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PEA-15 Is Inhibited by Adenovirus E1A and Plays a Role in ERK Nuclear Export and Ras-induced Senescence*

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Oncogenic *ras* activates multiple signaling pathways to enforce cell proliferation in tumor cells. The ERK1/2 mitogen-activated protein kinase pathway is required for the transforming effects of *ras*, and its activation is often sufficient to convey mitogenic stimulation. However, in some settings oncogenic *ras* triggers a permanent cell cycle arrest with features of cellular senescence. How the Ras/ERK1/2 pathway activates different cellular programs is not well understood. Here we show that ERK1/2 localize predominantly in the cytoplasm during *ras*-induced senescence. This cytoplasmic localization seems to be dependent on an active nuclear export mechanism and can be rescued by the viral oncoprotein E1A. Consistent with this hypothesis, we showed that E1A dramatically down-regulated the expression of the ERK1/2 nuclear export factor PEA-15. Also, RNA interference against PEA-15 restored the nuclear localization of phospho-ERK1/2 in Ras-expressing primary murine embryo fibroblasts and stimulated their escape from senescence. Because senescence prevents the transforming effect of oncogenic *ras*, our results suggest a tumor suppressor function for PEA-15 that operates by means of controlling the localization of phospho-ERK1/2.

Normal organisms keep stable cell numbers by regulating the process of cell proliferation and cell death. Cancer cells bypass these controls because of multiple genetic and epigenetic alterations that block the action of cell proliferation inhibitors or mimic growth factor activation (1). Fortunately, several fail-safe mechanisms have evolved to prevent the expansion of cells threatened by oncogenic stimuli. For example, the *myc* oncogene induces abnormal cell proliferation but promotes cell death as well (1, 2). Also, oncogenic *ras* leads to a permanent cell cycle arrest with features of cellular senescence (3). Tumor formation is therefore very much stimulated by the inactivation of key mediators of the apoptosis and senescence programs such as p53 and the Rb[1] tumor suppressor proteins (1, 2).

The induction of a senescent cell cycle arrest by oncogenic *ras* in normal fibroblasts contrasts with the well known mitogenic effects of Ras and the MAP kinase pathway in multiple cell lines and transgenic animals (4). Endogenous Ras proteins are required for cell proliferation (5), and Ras activation is part of the response to growth factors during normal development (6). In addition, constitutively active oncogenic alleles of *ras* are often found in human tumors (7) where they convey mitogenic signals. To explain this apparent paradox, it was proposed that normal cells interpret aberrant mitogenic signaling by activating a p53- and Rb-dependent senescence program that is absent in most tumor cell lines (3). Another explanation is that the ERK1/2 MAP kinase pathway activates different cellular programs depending on the strength and duration of its stimulation. For example, sustained activation of the ERK1/2 MAP kinase pathway by constitutively active Ras or nerve growth factor triggers growth arrest and differentiation in the PC12 pheochromocytoma cell line (8). In contrast, a transient activation of ERK1/2 by epidermal growth factor induces cell proliferation (8). To date, we do not know whether induction of senescence in normal fibroblasts by constitutive Ras activation involves sustained and strong ERK1/2 activity. As a matter of fact, it is equally possible that these cells activate feedback mechanisms to counteract constitutive Ras activation, blocking the mitogenic activity of the ERK1/2 MAP kinase cascade.

Here we report that constitutive Ras expression in IMR90 fibroblasts induced a senescent cell cycle arrest accompanied by a sustained phosphorylation of ERK1/2. Shortly after the introduction of oncogenic *ras*, ERK1/2 were phosphorylated and translocated to the nucleus. Upon establishment of the senescent phenotype, ERKs remained phosphorylated but relocalized to the cytoplasm. Blocking the senescent response to Ras by the viral oncoprotein E1A restored the nuclear localization of ERK1/2 normally observed in growing cells. The ERK nuclear export factor PEA-15 seems to be required for this relocalization of ERK and, in agreement, PEA-15 levels were reduced by E1A. Also, RNAi-mediated inactivation of PEA-15 in mouse fibroblasts rescued them from Ras-induced senescence. Hence, limiting ERK nuclear localization by PEA-15 keeps the aberrant mitogenic activity of oncogenic *ras* and the ERK1/2 pathway under control.

EXPERIMENTAL PROCEDURES

Cells and Retroviruses—Normal human diploid fibroblast IMR90 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% penicillin G/streptomycin sulfate (Invitrogen). Primary mouse embryo fibroblasts (MEFs) were obtained from 13.5-day-old embryos as described (9). The retroviruses pBabe, pBabeRas, pLPC, pLPCE1A, pWZL, and pWZLRas were de-

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[1] The abbreviations used are: Rb, retinoblastoma protein; BrdUrd, bromodeoxyuridine; ERK, extracellular signal-regulated kinase; ERK2*, ERK2-L73P, S151D; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; GST, glutathione *S*-transferase; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; PML, promyelocytic leukemia; RNAi, RNA interference; shRNA, short hairpin RNA; SA-β-gal, senescence-associated β-galactosidase.

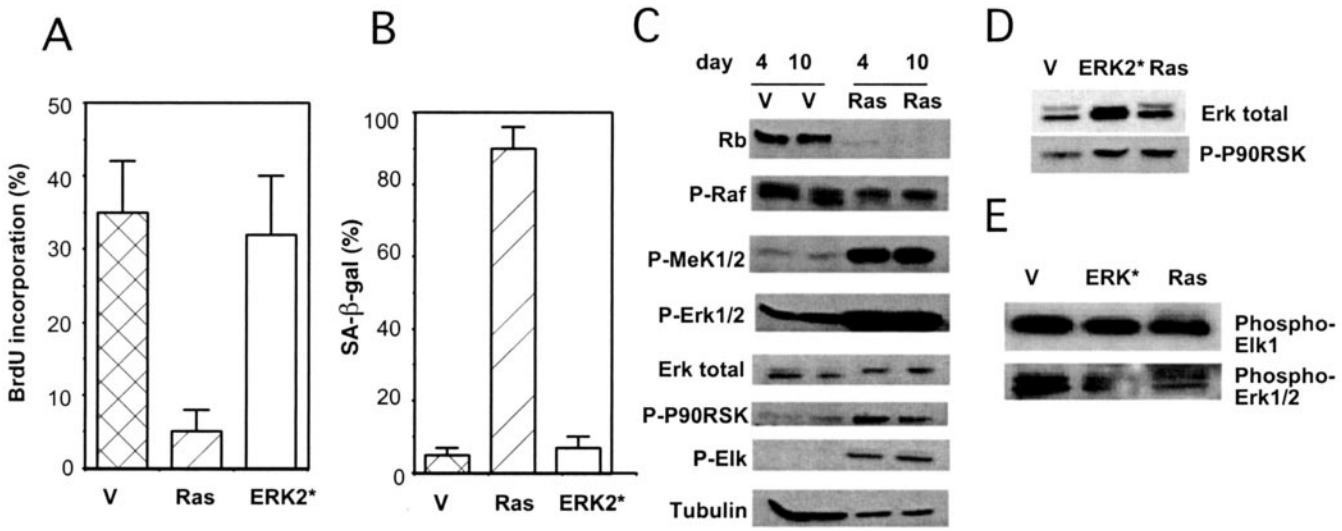

FIG. 1. **Sustained phosphorylation of several members of the ERK1/2 MAP kinase pathway in human fibroblasts during senescence.** *A*, BrdUrd (*BrdU*) incorporation. Cells were infected with the retroviral vector pBabe or its derivatives that express Ras or ERK2*. Six days after selection, cells were pulsed with BrdUrd for 3 h and then fixed and stained with an anti-BrdUrd antibody. The results are representative of three independent experiments and are presented as the percentage of nuclei that incorporate BrdUrd. *B*, SA-β-gal. Cells were fixed and stained 6 days after selection. The graph summarizes the percent of positive cells after counting 200 cells in three independent experiments. *C* and *D*, Western blots. Total Laemmli cell extracts were prepared on the indicated days. Twenty micrograms of total protein was used for SDS-PAGE, and individual proteins were visualized after immunoblotting using specific antibodies. *E*, immunoblots of an immunoprecipitation kinase assay using GST-Elk1 as substrate. *V*, vector control.

scribed previously (10). pBabeERK was constructed as follows. ERK2-L73P, S151D (abbreviated ERK2*) was obtained from pCMVERK2* (11) by XbaI and partial EcoRI digestion, blunted with Klenow, and ligated into the SnaBI site of pBabe. Retroviral-mediated gene transfer was performed as described previously (10).

RNA Hairpins Trigger RNA Interference—To construct the gene cassettes that express RNA hairpins (shRNAs) from the U6 polymerase III promoter, we used shagging-PCR as described (cshl.org/public/SCIENCE/hannon.html). shRNAs (primers are available upon request) were designed against nucleotide locations 1–29 and 295–324 of the mouse PEA-15 mRNA sequence (GenBank™ accession number NM_011063) with the computer-assisted RNAi Oligo Retriever program (cshl.org/public/SCIENCE/hannon.html). The common upstream primer U6-BglII (5′-TCTGCCAGATCTGATTTAGGTGACACTATAG3-′), complementary to a region upstream of the U6 promoter on the pGEMU6 plasmid (N. Hernandez, Cold Spring Harbor Laboratory), was used to amplify a PCR cassette containing the U6 promoter, a target-specific hairpin sequence, the Pol-III terminator, and two terminally placed restriction sites (BglII and EcoRI) for convenient directional cloning. The resulting 400-bp PCR product was cloned between the BglII and EcoRI sites of the retroviral vector pMSCVpuro (Clontech).

Cell Proliferation and Senescence Determination—To determine cell proliferation rates we used a BrdUrd incorporation assay as described (10). Senescence was scored by determining the percentage of the population exhibiting a senescence-associated β-galactosidase (SA-β-gal) activity (12).

Senescence Rescue Assay—Primary MEFs were co-infected with the retroviral vector pBabe or its derivative expressing oncogenic *ras* and with pMSCV or its derivatives expressing anti-PEA-15 RNA hairpins. Two days after double selection (2 μg/μl puromycin for 3 days and 75 μg/μl hygromycin for 5 days), all populations expressing oncogenic *ras* entered senescence, whereas the control cells remained growing for one additional week (two passages) and then entered into senescence induced by culture shock (13). We kept all of these cells for another 2 weeks in subconfluent cultures, changing media every 3 days. We used morphological criteria and the SA-β-gal assay to evaluate whether the cells remained senescent or resumed proliferation.

Protein Expression—To prepare total cell protein, cells were collected by trypsinization, washed with PBS, lysed in 100 μl of SDS sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol), and boiled for 5 min. For Western blots, 20 μg of total cell protein were separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). The primary antibodies used were anti-pRb (G3–245, 1:250; BD Biosciences), anti-p53 (CM5, 1:2000; Novocastra), anti-mouse PML (36-1-104, 1:5000; a gift from S. Lowe), anti-phosphoserine 259 Raf, anti-phospho-MEK1/2, anti-phospho ERK1/2, anti-phospho-p90Rsk, anti-phospho-Elk1 (Phospho-ERK1/2 Pathway sampler kit, all used 1:500; Cell Signaling), anti-total ERK (K-23. 1:1000; Santa Cruz Biotechnology), anti-PEA-15 (1:1000, kindly provided by Dr J. Ramos, Rutgers University), and anti-α-tubulin (B-5–1-2, 1:5000; Sigma). Signals were revealed after incubation with anti-mouse or anti-rabbit secondary antibodies coupled to peroxidase (Amersham Biosciences) by using enhanced chemiluminescence (ECL, Amersham Biosciences) or Lumi-Light Plus (Roche Applied Science). ERK1/2 kinase assays were performed according to an immunoprecipitation/kinase assay (Cell Signaling Technologies Inc.) using 5 μg of GST-Elk1 as substrate. Phosphorylated GST-Elk1 was detected by Western blot using the anti-phospho-Elk1 (Ser-383) antibody (Cell Signaling).

Fluorescence Microscopy—For fluorescence microscopy, 2×10^4 control IMR90 cells or Ras-senescent IMR90 cells were plated on coverslips placed on 12-well plates (Costar, Corning Inc.). Twenty-four hours after plating, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, the cells were washed in PBS and permeabilized using ice-cold 0.2% Triton X-100 in PBS/bovine serum albumin solution for 5 min. Then the cells were washed three times with PBS/bovine serum albumin and incubated for 1 h at room temperature with anti-phospho-ERK1/2 (9106, 1:300; Cell Signaling); anti-p53 (CM5, 1:50, Novocastra), or anti-mouse PML (36-1-104, 1:200) as primary antibodies. Next, the cells were washed and incubated for 1 h with Oregon-Green-conjugated secondary antibody (1:1000; Molecular Probes, Eugene, OR) or (Alexa 568 anti-mouse, 1:2000; Molecular Probes). Finally, the cells were washed three times with PBS, incubated in 300 nM 4′,6-diamidino-2-phenylindole for 10 min, and mounted on microscope slides. Images were obtained using MetaMorph software (Universal Imaging Corp.) and processed using Adobe Photoshop 6.0. For studying ERK localization in MEFs, cells were first serum-starved for 48 h and later fixed at different times after adding serum. The staining and image processing was as described above.

RESULTS

Oncogenic ras Induces a Constitutive Stimulation of the MAP Kinase Pathway—It is known that both proliferation and senescence induced by oncogenic *ras* (RasV12) require signaling through the ERK1/2 MAP kinases (14, 15). However, MAP kinase signaling is the subject of a negative feedback regulation by the ERK1/2-dependent induction of specific phosphatases that dephosphorylate ERK1/2 (16, 17). In particular, it has been reported that the levels of MKP-2 are increased after the induction of cellular senescence by serial passage (replica-

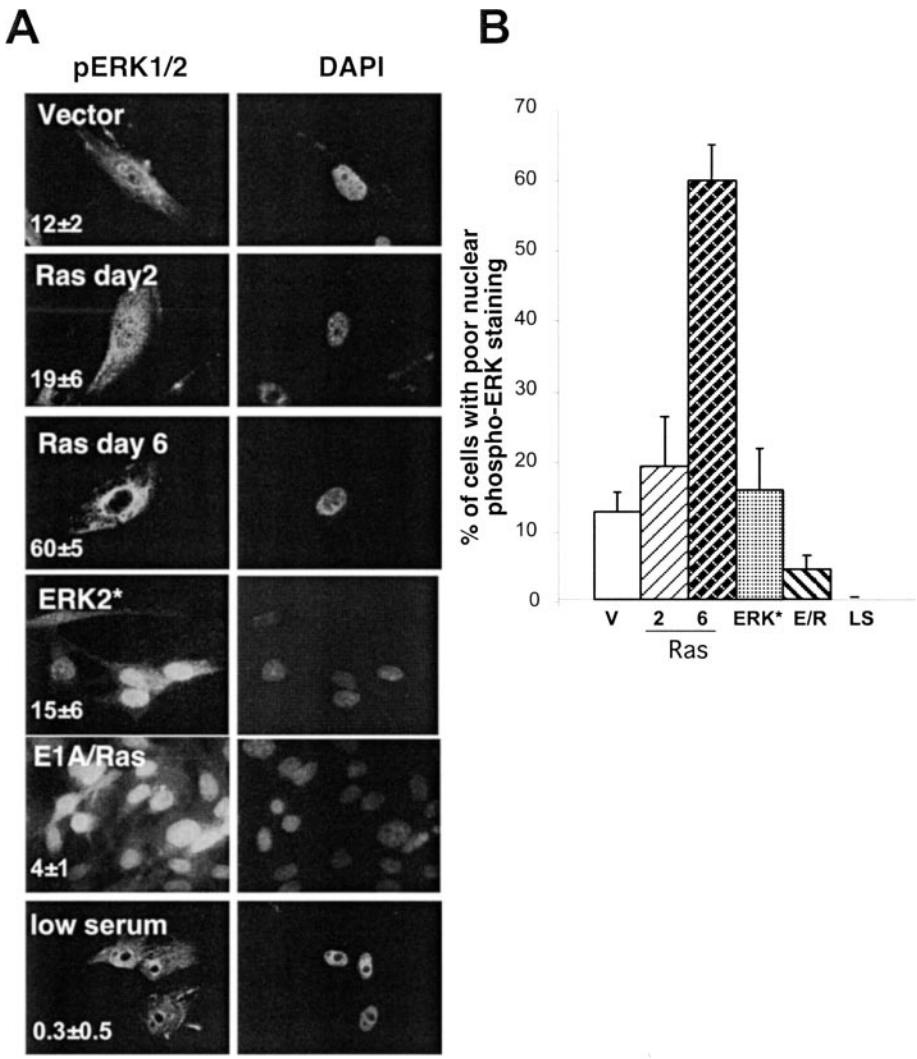

FIG. 2. **Localization of phospho-ERK1/2 in growing and senescent human fibroblasts.** *A*, immunofluorescence images of IMR90 fibroblasts infected with control retroviral vectors or derivatives expressing oncogenic *ras* or ERK2* or a combination of oncogenic *ras* and E1A (E1A/Ras). The cells were fixed 2 and 6 days after selection and then stained for phospho-ERK1/2 (*pERK1/2*) by indirect immunofluorescence. Only Ras-expressing cells are shown for day 2, because the other cells were similar at days 2 and 6. The *top row* represents cells expressing the empty vector that were serum-starved for 3 days and then treated with fresh serum for 5 min, fixed, and stained for phospho-ERK1/2. We quantified the percentage of cells that showed an apparent nuclear exclusion of phospho-ERK, and these numbers are indicated at the *bottom left* of each immunofluorescence section in the *left column*. DAPI, 4′,6-diamidino-3-phenylindole. *B*, quantification, in bar graph form, of the percentage of cells that showed an apparent nuclear exclusion of phospho-ERK. More than 100 cells were counted for each condition in three independent experiments. *V*, vector; *Ras,* oncogenic ras (on days *2* and *6*), *ERK**, constitutively active ERK2; *E/R*, E1A plus Ras; *LS*, low serum.

tive senescence) (18). Consequently, it is possible that cell cycle exit in response to oncogenic *ras* in primary fibroblasts is due to these feedback signals mediated by protein phosphatases. To test this idea, we infected human diploid fibroblast IMR90 cells with a retrovirus expressing oncogenic *ras* and a vector control. As shown previously (3, 10, 14, 19), Ras-expressing cells entered a permanent cell cycle arrest with features of cellular senescence (Fig 1, *A* and *B*). To verify whether ERK1/2 remained active during Ras-induced senescence or whether their activity is down-regulated by dephosphorylation, we performed a series of immunoblots with antibodies recognizing phosphorylated forms of ERK1/2, their upstream regulators (Raf and MEK1/2), and two of their substrates (Elk-1 and p90Rsk). The results of such an analysis are presented in Fig. 1*C*. In Ras-expressing cells, all members of the ERK1/2 pathway downstream of Ras displayed a pattern of phosphorylation consistent with constitutive signaling. Interestingly, the downstream target of Ras, Raf, was dephosphorylated at Ser-259 in cells expressing oncogenic *ras.* Phosphorylation of Raf at this residue is considered to inhibit its activity (20). Whether oncogenic *ras* mediates this dephosphorylation by activating a phosphatase or inhibiting a protein kinase requires further study. We concluded that the MAP kinases ERK1/2 remained active and phosphorylated both at the beginning of the senescent arrest induced by Ras (4 days after infection) and when the senescent arrest was well established (10 days after infection). Clearly, exit from the cell cycle in response to oncogenic *ras* is not the result of inactivation of the ERK1/2 pathway by dephosphorylation.

Constitutive Expression of Activated ERK Is Not Sufficient to Induce Senescence—Senescence can also be induced in primary fibroblasts by activated alleles of Raf and MEK, which, like Ras, activate ERK1/2 (14, 15). Because we showed that all the members of the Ras-ERK1/2 signal transduction cascade (Ras, Raf, MEK, and ERK1/2) are constitutively active during Ras-induced senescence, we decided to investigate whether an activated ERK2 allele also induces senescence. To do this experiment we used the constitutively active ERK2* described by Ahn and colleagues (11). This allele bears mutations that enhance the rate of autophosphorylation of ERK2 at Thr-183 and Tyr-185, which are the sites phosphorylated by the ERK activators MEK1/2. More importantly, expression of this constitutively active ERK2 allele in human embryonic kidney cells (293) led to phosphorylation of the ERK substrates Elk1 and Rsk as efficiently as does a constitutively active MEK1 allele (11). Surprisingly, activated ERK2* did not induce senescence (Fig 1, *A* and *B*) despite activating Rsk to a similar extent as Ras did (Fig. 1*D*). We also performed immunoprecipitation/kinase assays, using an excess of GST-Elk1 as a substrate. In this experiment, phospho-ERK, immunoprecipitated from ERK2*-expressing cells was as active as the phospho-ERK1/2 immunoprecipitated from control cells growing in serum or RasV12-expressing cells (Fig. 1*E*). Together, our results are consistent with those reported before about this ERK2* allele (11). However, we can not entirely eliminate the possibility that the two mutations present in ERK2* impair its interactions with key substrates that mediate cell cycle arrest. Alter-

natively, ERK2* and RasV12-activated ERK1/2 may differ in some other functions of the ERK1/2 MAP kinase pathway.

Phosphorylated ERKs Localized Predominantly to the Cytoplasm during Ras-induced Senescence—To further investigate ERK physiology during senescence and explain why activated Ras, Raf, or MEK but not ERK induced senescence, we performed localization studies by indirect immunofluorescence using antibodies specific for phosphorylated ERK1/2. We found that phosphorylated ERK1/2 (phospho-ERK) localized both in the in the nucleus and in the cytoplasm early after introducing oncogenic *ras* in primary fibroblasts, when cells are highly proliferative (Fig. 2). The same pattern of staining was observed for control cells bearing an empty vector. This situation is the result of the steady state equilibrium of ERK shuttling between the nucleus and the cytoplasm (21). However, by the time cells entered senescence (6 days after the introduction of Ras), a significant number of cells (~60%) displayed phospho-ERK mainly in the cytoplasm, apparently excluding the nucleus and often forming a perinuclear halo (Fig. 2). In contrast, the constitutively active ERK2*, which failed to induce senescence, was found predominantly localized in the nucleus (Fig. 2). In addition, blocking Ras-induced senescence with the adenoviral oncoprotein E1A was accompanied by a dramatic enrichment of phospho-ERK1/2 in the nucleus. A similar pattern of staining was also observed in cells that were serum-starved and then serum-stimulated (Fig. 2). Thus, senescence strongly correlated with the nuclear exclusion of phospho-ERK1/2.

We then treated Ras-senescent cells with the CRM1 (chromosome region maintenance 1) inhibitor leptomycin B, which blocks nuclear export. We reasoned that this drug should relocalize ERK to the nucleus in Ras-senescent cells if an active export mechanism was responsible for the observed cytoplasmic ERK localization. The results, shown in Fig. 3, indicate that leptomycin B induced the relocalization of phospho-ERK1/2 to the nucleus of Ras-senescent cells. The data are consistent with a model in which phospho-ERK1/2 freely enter the nucleus followed by an active export to the cytoplasm during Ras-induced senescence. However, it is also plausible that an ERK1/2-anchoring protein required nuclear export to localize in the cytoplasm and was therefore able to retain ERK1/2 in this cellular compartment.

E1A Inhibits Ras-induced Senescence in Human Cells and Blocks the Expression of the ERK Export Factor PEA-15—Ras-induced senescence in human cells can be efficiently bypassed by the oncoprotein E1A from the adenovirus (3, 10). E1A has multiple activities, including inhibition of the Rb and p53 tumor suppressors (22–24). However, inhibition of Rb and p53, using the oncoproteins E6 and E7 from human papillomavirus, did not rescue the cell cycle arrest induced by oncogenic *ras* in primary human fibroblasts (25), nor did it prevent ERK localization in the cytoplasm (not shown). Obviously, additional E1A targets are responsible for maintaining Ras-induced cell cycle arrest and ERK localization in the cytoplasm. One attractive hypothesis is that E1A affects proteins responsible for cytoplasmic localization of ERK1/2. Hence, E1A can be used as a tool to discover the mechanism responsible for cytoplasmic ERK localization in senescent cells.

We asked whether E1A targets factors known to mediate ERK nuclear export. An obvious candidate was PEA-15, recently characterized as an ERK nuclear export factor (26). PEA-15 binds ERK1/2 in the nucleus and uses CRM1-mediated nuclear export to localize them to the cytoplasm. Accordingly, PEA-15 null cells displayed increased cell proliferation and constitutive localization of ERK in the nucleus (26). Interestingly, E1A inhibited Ras-induced senescence (Fig 4, *A* and *B*) and dramatically reduced the expression of PEA-15 in human

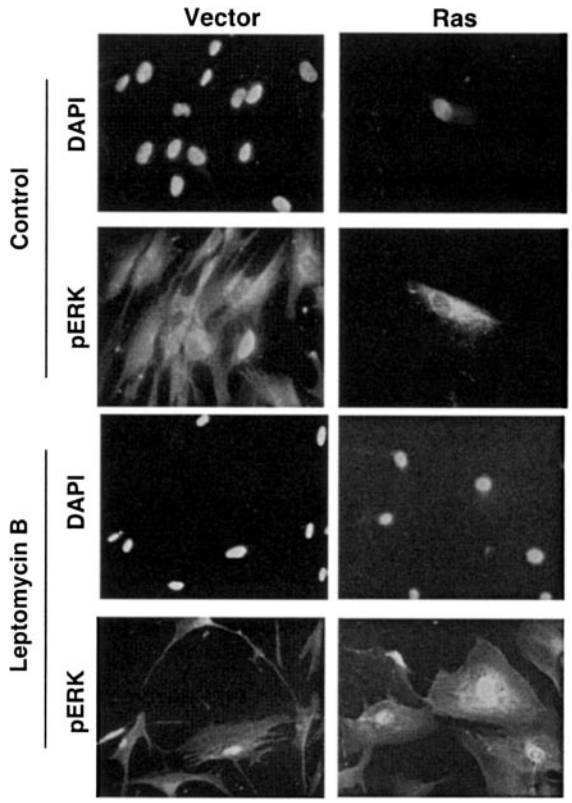

FIG. 3. **Leptomycin B relocalized ERK1/2 from the cytoplasm to the nucleus of senescent cells.** IMR90 fibroblasts were infected with an empty retroviral vector or its derivative expressing oncogenic *ras*. Actively growing control cells or Ras-senescent cells were plated on cover slips 6 days after infection and treated with 10 nM leptomycin B (Sigma) for 3 h. The cells were then fixed, and phospho-ERK (*pERK*) was visualized by indirect immunofluorescence. *DAPI*, 4′,6-diamidino-2-phenylindole.

fibroblasts (Fig 4*C*). This finding suggested that PEA-15 could control ERK localization during Ras-induced senescence.

RNAi-mediated Inactivation of PEA-15, an ERK1/2 Nuclear Export Factor, Rescued Primary Murine Embryo Fibroblasts from Ras-induced Senescence—To confirm a role for PEA-15 in cytoplasmic ERK localization during Ras-induced senescence, we blocked its expression using RNAi. To induce RNAi, we designed two retroviral vectors that express RNA hairpins with complementary sequences for mouse PEA-15 (Fig. 5*A*). Cells expressing one of these hairpins showed reduction of PEA-15 expression 2 days after infection (Fig. 5*B*). More importantly, although these cells entered into Ras-induced senescence, many of them escaped senescence, forming foci of highly proliferative cells that can be established as permanent, immortalized cultures. This ability to escape senescence induced by PEA-15 RNAi was scored in four independent experiments using four different MEF isolates. In contrast, cells with the non-functional hairpin or cells with an empty vector used as a control rarely did so (Fig. 5*C*).

In MEFs, Ras-induced senescence depends on p53 functions and the induction of p53 targets genes such as *PML* (3, 27). We used immunofluorescence and immunoblots to evaluate the status of p53 and PML at day 10 after infection. By this time, all cells expressing oncogenic *ras* were arrested and senescent, whereas cells expressing oncogenic *ras* and the anti-PEA-15 hairpin were divided into two populations, one of senescent cells and one of small cells that escaped senescence. Cells with the vector controls or with the anti-PEA-15 hairpin alone were also in senescence due to culture shock (13). All cell populations expressed p53 and PML immunoreactive material, but the

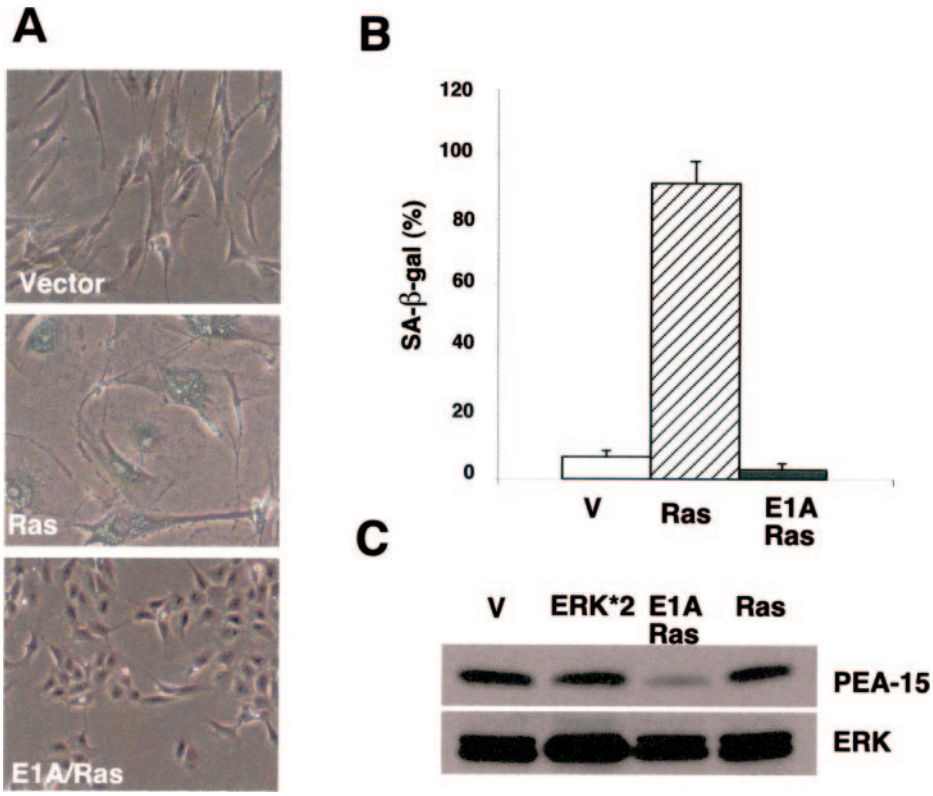

FIG. 4. **E1A inhibits Ras-induced senescence and reduces the expression of PEA-15.** *A*, pictures of cells stained for senescence-associated β-galactosidase (400× magnification). Cells expressing vector controls (pLPC and pWZL), oncogenic *ras* (*Ras*), or oncogenic *ras* plus E1A (*E1A/Ras*) were fixed for staining 8 days after infection. *B*, quantitation of SA-β-Gal staining. Results are the average and S.D. of four independent experiments. *C*, Western blots. IMR90 fibroblasts were co-infected with empty retroviral vectors (*V*) or their derivatives expressing ERK2*, E1A (pLPC-E1A), and oncogenic *ras* (pWZL-Ras). Total cell protein was prepared from cells lysed 8 days after infection.

strongest signal was found in cells expressing oncogenic *ras* alone. Blocking PEA-15 by RNA interference reduced p53 and PML levels in Ras-expressing cells to the levels seen in cells with vector alone (Fig. 5, *D* and *E*). This reduction was observed in all cells of the population (senescent and non-senescent), and, therefore, it was not a consequence of escaping senescence. Thus, escape from senescence of cells expressing an anti-PEA-15 RNA hairpin was not due to mutations that result in a loss of p53 expression. Altogether, our data suggest that PEA-15 is required to connect signals from oncogenic *ras* to p53 and maintain the state of senescence.

To investigate whether blocking PEA-15 by RNAi would increase the nuclear localization of ERK1/2, we studied ERK1/2 localization in cells expressing oncogenic *ras* or oncogenic *ras* plus the anti-PEA-15 hairpin. For this experiment we used cells at day 10 after infection. To limit the effects that the cell cycle and cell proliferation may have on ERK localization, we serum-starved the cells for 48 h, added serum for 1 h, and then fixed the cells for immunofluorescence. As shown previously, most cells expressing oncogenic *ras* alone displayed a cytoplasmic staining for phospho-ERK1/2. This finding is consistent with reports indicating that senescent cells do not respond to serum stimulation (28). However, more than half of the cells expressing the anti-PEA-15 hairpin exhibited a predominant nuclear staining of phospho-ERK1/2 (Fig. 6*B*). Hence, rescuing senescence by blocking PEA-15 expression correlates very well with an increase in the nuclear localization of phopho-ERK1/2.

To further characterize how blocking PEA-15 functions affects ERK localization, we developed a kinetic protocol to look for ERK nuclear export in cells expressing oncogenic *ras* alone or with an anti-PEA-15 RNA hairpin. Again, we used cells at day 10 after infection and treated them with leptomycin B to allow for nuclear accumulation of ERK1/2. Then we studied ERK localization at different times after the withdrawal of leptomycin B. In cells expressing oncogenic *ras* alone, ERK quickly moved out of the nucleus to accumulate in the cytoplasm. In cells expressing oncogenic *ras* and the anti-PEA-15 hairpin, ERKs were retained for a longer time in the nucleus. One hour after the withdrawal of leptomycin B, ERK1/2 was found both in the nucleus and in the cytoplasm (Fig. 7). Similar results were obtained with an anti-ERK1/2 antibody that recognizes non-phosphorylated ERK1/2. The main conclusion from this experiment is that PEA-15 plays an important role in controlling the nuclear export of phospho-ERK1/2. However, because ERK1/2 still manage to exit the nucleus in cells with very low levels of PEA-15, other factors might regulate ERK1/2 nuclear localization as well.

DISCUSSION

The ERK1/2 MAP kinase pathway conveys mitogenic signals from growth factor receptors. However, this pathway also mediates a senescent cell cycle arrest in normal fibroblasts expressing oncogenic *ras* (3) or in cells from transgenic mice that age prematurely due to expression of a short isoform of the p53 tumor suppressor protein (29). Here we present evidence indicating that the localization of ERK1/2 distinguishes mitogenic signaling from senescence. First, ERK1/2 localize mainly in the cytoplasm of senescent cells. Second, the viral oncoprotein E1A, which blocks Ras-induced senescence and stimulates transformation, prevents the cytoplasmic localization of ERK1/2. Third, E1A inhibits the expression of the ERK1/2 export factor PEA-15. Finally, RNAi-mediated inhibition of PEA-15 stimulated the escape from senescence in mouse fibroblasts expressing oncogenic *ras* and reduced the levels of p53 induced by oncogenic *ras*.

In quiescent cells, ERK1/2 are normally localized in the

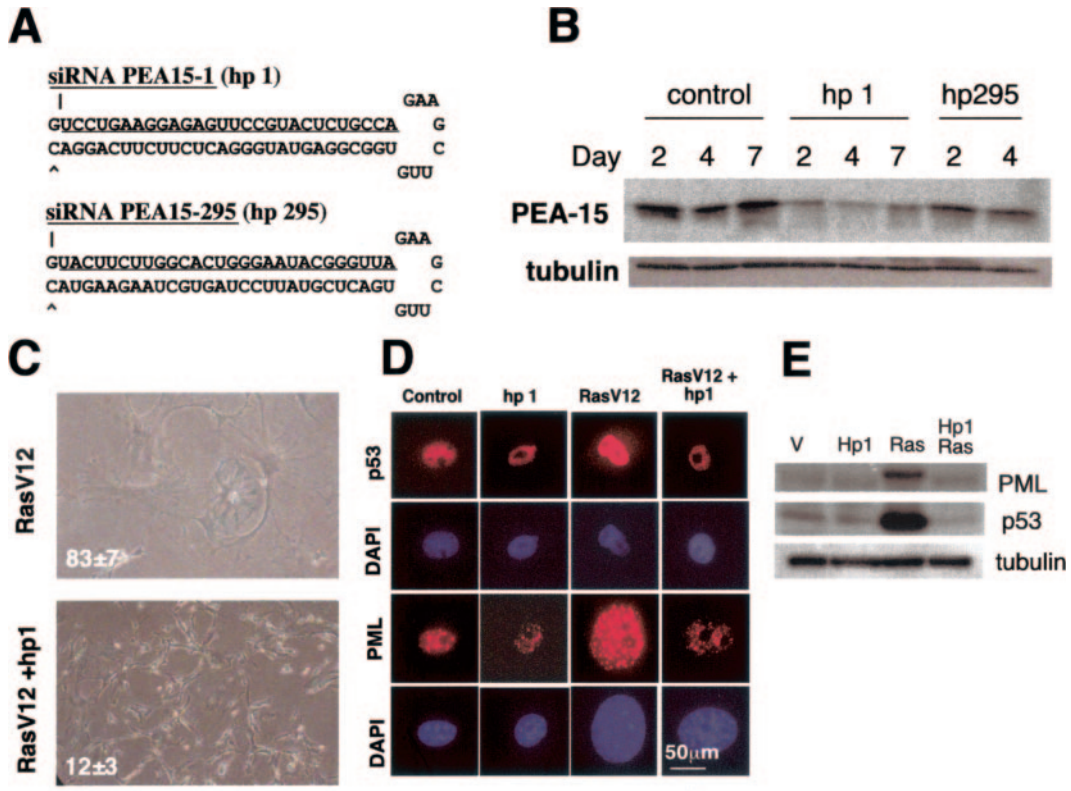

FIG. 5. **RNAi against PEA-15 rescue mouse fibroblasts from *ras*-induced senescence.** *A*, RNA hairpins designed to trigger RNAi against PEA-15. shRNA-PEA-15-1 (hp1) (*siRNA PEA15-1 (hp 1)*) targets the sequence between nucleotides 1 and 29. shRNA-PEA-15-295 (hp295) (*siRNA-PEA-15-295 (hp295)*) targets the sequence between nucleotides 295 and 324. *B*, Western blot. Total protein was prepared from cells after infection with the control vector pMSCV (*control*) or its derivative expressing a hairpin against PEA-15 (*hp1*). PEA-15 was visualized using an anti-PEA-15 antibody kindly provided by Dr J. Ramos. *hp295*, shRNA-PEA-15-295. *C*, pictures of SA-β-Gal-stained cells (100× magnification) fixed 2 weeks after infection with retroviral vectors expressing Ras and a vector control (pMSCV) or Ras and pMSCV expressing PEA-15-1 RNA hairpin. The number of senescence-associated β-galactosidase positive cells and the S.D. of three independent counts is indicated at the *bottom left* of the *panel sections*. *D*, MEFs expressing vector controls (*Control*), anti-PEA-15 RNA hairpin (*hp1*), oncogenic *ras* (*RasV12*), or oncogenic *ras* plus anti-PEA-15 RNA hairpin (*RasV12 + hp1*) were plated on coverslips 10 days after infection. After serum starvation (0.5% serum) for 2 days, fresh serum was added, and the cells were fixed for p53 immunostaining 1 h later. *DAPI*, 4′-6-diamidino-2-phenylindole. *E*, immunoblots for p53 and PML in MEFs expressing vector controls (*V*), anti-PEA-15 RNA hairpin (*Hp1*), oncogenic *ras* (*Ras*), or oncogenic *ras* plus anti-PEA-15 RNA hairpin (*Hp1 Ras*).

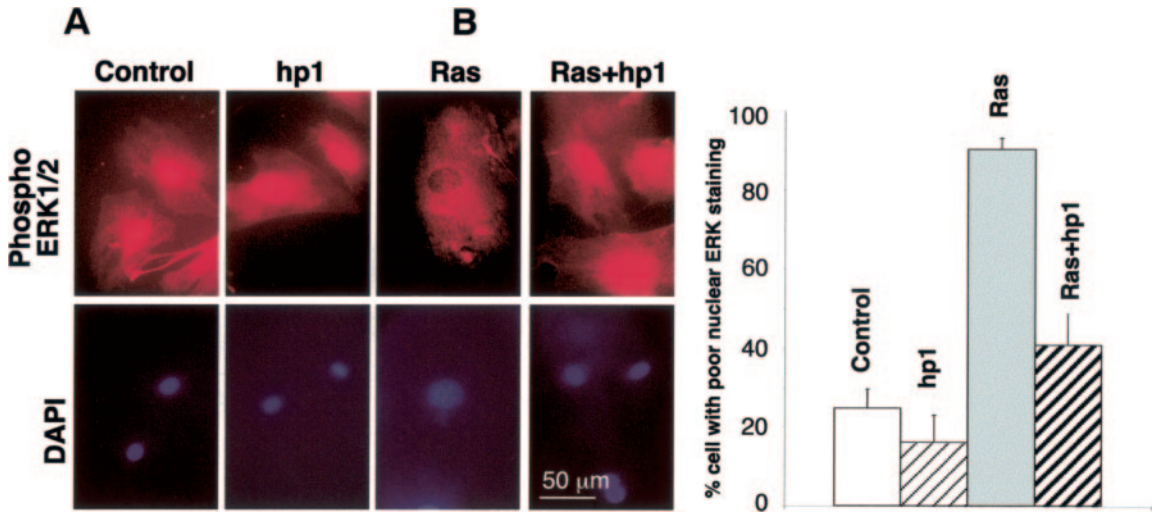

FIG. 6. **RNAi against PEA-15 increases nuclear localization of phospho-ERK1/2 in MEFs expressing oncogenic *ras*.** *A*, MEFs expressing vector controls (*Control*), anti-PEA15 hairpin 1 (*hp1*), oncogenic *ras* (*Ras*), or oncogenic *ras* plus anti-PEA-15 hairpin 1 (*Ras+hp1*) were plated on coverslips 10 days after infection. After serum starvation (0.5% serum) for 2 days, fresh serum was added, and the cells were fixed for phospho-ERK immunostaining 1 h later. *DAPI*, ′-6-diamidino-2-phenylindole. *B*, quantitation of the percentage of cells displaying exclusion of phospho-ERK1/2 from the nucleus. One hundred cells were counted from three independent experiments.

cytoplasm. Upon stimulation by growth factors, ERK1/2 are phosphorylated by MEK (MAPKK), which induces their translocation to the nucleus (30). It has been proposed that MEK acts as a cytoplasmic anchor for inactive ERK1/2 and that their interaction is disrupted after phosphorylation of ERK1/2 by MEK, allowing the nuclear import of ERK1/2 (31). However, we think it is unlikely that MEK anchors ERK1/2 in senescent cells. MEK and ERK1/2 remained constitutively phosphoryl-

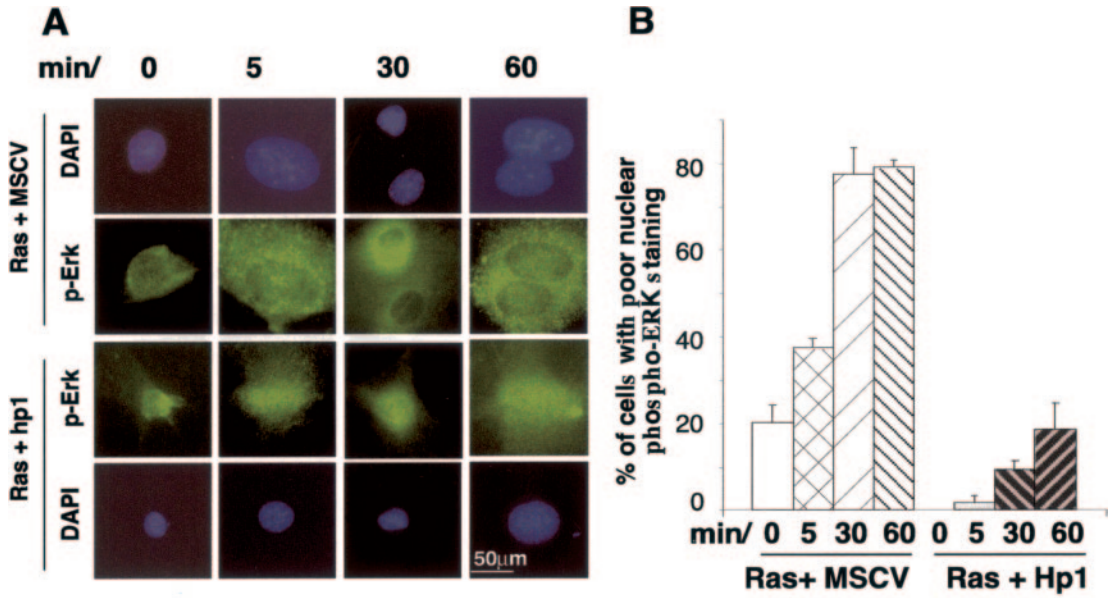

FIG. 7. **Kinetics of nuclear export after disabling PEA-15 with RNAi in MEFs.** MEFs expressing oncogenic *ras* (*Ras + MSCV*) or oncogenic *ras* plus anti-PEA-15 RNA hairpin (*Ras + hp1*) were plated on coverslips 10 days after infection. After serum starvation (0.5% serum) for 2 days, fresh serum was added, and the cells were treated with leptomycin B for 150 min. Then, leptomycin B was washed out with fresh medium, and the cells were fixed for phospho-ERK (*p-Erk*) immunostaining 5, 10, 30, and 60 min afterward. *A*, representative images. *DAPI*, ′-6-diamidino-2-phenylindole. *B*, quantitation of the percentage of cells displaying exclusion of phospho-ERK1/2 from the nucleus. More than 75 cells were analyzed at each time point.

ated during Ras-induced senescence and, therefore, were unable to form stable complexes in these cells. Cytoplasmic accumulation of phosphorylated ERKs was observed in cells stimulated with angiotensin II and treated with protein kinase C inhibitors (32). Interestingly, upon treatment with leptomycin B, ERK1/2 nuclear localization was restored (32). These observations are consistent with a model in which ERK1/2 are translocated to the nucleus after MEK-dependent phosphorylation and exported to the cytoplasm via CRM1 by a factor normally inhibited by protein kinase C activity. Intriguingly, PEA-15 is phosphorylated by protein kinase C (26), suggesting that it might be the protein kinase C-sensitive factor that controls ERK localization. Consistent with this idea, we have shown here that PEA-15 is required for ERK-nuclear export and for maintaining Ras-induced senescence. Also, the ability of E1A to block senescence correlated with a dramatic reduction of PEA-15 levels.

The factors controlling ERK localization are, for the most part, still unknown. In addition to nuclear export, several studies have indicated that ERKs can be retained in specific compartments by anchoring proteins. For example, the β-arrestins have been implicated in mediating a cytoplasmic sequestration of ERK1/2 (33), and unidentified nuclear anchoring proteins may sequester ERKs in the nucleus (34). For these reasons, it is not a surprise that knocking out PEA-15 expression in our experiments did not block entirely the process of ERK nuclear export or the ability of primary MEFs to enter oncogenic *ras*-induced senescence.

Genetically, MEFs with low PEA-15 levels behaved similarly to MEFs null for the candidate tumor suppressor Mnt. Mnt is a Max-interacting transcriptional repressor that suppresses Myc-dependent transactivation. Mnt null MEFs, like PEA-15-disabled MEFs, arrested proliferation in response to oncogenic *ras* but frequently escaped from senescence (35). Mnt-induced immortalization paralleled with inhibition of the expression of Myc target genes, including CDK4 (35), confirming the well known role of *myc* as an immortalizing oncogene (36). Whether PEA-15 or cytosolic ERK1/2 stimulate Mnt functions or block Myc activity requires further study.

A defective accumulation of ERK1/2 in the nucleus was reported previously in cells that entered senescence after serial passage (37, 38) or the introduction of oncogenic *ras* (37). In the study described by Park and colleagues, the reduction in nuclear ERK was accompanied by the nuclear accumulation of actin and Racl proteins and a marked induction of RhoA expression (37). To explain their observations, they suggested that senescent cells have a general defect in protein translocation across the nuclear membrane. This argument is in apparent conflict with our model proposing normal nuclear import followed by the active export of ERK1/2 from the nucleus by PEA-15. On the other hand, our results are entirely consistent with the idea proposed by both groups (37, 38) that a failure to accumulate ERK in the nucleus explains the lack of response to proliferative stimuli exhibited by senescence cells. It remains to be investigated whether cytoplasmic ERK signaling plays a role in senescence or cell cycle control.

Our work has important implications for the understanding of the interplay between factors supporting the transforming effect of oncogenic *ras* and factors that oppose it. It is well established that tumor suppressor pathways controlled by Rb, p53, and PML block Ras-dependent transformation via the establishment of a senescent cell cycle arrest (3, 10, 39–41). It is also well known that cellular transformation involves the suppression of the senescence program by different oncogenic activities (42, 43). One particularly powerful anti-senescence oncogene is the adenoviral oncoprotein E1A (3, 10). Unlike other oncoproteins such as papillomavirus E6/E7, E1A is sufficient to rescue normal fibroblasts from Ras-induced arrest (25). E1A, like E6, blocks the transcriptional activity of p53 (22) and, like E7, binds Rb blocking its ability to repress E2F-dependent promoters (23, 24, 44). Clearly, therefore, it would seem that E1A must have other activities conferring its unique ability to block Ras-induced senescence. Our data suggest that this new E1A activity results in down-regulation of the ERK1/2 nuclear export factor PEA-15. Thus, *PEA-15* is an E1A-modulated gene that normally provides a fail-safe mechanism to avoid constitutive activation of the MAP kinase pathway. As a consequence, we

predict that PEA-15 may have a tumor suppressor activity, particularly, in tumors driven by oncogenic *ras*.

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