Regulation of Ras–MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells

Elizabeth R. Smith, Jennifer L. Smedberg, Malgorzata E. Rula, and Xiang-Xi Xu

Ovarian Cancer and Tumor Cell Biology Programs, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111

In response to retinoic acid, embryonic stem and carcinoma cells undergo differentiation to embryonic primitive endoderm cells, accompanied by a reduction in cell proliferation. Differentiation does not reduce the activation of cellular MAPK/Erk, but does uncouple mitogen-activated protein kinase (MAPK) activation from phosphorylation/activation of Elk-1 and results in inhibition of c-Fos expression, whereas phosphorylation of the cytoplasmic substrate p90RSK remains unaltered. Cell fractionation and confocal immunofluorescence microscopy demonstrated that activated MAPK is restricted to the cytoplasmic compartment after differentiation. An intact actin and microtubule cytoskeleton appears to be required for the restriction of MAPK nuclear entry induced by retinoic acid treatment because the cytoskeletal disrupting agents nocodazole, colchicine, and cytochalasin D are able to revert the suppression of c-Fos expression. Thus, suppression of cell proliferation after retinoic acid–induced endoderm differentiation of embryonic stem and carcinoma cells is achieved by restricting nuclear entry of activated MAPK, and an intact cytoskeleton is required for the restraint.

Introduction

In vitro, multipotent F9 embryonic carcinoma (EC) and pluripotent embryonic stem (ES) cells undergo differentiation in response to treatment with retinoic acid (RA) to primitive endoderm-like cells (Sherman and Miller, 1978). The primitive endoderm lineage is an epithelial cell type present in the blastocyst stage of the early mouse embryo that gives rise to the extraembryonic tissues (Sherman and Miller, 1978; Robertson, 1987). RA-differentiated F9 and ES cells express endoderm markers GATA-4 (Arceci et al., 1993), GATA-6 (Morrisey et al., 1998; Koutsourakis et al., 1999), Disabled-2 (Cho et al., 1999; Morrisey et al., 2000; Smith et al., 2001b), tissue plasminogen activator, and components of the basement membrane, collagen type IV and laminin (Vasios et al., 1989; Faria et al., 1999). The cellular changes that accompany EC and ES cell differentiation closely follow those

that occur in the early embryo, and F9 cells are frequently used to study the effects of RA on differentiation, proliferation, and embryonic development (Boylan and Gudas, 1991).

In addition to changes in cellular morphology and gene expression, the differentiated F9 cells exhibit a suppressed or impaired response to growth factors or serum and a reduced growth rate (Sherman and Miller, 1978; Faria et al., 1999). In many mammalian cell types, the Ras–MAPK cascade is the principal mitogenic signaling pathway and MAPK activation is essential for cell growth (Brunet et al., 1999; Hochholdinger et al., 1999; Kim-Kaneyama et al., 2000). The Ras–MAPK signaling pathway mediates the cellular response to multiple growth factor receptor tyrosine kinases, and the transmittance of the signal from the extracellular receptor at the cell surface to changes in gene expression has been well described (Robinson and Cobb, 1997; Chang and Karin, 2001). Specifically, a linear pathway from receptor tyrosine kinase–Ras–Raf–MAPK kinase (MEK) results in phosphorylation and activation

of MAPK or extracellular signal–regulated kinase (ERK), p44/42 MAPK or ERK1 and 2, respectively, by MEK and subsequent phosphorylation/activation of, among several targets, the transcription factor Elk-1. Elk-1 is responsible for transcriptional activation of the immediate early gene *c-fos* through binding to the Ets/SRE element in the *c-fos* promoter (Gille et al., 1992; Marais et al., 1993; Yang et al., 1999). c-Fos interacts with the transcription factor Jun to form the AP-1 complex, which mediates the biological response, including cell cycle progression in serum-starved growth-arrested cells (Field et al., 1992). Moreover, c-Fos expression contributes to and is required for the malignant growth of solid tumors (Angel and Karin, 1991; Saez et al., 1995; Arteaga and Holt, 1996), and down-regulation of c-Fos expression interferes with the growth of tumor cells in vitro (Arteaga and Holt, 1996). Thus, c-Fos is likely a site of regulation in cell growth control (Altin et al., 1992; Brown et al., 1998; Vanhoutte et al., 2001). In F9 cells treated for 4 d with RA to induce endodermal differentiation, serum causes a rapid and significant activation of MAPK; however, c-Fos expression is consistently suppressed (Smith et al., 2001a,b). This uncoupling of MAPK activation from c-Fos expression occurs at the step of Elk-1 phosphorylation/activation by MAPK.

Both the duration and the localization of the Ras/MAPK signal are normally regulated during proliferation and differentiation of many cell types (Pouyssegur et al., 2002). Dual phosphorylation of MAPK on tyrosine and threonine by MEK occurs in the cytoplasm, and several nonspecific phosphoserine/phosphothreonine- and phosphotyrosine-specific phosphatases and a MAPK-specific phosphatase (MKP3) have been reported to dephosphorylate and inactivate p44/p42 MAPK/Erk (Camps et al., 1998; Keyse, 2000), effectively terminating the signal. Activated MAPK must translocate into the nucleus to phosphorylate Elk-1 and other nuclear targets. The MAPK-specific phosphatases MKP1 and MKP2, which are neosynthesized in response to MAPK pathway stimulation (Volmat et al., 2001), are also stabilized by MAPK-dependent phosphorylation (Brondello et al., 1999) and reside in the nucleus (Brondello et al., 1995), where they may also rapidly terminate MAPK activity acting in a feedback loop. Presumably, under resting conditions, nonphosphorylated MAPK is complexed with MEK in the cytoplasm, and upon phosphorylation disassociates from MEK and either freely diffuses as a monomer through nuclear pores (Adachi et al., 1999), homodimerizes and enters the nucleus via a carrier-free/nuclear pore–independent mechanism (Khokhlatchev et al., 1998), or interacts with the nuclear pore complex for entry (Matsubayashi et al., 2001; Whitehurst et al., 2002). In the nucleus, the signal must be terminated by dephosphorylation and MAPK relocated to the cytoplasm via a MEK-dependent active transport (Adachi et al., 2000).

To understand how endoderm differentiation of F9 EC cells altered growth factor–stimulated c-Fos expression, we focused on active MAPK and its sustained nucleocytoplasmic localization. Here, we report that in differentiated F9 EC cells, and to a similar extent in differentiated mouse ES cells, MAPK does not enter the nucleus upon serum stimulation but remains activated in the cytoplasm.
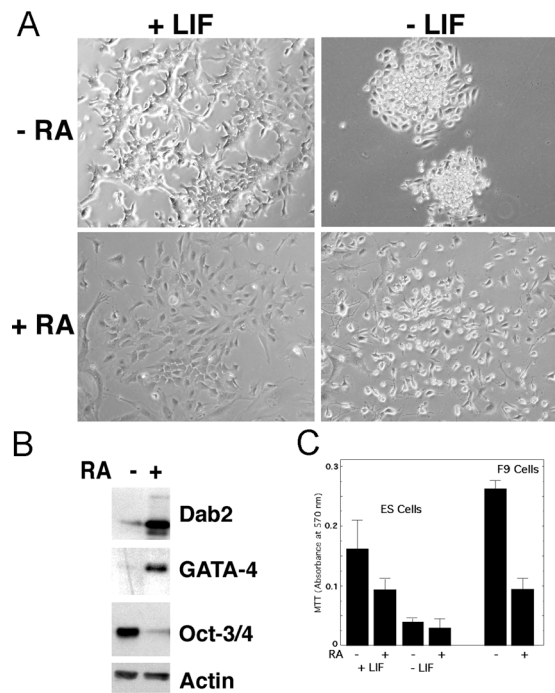

Figure 1. **RA induces differentiation and cell growth suppression similarly in ES and F9 cells.** Monolayer mouse ES or F9 cells on gelatin-coated plates were treated with 0.1 μM RA or DMSO solvent control for 4 d. Medium for ES cells is either with or without LIF. (A) ES cells were photographed. (B) ES cells cultured with or without RA and in the presence of LIF were analyzed by Western blot for Disabled-2 (Dab2), GATA-4, and Oct-3/4. β-actin was measured as a protein loading control. (C) Cell number was determined by MTT assay, measuring absorbance at 570 nm. Equal numbers (10^4) of ES or F9 cells were treated with or without RA and LIF for 4 d, and the cells were then incubated with MTT reagent for 2 h to measure cellular metabolism. Triplicate wells of cells in 96-well plates were used in each experiment, and the mean ± SD is reported here. The experiment was repeated three times with identical results.

Thus, in differentiated cells, the transcriptional-dependent (nuclear) and -independent (cytoplasmic) MAPK activation are uncoupled by the restriction of MAPK nuclear entry.

Results

RA-induced endodermal differentiation of ES and EC cells results in uncoupling of MAPK activation and c-Fos expression

The F9 EC cells originally derived from a spontaneous mouse testicular teratocarcinoma typically remain multipotent and undifferentiated until induced by RA and have served as a useful model for studying endoderm differentiation of ES cells (O'Shea, 2001). RA-induced F9 differentiation is accompanied by growth suppression, and the F9 cells have also been used as a common model to study the antiproliferative activity of RA (Faria et al., 1999). Recently, the mechanism of RA-induced growth suppression of F9 cells was uncovered; i.e., the differentiation results in uncoupling of MAPK activation and c-Fos expression (Smith et al., 2001a,b). It was of interest to determine whether or not similar regulation of the Ras–MAPK path-
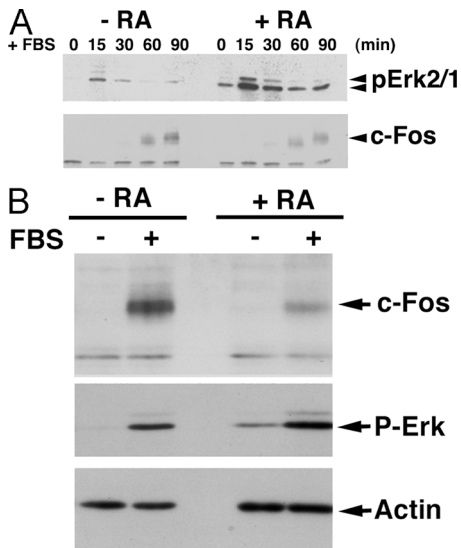
Figure 2. **RA-induced differentiation of ES cells elevates MAPK activation relative to c-Fos expression.** (A) Monolayer ES cells were treated with 0.1 μM RA for 4 d, serum starved overnight, and stimulated the next day with 15% FBS. At indicated time points, cells were disrupted in NP-40 lysis buffer and equal protein was loaded for each sample. Activated MAPK (pErk2/1) and c-Fos were determined by immunoblotting of lysates of undifferentiated (−RA) and differentiated (+RA) ES cells stimulated with 15% FBS for 0–90 min. The experiment was repeated three times with identical results. (B) Parallel experiments using F9 cells are shown for comparison. The results of the 0- and 90-min time points are shown.

Figure 3. **RA-induced differentiation of F9 cells suppresses phosphorylation of nuclear but not cytoplasmic MAPK substrates.** F9 cells were treated with 0.1 μM RA for 4 d, serum starved overnight, and stimulated with or without 15% FBS for 15 min. Another set of cells remained nonsynchronized (NS) by culturing in the presence of serum at all times. Cells were disrupted in lysis buffer, and equal protein was loaded for each sample. The cell lysates were analyzed by immunoblotting for phospho-pErk2/1, -Elk-1, -p90RSK, -AKT, and β-actin (Actin) in undifferentiated (−RA) and differentiated (+RA) F9 cells that had been serum starved (−FBS), serum stimulated (+FBS), or grown continuously in the presence of serum, or nonsynchronized (NS).

way occurs in ES cells, which also differentiate to endoderm cells with RA treatment (Rohwedel et al., 1999). Upon addition of RA for 4 d, ES cells in monolayer culture exhibit a morphological change (Fig. 1 A), and the cells are flattened and spread out. ES cells cultured without leukocyte inhibitory factor (LIF) rapidly degenerated either in the presence or absence of RA, and cell death was abundant. After RA treatment for 4 d, the endoderm markers Disabled-2 (Fig. 1, Dab2) and GATA-4 are induced, and the expression of the undifferentiated ES cell marker Oct-3/4 is lost (Fig. 1 B).

Similar to F9 cells, the proliferation/growth of the ES cells was reduced approximately twofold with RA treatment for 4 d, determined by MTT cell proliferation assay (Fig. 1 C). In medium without LIF to suppress spontaneous differentiation, the cell number was much reduced, most likely due to cell death and suppression of growth either in the presence or absence of RA. Also, we assessed the effect of RA differentiation of ES cells on downstream targets of MAPK activation, Elk-1 phosphorylation, and c-Fos expression. Serum stimulation resulted in a sustained, significant elevation in phosphorylated MAPK/Erk (pErk) levels in differentiated ES cells compared with control cells, ∼4.5-fold higher at the 15-min time point (Fig. 2 A). Despite an increase in total MAPK activation, c-Fos expression in differentiated cells at 90 min is slightly lower, 0.64 times that of the undifferentiated cells. Thus, similar to F9 cells (Fig. 2 B), RA-induced differentiation of ES cells causes an uncoupling of MAPK activation from c-Fos expression and reduced cell proliferation (Fig. 2 A).
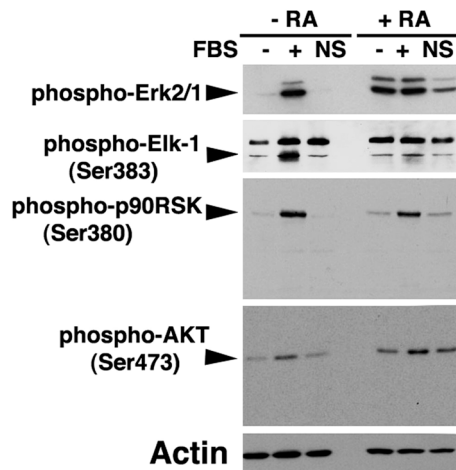
Endoderm differentiation differentially affects phosphorylation of MAPK nuclear and cytoplasmic substrates

Previously, we have determined that the dephosphorylation of Elk-1 by an Elk-1 phosphatase is not the mechanism accounting for the RA-induced uncoupling of MAPK activation to Elk-1 phosphorylation/activation and c-Fos expression (Smith et al., 2001b). To further investigate the mechanism for MAPK–Elk-1–c-Fos regulation, we examined additional MAPK substrates and related signaling pathways. Consistent with previous observations (Smith et al., 2001b), serum (FBS) stimulation resulted in phosphorylation/activation of the transcription factor Elk-1 in control or undifferentiated F9 cells (Fig. 3). In differentiated F9 cells, MAPK activation was significant, and in most experiments, phosphorylated MAPK is present even in nonstimulated (Fig. 3, FBS−) and nonsynchronized (Fig. 3, NS) cells grown continuously in the presence of serum. However, serum stimulation and MAPK activation failed to phosphorylate/activate Elk-1 in those cells (Fig. 3). Differentiation did not alter the response of another growth factor–responsive pathway to serum stimulation because there was no significant difference in the amount of AKT phosphorylated in undifferentiated or differentiated F9 cells (Fig. 3). This result indicates that the alteration in serum responsiveness is specific to the MAPK–Elk-1–c-Fos signaling pathway. Moreover, differentiation does not suppress pan-MAPK activity because phosphorylation of p90RSK, a cytoplasmic MAPK substrate (Formstecher et al., 2001), was not suppressed by RA treatment (Fig. 3). Thus, it appears that the phosphorylation of MAPK substrates is differentially regulated; the phosphorylation of

Figure 4. **Activated MAPK is localized in the cytoplasmic fraction of differentiated F9 cells, determined by fractionation.** F9 cells, induced to differentiate to endoderm-like cells with 0.1 μM RA for 4 d, were serum starved (−FBS), serum restimulated for 15 min (+FBS), or maintained nonsynchronized (NS). Cells were fractionated to obtain nuclear and cytoplasmic fractions, and equal amounts of sample protein were separated on SDS–polyacrylamide (7.5%) gels and immunoblotted for (A) cytosolic (Clathrin) and nuclear (GATA-4) markers or (B) Elk-1, phospho-Elk-1 (P-Elk-1), MAPK/Erk (Erk), and phospho-MAPK/Erk (P-Erk). (C) The nucleocytoplasmic distribution of pErk, pAKT, p90RSK (S380 and T359/S363), and β-actin is shown. Cell fractionation experiments were done four times with similar conclusions.
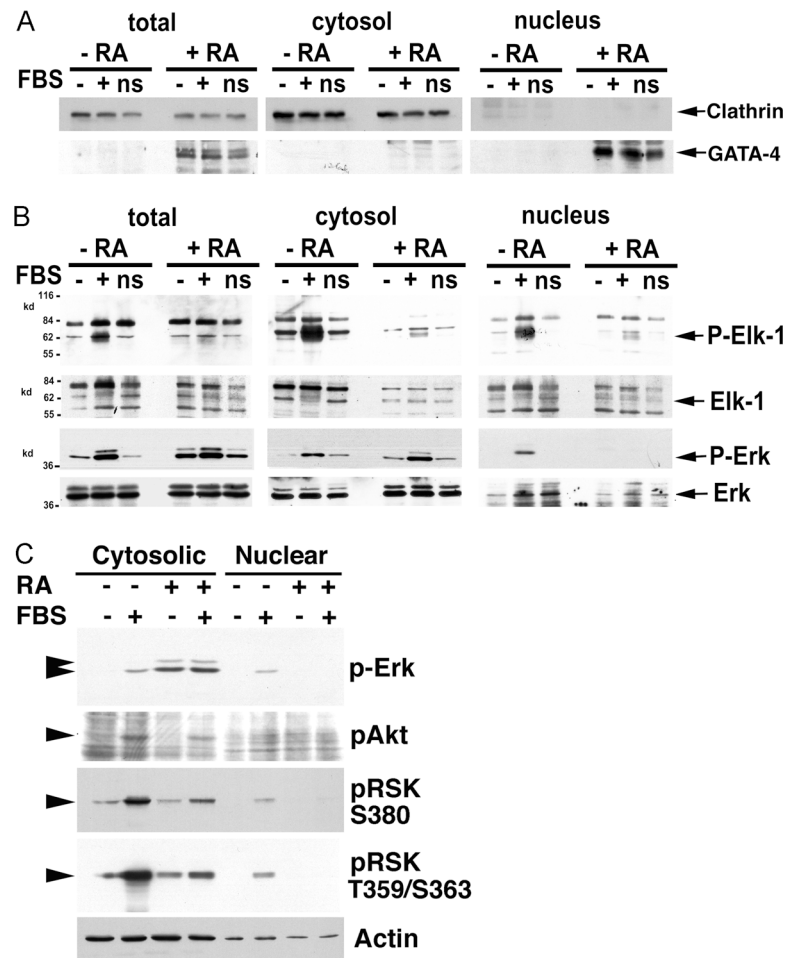
MAPK nucleocytoplasmic distribution determined in cell fractions

Because phosphorylation/activation of nuclear Elk-1 depends on the presence of MAPK in the nucleus, we examined whether or not differentiation alters the subcellular distribution of active MAPK (pErk). We separated F9 cells into nuclear and cytosolic fractions by differential centrifugation and verified the purity of the fractions by assaying for markers of both compartments (Fig. 4 A). Determination by Western blot of the endocytic vesicle-coating protein clathrin as a cytosolic marker and the GATA-4 transcription factor as a nuclear marker indicates that there is no significant cross-contamination between cytosolic and nuclear components in the fractions. The same fractions were used to determine the distribution of the phosphorylated and unphosphorylated MAPK and Elk-1 (Fig. 4 B). In undifferentiated cells, phosphorylated or activated MAPK and Elk-1 are present in both nuclear and cytosolic fractions, as shown by immunoblots of isolated nuclear and cytoplasmic fractions of F9 cells. After differentiation, the active pErk is abundant in the cytosolic fraction but is greatly reduced in the nuclear fraction. Thus, cell fractionation indicates that activated MAPK is no longer located in the nucleus in RA-differentiated F9 cells. In undif-

ferentiated cells, total Erk level increases in the nucleus upon serum stimulation (Fig. 4 B), indicating nuclear translocation upon activation of the kinase. In differentiated cells, nuclear entry of total MAPK is greatly reduced (Fig. 4 B).

The nucleocytoplasmic distribution of the total Elk-1 level appears not to change significantly by serum stimulation or RA-induced differentiation (Fig. 4 B). Interestingly, compared with most transiently transfected cell lines, in F9 cells as in adult rat brain (Sgambato et al., 1998), a large fraction of Elk-1 is cytoplasmic and phosphorylated after serum addition. Although an equal amount of Elk-1 is located in the nucleus in differentiated and undifferentiated F9 cells, the amount of activated/phosphorylated Elk-1 located in the nucleus is 5.6 times greater in undifferentiated cells (Fig. 4 B).

In another experiment, the fractionated cellular components were further analyzed for additional proteins (Fig. 4 C). AKT is mainly cytoplasmic. The nucleocytoplasmic distribution of p90RSK phosphorylated either dually at Thr 359/Ser 363 or on Ser 380 correlates well with the distribution of phosphorylated MAPK. In undifferentiated F9 cells, there is a small fraction of phospho-p90RSK in the nucleus, which is absent in differentiated cells. Thus, it appears that p90RSK has a separate larger nuclear and smaller cytoplasmic pool. These results can be explained by the assumption that MAPK is no longer able to enter the nucleus to phosphorylate nuclear p90RSK in differentiated cells, and only cytoplasmic p90RSK is phosphorylated.
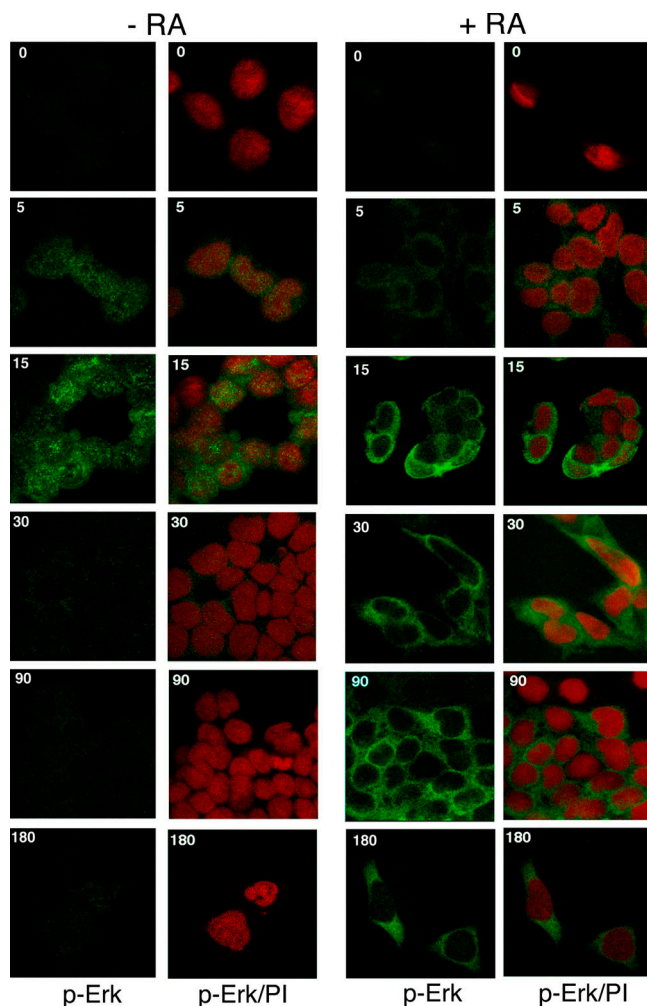
Figure 5. **Immunofluorescence microscopic localization of activated MAPK in F9 cells.** F9 cells in monolayer culture, with or without differentiation by RA for 4 d, were serum starved overnight and stimulated with serum. At the indicated times, cells were fixed with ice-cold methanol and stained with an antiactivated/phosphorylated p42/p44 MAPK mAb (p-Erk) and analyzed by immunofluorescence confocal microscopy. Control cells were treated with DMSO vehicle and processed identically. Control (−RA) and differentiated (+RA) F9 cells were serum stimulated for 0–180 min, and phospho-MAPK (Cy-2–labeled; green) is shown. Nuclear PI staining is overlayed (p-Erk/PI) with activated MAPK (pErk). The experiment was performed four times with similar results.

MAPK nucleocytoplasmic location determined by immunofluorescence microscopy

To confirm that RA-induced differentiation alters MAPK nucleocytoplasmic localization, we examined activated MAPK (pErk) localization using indirect immunofluorescence confocal microscopy. In undifferentiated F9 cells, nuclear localized pErk was detectable within 5 min, reached maximum by 15 min, and remained visible 30–60 min after serum addition (Fig. 5). This time course closely corresponds to MAPK activation in total F9 cell lysates determined by immunoblot analysis (Smith et al., 2001a,b; Smedberg et al., 2002). In RA-treated cells, activated MAPK could be readily detected 5 min after serum addition, reached maximum by 15 min, and remained elevated after 3 h. However, the pattern of cellular distribution of pErk is strikingly different between differentiated and undifferentiated F9 cells. In undifferentiated cells, pErk staining exhibits a punctate pattern throughout the cell body. In contrast, in differentiated cells, pErk staining is exclusively in the outer ring of the cells, and no significant pErk was detectable in the nucleus (Fig. 5). The nuclear exclusion of the active MAPK is best illustrated by overlapping of pErk staining with propidium iodide (PI) to show the nucleus (Fig. 5).

For ES cells, the difference of MAPK nuclear translocation between undifferentiated and differentiated cells is also detectable by confocal immunofluorescence microscopy. As shown in immunofluorescence staining of phospho-Erk in ES cells (Fig. 6 A), MAPK activation occurred within 5 min of serum addition and corresponded to nuclear-localized phosphorylated MAPK (pErk) in undifferentiated ES cells. The signal diminished rapidly thereafter, and any of the low level of pErk remaining was primarily cytoplasmic by 15–30 min. In contrast, phospho-MAPK/pErk in RA-treated ES cells was mainly cytoplasmic at all times after serum addition, although the signal persisted and remained augmented after 1 h (Fig. 6 B). Thus, nuclear translocation of phosphorylated/activated MAPK is impaired in differentiated F9 and ES cells.

Inhibition of nuclear export does not alter c-Fos expression in F9 cells

The above observations establish that differentiated EC and ES cells have the ability to prevent an accumulation of activated MAPK in the nucleus despite an increased cytoplasmic MAPK activation. It is possible that differentiation alters the rate of export of the activated MAPK from the nucleus, such that export is accelerated or an anchoring protein required for p-MAPK nuclear retention is reduced, and the amount of nuclear p-MAPK at a given time after serum stimulation is lowered in differentiated cells. If this were the case, inhibition of export would be predicted to retain active MAPK in the nucleus and to elevate c-Fos expression. However, when F9 cells were treated with leptomycin B, a specific inhibitor of nuclear export of proteins that has been shown to prevent MAPK translocation to the cytoplasm in *Xenopus laevis* A6 and rat 3Y1 cells (Adachi et al., 2000), we found no increases in c-Fos expression either in RA-treated or -untreated F9 cells (Fig. 7 A) and no effect on MAPK activation. Moreover, as shown by immunofluorescence microscopy for activated MAPK/phospho-Erk (Fig. 7 B), leptomycin B did not alter the distribution of the activated kinase in differentiated F9 cells, p-MAPK remained cytoplasmic in the presence of leptomycin B in RA-treated cells. Therefore, RA-induced differentiation does not increase the rate of export of the activated MAPK from the nucleus. Nevertheless, leptomycin B treatment did result in the nuclear accumulation of total MAPK in the nucleus of both differentiated and undifferentiated cells (Fig. 7 C). We interpret this result to mean that the nucleocytoplasmic trafficking of unphosphorylated MAPK is not perturbed by RA treatment, and once export of unphosphorylated MAPK is inhibited by leptomycin B, total MAPK accumulates as unregulated MAPK nuclear import continues. Previously, we have also ruled out that RA increases the activity of a phosphatase (Smith et al., 2001b). Thus, the data suggest that an increased rate of nuclear export of the activated MAPK is unlikely the affected step; in-
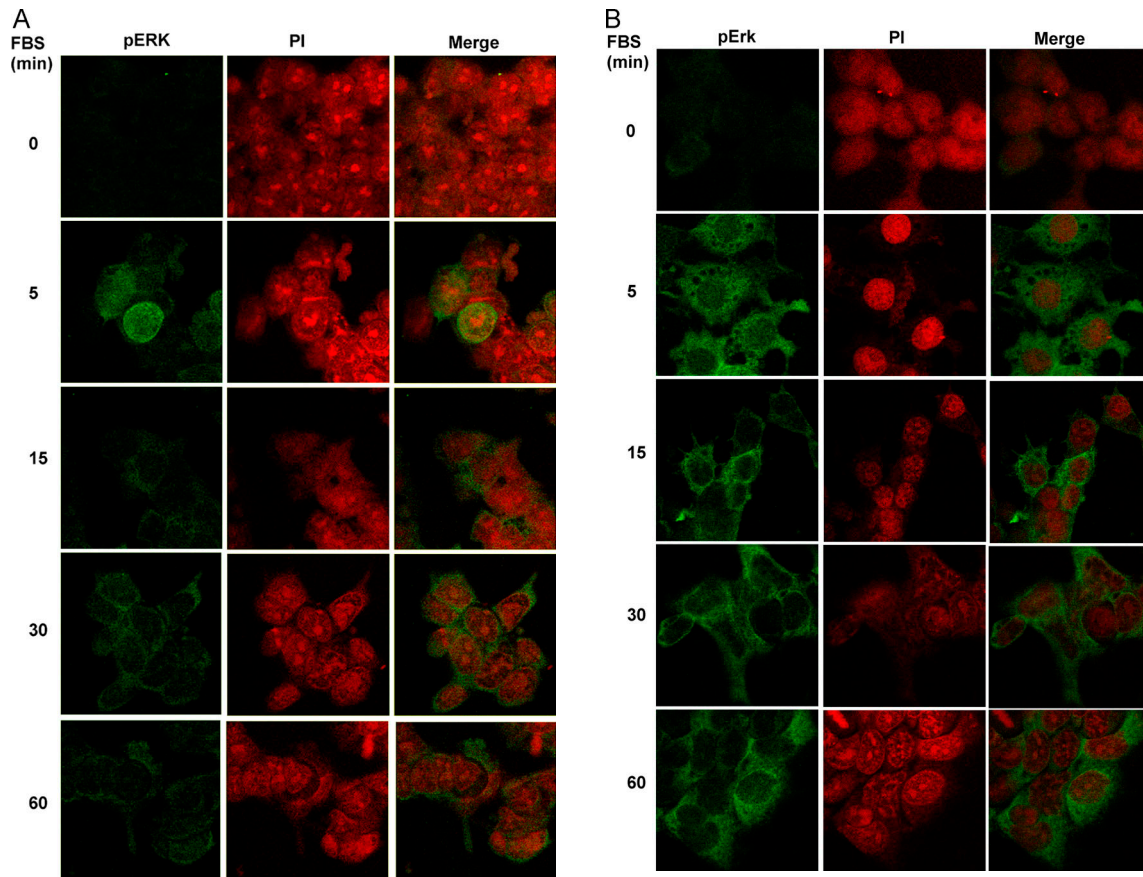
Figure 6. **Cellular distribution of activated MAPK in ES cells determined by immunofluorescence microscopy.** ES cells, induced with RA for 4 d, were serum starved overnight and restimulated with serum. At the indicated time points, cells were fixed with ice-cold methanol, stained with an antiactivated p42/p44 MAPK mAb, and analyzed by immunofluorescence confocal microscopy. Control ES cells with added DMSO vehicle without RA were processed identically. (A) Control (−RA) and (B) differentiated (+RA) ES cells were serum stimulated for 0–60 min, and phospho-MAPK (Alexa 488–labeled; green) and nuclear PI (red) are shown individually and merged. The experiment was performed three times with similar results.

stead, nuclear entry of activated MAPK is suppressed after RA-induced endoderm differentiation of ES and EC cells.

Disruption of the cellular cytoskeleton abolishes suppression of c-Fos expression in RA-differentiated F9 cells

Previous papers have reported that MAPK associates with the microtubule network (Reska et al., 1995) and that the cell cytoskeleton regulates adhesion-related MAPK activity and nucleocytoplasmic localization (Aplin et al., 2001). To determine the influence of the cell cytoskeleton on serum-stimulated c-Fos expression and MAPK activation, F9 cells were differentiated with RA for 4 d and preincubated for 30 min with various cytoskeleton-depolymerizing agents before stimulating with serum for 90 min. Nocodazole (10 μM) and colchicine (10 μM), both destabilizers of microtubules, appeared to increase the levels of activated MAPK slightly (2.4- and 1.5-fold, respectively). Independently of the differentiation state, c-Fos expression is augmented in the presence of nocodazole or colchicine (Fig. 8 A). Similarly, inhibition of actin polymerization and barbed-end filament growth by cytochalasin D (Cooper, 1987) slightly elevated phosphorylated MAPK (2.6-fold) and eliminated c-Fos suppression by RA-induced differentiation (Fig. 8 A). Although

disruption of the microtubule and actin cytoskeleton by nocodazole, colchicine, or cytochalasin D treatment causes observable changes in cellular morphology, most striking is that the cells round up and shrink, the activation of MAPK and expression of c-Fos are enhanced, and the suppression of c-Fos expression by RA-induced differentiation is prevented. Thus, it appears that an intact microtubule and actin cytoskeleton is required for restriction of MAPK nuclear entry in differentiated F9 cells. Furthermore, we show that the cytoskeleton disrupting agent-stimulated activation and c-Fos expression is MAPK dependent because the MEK inhibitor U0126 suppressed MAPK activation and c-Fos expression in both the absence or presence of cytochalasin D (Fig. 8 B).

Discussion

In the past 30 yr, many laboratories have investigated the phenomenon that RA can suppress the growth of F9 cells. The RA-induced F9 growth suppression and endoderm differentiation has been used as a popular model to investigate the mechanism of the antiproliferative activity of RA, yet the cause of growth suppression has not been determined for certain. In several of our recent investigations, we found that upon RA-induced differentiation c-Fos expression is sup-
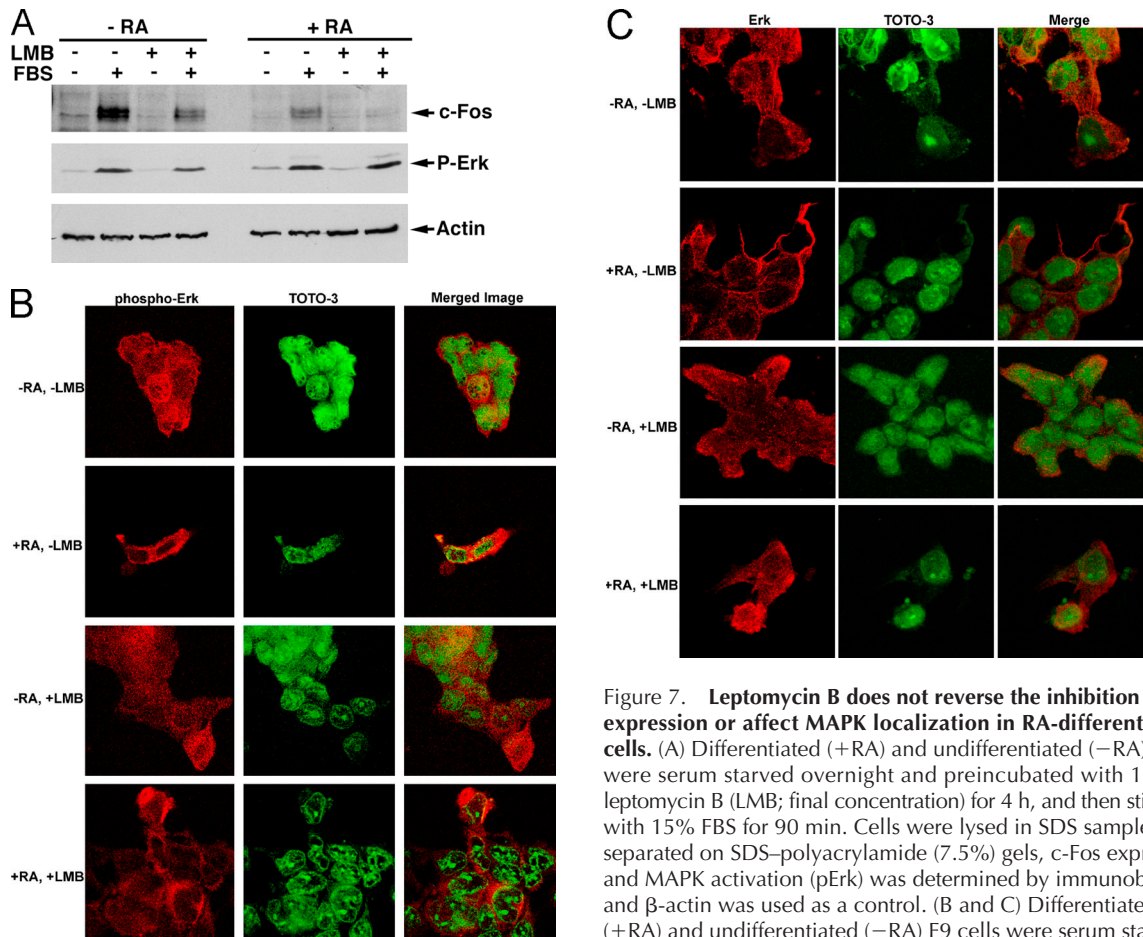
Figure 7. **Leptomycin B does not reverse the inhibition of c-Fos expression or affect MAPK localization in RA-differentiated F9 cells.** (A) Differentiated (+RA) and undifferentiated (−RA) F9 cells were serum starved overnight and preincubated with 10 nM leptomycin B (LMB; final concentration) for 4 h, and then stimulated with 15% FBS for 90 min. Cells were lysed in SDS sample buffer separated on SDS–polyacrylamide (7.5%) gels, c-Fos expression and MAPK activation (pErk) was determined by immunoblotting, and β-actin was used as a control. (B and C) Differentiated (+RA) and undifferentiated (−RA) F9 cells were serum starved overnight and incubated with 10 nM LMB for 30 min before and 10 min during stimulation with 15% FBS as described in Adachi et al. (2000). Activated MAPK (B, phospho-Erk) or total MAPK (C, Erk) were detected by rhodamine red–conjugated secondary antibodies (red) and analyzed by immunofluorescence confocal microscopy. Nuclei were counterstained with TOTO-3 (green).

pressed and uncoupled from MAPK activation (Smith et al., 2001a,b; Smedberg et al., 2002). This paper further shows that the mechanism is the restriction of MAPK nuclear localization upon differentiation, and both ES and EC cells behave similarly. Thus, we conclude that in RA-differentiated ES and EC (F9) cells, the transcription-dependent (nuclear) but not the transcription-independent (cytoplasmic) activity of MAPK is restricted, leading to growth suppression (Fig. 9).

Mechanism of RA-induced growth suppression of EC and stem cells

Several mechanisms have been proposed to explain the reduction in cell proliferation and impaired growth factor responsiveness that accompany differentiation. A reduction in expression of the protooncogene c-myb RNA and c-Src RNA (Lockett and Sleigh, 1987) and c-myc RNA (Sejersen et al., 1985; Dean et al., 1986) seen during F9 endoderm differentiation has been correlated with the reduced growth rate. Alternatively, targeted disruption of both alleles of the RA receptor RARβ$_2$ results in loss of RA-associated growth arrest, suggesting that RARβ$_2$ is essential for reduced cell proliferation rate (Faria et al., 1999). This result has been related to a reduction in maximal expression of RA-responsive genes at a later time after RA treatment (>24 h) or a reduced differentiation response. Genetic deletion of the retinoic X receptor α (RXRα) in F9 cells also abolishes endodermal differentiation and impairs the antiproliferative response to RA and down-regulation of p21^{CIP} and p27^{KIP}, cyclin-dependent kinase inhibitors that function in the control of cell cycle progression (Bastien et al., 2002). Alterations in the cell cycle have also been thought to be responsible for the reduced proliferation rate, where differentiation appears to lengthen the G$_1$ phase and decrease the S phase components (Kurki et al., 1989). These findings are not necessarily mutually exclusive. This work finds that RA-induced differentiation uncouples MAPK activation from c-Fos expression by suppressing serum-stimulated nuclear translocation of activated MAPK. Because proliferation is MAPK dependent and c-Fos influences cell cycle progression and cyclin D1 expression after serum-induced reentry into the cell cycle (Brown et al., 1998), cell growth rate is decreased. It should be noted that a reduced c-Fos expression coincides with the fact that cell cycle transit is lengthened (Kurki et al., 1989). We conclude that the restriction of nuclear entry of activated MAPK is the causative mechanism of the growth suppression of F9 cells treated with RA. In differentiated F9 cells, Ras/MAPK activation is enhanced;
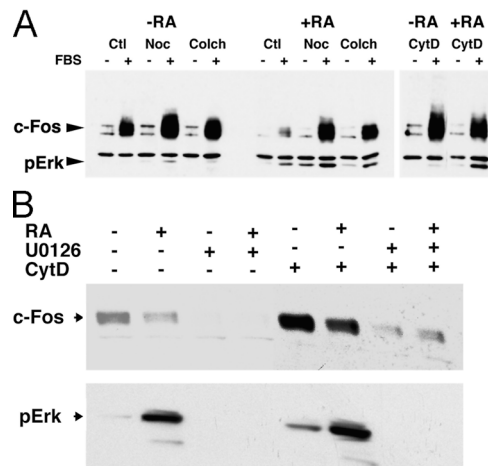
Figure 8. **Cytoskeleton-disrupting agents elevate MAPK activation and c-Fos expression in F9 cells.** (A) Undifferentiated F9 cells (−RA) and cells differentiated with RA for 4 d (+RA) were serum starved overnight and pretreated with 10 μM nocodazole (Noc), colchicine (Colch), or cytochalasin D (CytD), and solvent control (Ctl) for 30 min before stimulating with 15% serum for 90 min. Serum-containing medium also contained the inhibitors at the same concentration. Equal protein for each sample was separated on 7.5% PAGE gels, and c-Fos and activated MAPK (pErk) were determined by immunoblotting. In this blot, the membranes were incubated with a mixture of antibodies to c-Fos and pErk to determine both signals simultaneously. Shown is a representative experiment repeated in triplicate. (B) F9 cells, with or without RA treatment, with or without cytochalasin D and/or MEK inhibitor U0126 (10 μM), were first serum starved overnight, and then stimulated with serum for 90 min. The cells were harvested to analyze for c-Fos expression and MAPK activation (pErk) by Western blotting.

Figure 9. **Model for regulation of nucleocytoplasmic MAPK activity in endoderm differentiation of ES and EC cells.** MAPK is a downstream effector of the Ras pathway regulated by mitogen such as serum or growth factors. MAPK phosphorylates the cytoplasmic substrate p90 ribosomal S6 kinase (p90RSK) and the nuclear substrate Elk-1. The nuclear activity of MAPK is the transcription-dependent signal and is required for mitogen-stimulated cell proliferation. Endoderm differentiation of ES and EC cells results in cell cytoskeleton-dependent enhanced activation but also restriction of MAPK nuclear entry and suppression of cell growth.

however, the transcription-dependent activity of MAPK in the nucleus, which is required for mitogenesis, is suppressed. The cytoplasmic MAPK activity is not suppressed upon differentiation, allowing the differentiated endoderm cells to respond to the Ras/MAPK signal that is unrelated to cell division (Fig. 9).

Regulation of nuclear translocation of MAPK

Nucleocytoplasmic localization of MAPK is a pivotal point in regulation of its downstream targets, including those involved in growth regulation, differentiation, and cell function. Nuclear accumulation of p42/p44 MAPK is observed after mitogenic stimulation, which may persist for up to 6 h (Volmat et al., 2001), and is believed to be accompanied by neosynthesis of nuclear anchoring proteins. The nucleus has been proposed to act as an "anchoring and inactivating center," restricting MAPK from activation by MEK in the cytoplasm (Volmat et al., 2001; Pouyssegur et al., 2002).

Although the mechanisms regulating nucleocytoplasmic translocation of MAPK are fairly well described, our finding in F9 EC and ES cells that differentiation restricts nuclear access of activated MAPK is only the second physiological example of this kind of regulation. Human diploid fibroblasts that have undergone replicative senescence, which is characterized by a loss of proliferative capacity and impaired expression of c-Fos and other immediate early genes in response to mitogens and serum, show a reduced abundance of nuclear activated MAPK (Tresini et al., 2001). The de-
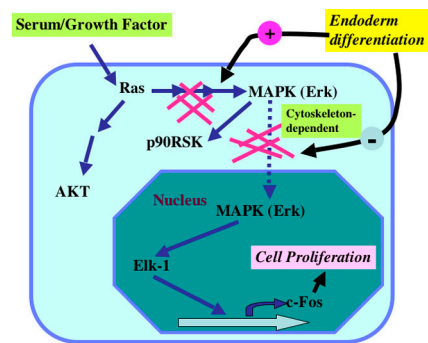
creased nuclear abundance of active MAPK is accompanied by decreased activation of Elk-1 and reduced c-Fos expression, but the senescent fibroblasts differ from the F9 and ES cells in that total number of MAPK molecules is increased. In the senescent fibroblasts, it was suggested that the abundant unphosphorylated MAPK molecules compete for phosphorylated MAPK for dimerization and nuclear translocation, resulting in a reduced fraction of activated MAPK in the nucleus (Tresini et al., 2001). In general, systems that are aging affected, including primary rat hepatocytes in culture, mouse T lymphocytes and macrophages, human lymphocytes, melanocytes, and fibroblasts, also exhibit aging- or senescent-associated changes in MAPK signaling (Tresini et al., 2001). We did not observe a change in the total amount of MAPK protein in RA-differentiated F9 and ES cells, but rather a reduction of total MAPK located in the nucleus. Instead, we conclude that the reduced amount of activated MAPK in differentiated F9 and ES cells is the result of an actin- and microtubule-dependent inhibitory mechanism on nuclear translocation of the activated MAPK imposed by the cells after differentiation (Fig. 9). This restriction of MAPK nuclear entry may be a general mechanism for suppression of proliferation in cell differentiation, as we have begun to observe the restriction of MAPK nuclear entry in other types of differentiated cells in tissues and primary cultures (unpublished data).

Role of cytoskeleton in regulation of MAPK nuclear entry

The regulated coupling of MAPK activation and c-Fos expression may be related to morphological changes in the cytoskeleton that accompany endodermal differentiation of F9 cells (Kurki et al., 1989; Burdsal et al., 1994), such that destabilization may reconfigure a situation similar to the undifferentiated cells. Moreover, the data reported here indicate that serum-induced MAPK activation and nuclear import in F9 cells do not depend on the microtubule and microfilament network and regulation of the cytoskeleton. Rather, disruption of the cell cytoskeleton results in an enhanced

MAPK activation and c-Fos expression, and suppression of c-Fos expression by RA-induced differentiation is prevented without an organized cytoskeleton. The enhanced MAPK activation by inhibition of protein synthesis and disruption of cell cytoskeleton has been previously observed (Kyriakis et al., 1994). We conclude that the restriction of MAPK activation and nuclear entry requires an intact actin and microtubule cytoskeleton.

The mechanism for the uncoupling of MAPK activation and c-Fos expression has been investigated. The reduction in activated MAPK in the nucleus of the differentiated F9 or ES cells is not due to higher nuclear export because leptomycin B had no effect nor to an increased dephosphorylation in the nucleus because active MAPK actually remains elevated for a longer period than in control cells and appears to be severalfold more abundant, especially in ES cells. It is not known if a protein that actively binds MAPK is expressed in ES and EC cells, although one such protein, PEA-15, which has been found to retain MAPK in cytoplasm in neural cells (Formstecher et al., 2001), was not found in F9 cells induced by RA (unpublished data).

When F9 cells undergo differentiation, a striking phenotype is the rearrangement of cell cytoskeleton (Kurki et al., 1989; Burdsal et al., 1994). We favor the idea that in differentiated cells, MAPK is tethered to actin and/or microtubule filaments themselves or associates with a protein complex hinged on endocytic cargos traveling along an actin or microtubule track. The arrangement of the cytoskeleton or the directional transport of endocytic cargos may restrict the access of MAPK for nuclear entry, leading to suppression of its nuclear transcriptional-dependent activity, thus growth suppression (Fig. 9). RA may induce the expression of a set of proteins such as adaptors and regulators to allow such organization. This mechanism may underlie the importance of cell adhesion and the cytoskeleton structure in the regulation of mitogenic signaling and cell proliferation.

Materials and methods

Materials
RA (all-trans-RA) was purchased from Sigma-Aldrich and stock solutions were prepared in DMSO. Tissue culture supplies were obtained from Fisher Scientific; DME and FBS were purchased from Mediatech; and the ECL Super-Signal West Dura extended duration substrate immunodetection reagents came from Pierce Chemical Co. Antibodies were obtained from multiple sources: Dab2/p96 monoclonal, clathrin heavy chain monoclonal, and Erk1 monoclonal (BD Biosciences); Elk-1 polyclonal, phospho-Elk-1 monoclonal, c-Fos polyclonal, and PEA-15 polyclonal (Santa Cruz Biotechnology, Inc.); phospho-Erk2/1 monoclonal and actin monoclonal (Sigma-Aldrich); and Erk2/1 polyclonal, phospho-p90RSK (Ser 380) polyclonal, phospho-p90RSK (Thr 359/Ser 363) polyclonal, and phospo-AKT (Ser 473) polyclonal (Cell Signaling Technology). Cy2-, FITC-, and Texas red–conjugated donkey anti–rabbit and anti–mouse IgGs came from Jackson ImmunoResearch Laboratories. SlowFade and ProLong antifade mounting media, Alexa488-conjugated donkey anti–mouse IgG, rhodamine red–conjugated goat anti–mouse IgG, DAPI, TOTO-3, and PI were purchased from Molecular Probes. All other chemicals and supplies were obtained from Fisher Scientific or Sigma-Aldrich and were reagent grade or higher.

Cell culture
F9 mouse EC cells were purchased from the American Type Culture Collection and cultured on gelatin-coated tissue culture plates in DME containing 10% heat-inactivated FBS and 1× antibiotic-antimycotic solution. The plates were coated with an autoclaved 0.1% gelatin solution overnight at 4°C and washed with PBS before use. All-trans-RA (referred to as RA) was added from a 1-mM stock solution in DMSO to a final concentration of 0.1 mM, generally, 24 h after plating. Control cultures received an equal volume of DMSO vehicle, which was generally ≤0.01% of the final culture volume. Mouse RW-4 ES cells were maintained on a layer of irradiated murine embryonic fibroblasts or feeder layer in ES cell medium with LIF (ESGRO; Chemicon; Robertson, 1987). For experiments, the cells were trypsinized and plated on gelatinized tissue culture plates without feeder cells for 2 d in ES cell medium containing LIF, and then trypsinized and plated in the presence of 0.1 mM RA for 4 d.

Cell fractionation
F9 cells were cultured 4 d in the presence of RA or DMSO vehicle, and then incubated overnight (18–24 h) in DME containing 0.1% FBS and antibiotics/antimycotics (serum-depleted medium). For serum-stimulation experiments, serum-depleted medium was removed and replaced with 15–20% FBS-containing medium for various times (0–90 min) at 37°C, 5% CO_2. At the end of the treatment, cells were washed three times with ice-cold PBS containing 0.5 mM sodium orthovanadate, scraped into hypotonic lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, and a protease inhibitor cocktail; Sigma-Aldrich), and incubated on ice for 10 min (Fazioli et al., 1993). The lysate was Dounce homogenized (40 strokes) with a tight-fitting pestle and designated the total homogenate. To obtain the nuclear fraction, the homogenate was centrifuged at 375 g for 5 min, and the pellet (nuclear fraction) was washed five times with hypotonic lysis buffer containing 0.1% NP-40 to remove membrane and cytosolic contamination and resuspended in lysis buffer containing 0.5% sodium deoxycholate, 0.1% SDS, and 0.2% NP-40. The soluble fraction was centrifuged twice at 375 g to remove nuclear contamination and was designated the cytosolic fraction. Fractions were centrifuged at 12,000 g to remove insoluble material. All procedures were performed on ice. Protein was quantitated using DCC assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Immunoblotting was performed according to standard procedures, as described previously (Smith et al., 2001b).

Immunofluorescence microscopy
F9 cells were first differentiated with RA and reseeded at 10^4 cells/well onto gelatin-coated Permanox 4-well slide chambers (Lab-Tek). For some experiments, cells were seeded directly onto and differentiated in the slide chambers. To process for immunocytochemistry, after treatment, cells were washed two times with PBS and fixed and lysed in ice-cold methanol at −20°C for 5 min (Edwards et al., 1988), washed two times in PBS, and blocked in PBS containing 3% BSA for 1 h at RT. The primary antibody was diluted (1:200) into 3% BSA in PBS containing 0.1% Tween-20 and incubated with the slides for 1 h at 37°C in a humidified chamber (Osborn and Weber, 1982). The slides were washed three times with PBS containing 1% BSA and 0.1% Tween-20 and incubated with the appropriate secondary antibody (1:100–1:200) in 3% BSA at 37°C for 1 h. Slides were washed four times with PBS, stained with PI (1:3,000 dilution of a 10-mg/ml stock in water) for 2 min at RT, and washed three more times with PBS. The slides were mounted in SlowFade or ProLong media according to the manufacturer's directions. Cells were viewed on a laser scanning confocal microscope (model 200; Bio-Rad Laboratories) using a 60× water objective, and images were deconvoluted using the Laser Sharp software (Bio-Rad Laboratories) and analyzed using Adobe Photoshop.

To determine the effect of leptomycin B on MAPK and phospho-MAPK localization, serum-starved cells were incubated with 10 nM leptomycin B for 30 min in serum-free medium and stimulated with 15% FBS for 10 min in the continued presence of leptomycin B. Cells were processed for immunocytochemistry as described in the previous paragraph. Antibody controls (minus the primary antibody) demonstrated little to no fluorescence staining, indicating the specificity of the antibodies. The Erk1 mAb (BD Biosciences) was used at 1:50 dilution, and the secondary antibody, rhodamine red–conjugated goat anti–mouse IgG (Molecular Probes), was used at 1:300. Cell nuclei were counterstained with TOTO-3 diluted 1:10,000 in PBS for 2 min at RT.

References

Adachi, M., M. Fukuda, and E. Nishida. 1999. Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. *EMBO J.* 18:5347–5358.

Adachi, M., M. Fukuda, and E. Nishida. 2000. Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism. *J. Cell Biol.* 148:849–856.

Altin, J.G., R. Wetts, K.T. Riabowol, and R.A. Bradshaw. 1992. Testing the in vivo role of protein kinase C and c-fos in neurite outgrowth by microinjection of antibodies into PC12 cells. *Mol. Biol. Cell.* 3:323–333.

Angel, P., and M. Karin. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta.* 1072:129–157.

Aplin, A.E., S.A. Stewart, R.K. Assoian, and R.L. Juliano. 2001. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J. Cell Biol.* 153:273–281.

Arteaga, C.L., and J.T. Holt. 1996. Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer zenografts in nude mice. *Cancer Res.* 56: 1098–1103.

Arceci, R.J., A.A. King, M.C. Simon, S.H. Orkin, and D.B. Wilson. 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* 13:2235–2246.

Bastien, J., S. Adam-Stitah, J.-L. Plassat, P. Chambon, and C. Rochette-Egly. 2002. The phosphorylation site located in the A region of retinoic X receptor α is required for the antiproliferative effect of retinoic acid (RA) and the activation of RA target genes in F9 cells. *J. Biol. Chem.* 277:28683–28689.

Boylan, J.F., and L.J. Gudas. 1991. Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. *J. Cell Biol.* 112:965–979.

Brondello, J.M., F.R. McKenzie, H. Sun, N.K. Tonks, and J. Pouyssegur. 1995. Constitutive MAP kinase phosphatase (MKP-1) expression blocks G1 specific gene transcription and S-phase entry in fibroblasts. *Oncogene.* 10:1895–1904.

Brondello, J.M., J. Pouyssegur, and F.R. McKenzie. 1999. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science.* 286:2514–2517.

Brown, J.R., E. Nigh, R.J. Lee, H. Ye, M.A. Thompson, F. Saudou, R.G. Pestell, and M.E. Greenberg. 1998. Fos family members induce cell cycle entry by activating cyclin D1. *Mol. Cell. Biol.* 18:5609–5619.

Brunet, A., D. Roux, P. Lenormand, S. Dowd, S. Keyse, and J. Pouyssegur. 1999. Nuclear translocation of p42/p44-mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J.* 18:664–674.

Burdsal, C.A., M.M. Lotz, J. Miller, and D.R. McClay. 1994. Quantitative switch in integrin expression accompanies differentiation of F9 cells treated with retinoic acid. *Dev. Dyn.* 201:344–353.

Camps, M., A. Nichols, C. Gillieron, B. Antonsson, M. Muda, C. Chabert, U. Boschert, and S. Arkinstall. 1998. Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science.* 280:1262–1265.

Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature.* 410:211–218.

Cho, S.Y., S.Y. Cho, S.H. Lee, and S.S. Park. 1999. Differential expression of mouse Disabled 2 gene in retinoic acid-treated F9 embryonal carcinoma cells and early mouse embryos. *Mol. Cells.* 9:179–184.

Cooper, J.A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105: 1473–1478.

Dean, M., R.A. Levine, and J. Campisi. 1986. c-myc regulation during retinoic acid-induced differentiation of F9 cells is posttranscriptional and associated with growth arrest. *Mol. Cell. Biol.* 6:518–524.

Edwards, S.A., A.Y.K. Rundell, and E.D. Adamson. 1988. Expression of c-fos antisense RNA inhibits the differentiation of F9 cells to parietal endoderm. *Dev. Biol.* 129:91–102.

Faria, T.N., C. Mendelsohn, P. Chambon, and L.J. Gudas. 1999. The targeted disruption of both alleles of RARβ₂ in F9 cells results in the loss of retinoic acid-associated growth arrest. *J. Biol. Chem.* 274:26783–26788.

Fazioli, F., L. Minichiello, B. Matoskova, W.T. Wong, and P.P. Di Fiore. 1993. eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol. Cell. Biol.* 13:5814–5828.

Field, S.J., R.S. Johnson, R.M. Mortensen, V.E. Papaioannou, B.M. Spiegelman, and M.E. Greenberg. 1992. Growth and differentiation of embryonic stem cells that lack an intact c-fos gene. *Proc. Natl. Acad. Sci. USA.* 89:9306–9310.

Formstecher, E., J.W. Ramos, M. Fauquet, D.A. Calderwood, J.-C. Hsieh, B. Canton, X.-T. Nguyen, J.-V. Varnier, J. Camonis, M.H. Ginsberg, and H. Chneiweiss. 2001. PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. *Dev. Cell.* 1:239–250.

Gille, H., A.D. Sharrocks, and P.E. Shaw. 1992. Phosphorylation of transcription factor p62^{TCF} by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature.* 358:414–424.

Hochholdinger, F., G. Baier, A. Nogalo, B. Bauer, H.H. Grunicke, and F. Uberall. 1999. Novel membrane-targeted ERK1 and ERK2 chimeras which act as dominant negative, isotype-specific mitogen-activated protein kinase inhibitors of Ras-Raf-mediated transcriptional activation of c-fos in NIH 3T3 cells. *Mol. Cell. Biol.* 19:8052–8056.

Keyse, S.M. 2000. Protein phosphatases, the regulation of mitogen-activated protein kinase signalling. *Curr. Opin. Cell Biol.* 12:186–192.

Khokhlatchev, A.V., B. Canagarajah, J. Wilsbacher, M. Robinson, M. Atkinson, E. Goldsmith, and M.H. Cobb. 1998. Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell.* 93: 605–615.

Kim-Kaneyama, J., K. Nose, and M. Shibanuma. 2000. Significance of nuclear relocalization of ERK1/2 in reactivation of c-fos transcription and DNA synthesis in senescent fibroblasts. *J. Biol. Chem.* 275:20685–20692.

Koutsourakis, M., A. Langeveld, R. Patient, R. Beddington, and F. Grosveld. 1999. The transcription factor GATA6 is essential for early extraembryonic development. *Development.* 126:723–732.

Kurki, P., A. Laasonen, E.M. Tan, and E. Lehtonen. 1989. Cell proliferation and expression of cytokeratin filaments in F9 embryonal carcinoma cells. *Development.* 106:635–640.

Kyriakis, J.M., P. Banerjee, E. Nikolakaki, T. Dai, E.A. Rubie, M.F. Ahmad, J. Avruch, and J.R. Woodgett. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature.* 369:156–160.

Lockett, T.J., and M.J. Sleigh. 1987. Oncogene expression in differentiating F9 mouse embryonal carcinoma cells. *Exp. Cell Res.* 173:370–378.

Marais, R., J. Wynne, and R. Treisman. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell.* 65:663–675.

Matsubayashi, Y., M. Fukuda, and E. Nishida. 2001. Evidence for existence of a nuclear pore complex-mediated, cytosol-independent pathway of nuclear translocation of ERK MAP kinase in permeabilized cells. *J. Biol. Chem.* 276: 41755–41760.

Morrisey, E.E., Z. Tang, K. Sigrist, M.M. Lu, F. Jian, H.S. Ip, and M.S. Parmacek. 1998. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* 12:3570–3590.

Morrisey, E.E., S. Musco, M.Y. Chen, M.M. Lu, J.M. Leiden, and M.S. Parmacek. 2000. The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. *J. Biol. Chem.* 275:19949–19954.

O'Shea, K.S. 2001. Directed differentiation of embryonic stem cells: genetic and epigenetic methods. *Wound Repair Regen.* 9:443–459.

Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. *Methods Cell Biol.* 24:97–132.

Pouyssegur, J., V. Volmat, and P. Lenormand. 2002. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochem. Pharmacol.* 64:755–763.

Reska, A.A., R. Seger, C.D. Diltz, E.G. Krebs, and E.H. Fischer. 1995. Association of mitogen-activated protein kinase with the microtubule cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 92:8881–8885.

Robertson, E.J. 1987. Embryo-derived stem cell lines. *In* Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E.J. Robertson and R.J. Robertson, editors. IRL Press Limited, Oxford, UK. 71–112.

Robinson, M.J., and M.H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9:180–186.

Rohwedel, J., K. Guan, and A.M. Wobus. 1999. Induction of cellular differentiation by retinoic acid in vitro. *Cells Tissues Organs.* 165:190–202.

Saez, E., S.E. Rutberg, E. Mueller, H. Oppenheim, J. Smoluk, S.H. Yuspa, and B.M. Spiegelman. 1995. c-fos is required for malignant progression of skin tumors. *Cell.* 82:721–732.

Sejersen, T., J. Sumegi, and N.R. Ringertz. 1985. Expression of cellular oncogenes in teratoma-derived cell lines. *Exp. Cell Res.* 160:19–30.

Sgambato, V., P. Vanhoutte, C. Pages, M. Rogard, R. Hipskind, M.-J. Besson, and J. Caboche. 1998. *In vivo* expression and regulation of Elk-1, a target of the extracellular-regulated kinase signaling pathway, in the adult rat brain. *J.*

Neurosci. 18:214–226.

Sherman, M.I., and R.A. Miller. 1978. F9 embryonal carcinoma cells can differentiate into endoderm-like cells. *Dev. Biol.* 63:27–34.

Smedberg, J.L., E.R. Smith, C.D. Capo-chichi, A. Frolov, D.-H. Yang, A.K. Godwin, and X.-X. Xu. 2002. Ras/MAPK pathway confers basement membrane dependence upon endoderm differentiation of embryonic carcinoma cells. *J. Biol. Chem.* 277:40911–40918.

Smith, E.R., C.D. Capo-chichi, J. He, J.L. Smedberg, D.-H. Yang, A.H. Prowse, A.K. Godwin, T.C. Hamilton, and X.-X. Xu. 2001a. Disabled-2 mediates c-Fos suppression and the cell growth regulatory activity of retinoic acid in embryonic carcinoma cells. *J. Biol. Chem.* 276:47303–47310.

Smith, E.R., J.L. Smedberg, M.E. Rula, T.C. Hamilton, and X.-X. Xu. 2001b. Disassociation of MAPK activation and c-Fos expression in F9 embryonic carcinoma cells following retinoic acid-induced endoderm differentiation. *J. Biol. Chem.* 276:32094–32100.

Tresini, M., A. Lorenzini, L. Frisoni, R.G. Allen, and V.J. Cristofalo. 2001. Lack of Elk-1 phosphorylation and dysregulation of the extracellular regulated kinase signaling pathway in senescent human fibroblast. *Exp. Cell Res.* 269:

287–300.

Vanhoutte, P., J.L. Nissen, B. Brugg, B.D. Gaspera, M.-J. Besson, R.A. Hipskind, and J. Caboche. 2001. Opposing roles of Elk-1 and its brain-specific isoform, short Elk-1, in nerve growth factor-induced PC12 differentiation. *J. Biol. Chem.* 276:5189–5196.

Vasios, G.W., J.D. Gold, M. Petkovich, P. Chambon, and L.J. Gudas. 1989. A retinoic acid-responsive element is present in the 5′ flanking region of the laminin B1 gene. *Proc. Natl. Acad. Sci. USA.* 86:9099–9103.

Volmat, V., M. Camps, S. Arkinstall, J. Pouyssegur, and P. Lenormand. 2001. The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *J. Cell Sci.* 114:3433–3443.

Whitehurst, A.W., J.L. Wilsbacher, Y. You, K. Luby-Phelps, M.S. Moore, and M.H. Cobb. 2002. ERK2 enters the nucleus by a carrier-independent mechanism. *Proc. Natl. Acad. Sci. USA.* 99:7491–7501.

Yang, S.H., P. Shore, N. Willingham, J.H. Lakey, and A.D. Sharrocks. 1999. The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1. *EMBO J.* 18:5666–5674.