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ERK Activity and G1 Phase Progression: Identifying Dispensable Versus Essential Activities and Primary Versus Secondary Targets[D]

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The ERK subfamily of MAP kinases is a critical regulator of S phase entry. ERK activity regulates the induction of cyclin D1, and a sustained ERK signal is thought to be required for this effect, at least in fibroblasts. We now show that early G1 phase ERK activity is dispensable for the induction of cyclin D1 and that the critical ERK signaling period is restricted to 3–6 h after mitogenic stimulation of quiescent fibroblasts. Similarly, early G1 phase ERK activity is dispensable for entry into S phase. Moreover, if cyclin D1 is expressed ectopically, ERK activity becomes dispensable throughout the G1 phase. In addition to its effect on cyclin D1, ERK activity is thought to contribute to the down-regulation of $p27^{kip1}$. We found that this effect is restricted to late G1/S phase. Mechanistic analysis showed that the ERK effect on $p27^{kip1}$ is mediated by Skp2 and is secondary to its effect on cyclin D1. Our results emphasize the importance of mid-G1 phase ERK activity and resolve primary versus secondary ERK targets within the G1 phase cyclin-dependent kinases.

INTRODUCTION

Cell cycle progression through G1 phase is carefully controlled in cells, and deregulation of the G1 phase cell cycle machinery is a common feature of cancers. The rate of cell cycle progression through G1 phase is, in large part, determined by the production of cyclin D1, typically the rate-limiting step in the formation of cyclin D-cdk4/6 complexes as quiescent cells reenter the cycle. Formation of the cyclin D1-cdk4/6 complex results in the sequestration of the cdk inhibitors, $p21^{cip1}$ and $p27^{kip1}$ (hereafter called p27) and thereby contributes to the late G1 phase activation of cyclin E-cdk2 (Sherr, 1994; Sherr and Roberts, 1999). Active cdk4/6 and cdk2 phosphorylate the pocket proteins, comprising the retinoblastoma protein Rb, p107, and p130 (Weinberg, 1995; Takahashi *et al.*, 2000). Phosphorylated pocket proteins release associated E2Fs that, in turn, allows for transcription of the E2F-dependent genes required for further cell cycle progression. Cyclin A is one of those E2F-dependent genes; the induction of cyclin A and formation of active cyclin A-cdk2 complexes is required for S phase entry. This established sequence of events emphasizes the central role of cyclin D1 as a primary target of mitogenic signaling.

ERK activity is also required for cell cycle progression through G1 phase. Early studies indicated that the persistence of ERK activity in mid-G1 phase correlated with efficient S phase entry (Meloche *et al.*, 1992). Subsequent studies showed that sustained ERK signal of 5–6 h is associated with the induction of cyclin D1 mRNA and protein in mid-G1 phase (~8–9 h after mitogenic stimulation of quiescent cells; Albanese *et al.*, 1995; Lavoie *et al.*, 1996; Weber *et al.*, 1997; Balmanno and Cook, 1999; Welsh *et al.*, 2001). Additionally, early G1 phase ERK activity stimulates a transient induction of $p21^{cip1}$ (Bottazzi *et al.*, 1999), an effect that may contribute to assembly of cyclin D-cdk4/6 complexes (Sherr and Roberts, 1999). Interestingly, intense ERK signals (e.g., resulting from overexpression of activated Raf) can lead to a persistent up-regulation of $p21^{cip1}$ and G1 phase arrest (Sewing *et al.*, 1997; Woods *et al.*, 1997).

Finally, several studies have indicated that ERK activity also contributes to the down-regulation of p27 (Kerkhoff and Rapp, 1997; Rivard *et al.*, 1999; Delmas *et al.*, 2001; Kortylewski *et al.*, 2001; Foster *et al.*, 2003; Bhatt *et al.*, 2005). In this report, we used mitogen stimulation of quiescent fibroblasts to study the role of endogenous ERK activity in G1 phase progression, map its temporal effects, identify its essential targets, and resolve its primary versus secondary cell cycle effects.

MATERIALS AND METHODS

Cell Culture

Spontaneously immortalized mouse embryo fibroblasts (MEFs) and early passage wild-type and $p27^{T187A}$ MEFs were grown to near confluence in DMEM, 10% FBS and then serum-starved for 48 h in DMEM, 1 mg/ml fatty acid–free BSA (DMEM-BSA). Early passage human foreskin fibroblasts were maintained and starved as described (Bohmer *et al.*, 1996). For experimentation, serum-starved cells were trypsinized, resuspended in DMEM-BSA, and collected by centrifugation. MEFs (~10^6 cells in 10 ml) and human fibroblasts (~1.5×10^6 cells in 20 ml) were then resuspended in DMEM-BSA and preincubated in suspension with 50 μM U0126 (Promega, Madison, WI) or DMSO (vehicle) for 30 min at 37°C before being reseeded in 100-mm (MEFs) or 150-mm (human fibroblasts) dishes and stimulated with 10% FBS in the continued presence of DMSO or 50 μM U0126. When indicated, U0126 was washed-out by rinsing monolayers twice with cold DMEM and then refeeding the cultures fresh DMEM-10% FBS. MEFs expressing a tetracycline-repressible cyclin D1 (Tet-D1-MEFs) were prepared as described (Zhu *et al.*, 2000) and cultured similarly except that they were maintained in the presence of 2 μg/ml tetracycline and then serum-starved and stimulated in the absence

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or presence of 2 μg/ml tetracycline. Primary MEFs from wild-type and p27T187A knockin mice (Malek *et al.*, 2001) were maintained in 10% FCS and used at passages 2–5.

Adenoviral Infection and Small Interfering RNA

For adenoviral infection, confluent MEFs were incubated in serum-free DMEM-1 mg/ml BSA for 12 h, infected overnight with adenoviruses encoding LacZ or cyclin D1 (kind gift of Jeffrey Albrecht) and then incubated in fresh DMEM-BSA for an additional 24 h. For cyclin D1 knockdown, MEFs ($\sim 10^6$) were seeded in 100-mm dishes with antibiotic-free DMEM-10% FBS and incubated overnight. The cultures were washed three times with serum-free/antibiotic free OptiMEM (Invitrogen, Carlsbad, CA) before transfection with 100 nM of an irrelevant small interfering RNA (siRNA; human E cadherin; GAGUGAAUUUUGAAGAUUGtt with 4/19 mismatches relative to the homologous mouse sequence) or siRNA specific to mouse cyclin D1 (GCGGUAGGGAUGAAAUAGUtt). The transfection used Lipofectamine 2000 (Invitrogen) and a final volume of 4 ml OptiMEM and generally followed the manufacturer's protocol. After a 4–6 h, the medium was replaced with DMEM-BSA, and the cells were starved for 2 d.

Analysis of mRNA and Protein Expression

Cell pellets were extracted in Trizol (Invitrogen; 1 ml/1–1.5 × 10^6 cells) for isolation of total RNA. Northern blotting of total RNA used random-primed cDNA probes and standard procedures. Quantitative real-time RT-PCR (QPCR) was performed as described (Stewart *et al.*, 2004). Mouse p27 and cyclin E mRNAs were quantified using Assay on Demand primer-probe sets Mm00438167_g1 and Mm00432367_ml, respectively (Applied Biosystems, Foster City, CA). The primers and probe used to detect Skp2 mRNA have been described (Stewart *et al.*, 2004), and those used for 18S rRNA were as follows: 5′-CCT GGT TGA TCC TGC CAG TAG (forward primer, 150 nM), 5′-CCG TGC GTA CTT AGA CAT GCA (reverse primer, 150 nM), and VIC-TGC TTG TCT CAA AGA TTA-MGB-NFQ (probe, 100 nM). QPCR results show the mean ± SD of duplicate PCR reactions.

When cells were analyzed by Western blotting, they were washed with cold PBS, 1 μM Na_3VO_4, scraped, collected by centrifugation, and quick-frozen in dry ice before lysis. Western blotting used antibodies specific for ERK1/2 (BD Transduction Laboratories, Lexington, KY; M12320), dually phosphorylated (active) ERK1/2 (pERK1/2; Cell Signaling, Danvers, MA; 9101S), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA; SC-8396), p27 (BD Transduction Laboratories; K25020), Skp2 (Zymed Laboratories, South San Francisco, CA; 32-3400), cdk4 (Santa Cruz, SC-260), Rb (Zymed Laboratories; 28-0007), AKT1 (gift of Morris Birnbaum), phospho-AKT^{Ser473} (Cell Signaling; 9271S), and actin (Santa Cruz; SC-8432). The rabbit polyclonal cyclin A antibody was prepared in this laboratory using recombinant cyclin A as immunogen. Western blot signals were detected by enhanced chemiluminescence. The images were digitized by scanning, and figures were assembled using Adobe Photoshop (San Jose, CA).

Analysis of Cyclin D1-cdk4 Complexes

Cell pellets were lysed in 100 μl of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM NaF, and 10 mM Na_3VO_4. Equal amounts of lysate (200 μg) were incubated with 5 μg of anti-cdk4 (BioSource, Camarillo, CA; AH20202) for 2 h at 4°C followed by incubation with 50 μl washed anti-mouse conjugated agarose (Invitrogen) with rocking for 2 h at 4°C. Collected immunoprecipitates were washed five times with the lysis buffer and resuspended in 50 μl of reducing SDS sample buffer. The immunoprecipitates were fractionated on SDS polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-cyclin D1 (Santa Cruz; SC-8396) and anti-cdk4 (Santa Cruz; SC-260).

Immunostaining

S phase entry was determined by performing the serum stimulation in dishes containing coverslips and 3 μg/ml bromodeoxyuridine (BrdU; GE Healthcare, Piscataway, NJ). Coverslips were removed at selected times, washed with PBS, fixed (15 min) in 3.7% paraformaldehyde, incubated (10 min) in 50 mM ammonium chloride, and permeabilized (5 min) with 0.2% Triton X-100 in PBS. Permeabilized cells were incubated at room temperature sequentially with DNase (500 U/ml) and 2% BSA in PBS for 1 h, anti-BrdU (BIODESIGN, Saco, ME; M20105S; 250-fold dilution) and DNAse in PBS for 1 h, and TRITC-conjugated donkey anti-sheep antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 200-fold dilution) in PBS for 1 h. Cell nuclei were stained with DAPI (Sigma, St. Louis, MO; 2 μg/ml in PBS) for 10 min. BrdU-positive cells were identified using epifluorescence microscopy, usually counting 150–200 DAPI-positive cells per sample.

RESULTS

We determined exactly when endogenous ERK activity was required for G1 phase induction of cyclin D1 and S phase entry in MEFs by adding (Figure 1A) or washing-out (Figure 1D) the MEK inhibitor, U0126, at selected times after serum stimulation of quiescent cells. U0126 inhibits activation of ERK1, ERK2, and ERK5 (Kamakura *et al.*, 1999; Mody *et al.*, 2001). Although all three ERK isoforms have been linked to

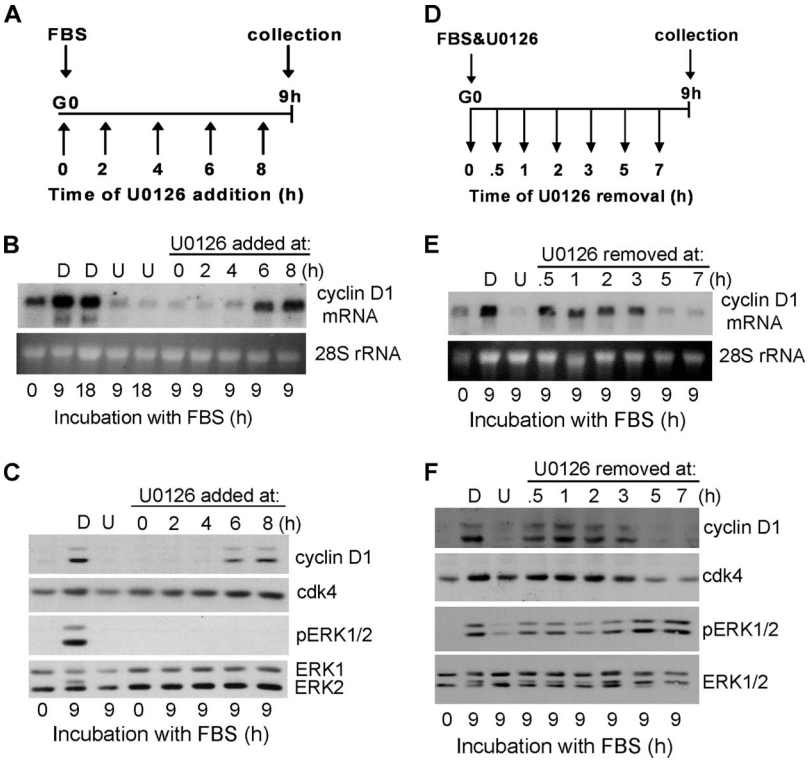

Figure 1. Early G1 phase ERK activity is dispensable for cyclin D1 gene expression. (A–C) Quiescent MEFs were stimulated with 10% FBS and treated with DMSO or 50 μM U0126 at 0, 2, 4, 6, and 8 h after serum stimulation. All of these samples were collected after 9 h of incubation with 10% FBS. Controls received 10% FBS alone (DMSO; D) or were continuously exposed to U0126 (U). (D–F) Quiescent MEFs were trypsinized and preincubated with DMSO or 50 μM U0126 for 30 min at 37°C before being reseeded in 100-mm dishes and stimulated with 10% FBS in the continued presence of U0126. After 0.5, 1, 2, 3, 5, or 7 h, U0126 was washed-out by rinsing monolayers twice with cold DMEM and then refeeding the cultures with fresh DMEM-10% FBS. All of these samples were collected after 9 h of total incubation and lysed. Controls received 10% FBS alone (DMSO; D) or were continuously exposed to U0126 (U). Equal amounts of total RNA (B and E) or protein (C and F) from collected cells were analyzed by Northern blotting for cyclin D1 mRNA and 28S rRNA (loading control) or Western blotting for cyclin D1, pERK1/2, ERK1/2, and cdk4 (loading control).

the induction of cyclin D1, the roles of endogenous ERK1 and ERK2 are well established, but the role of ERK5 is less clear (Squires *et al.*, 2002; Mulloy *et al.*, 2003).

Cyclin D1 mRNA and protein are induced in mid-G1 phase (~9 h after serum stimulation of quiescent fibroblasts) (Welsh *et al.*, 2001; Figure 1B; DMSO). U0126 blocked the induction of cyclin D1 mRNA and protein when added between 0 and 6 h after mitogenic stimulation, but addition of U0126 at 6 h and beyond was without effect (Figure 1, B and C; U0126). Although this result supports previous studies showing that a sustained ERK signal of 5–6 h is associated with the mid-G1 phase induction of cyclin D1 (Weber *et al.*, 1997; Balmanno and Cook, 1999; Welsh *et al.*, 2001), U0126 washout experiments showed that cyclin D1 mRNA and protein were expressed normally even when ERK activity was inhibited through the first 3 h after mitogenic stimulation (Figure 1, E and F). Controls showed that ERK activity was blocked within 5 min after addition of U0126 and restored within 5 min of removing U0126; these effects were specific (Supplementary Figure 1, A and B, respectively). Thus, although a sustained ERK signal of 5–6 h does correlate with mid-G1 phase induction of cyclin D1 mRNA, the first 3 h of ERK signaling are, in fact, dispensable. The critical ERK signaling period is ~3–6 h after mitogenic stimulation of quiescent cells.

Remarkably, inhibition of MEK/ERK signaling with U0126 for the first 3 h of G1 phase did not affect maximal S phase entry in quiescent MEFs stimulated with serum (Figure 2A; U0 washout [wo] 3 h) and only had a small effect on the kinetics of S phase entry (Figure 2B). In contrast, inhibition of MEK/ERK signaling for the first 5 h of G1 phase effectively prevented S phase entry (Figure 2A; U0 wo 5 h). These results reveal a tight correlation between ERK-dependent cyclin D1 induction and ERK-dependent S phase entry. This finding was not restricted to MEFs; U0126 washout experiments showed that the first 3 h of ERK signaling are also dispensable for S phase entry and cyclin D1 induction in early passage human fibroblasts (Supplementary Figure 2). Thus, cyclin D1 induction and S phase entry share the requirement for mid-G1 phase ERK activity, raising the possibility that cyclin D1 represents the major ERK cell cycle target in G1 phase.

MEFs expressing tetracycline-repressible cyclin D1 (Tet-D1-MEFs) were then used to supply cyclin D1 ectopically and determine if there were other essential ERK targets in G1 phase. Quiescent tet-D1-MEFs stimulated with serum in the presence of tetracycline behaved like the nontransfected parents: ERK activity was sustained, cyclin D1 was induced in mid-G1 phase and cyclin A (a marker for the end of G1 phase) was expressed in the absence of U0126 (Figure 3A, left panels). All these events were inhibited by continuous exposure to U0126 (Figure 3A, middle panels). When we removed tetracycline, cyclin D1 was expressed ectopically (2.5–3-fold above endogenous levels; see legend to Figure 3). Despite continuous ERK/MEK inhibition with U0126, the ectopically expressed cyclin D1 restored a near normal rate of progression through G1 phase as assessed by the time-dependent hyperphosphorylation of Rb (Figure 3B) and entry into S phase as judged by the temporal induction of cyclin A (Figure 3A; compare left and right panels) and incorporation of BrdU (Figure 3C). Others (Cheng *et al.*, 1998; Treinies *et al.*, 1999) have reported that ERK is involved in assembly of cyclin D1-cdk4 complexes, but we found that ectopically expressed cyclin D1 bound to cdk4 in serum-stimulated MEFs, both in the absence and presence of U0126 (Figure 3D). In fact, the absence of an ERK requirement for cyclin D1-cdk4 complex formation was expected given the normal kinetics of Rb phosphorylation in the U0126-treated cyclin D1 expressors. Thus, ERK activity is dispensable for G1 phase progression of mitogen-treated MEFs expressing cyclin D1.

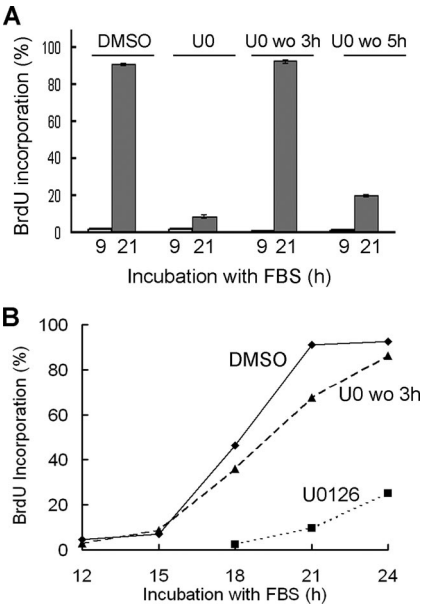

Figure 2. Early G1 phase ERK activity is dispensable for S phase entry. (A) Quiescent MEFs were preincubated with DMSO (vehicle) or 50 μM U0126 (U0) before being reseeded in 100-mm dishes containing coverslips. The cells were stimulated with 10% FBS with BrdU in the continued presence of U0126. U0126 was washed-out (wo) at the indicated times, and the cells were refed DMEM-10% FBS and BrdU without U0126. Coverslips were collected after 9 and 21 h of total incubation. Entry into S phase was measured by incorporation of BrdU using epifluorescence microscopy. Results show the mean ± SD of three experiments. (B) Quiescent MEFs preincubated with DMSO or U0126 as in A, were reseeded onto 35-mm dishes containing coverslips and stimulated with 10% FBS in the presence of BrdU. U0126 was either maintained throughout the mitogenic stimulation (dotted line) or washed-out after 3 h of mitogenic stimulation (dashed line). In the latter case, the cells were refed DMEM-10% FBS and BrdU without U0126. Coverslips were collected at the indicated times, and S phase entry was determined by BrdU incorporation.

Conversely, we found that siRNA-mediated knockdown of cyclin D1 inhibited Rb phosphorylation (Figure 4A) and S phase entry (Figure 4B) despite normal activation of ERK (Figure 4A). A distinct cyclin D1 RNA interference (RNAi) gave similar results (data not shown). In general, the inhibitory effect of cyclin D1 siRNA on S phase entry was somewhat less than the effect of U0126 (Figure 4B; also see *Discussion*). Nevertheless, it is clear that the mitogenic effect associated with sustained ERK activity can be blocked by knockdown of cyclin D1 (Figure 4), and ectopic expression of cyclin D1 can overcome the requirement for G1 phase ERK activity (Figure 3). Together these results indicate that cyclin D1 is a major ERK target as MEFs progress from quiescence to S phase.

The effect of ERK on p27 Levels Is a Downstream Consequence of Cyclin D1 Induction

Others have reported that ERK signaling contributes to the down-regulation of p27 (Kerkhoff and Rapp, 1997; Rivard *et al.*, 1999; Delmas *et al.*, 2001; Kortylewski *et al.*, 2001; Foster *et al.*, 2003; Bhatt *et al.*, 2005), and p27 down-regulation, in turn, contributes to S phase entry by facilitating the activa-

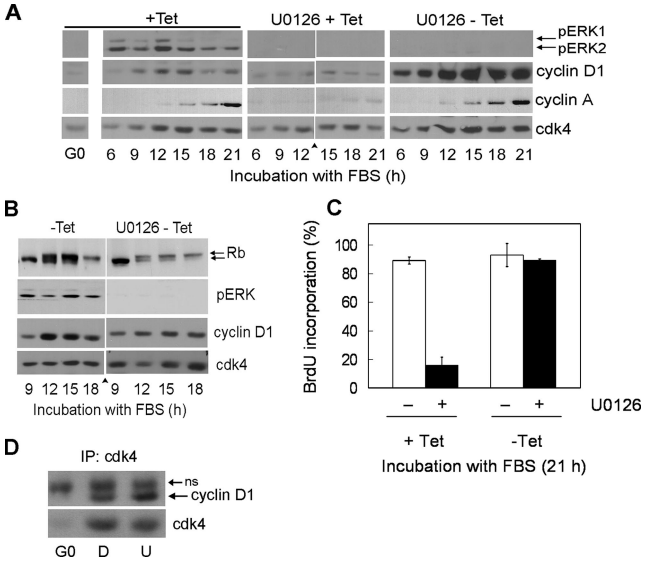

Figure 3. Enforced expression of cyclin D1 overcomes the need for ERK signaling in G1 phase. MEFs expressing a tetracycline-repressible cyclin D1 were maintained in the presence of 2 μg/ml tetracycline (Tet). Near-confluent cells were serum-starved, preincubated with DMSO or U0126, replated at subconfluence in dishes containing coverslips, and stimulated with 10% FBS, all in the absence or presence of Tet. (A) Collected cells were analyzed by Western blotting with antibodies to pERK, cyclin D1, cyclin A, or cdk4 (loading control). Cyclin D1 overexpression (U0126-tet relative to +tet; 9–15 h) was determined with Image J and normalized to the matched cdk4 loading control. (B) FBS-stimulated cells incubated without Tet were analyzed by Western blotting for the hyperphosphorylation of Rb by gel-shift (the upper and lower arrows, respectively, show hyper- and hypophosphorylated Rb), and the levels of cyclin D1, pERK, and cdk4 (loading control). The samples in A and B were run and analyzed by Western blotting at the same time, but they were divided into different gels as needed to accommodate the number of samples. The small arrowheads indicate where samples were divided into two identical gels. The thick space between the G0 and 6-h samples in A indicates removal of extraneous information. (C) S phase entry was determined from coverslips collected after 21 h of mitogenic stimulation. The results show the mean ± SD of two experiments. (D) Equal amounts of total cell protein from samples collected at G0 or after a 9-h incubation with FBS and DMSO (D) or FBS and 50 μM U0126 (U) were immunoprecipitated (IP) with anti-cdk4 and then blotted with anti-cyclin D1 and anti-cdk4. ns, nonspecific.

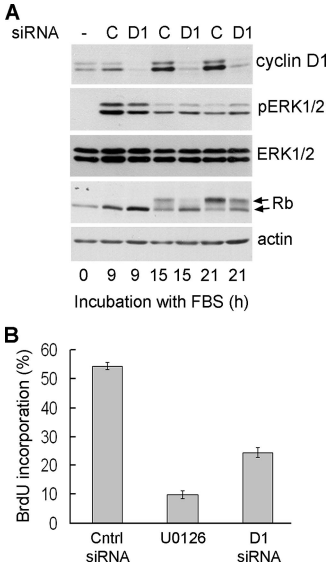

Figure 4. Effect of cyclin D1 siRNA on G1 phase progression. MEFs were either serum-starved and pretreated with 50 μM U0126 or transfected with irrelevant control or cyclin D1 siRNA before serum starvation. The cells were plated at subconfluence in dishes containing coverslips and stimulated with 10% FBS in the presence of BrdU. (A) Western blot of cell lysates treated with control (C) or cyclin D1 (D1) siRNA and probed with antibodies to cyclin D1, pERK1/2, ERK1/2, Rb, and actin (loading control). The upper and lower arrows in the Rb blot indicate hyper- and hypophosphorylated Rb, respectively. (B) BrdU incorporation determined from coverslips collected after 21-h stimulation with 10% FBS.

tion of cyclin-cdk2 complexes. Some of these studies concluded that the ERK effect is posttranscriptional, but the exact mechanism by which ERK regulates p27 has not been resolved.

We first examined the effect of endogenous ERK inhibition on the levels of p27 as serum-stimulated MEFs progressed from G0 to S phase. U0126 inhibited the down-regulation of p27 protein in MEFs (Figure 5A), but this effect was incomplete and was not detected until 18 h, when serum-stimulated MEFs are progressing through S phase (refer to Figure 2B). Thus, the ERK effect on p27 is less pronounced than the ERK effect on cyclin D1. U0126 did not block the mid- to late G1 phase down-regulation of p21 (data not shown), consistent with our previous studies, which concluded that p21 down-regulation is ERK-independent (Bottazzi *et al.*, 1999).

p27 levels are typically controlled posttranscriptionally by ubiquitin-mediated proteolysis involving two E3 ligases: KPC (Kamura *et al.*, 2004) and SCF^{Skp2} (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999). KPC is thought to act in G0/G1 phase, whereas Skp2 acts in late G1/S phase (Kamura *et al.*, 2004). Because ERK inhibition selectively affected p27 levels in late G1/S phase (refer to Figure 5A), we asked if the ERK effect on p27 required the action of SCF^{Skp2}. Indeed we found that late G1/S phase p27 levels were unaffected by U0126 in knockin MEFs that express $p27^{T187A}$ (Malek *et al.*, 2001), a p27 mutant that cannot be recognized by SCF^{Skp2} (Figure 5B; compare 21 h ± U0126 in WT and T187A cells). Thus, when SCF^{Skp2}-dependent degradation of p27 is precluded, ERK loses its ability to regulate p27. We conclude that the ERK effect on p27 is mediated by SCF^{Skp2}. Note that down-regulation of p27 persists in T187-MEFs (Figure 5B; G0 vs. 21 h), even after ERK inhibition. This result implies that SCF^{Skp2}-independent degradation of p27 is also independent of ERK activity (see *Discussion*).

SCF^{Skp2} is comprised of core components (Skp1, Cul-1, and Rbx/Roc1 or Ro52) and the F-box protein, Skp2, which is the substrate-recognizing component of the complex (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999; Sabile *et al.*, 2006). Because the core components are typically constitutively expressed, whereas Skp2 is an E2F-regulated gene (Markey *et al.*, 2002; Vernell *et al.*, 2003; Zhang and Wang, 2006 and our unpublished results), we reasoned that the effect of U0126 on p27 degradation might be a downstream consequence of a primary ERK effect on cyclin D1, with consequent inactivation of Rb (or other pocket proteins), release of E2Fs, and induction of Skp2. Indeed, we found that the inhibitory effect of U0126 on G1/S phase down-regulation of p27 correlated well with the inhibitory effect of U0126 on Rb hyperphosphorylation and Skp2 expression (Figure 5C). Moreover, U0126 blocked the induction of Skp2 mRNA but did not affect the levels of p27 mRNA (Figure 5D), indicating that the ERK effect on p27 is posttranscriptional.

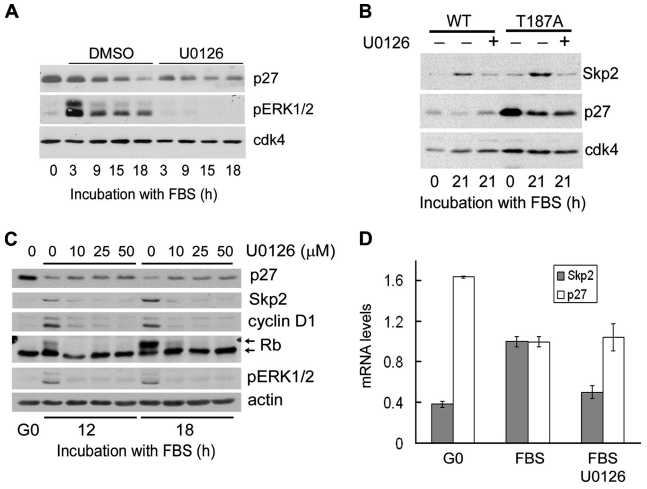

Figure 5. Regulation of p27 at late G1/S phase by ERK. (A) Quiescent MEFs pretreated with DMSO or 50 μM U0126 were plated at subconfluence and stimulated with 10% FBS; DMSO or U0126 remained throughout the FBS incubation period. Cells collected at different times throughout G1 and S phases were analyzed by Western blotting for p27, pERK, and cdk4 (loading control). (B) Serum-starved early passage wild-type or p27^{187A} MEFs pretreated with DMSO or 50 μM U0126 were reseeded at subconfluence and stimulated with 10% FBS for 21 h. Collected cells were analyzed by Western blotting for Skp2, p27, and cdk4 (loading control). (C) MEFs incubated with selected concentrations of U0126 for 12 or 18 h were collected and analyzed by Western blotting for p27, Skp2, Rb, cyclin D1, pERK1/2, and actin (loading control). The upper and lower arrows in the Rb blot show hyper- and hypophosphorylated Rb, respectively. (D) MEFs treated with 10% FBS ± 50 μM U0126 for 21 h were collected and analyzed by QPCR for p27 mRNA, Skp2 mRNA, and 18S rRNA. The figure shows p27 and Skp2 mRNA levels plotted relative to 18S rRNA and normalized to the mRNA levels seen in the FBS-stimulated cells.

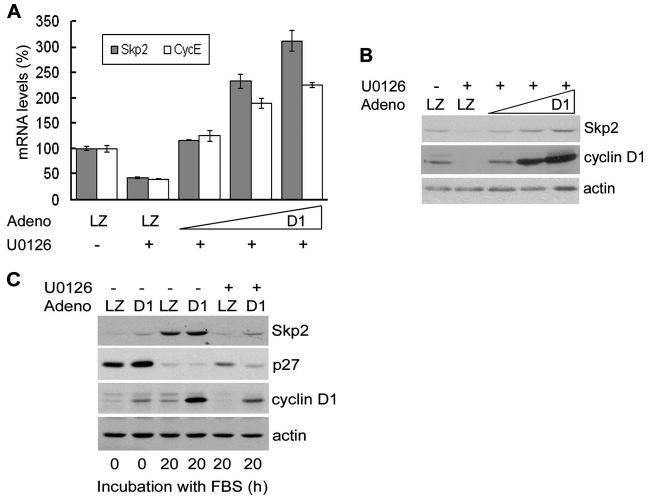

Figure 7. Ectopic cyclin D1 rescues Skp2 expression and p27 degradation in MEK/ERK-inhibited cells. (A and B) Serum-starved MEFs infected with 300, 900, or 2700 moi Ad-cyclin D1 (or 2700 moi Ad-LacZ as control; LZ) were pretreated with DMSO or 50 μM U0126, reseeded at subconfluence, and stimulated with 10% FBS for 21 h. Collected cells were analyzed by QPCR for Skp2 mRNA, cyclin E mRNA and 18S rRNA (A), or by Western blotting for Skp2, cyclin D1, and actin (B). The levels of Skp2 and cyclin E mRNAs in A are plotted relative to 18S rRNA and normalized to mRNA levels in the LacZ-infected cells lacking U0126. (C) Serum-starved MEFs infected at 600 moi with Ad-LacZ (LZ) or Ad-cyclin D1 (D1) were serum-starved, pretreated with DMSO or 50 μM U0126, and either immediately collected (0) or replated with 10% FBS for 20 h before collection. The collected cells were analyzed by Western blotting with antibodies to Skp2, p27, cyclin D1, and actin (loading control). Image J was used to quantify the degree of Skp2 expression in Ad-cyclin D1 infected cells treated with U0126, relative to Lac Z–infected cells incubated for 20 h with 10% FBS. The degree of rescue was 40–85%, n = 4.

To directly test whether the ERK effects on cyclin D1, Skp2, and p27 were causally related, we used siRNA and ectopic expression to manipulate cyclin D1 levels downstream of ERK activation. Cyclin D1 knockdown inhibited, although not completely, the mitogen-dependent induction of Skp2 mRNA (Figure 6A) and protein (Figure 6B), and it similarly inhibited the down-regulation of p27 in late G1/S phase (~50% relative to actin; Figure 6C). Conversely, ectopic cyclin D1 rescued the expression of Skp2 mRNA (Figure 7A) and protein (Figure 7B) in a dose-dependent manner in U0126-treated MEFs, and the rescue (40–85% of endogenous expression; see Figure 7 legend) could be detected with near normal levels of ectopic cyclin D1. This effect was transcriptional and consistent with cyclin D1–dependent Rb inactivation and E2F release because cyclin E (a prototype E2F1-induced gene; DeGregori *et al.*, 1995) and Skp2 mRNA were similarly rescued by ectopic cyclin D1 expression (Figure 7A). Moreover, the forced expression of cyclin D1 restored p27 down-regulation in MEK/ERK-inhibited cells (Figure 7C; LZ+U0126 vs. D1+U0126). On the basis of the results in Figures 6 and 7, we conclude that the ERK effects on Skp2 and p27 are secondary consequences of the ERK effect on cyclin D1.

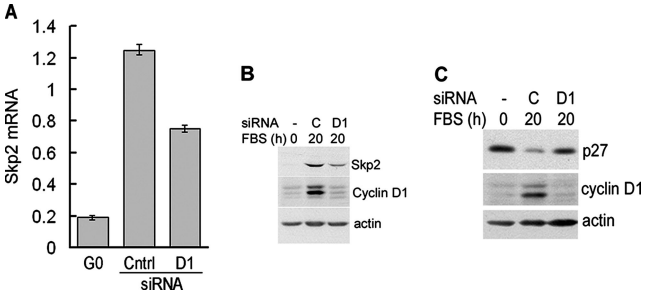

Figure 6. Regulation of Skp2 and p27 by cyclin D1 RNAi. MEFs transfected with control (C) or cyclin D1 siRNA were serum-starved and then replated at subconfluence with 10% FBS for 20 h. (A) A portion of the collected cells were analyzed by QPCR for Skp2 mRNA and 18S rRNA; the level of Skp2 mRNA plotted relative to 18S rRNA is shown. (B) The remainder of the cells was analyzed by Western blotting for Skp2, cyclin D1, and actin (loading control). (C) Collected cell lysates from an independent experiment were Western blotted for p27, cyclin D1, and actin.

DISCUSSION

Many studies exploring the relationship between ERK signaling and cell cycle progression have focused on events occurring rapidly after mitogenic stimulation. Our results indicate that these short-term effects would not be required for mitogenesis if they do not persist into mid-G1 phase. One study using discontinuous mitogenic stimulation concluded that early-G1 phase ERK activity is required for G1 phase progression (Jones and Kazlauskas, 2001) and another concluded that ERK activity was required throughout most of

G1 phase (Yamamoto *et al.*, 2006). Our results, indicating that only mid-G1 phase ERK activity is required for mitogenesis in fibroblasts, agree well with Fassett *et al.* (2003), who studied the relationships between ERK activity, cyclin D1 expression, and G1 phase progression in rat hepatocytes. Similarly, others have shown that the induction of cyclin D1 and DNA synthesis correlates with sustained, mid-G1 phase ERK activity and the mid-G1 phase expression of Fra-1 and Fra-2 rather than transient/early G1 phase ERK activity and expression of c-fos (Balmanno and Cook, 1999; Cook *et al.*, 1999). Finally, the correlation we observe between the inhibitory effect of U0126 on cyclin D1 expression and S phase entry is consistent with studies (Peeper *et al.*, 1997; D'Abaco *et al.*, 2002), showing that Ras and ERK activities are dispensable for S phase entry in Rb-null cells. Thus, although a sustained ERK signal correlates with cyclin D1 induction and S phase entry in fibroblasts, only the mid-G1 phase ERK signal is required. This finding can explain why cyclin D1 gene expression in fibroblasts requires coordinated signaling by growth factor receptor tyrosine kinases and integrins, because growth factors do not sustain the ERK signal into mid-G1 phase in the absence of a concomitant integrin signal (Roovers *et al.*, 1999).

Two studies (Cheng *et al.*, 1998; Treinies *et al.*, 1999) using conditional expression of activated MEK have concluded that MEK/ERK activity is sufficient to stimulate complex formation between cyclin D1 and cdk4. Although our results do not contradict these findings, our data with conditional cyclin D1 expression shows that MEK/ERK activity is not *required* for assembly of a cyclin D1-cdk4 complex in mitogen-treated MEFs. Other kinases may facilitate assembly in these conditions, or assembly might be mediated by $p21^{cip1}$ or p27, both of which are present in MEK/ERK-inhibited MEFs. These cdk inhibitors are established mediators of cyclin D1-cdk4 complex formation (reviewed in Sherr and Roberts, 1999).

In addition to cyclin D1, several studies have indicated that p27 accumulates when endogenous ERK activity is inhibited (Rivard *et al.*, 1999; Delmas *et al.*, 2001; Kortylewski *et al.*, 2001; Foster *et al.*, 2003; Bhatt *et al.*, 2005). In most of these studies, the effect of ERK on p27 was posttranscriptional. Our data extend these observations by showing that the ERK effect on p27 is restricted to late G1/S phase, is mediated by SCF^{Skp2}, and is due (at least in large part) to an ERK-dependent induction of Skp2 gene expression. However, we also show that the ERK effect on p27 is incomplete (because p27 continues to down-regulate in MEK/ERK-inhibited cells) and secondary to its effect on cyclin D1. In particular, we find that ERK regulates p27 levels after it stimulates cyclin D1 expression. Cyclin D1 depletion is sufficient to inhibit late G1/S phase Skp2 induction and p27 down-regulation, whereas ectopic cyclin D1 expression rescues these events in MEK/ERK-inhibited cells. We note, however, two studies that reported ERK-mediated effects on p27 that were independent of Skp2 (Delmas *et al.*, 2001; Foster *et al.*, 2003).

Our findings can explain why ERK selectively regulates p27 degradation in late G1/S phase: its effect is secondary to cyclin D1 expression, pocket protein inactivation, and Skp2 induction. G1 phase p27 levels decreased in U0126-treated MEFs and even in T187A-MEFs, showing that SCF^{Skp2}-independent p27 down-regulation persists in ERK-inhibited cells. KPC is thought to regulate p27 degradation in the G1 portion of the cell cycle (Kamura *et al.*, 2004). The overall ERK effect on p27 levels may therefore be partial because KPC-dependent p27 degradation is ERK independent.

The degree of Skp2 and S phase inhibition by cyclin D1 siRNA was somewhat less than that seen after inhibition of ERK with U0126. This result was expected because cyclin D1 siRNA also had a somewhat reduced inhibitory effect on Rb phosphorylation, relative to U0126. Incomplete transfection, residual cyclin D1, or the cyclin D2, which is ERK-dependent and expressed in MEFs (Dey *et al.*, 2000; Piatelli *et al.*, 2002; Huang *et al.*, 2006), most likely account for the residual hyperphosphorylation of Rb and induction of Skp2 in MEFs transfected with cyclin D1 siRNA. Nevertheless, our results cannot exclude the possibility that other component of SCF^{Skp2} complexes, such as Ro52 (Sabile *et al.*, 2006) or cks1 (Ganoth *et al.*, 2001) may also be involved in ERK-dependent p27 degradation.

Overall, we conclude that early G1 phase ERK activity is dispensable for both cyclin D1 gene expression and S phase entry when quiescent fibroblasts are mitogen-stimulated to reenter the cell cycle. In stark contrast, sustained ERK activity in mid-G1 phase is required for these events. We also conclude that cyclin D1 is a primary and essential cell cycle target of mid-G1 phase ERK signaling during cell cycle reentry of mitogen-stimulated fibroblasts. In contrast, the effects of ERK on Skp2 and p27 levels are, at least in large part, downstream consequences of ERK-dependent cyclin D1 induction. Our results clarify the importance of sustained ERK signaling in G1 phase and suggest that the primary ERK-regulated cell cycle targets required for mitogenesis in mesenchymal cells may be more limited than generally thought.

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REFERENCES

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem. *270*, 23589–23597.

Balmanno, K., and Cook, S. J. (1999). Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. Oncogene *18*, 3085–3097.

Bhatt, K. V., Spofford, L. S., Aram, G., McMullen, M., Pumiglia, K., and Aplin, A. E. (2005). Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling. Oncogene *24*, 3459–3471.

Bohmer, R. M., Scharf, E., and Assoian, R. K. (1996). Cytoskeletal integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. Mol. Biol. Cell *7*, 101–111.

Bottazzi, M. E., Zhu, X., Bohmer, R. M., and Assoian, R. K. (1999). Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. J. Cell Biol. *146*, 1255–1264.

Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Cheng, M., Sexl, V., Sherr, C. J., and Roussel, M. (1998). Assembly of cyclin D-dependent kinase and titration of $p27^{kip1}$ regulated by mitogen-activated protein kinase kinase (MEK1). Proc. Natl. Acad. Sci. USA *95*, 1091–1096.

Cook, S. J., Aziz, N., and McMahon, M. (1999). The repertoire of fos and jun proteins expressed during the G1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. Mol. Cell Biol. *19*, 330–341.

D'Abaco, G. M., Hooper, S., Paterson, H., and Marshall, C. J. (2002). Loss of Rb overrides the requirement for ERK activity for cell proliferation. J. Cell Sci. *115*, 4607–4616.

DeGregori, J., Kowalik, T., and Nevins, J. R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol. Cell. Biol. *15*, 4215–4224.

Delmas, C., Manenti, S., Boudjelal, A., Peyssonnaux, C., Eychene, A., and Darbon, J. M. (2001). The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells. J. Biol. Chem. *276*, 34958–34965.

Dey, A., She, H., Kim, L., Boruch, A., Guris, D. L., Carlberg, K., Sebti, S. M., Woodley, D. T., Imamoto, A., and Li, W. (2000). Colony-stimulating factor-1 receptor utilizes multiple signaling pathways to induce cyclin D2 expression. Mol. Biol. Cell *11*, 3835–3848.

Fassett, J. T., Tobolt, D., Nelsen, C. J., Albrecht, J. H., and Hansen, L. K. (2003). The role of collagen structure in mitogen stimulation of ERK, Cyclin D1 expression, and G1-S progression in rat hepatocytes. J. Biol. Chem. *278*, 31691–31700.

Foster, J. S., Fernando, R. I., Ishida, N., Nakayama, K. I., and Wimalasena, J. (2003). Estrogens down-regulate p27Kip1 in breast cancer cells through Skp2 and through nuclear export mediated by the ERK pathway. J. Biol. Chem. *278*, 41355–41366.

Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001). The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat. Cell Biol. *3*, 321–324.

Huang, W., Chang, H. Y., Fei, T., Wu, H., and Chen, Y. G. (2006). GSK3beta mediates suppression of cyclin D2 expression by tumor suppressor PTEN. Oncogene. Epub ahead of print.

Jones, S. M., and Kazlauskas, A. (2001). Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat. Cell Biol. *3*, 165–172.

Kamakura, S., Moriguchi, T., and Nishida, E. (1999). Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J. Biol. Chem. *274*, 26563–26571.

Kamura, T., Hara, T., Matsumoto, M., Ishida, N., Okumura, F., Hatakeyama, S., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2004). Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. Nat. Cell Biol. *6*, 1229–1235.

Kerkhoff, E., and Rapp, U. R. (1997). Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. Mol. Cell. Biol. *17*, 2576–2586.

Kortylewski, M., Heinrich, P. C., Kauffmann, M. E., Bohm, M., MacKiewicz, A., and Behrmann, I. (2001). Mitogen-activated protein kinases control p27kip1 expression and growth of human melanoma cells. Biochem. J. *357*, 297–303.

Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem. *271*, 20608–20616.

Malek, N. P., Sundberg, H., McGrew, S., Nakayama, K., Kyriakides, T. R., and Roberts, J. M. (2001). A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature *413*, 323–327.

Markey, M. P., Angus, S. P., Strobeck, M. W., Williams, S. L., Gunawardena, R. W., Aronow, B. J., and Knudsen, E. S. (2002). Unbiased analysis of RB-mediated transcriptional repression identifies novel targets and distinctions from E2F action. Cancer Res. *62*, 6587–6597.

Meloche, S., Seuwen, K., Pages, G., and Pouyssegur, J. (1992). Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Mol. Endocrinol. *6*, 845–854.

Mody, N., Leitch, J., Armstrong, C., Dixon, J., and Cohen, P. (2001). Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. FEBS Lett. *502*, 21–24.

Mulloy, R., Salinas, S., Philips, A., and Hipskind, R. A. (2003). Activation of cyclin D1 expression by the ERK5 cascade. Oncogene *22*, 5387–5398.

Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A., and Ewen, M. E. (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature *386*, 177–181.

Piatelli, M. J., Doughty, C., and Chiles, T. C. (2002). Requirement for a hsp90 chaperone-dependent MEK1/2-ERK pathway for B cell antigen receptor-induced Cyclin D2 expression in mature B lymphocytes. J. Biol. Chem. *277*, 12144–12150.

Rivard, N., Boucher, M. J., Asselin, C., and L'Allemain, G. (1999). MAP kinase cascade is required for p27 downregulation and S phase entry in fibroblasts and epithelial cells. Am. J. Physiol. *277*, C652–C664.

Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E., and Assoian, R. K. (1999). Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. Mol. Biol. Cell *10*, 3197–3204.

Sabile, A., Meyer, A. M., Wirbelauer, C., Hess, D., Kogel, U., Scheffner, M., and Krek, W. (2006). Regulation of p27 degradation and S-phase progression by Ro52 RING finger protein. Mol. Cell. Biol. *26*, 5994–6004.

Sewing, A., Wiseman, B., Lloyd, A. C., and Land, H. (1997). High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Mol. Cell. Biol. *17*, 5588–5597.

Sherr, C. J. (1994). G1 phase progression: cycling on cue. Cell *79*, 551–555.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1 phase progression. Genes Dev. *13*, 1501–1512.

Squires, M. S., Nixon, P. M., and Cook, S. J. (2002). Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. Biochem. J. *366*, 673–680.

Stewart, S. A., Kothapalli, D., Yung, Y., and Assoian, R. K. (2004). Antimitogenesis linked to regulation of Skp2 gene expression. J. Biol. Chem. *279*, 29109–29113.

Takahashi, Y., Rayman, J. B., and Dynlacht, B. D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. *14*, 804–816.

Treinies, I., Paterson, H. F., Hooper, S., Wilson, R., and Marshall, C. J. (1999). Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal to stimulate DNA synthesis. Mol. Cell. Biol. *19*, 321–329.

Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr. Biol. *9*, 661–664.

Vernell, R., Helin, K., and Muller, H. (2003). Identification of target genes of the p16INK4A-pRB-E2F pathway. J. Biol. Chem. *278*, 46124–46137.

Weber, J. D., Raben, D. M., Philips, P. J., and Baldassare, J. J. (1997). Sustained activation of extracellular-signal regulated kinase I (ERK1) is require for the continued expression of cyclin D1 in G1 phase. Biochem. J. *326*, 61–68.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Welsh, C. F., Roovers, K., Villanueva, J., Liu, Y., Schwartz, M. A., and Assoian, R. K. (2001). Timing of cyclin D1 expression within G1 phase is controlled by Rho. Nat. Cell Biol. *3*, 950–957.

Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997). Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol. Cell. Biol. *17*, 5598–5611.

Yamamoto, T., Ebisuya, M., Ashida, F., Okamoto, K., Yonehara, S., and Nishida, E. (2006). Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow for cell-cycle progression. Curr. Biol. *16*, 1171–1182.

Zhang, L., and Wang, C. (2006). F-box protein Skp2, a novel transcriptional target of E2F. Oncogene *25*, 2615–2627.

Zhu, X., Scharf, E., and Assoian, R. K. (2000). Induction of anchorage-independent growth by TGF-beta linked to anchorage-independent expression of cyclin D1. J. Biol. Chem. *275*, 6703–6706.