at 7 hr after stimulation. Quantitative RT-PCR analysis of all of the genes in Table 1 confirmed the array data (Figure 3A and Figure S1). This list includes *JunD* [26], *Gadd45α* [27], *Ddit3* [28], and *Tob1* [29, 30], which are known to regulate cell-cycle progression negatively. In particular, Tob1 and Ddit3, also known as CHOP or GADD153, were shown to inhibit cell-cycle progression

Table 1. ERK-Dependent Downregulated Genes Whose Expression Is Rapidly Upregulated by ERK Inactivation

Genbank Accession No.	Gene	Description	Antiproliferative Ability[a]
AJ010605	Sox6	SRY box containing gene 6	+
BB548889	Gpr133	G protein-coupled receptor 133	
BC027250	1110018M03Rik		
NM_011018	Sqstm1	sequestosome 1	
BQ175796	Pak3	p21 (CDKN1A)-activated kinase 3	−
NM_010592	Jund1	Jun proto-oncogene related gene d1	+
BB417145	Gpr23	G protein-coupled receptor 23	
BM120193	Nudt18	nudix (nucleoside diphosphate linked moiety X)-type motif 18 (MGC38179)	−
BM117918	Dtna	dystrobrevin α	
NM_007837	Ddit3	DNA damage-inducible transcript 3	+
NM_007836	Gadd45a	growth arrest- and DNA damage-inducible 45 α	+
BE945188	1110002M09Rik		
AK020810	A930005F02Rik		
AK018112	6330404C01Rik		
AI267126	Klf9	Kruppel-like factor 9	−
BB408123	RGC32	response gene to complement 32	−
BB383709	C230029M16		
BG070342	Cpeb4	cytoplasmic polyadenylation element binding protein 4	+
NM_133943	Hsd3b7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	
AI426175	Hsc20	J-type cochaperone HSC20	
AK017272	Lpl	lipoprotein lipase	
NM_008606	Mmp11	matrix metalloproteinase 11	−
BC026841	Mef2c	myocyte enhancer factor 2C	+
AV023018	1190030G24		
NM_008608	Mmp14	matrix metalloproteinase 14	−
AA242096	Tob1	transducer of ErbB-2.1	+
NM_010286	Gilz	glucocorticoid-induced leucine zipper	+
BC003438	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	
BG067878	Arfgap3	ADP-ribosylation factor GTPase activating protein 3	−

[a] Fifteen genes in this list were cloned and injected into serum-starved NIH3T3 cells, and the effect on the FGF-induced S phase entry of the cells was examined. + denotes the genes that inhibited the S phase entry. − denotes the genes that failed to inhibit the S phase entry.

from G0/G1 to S phase [28–30]. Therefore, we hypothesized that other genes in this list could also have the ability to inhibit cell-cycle progression from G0/G1 to S phase. To test this idea, we picked up 15 genes from this list and examined their ability to inhibit S phase entry. Serum-starved NIH3T3 cells were microinjected with an expression plasmid for each of these 15 genes and stimulated with FGF. Cells expressing each gene were detected by the coinjected GFP expression, and whether or not the cells entered the S phase at 24 hr was determined by BrdU incorporation. Representative results are shown in Figure 2B, which demonstrates that JunD, Gadd45α, Sox6, Ddit3, Gilz, Cpeb4, and Mef2c have the ability to inhibit the S phase entry, whereas neither Pak3, Nudt18 (MGC38179), nor Klf9 inhibits S phase entry. At least three independent experiments for the 15 genes gave essentially the same results (Figure 2B and Table 1). Thus, in total, eight out of fifteen genes in the list are found to be antiproliferative genes (Table 1). We then performed siRNA experiments. We designed and synthesized three siRNA duplexes that target each of the all eight genes. Our results showed that each siRNA successfully reduced the target mRNA levels significantly (Figure S2A and data not shown). Then we examined the effect of individual siRNA treatment on S phase entry in the presence of EGF, which is not a potent mitogen in NIH3T3 cells. The obtained results showed that all the three siRNA duplexes for each of Cpeb4, Gadd45α, and Tob1 enhanced S phase entry (Figure S2B). These results suggest that downregulation of these antiproliferative genes facilitates G1 phase progression to S phase. Next, to determine where in G1 phase these antiproliferative genes have an inhibiting effect, we examined the state of Rb phosphorylation at Ser807/Ser811 in FGF-stimulated NIH3T3 cells to which each of these eight antiproliferative genes was microinjected. The repeated experiments demonstrated that JunD, Gadd45α, and Cpeb4 markedly inhibited the Rb phosphorylation, and Mef2c and Tob1 inhibited it moderately (Figure S3). All these results have shown that some of the ERK-dependent downregulated genes are antiproliferative genes and thus led to the idea that successful S phase entry may require the decreased expression levels of these antiproliferative genes until the onset of S phase.

Continuous ERK Activation throughout G1 Phase Induces and Maintains the Decreased Expression Levels of Antiproliferative Genes to Allow S Phase Entry

To test this idea, we first measured the time course of the change in the expression levels of these antiproliferative genes by quantitative RT-PCR in more detail. FGF and PDGF induced the marked decrease in mRNA levels of these eight antiproliferative genes, and their decreased expression levels were maintained throughout G1 phase (Figure 3A and data not shown). In contrast, TPA, which induced shorter ERK activation than FGF (Figure S4A), also induced the decrease in their expression levels, but the decrease was transient, and the expression levels were not maintained (Figure S4B). Moreover, EGF or IGF1, which induced only very short ERK

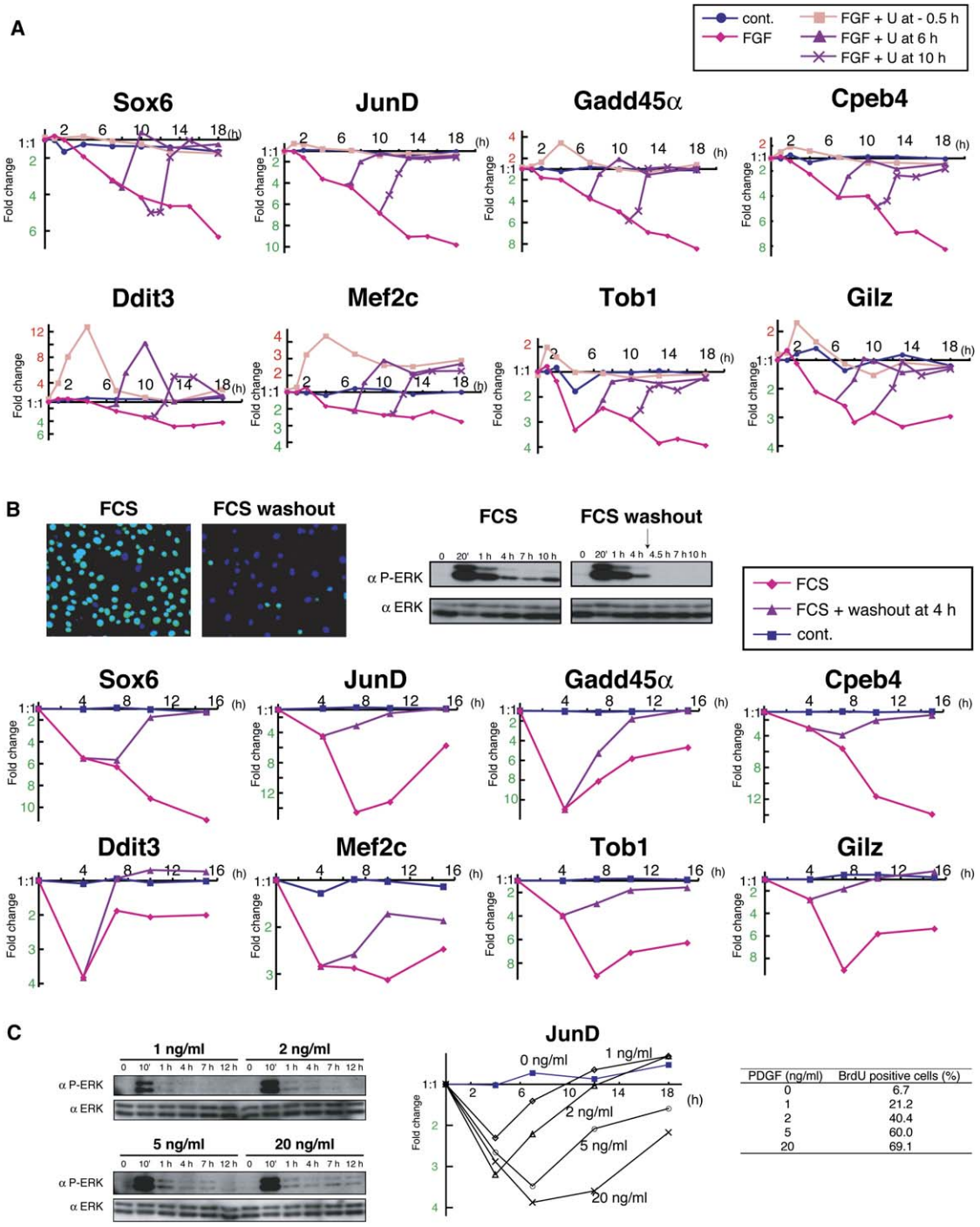

Figure 3. Sustained ERK Activation Is Required for Induction and Maintenance of the Decreased Expression Levels of a Set of Genes

(A) Serum-starved NIH3T3 cells were stimulated with bFGF (50 ng/ml) and treated with U0126 (20 μM) at 30 min before or 6 hr or 10 hr after stimulation. At indicated times after initial FGF stimulation, relative mRNA levels of the indicated genes were determined by quantitative RT-PCR.

(B) Serum-starved NIH3T3 cells were stimulated with 10% FCS. At 4 hr after stimulation, the cells were acid washed and then cultured in serum-free medium. Cells were incubated for 24 hr after initial stimulation in the presence of BrdU, fixed, and stained with anti-BrdU antibody (green). Cell nuclei were also visualized with Hoechst (blue). At the same time, the cell extracts were subjected to immunoblot analysis with antibodies against ERK and phosphorylated ERK (p-ERK), and relative mRNA levels of the indicated genes were determined.

(C) Serum-starved NIH3T3 cells were stimulated with PDGF at each concentration (1, 2, 5, 20, ng/ml). At indicated times after stimulation, the cell extracts were subjected to immunoblot analysis with antibodies against ERK and phosphorylated ERK (p-ERK) (left), and relative mRNA levels of JunD were determined (middle). The percentages of the BrdU-positive cells for 24 hr after stimulation were shown (right).

activation, did not decrease their expression levels (Figure S4). The decreases by FGF were completely inhibited by the U0126 pretreatment. Moreover, the addition of U0126 at 6 hr or 10 hr after FGF stimulation resulted in a rapid increase in their expression levels, reaching the levels that were attained by the U0126

treatment before stimulation (Figure 3A). Essentially the same results were obtained when we used PD098059, another MEK inhibitor, instead of U0126. Therefore, continuous ERK activation is required for induction and maintenance of the decreased expression levels of these genes. Moreover, it should be pointed out that mitogenic signal-dependent downregulation of these antiproliferative genes was commonly observed in other cell types, including normal rat kidney (NRK) cells and human embryonic WI-38 cells. For example, stimulation with 10% FCS induced ERK-dependent downregulation of all eight genes and six genes (except for Cpeb4 and Mef2c) in NRK cells and WI38 cells, respectively (data not shown).

It is well known that continuous exposure to growth factor is required for quiescent cells to enter S phase. In our experiments, readdition of 10% FCS to quiescent NIH3T3 cells induced sustained ERK activation and S phase entry (Figure 3B, upper, left). FCS withdrawal at 4 hr resulted in rapid ERK inactivation and cessation of S phase entry (Figure 3B, upper, right). While FCS, like FGF and PDGF, induced and maintained the decreased expression levels of the eight antiproliferative genes, the FCS withdrawal at 4 hr led to a rapid increase in the expression levels of all these downregulated genes (Figure 3B, lower). Therefore, sustained ERK activation was required for the maintenance of the decreased expression levels of the antiproliferative genes. It has been reported that the ERK activity duration and the S phase entry depend on the PDGF concentration [22]. Then, we examined the JunD mRNA level as well as ERK activity and S phase entry in the presence of varying concentrations of PDGF. The result showed that both the ERK activity duration and the duration of the decreased expression level of JunD mRNA become longer with increased PDGF concentration, in parallel with the increase in the S phase entry (Figure 3C). Thus, there was a good correlation between the ERK activity duration and the continuous downregulation of antiproliferative genes.

To evaluate the physiological role of the ERK-dependent downregulation of antiproliferative genes in cell-cycle progression, it is important to know whether endogenous expression levels of these genes block S phase entry. Anti-JunD antibody is available to estimate the JunD protein level in individual cells. Both immunoblotting and cell staining with the anti-JunD antibody showed that the changes of endogenous JunD protein level in response to FGF and U0126 roughly paralleled those of the mRNA level (Figure 4A and data not shown). Thus, JunD protein is downregulated by ERK activity and increases to the original level or a slightly higher level soon after ERK inactivation. As the expressed protein level depends on the plasmid concentration that is injected into the nucleus, the experiment with increasing the concentrations of the JunD expression plasmid enables us to estimate the relationship between the injected plasmid concentration and the expressed protein level. Our data indicated that endogenous JunD protein level in the presence of U0126 15 hr after FGF stimulation roughly corresponds to the expressed JunD protein level resulting from the injection of 10 μg/ml JunD plasmid (Figure 4B). Figure 4C shows that JunD inhibited FGF-stimulated S phase entry in a dose-dependent manner, and at the JunD expression plasmid concentration of 10 μg/ml the S phase entry was inhibited by about 40%. Thus, nearly endogenous levels of JunD significantly suppress S phase entry. Moreover, we have found that JunD and Gadd45α have an additive inhibitory effect on S phase entry. JunD is thought to antagonize other Jun family proteins such as c-Jun to inhibit the AP-1 activity in NIH3T3 cells [26]. Our microinjection experiment has shown that coexpression of c-Fos markedly suppresses the S phase entry-inhibiting ability of JunD, but not that of Gadd45α (data not shown). This suggests that these two antiproliferative genes differ in their respective target to inhibit S phase entry. In fact, coexpression of JunD and Gadd45α showed an additive effect (Figure 4C), although the antiproliferative effect of JunD or Gadd45α alone was modest at low doses (Figure 4C).

Importantly, we have further found that forced expression of these antiproliferative genes at low doses even in late G1 phase inhibits S phase entry. When JunD and Gadd45α plasmids at low doses were coinjected at 2 hr or 8 hr after FGF stimulation, S phase entry was strongly inhibited: the extent of the inhibition by injection at 2 hr or 8 hr was almost the same as that by injection before FGF stimulation (Figure 4D). This result suggests that if the downregulation of the antiproliferative genes is broken during G1 phase and their expression levels return to their original levels, the cells should cease to enter S phase. Thus, all these results, taken together, strongly suggest that successful S phase entry requires the decreased expression levels of these antiproliferative genes throughout G1 phase, which are induced and maintained by sustained ERK activation.

ERK Activation Alone Is Capable of Inducing Downregulation of Several Antiproliferative Genes

We then examined whether the activation of the ERK pathway alone is able to decrease the expression levels of these antiproliferative genes. We used NIH3T3 cells expressing ΔB-Raf:ER (estrogen receptor) [31]. B-Raf is known to function as a specific and direct activator of MEK, and in ΔB-Raf:ER cells the addition of estrogen or 4-hydroxy tamoxifen (4-HT) induces immediate and continuous ERK activation (data not shown). The addition of 4-HT resulted in a significant decrease in the expression levels of five out of eight antiproliferative genes (Figure 5A). It should be noted that 4-HT induces neither ERK activation nor downregulation of these genes in normal NIH3T3 cells (data not shown). Thus, sustained ERK activation is sufficient for induction and maintenance of the decreased expression levels of part of the ERK-dependent downregulated genes.

Downregulation of the Antiproliferative Genes Mostly Requires AP-1 Activity

In our preliminary experiment, treatment with cycloheximide inhibited completely the FGF-dependent downregulation of JunD and Gadd45α mRNA (data not shown), indicating requirement of de novo protein synthesis for the downregulation. In addition, our analyses with the chromatin immunoprecipitation (ChIP) assay with anti-RNA polymerase II antibody and the reporter assay have suggested that ERK activation leads to downregulation of JunD and Gadd45α by inducing transcriptional

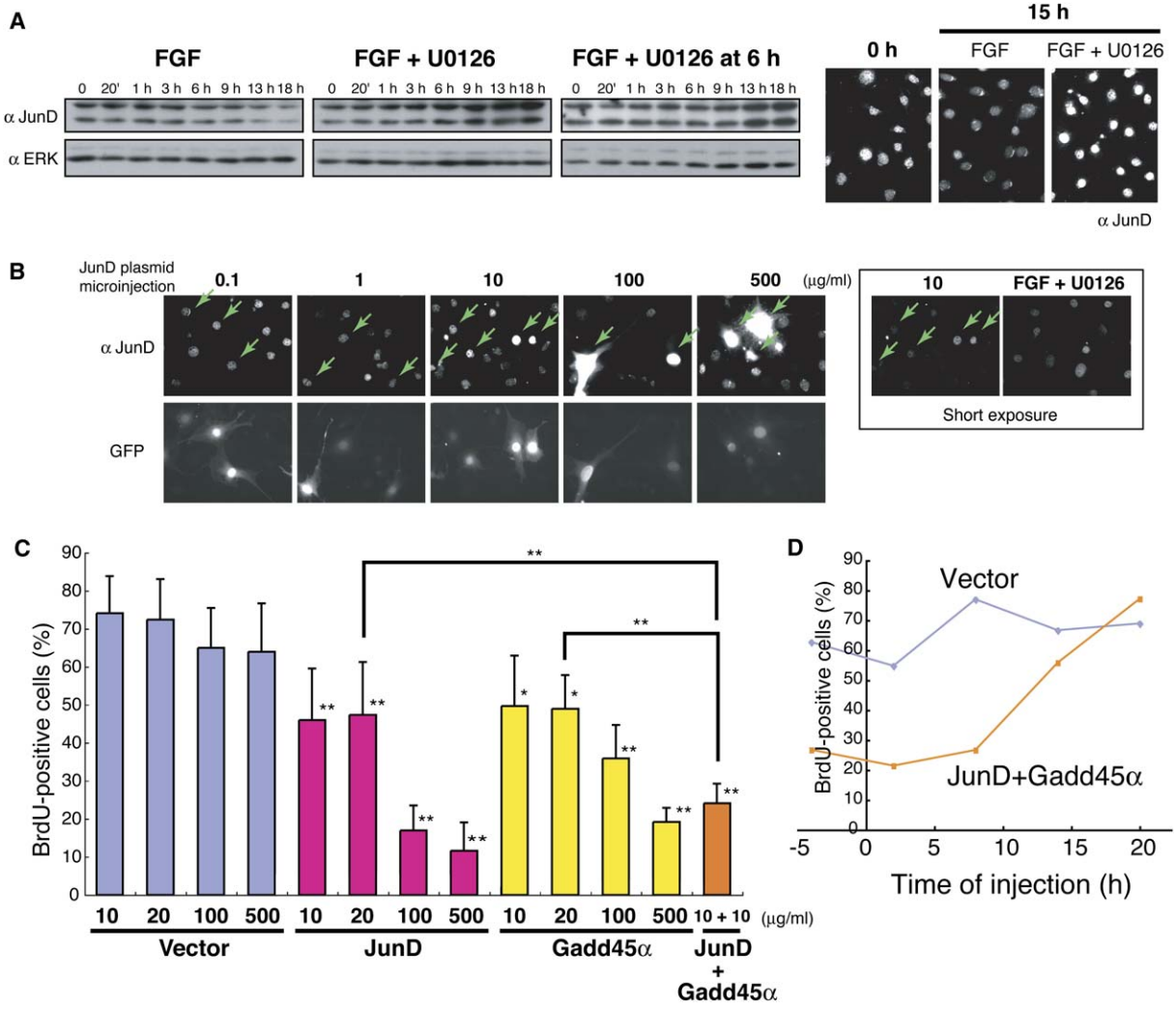

Figure 4. ERK Promotes the G1 Phase Cell-Cycle Progression by Downregulating Antiproliferative Genes

(A) Serum-starved NIH3T3 cells were stimulated as in Figure 3A. At indicated times, the cell extracts were subjected to immunoblot analysis with anti-JunD and anti-ERK antibodies, and the cells were stained with anti-JunD antibody.

(B) An expression plasmid for Myc-tagged JunD (0.1, 1, 10, 100, or 500 μg/ml), together with the GFP expression plasmid, was injected as in Figure 2B. After 4 hr, cells were stimulated with bFGF (50 ng/ml) and incubated for 15 hr. Cells were fixed and stained with anti-JunD antibody.

(C) An empty vector, Myc-tagged JunD or Myc-tagged Gadd45α (10, 20, 100, or 500 μg/ml) or both Myc-tagged JunD and Myc-tagged Gadd45α (10 μg/ml for each) were injected as in Figure 2B. The percentages of BrdU-positive cells were measured. The values (means ± SD) were obtained from three to seven independent series of experiments. Significant differences from the control group (empty vector) or between indicated samples are indicated with a single ($p < 0.05$, Student's t test) or double ($p < 0.01$, Student's t test) asterisk.

(D) Both Myc-tagged JunD and Myc-tagged Gadd45α at low doses (10 μg/ml for each) were injected into nuclei of NIH3T3 cells at various time points after FGF stimulation. Cells were incubated for 24 hr after FGF stimulation in the presence of BrdU, fixed, and stained with anti-BrdU antibody. The graph shows the percentages of BrdU-positive cells in the injected cells.

repression, rather than enhancing mRNA degradation (data not shown). We supposed then that the downregulation might depend on AP-1 activity, as AP-1 transcription factors are shown to be differentially regulated by the different ERK activity duration [21, 22, 32]. To investigate requirement of AP-1 activity for the downregulation of these antiproliferative genes, we used a lentivirus harboring A-Fos, a dominant-negative form of c-Fos. A-Fos contains a substitution of the basic DNA binding domain with an acidic sequence that enhances the stability of interaction with Jun partners but abolishes DNA binding [33]. Thus, A-Fos can inhibit the activity of Fos family members. Indeed, our reporter assay demonstrated that A-Fos expression by the lentivirus infection inhibited AP-1 activity by more than 90%, and coexpression of c-Fos was able to overcome the inhibitory effect of A-Fos (Figure 5B, left). The effect of A-Fos expression on the FGF-dependent downregulation of the eight antiproliferative genes was examined by quantitative RT-PCR. A-Fos expression almost completely inhibited the downregulation of Sox6, JunD, Ddit3, Gadd45α, and Cpeb4; partially inhibited that of Mef2c and Tob1; and failed to inhibit that of Gilz (Figure 5B, right), indicating that the ERK-dependent downregulation of these antiproliferative genes mostly requires AP-1 activity. Among these AP-1-regulated genes (Sox6, JunD, Ddit3, Gadd45α, and Cpeb4), JunD and Ddit3 promoters were found to contain putative consensus sequences that

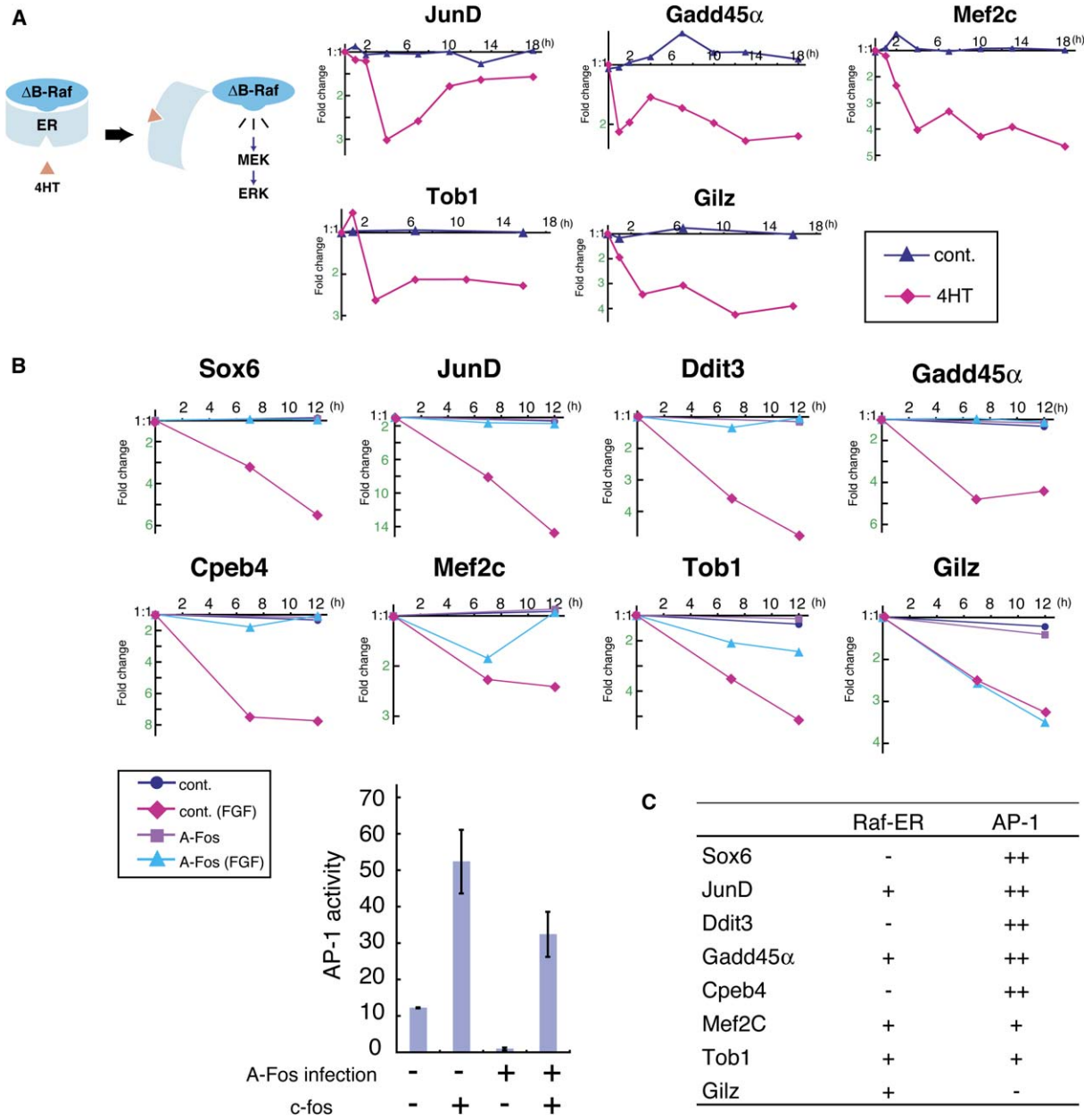

Figure 5. The ERK Pathway Sufficiency and the AP-1 Activity Requirement for the Downregulation of the Antiproliferative Genes

(A) Serum-starved ΔB-Raf:ER NIH3T3 cells were treated with 4-HT. At indicated times after 4-HT treatment, relative mRNA levels of the indicated genes were determined.

(B) NIH3T3 cells were infected with an A-Fos expression vector or a control vector by lentivirus followed by cotransfection with a reporter plasmid (pAP-1-Luc) and a c-Fos expression plasmid. The luciferase activity was measured 24 h after transfection. The data represent means and standard deviations in triplicate (left). Serum-starved NIH3T3 cells, which had been infected with an A-Fos expression vector or a control vector by lentivirus, were treated with FGF. At indicated times after FGF treatment, relative mRNA levels of the indicated genes were determined (right).

(C) Summary of (A) and (B). Raf-ER; + denotes the gene that was significantly downregulated by 4-HT in ΔB-Raf:ER NIH3T3 cells. AP-1; ++ and + denote the genes whose downregulation was inhibited completely or partially by A-Fos, respectively.

could bind to AP-1 family members (data not shown), as was reported previously [34, 35]. To determine whether or not AP-1 directly affects any of the target genes, we carried out the ChIP assay using anti-c-Fos and anti-c-Jun antibodies. The obtained results demonstrated that c-Fos and c-Jun could bind to the promoter regions in endogenous JunD and Ddit3 genes (Figure S5). Therefore, these two genes could be regulated by AP-1 directly. Figure 5C summarizes the results of the ΔB-Raf:ER and the A-Fos expression experiments. No correlation was found between the ERK pathway sufficiency and the AP-1 activity requirement among the eight genes in terms of their downregulation mechanisms. Thus, there may be several different mechanisms that mediate the ERK-dependent downregulation of antiproliferative genes.

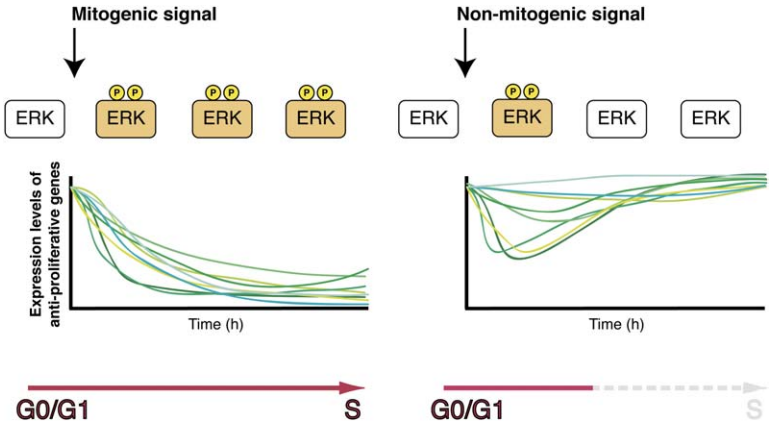

Figure 6. A Model for the Novel Role of Continuous ERK Activation in G1 Phase Progression

Sustained ERK activation throughout G1 phase in response to mitogenic signals induces and maintains decreased expression levels of antiproliferative genes and thus allows S phase entry (left). Transient ERK activation does not induce or maintain decreased expression levels of antiproliferative genes and thus is unable to cause S phase entry (right).

Discussion

The duration of ERK activity has been implicated in diverse biological processes as a crucial factor for determining cell fate decisions [3]. In PC12 cells, sustained ERK activation induced by NGF leads to differentiation into sympathetic-like neurons, whereas transient activation induced by EGF results in proliferation of the cells [36–38]. On the other hand, sustained ERK activation has been shown to be required for growth factor-stimulated G1 phase progression to S phase in fibroblastic cells [18–20]. However, how long ERK activity should be sustained has not been precisely determined. Moreover, it has remained unclear how sustained ERK activation regulates gene expression programs in mid or later stages of G1 phase to promote cell-cycle progression. In this study, we have first demonstrated that the long and continuous ERK activation throughout G1 phase in response to mitogenic signals is required for S phase entry. Our results have shown that when ERK is inactivated even in late G1 phase, the cells cease to enter S phase. Our results have further demonstrated that there are ERK-dependent downregulated genes, some of which are found to have the ability to suppress S phase entry. Their downregulation is induced and maintained by continuous ERK activation throughout G1 phase, and their decreased expression levels return to the original levels rapidly after ERK inactivation (Figure 6). Furthermore, their forced expression to endogenous levels before growth factor stimulation even in late G1 phase blocks S phase entry. Therefore, our results reveal not only the requirement of continuous ERK activation throughout G1 phase for successful S phase entry but also a novel role of continuous ERK activation in G1 phase progression, continuous downregulation of antiproliferative genes throughout G1 phase to allow cell-cycle progression (Figure 6). This mechanism has been hitherto unidentified, partly because previous studies have focused on ERK-dependent upregulated genes such as immediate-early genes and cyclins, which play an essential role in driving cell-cycle progression. Thus, growth factor-stimulated continuous ERK activation may function to ensure G1 phase progression by not only upregulating proliferation-promoting genes but also downregulating antiproliferative genes.

An intriguing idea is that cells also utilize this novel mechanism, that is, the ERK-dependent downregulation of antiproliferative genes as a system to monitor extracellular stimuli. The cells receive not only mitogenic signals but also diverse stimuli such as environmental stresses that induce transient ERK activation. As transient ERK activation does not induce sustained downregulation of antiproliferative genes, these inappropriate stimuli do not cause cell proliferation. Thus, this mechanism may work as a fail-safe mechanism, which prevents inappropriate stimuli from causing cell-cycle progression. In this study, we presented several such examples. Cessation of growth factor stimulation (FCS withdrawal) at mid G1 phase or insufficiency of mitogen doses (PDGF concentrations) resulted in transient ERK activation that could not maintain the decreased expression levels of antiproliferative genes and thereby led to the cessation of S phase entry. In addition, recent studies have shown that loss of adhesion to an extracellular matrix (ECM) causes the transient ERK activation and the failure of S phase entry even after strong mitogenic stimuli [12]. Therefore, cells progressing through G1 phase may monitor environmental conditions through the ERK activation status, by which gene expression programs are controlled to generate an appropriate response.

Recently, it has been shown that several transcription factors including the Fos family proteins and other immediate-early gene products, which have a docking site for ERK, termed the FXFP (DEF) domain, detect the ERK activity duration. The increase in the ERK activity duration is reflected by the increased and sustained phosphorylation of these transcription factors, which increases their stability and activity to elicit the biological outcome, such as induction of cyclin D1 expression [21, 22]. Our results have shown that AP-1 activity is required for the downregulation of most of the ERK-dependent downregulated, antiproliferative genes identified here. Therefore, continuous downregulation of these genes throughout G1 phase may act as one of the downstream effectors of the Fos family proteins that are stabilized by sustained ERK activity. It should be pointed out, however, that the extent of the dependence on AP-1 activity varies among these downregulated genes, and the efficiency of ERK activity for downregulation also differs among these genes. Thus, how growth factor stimulation downregulates these antiproliferative genes may not be accounted for by a single mechanism. Future work should address the detailed

molecular mechanisms for the downregulation of respective genes.

It is well known that CDK inhibitors function as negative regulators of cell-cycle progression [13]. In particular, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$, members of the Cip/Kip family, have been shown to be associated with G1/S transition in response to growth factor stimulation. It has been shown that high intensity of sustained ERK activation, which is achieved by high level expression of active Raf-1, induces cell-cycle arrest through $p21^{WAF1/CIP1}$ upregulation [39, 40]. In contrast, moderate or low ERK activation, which is seen in normal mid or late G1 phase, is not sufficient to maintain the induction of $p21^{WAF1/CIP1}$ expression. Therefore, $p21^{WAF1/CIP1}$ expression is thought to be a sensor of the magnitude, not the duration, of ERK activation. On the other hand, growth factor stimulation leads to $p27^{KIP1}$ downregulation through transcriptional and posttranscriptional mechanisms, which are mediated by the PI3K-AKT and ERK pathways. Our microarray and quantitative RT-PCR analyses have shown that FGF stimulation reduces the expression level of $p27^{KIP1}$ in an ERK-independent manner (data not shown), although previous reports have suggested that the ERK pathway regulates $p27^{KIP1}$ expression at the posttranslational level [41]. Our present study has identified a number of antiproliferative genes whose mRNA expression levels are downregulated in an ERK-dependent manner. These genes do not include CDK inhibitors and had not been clearly demonstrated to have the ability to inhibit S phase entry, except for Tob1 and Ddit3. The next challenge, therefore, will be elucidation of the molecular mechanisms by which these antiproliferative genes block S phase entry. The action mechanism of Tob1 has been relatively well studied, and Tob1 has been shown to regulate cyclin D1 expression negatively by recruiting histone deacetylase (HDAC) to the cyclin D1 promoter [42]. JunD was also shown to inhibit cyclin D1 expression, and it may exert this effect, probably through antagonizing the function of c-Jun [43]. Gadd45α and Ddit3, also known as Gadd153 or CHOP, which are both induced by DNA damaging agents and growth arrest signals, have been suggested to be involved in regulation of G1/S transition as well, but little is known about their target molecules in leading to G1/S arrest [28, 44]. Our result that c-Fos expression cancels the inhibitory effect of JunD, but not that of Gadd45α, has suggested that JunD and Gadd45a differ in their respective target to suppress G1 phase progression. Gilz was shown to suppress ERK activation by inhibiting phosphorylation of Raf-1 [45], implying that the ERK pathway may form a positive feedback loop by downregulating the inhibitor of its own pathway. Two transcription factors, MEF2C and Sox6, which are known to regulate cell differentiation [46, 47], are here also shown to have the ability to suppress G1 phase progression. Although cell differentiation is often associated with the cell-cycle arrest, cell differentiation-regulating genes do not necessarily have the ability to inhibit cell-cycle progression. So both Mef2C and Sox6 might inhibit G1 phase progression directly through acting on their specific target molecules, which are not known at present. Little is known about the function of Cpeb4 [48], a member of cytoplasmic polyadenylation element binding (CPEB) proteins. Thus, all the identified antiproliferative genes may differ in their target molecules to suppress G1 phase progression. Identification of these target molecules or genes will provide new insights into G1 phase progression.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/12/1171/DC1/.

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Accession Numbers

The GEO accession number for the Affymetrix array data reported in this paper is GSE4739.