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Involvement of MINK, a Ste20 Family Kinase, in Ras Oncogene-Induced Growth Arrest in Human Ovarian Surface Epithelial Cells

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Summary

The ability of activated Ras to induce growth arrest of human ovarian surface epithelial (HOSE) cells via induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ has been used to screen for Ras pathway signaling components using a library of RNA interference (RNAi) vectors targeting the kinome. Two known Ras-regulated kinases were identified, phosphoinositide 3-kinase p110α and ribosomal protein S6 kinase $p70^{S6K1}$, plus the MAP kinase kinase kinase kinase MINK, which had not previously been implicated in Ras signaling. MINK is activated after Ras induction via a mechanism involving reactive oxygen species and mediates stimulation of the stress-activated protein kinase p38 MAPK downstream of the Raf/ERK pathway. p38 MAPK activation is essential for Ras-induced $p21^{WAF1/CIP1}$ upregulation and cell cycle arrest. MINK is thus a distal target of Ras signaling in the induction of a growth-arrested, senescent-like phenotype that may act to oppose oncogenic transformation in HOSE cells.

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Introduction

Activating mutations in the *RAS* oncogenes are involved in the formation of as many as a third of all human tumors (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Whereas the early signaling events triggered by Ras protein in its active, GTP bound state have been studied in great detail (Downward, 2003), later steps in the Ras-regulated pathways are less well understood. In order to identify components of signaling pathways that play essential functions in changing cellular phenotype, RNAi (Downward, 2004a) has been employed recently as a tool for genetic screens in mammalian cells in tissue culture (Downward, 2004b). Two large-scale retroviral libraries of RNAi vectors have been created that target sizeable fractions of the human genome (Berns et al., 2004; Paddison et al., 2004). These have been employed in a high throughput, gene-by-gene analysis of the function of the proteasome (Paddison et al., 2004) and in a selective screen by using large pools of vectors targeting different genes to seek novel regulators of p53 signaling (Berns et al., 2004). Synthetic short interfering RNA oligonucleotide collections have also been used in high throughput screens, for example to study apoptosis regulation (Aza-Blanc et al., 2003).

It has recently proved possible to identify potential negative regulators of transformation by the application of selective RNAi screens to normal immortalized cells, looking for appearance of cells capable of anchorage-independent growth (Kolfschoten et al., 2005; Westbrook et al., 2005). We decided to use a different end result of the Ras signaling system as the basis for a selective screen: oncogene-induced growth arrest. In some untransformed cell types in tissue culture, ectopic expression of activated mutant Ras can lead not to transformation but to a profound growth arrest (Serrano et al., 1997). This can show some similarity to replicative senescence and has on occasion been termed premature senescence or stasis (Campisi, 2005).

Ras oncogene-induced growth arrest has been reported to involve upregulation of various cell cycle regulators and checkpoint proteins, including p53, $p16^{INK4A}$, ARF, and $p21^{WAF1/CIP1}$ (Lowe et al., 2004). The exact mechanism involved varies significantly between cell types and between species, particularly human and mouse (Drayton et al., 2004; Serrano and Blasco, 2001). There exists continuing controversy as to whether oncogene-induced growth arrest is a physiological protective mechanism for reducing the incidence of cancer in mammals by disabling cells that have begun to acquire some of the multiple mutations required to establish tumorigenesis, or whether it is just an artifact of overexpression of potent signaling molecules in serum and oxygen-rich tissue culture systems (Campisi, 2005), although recent evidence has strengthened the case for its in vivo significance (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). Whichever scenario turns out to be correct, Ras oncogene-induced growth arrest can be used as a powerful selection to allow screening for novel molecules that may be involved

either in Ras-induced signaling or in the establishment of senescence after cellular stress.

We report here a HOSE cell system in which the inducible expression of oncogenic Ras leads to irreversible growth arrest. These cells have been used in a selective RNAi screen for resistance to Ras-induced growth arrest by using a subset of the Netherlands Cancer Institute RNAi library (Berns et al., 2004) targeting 654 human genes, including all known protein kinases. Of three verified hits, two represent known direct or indirect targets of Ras signaling: phosphoinositide 3-OH (PI 3) kinase p110α and ribosomal protein S6 kinase p70^{S6K1}. The third hit, MINK, a Ste20/germinal center kinase family MAP kinase kinase kinase kinase, has not been implicated in Ras signaling previously. A pathway is elucidated in ovarian epithelial cells whereby Ras activates MINK via Raf/ERK with delayed kinetics through mechanisms involving reactive oxygen species. MINK then upregulates the activity of the p38 stress-activated kinase through MAP3K5/8 and MKK3/6, leading to p53-independent induction of p21$^{WAF1/CIP1}$ and permanent cell cycle arrest. Whereas PI 3-kinase and S6 kinase are both involved in Ras-induced transformation as well as growth arrest, MINK appears to play a role only in the growth arrest response and thus could potentially have tumor suppressor function.

Results

Expression of Oncogenic Ras Induces Permanent Growth Arrest in HOSE Cells

HOSE cells expressing the human papilloma virus oncogenes E6 and E7 have a lifespan of about 25 population doublings (PD) (Mok et al., 1996; Tsao et al., 1995). To prolong this lifespan, we introduced hTERT into HOSE 642-1 cells at passage five. The resulting cells grew continuously for more than 100 PD (data not shown). We cotransfected these cells with pcDNA6/TR and pcDNA4/TO-V12 H-Ras and thereby obtained cell clones in which the expression of V12 H-Ras was under the control of a tetracycline-inducible promoter (referred to as HOSE V12 H-Ras cells). Unexpectedly, inducible expression of constitutively active H-Ras in HOSE cells expressing hTERT, E6, and E7 led to a flat, enlarged, and vacuolated cell morphology within 48 hr in the majority of the cells (Figure 1A). These are known signs of a senescent-like phenotype. This change in morphology is accompanied by a profound cell proliferation arrest, with accumulation of cells in both G1 and G2/M phases of the cell cycle (Figure 1B). Long-term treatment of HOSE V12 H-Ras cells with doxycycline (dox) to induce expression of V12 H-Ras caused a permanent growth arrest as shown by counting cell numbers and by clonogenic assays (Figure 1C). Removal of dox did not result in a significant number of arrested HOSE V12 H-Ras cells reentering the cell cycle (data not shown).

p42/44 ERK, Akt, and GSK3 are all phosphorylated after a similar time course to the induction of Ras expression (Figure 1D), suggesting that expression of V12 H-Ras indeed activates known Ras signaling pathways such as Raf/ERK and PI 3-kinase/Akt in HOSE cells. To investigate the mechanism of the Ras-induced cell cycle arrest, the expression of various cell cycle regulators was studied after induction of Ras expression (Figure 1E). V12 H-Ras expression caused dephosphorylation of pRb, a decrease in cyclin A, and an increase in p21$^{WAF1/CIP1}$ levels, an effect first reported with Raf in murine fibroblasts (Sewing et al., 1997) and Schwann cells (Lloyd et al., 1997). Other cell cycle regulators known to play a role in oncogene-induced growth arrest in diploid fibroblasts include p53 and p16 (Lloyd et al., 1997; Serrano et al., 1997). However, in the HOSE cell system used here, p53 levels are low, presumably due to the presence of HPV E6 and E6AP; if anything, induction of V12 H-Ras expression leads to a slight decrease in p53 protein levels. There are also no significant changes in the levels of p16 and p15, whereas expression of another known Cdk inhibitor, p27, is decreased after induction of V12 H-Ras in HOSE cells.

Inhibition of the Ras-Induced Increase in p21$^{WAF1/CIP1}$ Overcomes Growth Arrest in HOSE Cells

To determine the role of p21$^{WAF1/CIP1}$ in the Ras-induced growth arrest in HOSE cells and to explore the possibility that it could be used as a positive control in an RNAi library screen for escape from Ras-induced growth arrest, we infected HOSE V12 H-Ras cells with pRETRO SUPER (pRS) retroviruses encoding short hairpin (sh) RNAs targeting p21$^{WAF1/CIP1}$ mRNA. After selection for retroviral integration, resistant cells were treated with dox to induce Ras expression for 14 days and analyzed after an additional 3 weeks of dox treatment. We found that pRS-p21$^{WAF1/CIP1}$-infected cells could bypass Ras-induced growth arrest relative to mock-infected cells, as seen by analyzing cell number and colony formation (Figures 2A and 2B). On analysis of the level of p21$^{WAF1/CIP1}$ mRNA and protein levels, it was clear that basal expression of p21$^{WAF1/CIP1}$ was only slightly reduced by the pRS-p21$^{WAF1/CIP1}$, with mRNA levels down by about 18% (Figure 2C). However, the p21$^{WAF1/CIP1}$ RNAi vectors did prevent the increase of p21$^{WAF1/CIP1}$ mRNA and protein levels induced after V12 H-Ras expression (Figures 2C and 2D). Presumably, this effect on Ras-induced, but not basal, p21$^{WAF1/CIP1}$ levels reflects the selective pressure on cells expressing Ras, which favors the outgrowth of cells having significant knockdown of p21$^{WAF1/CIP1}$. Thus, we conclude that the induction and maintenance of the V12 Ras-induced permanent growth arrest in HOSE cells is at least partially dependent on the upregulation of p21$^{WAF1/CIP1}$ through a p53-independent pathway.

A Selective RNAi Screen for Abrogation of Ras-Induced Growth Arrest in HOSE Cells

We used a retroviral RNAi library, constructed in pRS, with the HOSE V12 H-Ras cells as a system to identify genes required for Ras-induced growth arrest. The library used was a subset of the Netherlands Cancer Institute (NKI) library (Berns et al., 2004) targeting 654 human genes, including all known protein kinases (see Table S1 available in the Supplemental Data with this article online). Each gene was targeted by three different shRNA sequences. HOSE V12 H-Ras cells were infected with pools of retroviral mixtures targeting 96 mRNAs each. Cells were selected for expression of the shRNA vector by using puromycin for 7 days, and resistant cells were then treated with a single dose of 1 μg/ml dox. Whereas empty vector-infected control cells remained completely

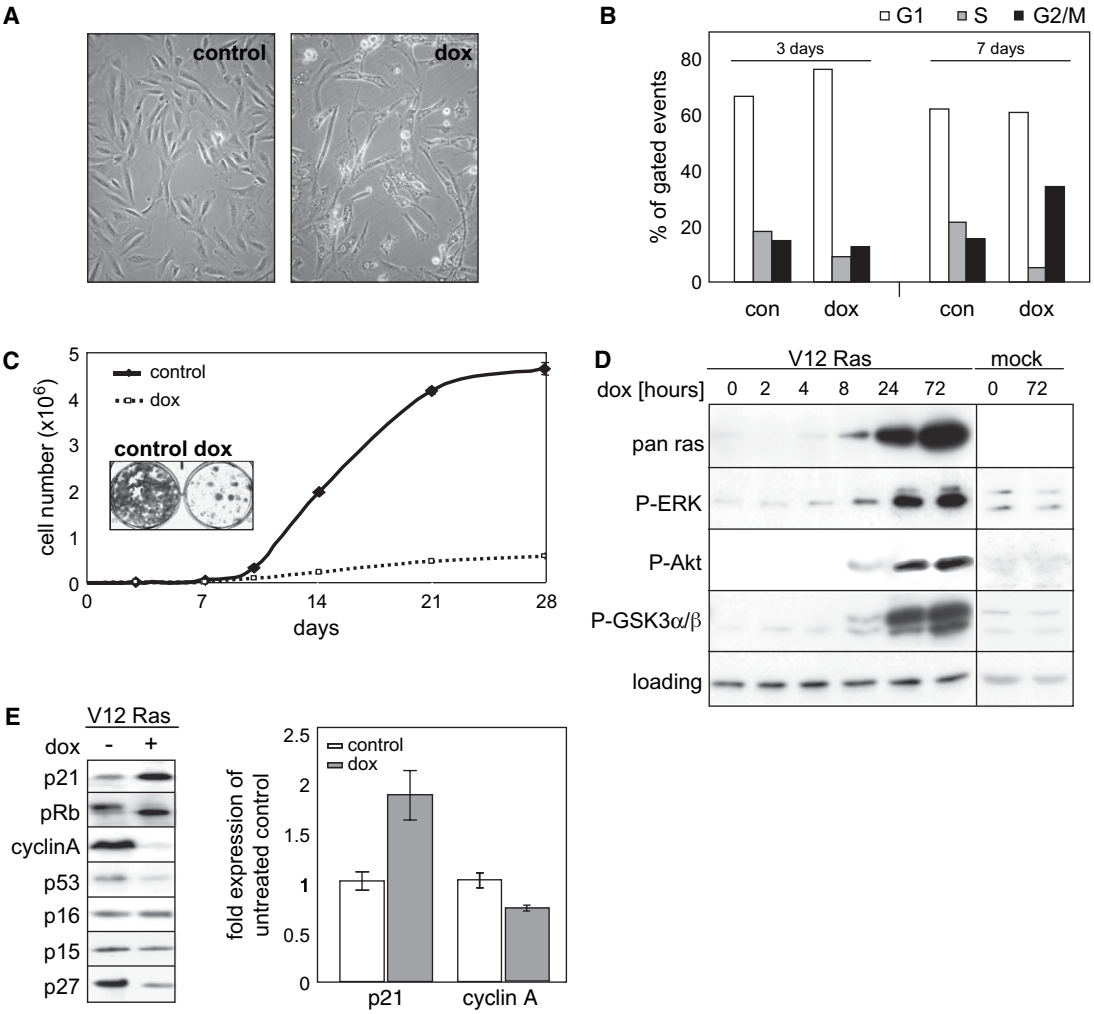

Figure 1. Expression of V12 H-Ras Induces Cell Cycle Arrest in Human Ovarian Surface Epithelial Cells

(A) Phase contrast micrographs of HOSE V12 H-Ras cells after 72 hr treatment with or without 1 ng/ml doxycycline (dox).

(B) Cell cycle distribution in HOSE cells after Ras induction. HOSE V12 H-Ras cells were treated with 1 ng/ml dox for the indicated times, and DNA content was analyzed by FACS. (Data shown are representative of three independent experiments.)

(C) HOSE V12 H-Ras cells were treated with or without 1 ng/ml dox, and cell numbers were counted at indicated time points. Inset, 500 HOSE V12 H-Ras cells were plated and treated with or without 1 ng/ml dox. After 21 days, cells were stained with crystal violet.

(D) HOSE V12 H-Ras cells were treated with or without 1 ng/ml dox for indicated times. Total extracts were immunoblotted with the indicated antibodies.

(E) HOSE V12 H-Ras cells were treated with 1 ng/ml dox for 48 hr. Total cell extracts were immunoblotted. Total RNA was used in quantitative RT-PCR analysis by using $p21^{WAF1/CIP1}$ and cyclin A-specific primers.

Error bars show standard deviation of the population from the arithmetic mean of at least three independent experiments.

growth arrested 3 weeks after treatment, we found five single cell clones growing out of the nonproliferating background. Sequencing of the retroviral shRNA inserts in these cells revealed that three of the five clones contained single retroviral integrations targeting the genes *RPS6KB1* (encoding ribosomal protein S6 kinase $p70^{S6K1}$), *PIK3CA* (encoding phosphoinositide 3-OH kinase catalytic subunit p110α), and *MINK* (encoding the Ste20/germinal center kinase family MAP kinase kinase kinase kinase MINK). The other clones contained multiple integrations, including the same sequence targeting *RPS6KB1* in one case. p110α is a known direct target of Ras (Rodriguez-Viciana et al., 1994), whereas $p70^{S6K1}$ is an indirect target that is regulated by phosphoinositide 3-OH kinase (PI 3-kinase) (Chung et al., 1994). MINK has not previously been implicated in Ras signaling (Dan et al., 2000, 2001).

To validate this result, we subcloned the identified shRNAs into pMig, a pRS derivative in which EGFP is expressed from an IRES after the puromycin resistance gene. These vectors were used to infect HOSE V12 H-Ras cells with viruses containing only the relevant sequences found in the Ras-resistant colonies. Puromycin-selected cell populations were FACS sorted, and high GFP fluorescence expressing cells were collected and analyzed for the efficiency of knockdown of their respective gene targets. A quantitative RT-PCR analysis (Figure 3A) showed that the shRNA sequences identified in the screen are indeed able to suppress target mRNA expression, although in the case of p110α and MINK,

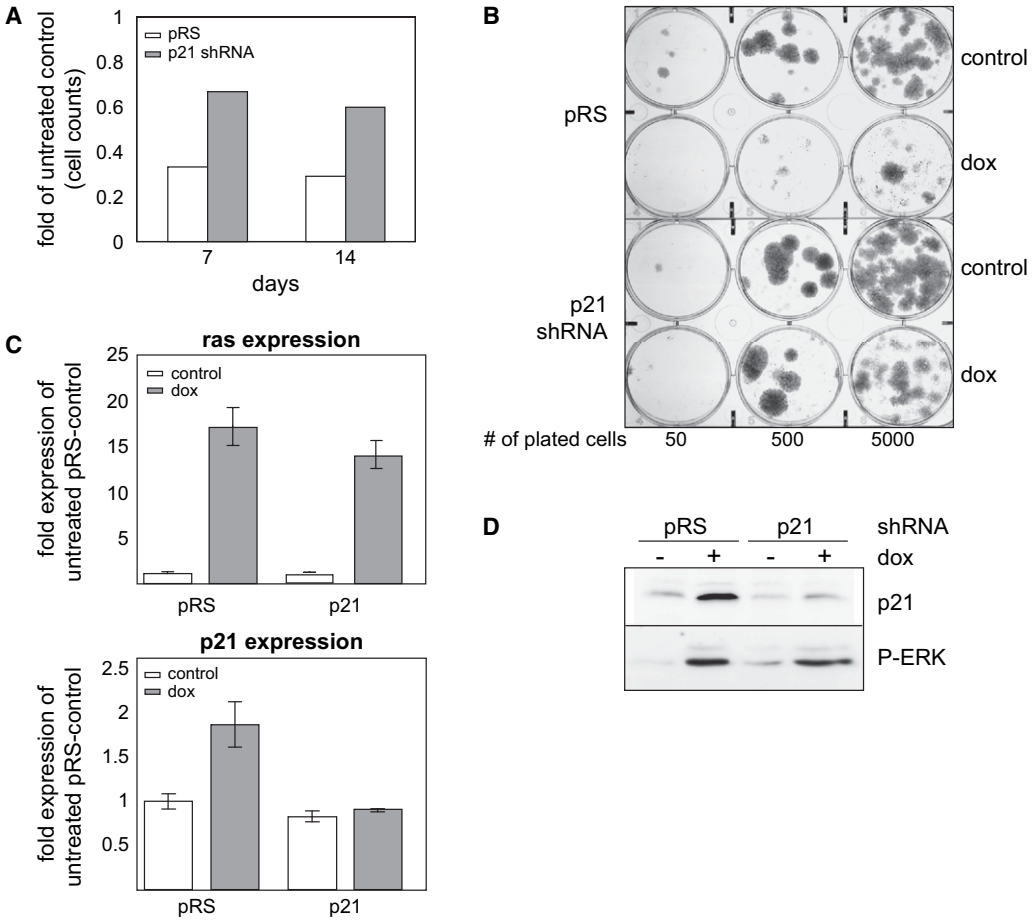

Figure 2. Reduction of p21$^{WAF1/CIP1}$ Expression Partially Prevents Ras-Induced Growth Arrest in HOSE Cells

HOSE V12 H-Ras cells were infected with a retroviral mixture targeting p21$^{WAF1/CIP1}$, selected for shRNA expression, then treated with 1 μg/ml dox for 14 days. Cells were then replated as appropriate: either at 200 cells/6 well for cell counting assay, at indicated cell numbers for clonogenic assays, or at 20,000 cells/10 cm plate for quantitative RT-PCR and Western blot analysis.

(A and B) HOSE V12 H-Ras cells expressing p21$^{WAF1/CIP1}$ siRNA were treated with or without 1 ng/ml dox, and cell numbers were counted at the indicated time points (A), or cells were stained with crystal violet after 21 days (B).

(C and D) HOSE V12 H-Ras cells were treated with 1 ng/ml dox for 21 days, and total RNA was used to perform quantitative RT-PCR (C) and total cell lysates to perform Western blot analysis (D) to detect p21$^{WAF1/CIP1}$ expression and ERK phosphorylation.

Error bars show standard deviation of the population from the arithmetic mean of at least three independent experiments.

the knockdown in the puromycin and GFP-selected pools at this stage is relatively modest, possibly reflecting heterogeneity within the population. To ensure that treatment of empty vector control and shRNA expressing cell populations with dox led to induction of comparable levels of Ras expression and subsequent phosphorylation of ERK1/2, we performed a Western blot analysis (Figure 3B). Next, we dox treated the selected cell populations and analyzed cell growth by clonogenic assay or cell number counting. We found that only about 1% of the control empty vector-infected cells had been able to bypass Ras-induced growth arrest, whereas 44% ± 15% of the p70^{S6K1} knockdown, 28% ± 11% of the p110α knockdown, and 43% ± 6% of the MINK knockdown cells proliferated despite activated Ras expression (Figures 3C and 3D). Checking the shRNA-induced knockdown of MINK after 5 weeks of dox treatment, we found that MINK expression is induced by Ras activation by about 2-fold and this upregulation is completely prevented by MINK-specific shRNA (Figure 3E). For dox-treated cells, the MINK shRNA containing cells express 26% of the level of MINK mRNA of control cells. The process of Ras induction has thus markedly selected for cells with more efficient knockdown of MINK, relative to the starting population of MINK shRNA expressing cells analyzed in Figure 3A.

To ensure that the observed phenotypic changes produced by the shRNA vectors were not caused by off-target effects, we employed predesigned synthetic double-stranded RNA oligonucleotides targeting p70^{S6K1}, p110α, and MINK. These siRNAs were able to transiently reduce the level of their target mRNAs (Figure 3F). We studied the effect of these siRNAs on Ras-induced inhibition of cell cycle progression in a short-term ELISA for BrdU incorporation. HOSE V12 H-Ras cells were transfected with the specific siRNAs or an siRNA targeting GFP as a negative control, and 48 hr posttransfection, cells were treated with dox for an additional 72 hr. In control cells, Ras induction caused an inhibition of BrdU incorporation of 65% ± 4% (Figure 3G). Cells transfected with siRNA targeting p70^{S6K1}, p110α, or MINK had significantly less inhibition of BrdU incorporation

($p70^{S6K1}$, 23% ± 9%; p110α, 31% ± 5%; and MINK, 32% ± 8%). Furthermore, we found that inhibiting Ras-controlled signaling pathways by pretreating the cells with the chemical inhibitors LY294002, PD98059, or Rapamycin could also almost completely prevent the Ras-induced inhibition of BrdU incorporation (Figure 3H). These inhibitors implicate PI 3-kinase (LY294002), S6K (LY294002 and Rapamycin), and MEK (PD98059) in Ras-induced growth arrest in HOSE cells, independently confirming two of the three genes identified in the screen. The effects of the drugs in countering the short term growth arrest induced by Ras are more complete than with the siRNAs, probably reflecting redundancy at the protein level due to the presence of other isoforms of the genes targeted.

MINK belongs to a subgroup of GCK/STE20-like kinases, the other very closely related kinases being MAP4K4/HGK and TNIK. TNIK is 65% identical to MINK, with 89% and 90% identity in the kinase and citron-homology domains, respectively. Knocking down expression of TNIK by using siRNA oligos also greatly reduces Ras-induced growth arrest (Figure S1). It therefore appears highly likely that there is some redundancy between MINK and TNIK in the activation of Ras oncogene-induced arrest in HOSE cells.

Expression and Activation of MINK Is Induced by Ras Expression

Whereas PI 3-kinase p110α and $p70^{S6K1}$ are known to be activated downstream of Ras, MINK has not previously been associated with Ras signaling. Therefore, we examined the effects of expression of activated Ras on MINK mRNA levels and kinase activity in HOSE cells. Dox treatment of HOSE V12 H-Ras cells to induce Ras expression led to an increase in MINK mRNA expression and protein levels by about 2-fold within 72 hr (Figures 4A and 4B). Additionally, in vitro kinase assays of MINK from HOSE cells exogenously expressing HA-tagged wt full-length MINK showed that V12 H-Ras expression also increases MINK kinase activity 2- to 3-fold (Figures 4C and 4D). Immunoprecipitates of a kinase-inactive mutant of MINK failed to phosphorylate the MBP substrate (data not shown). Thus, there may be independent stimulatory effects of Ras on both the expression and the kinase activity of MINK. To elucidate which signaling pathways might be involved in the Ras-induced activation of MINK, we performed in vitro kinase assays by using lysates from cells pretreated with the chemical inhibitors that we had found to prevent Ras-induced blockage of cell cycle progression (Figure 3H). UO126, another inhibitor of MEK, abrogated Ras-induced activation of MINK (Figure 4E), but both LY294002 and Rapamycin did not significantly affect the activation of MINK by Ras. Thus, activation of MINK after expression of activated Ras is mediated principally through the Raf/MEK/ERK signaling pathway in HOSE cells.

To determine whether activation of the Raf pathway was sufficient to induce MINK activation, we created HOSE cells with an inducible Raf construct, ΔRaf:ER*, the kinase activity of which is stimulatable by 4HT, but not natural estrogens (Bosch et al., 1997). Activation of Raf in HOSE cells increased MINK kinase activity significantly in a time-dependent manner (Figure 4F), supporting the model of Ras-induced activation of MINK via the Raf/MEK/ERK signaling pathway.

MINK Controls Ras-Induced Growth Arrest through p38 Stress-Activated Kinase

How might MINK be involved in Ras-induced growth arrest in HOSE cells? MINK has been shown to be able to activate the p38 stress-activated protein kinase (Dan et al., 2000). Activation of p38 MAPK has been implicated in mediating premature senescence in response to high intensity oncogenic Ras signals in primary human fibroblasts (Deng et al., 2004). In our cell system, we could detect a significant increase in phosphorylation of p38 MAPK after activated Ras expression for 3 days, which is slightly delayed relative to the kinetics of ERK phosphorylation (Figure 5A). The induction of p38 MAPK phosphorylation followed the gradual increase in MINK protein levels (Figure 5A) and was inhibited by the knockdown of MINK by using MINK-specific siRNAs (Figure 5B). Knocking down MINK also markedly reduced the phosphorylation of the p38 MAPK activating kinases MKK3/6 (Figure 5B), but not ERK phosphorylation or Ras expression. Looking at the effects of MINK knockdown on the expression of cell cycle regulators, there was partial inhibition of both the Ras-induced increase in $p21^{WAF1/CIP1}$ and decrease in cyclin A levels (Figure 5B). These data implicate a role for MINK in the inhibition of proliferation by activated Ras in HOSE cells through its ability to activate the p38 MAPK stress kinase and increase $p21^{WAF1/CIP1}$ expression.

To elucidate further the role of p38 MAPK activation in the Ras-induced growth arrest in HOSE cells, we transfected HOSE V12 H-Ras cells with specific siRNAs targeting both MKK3 and MKK6, the two MAP kinase kinases responsible for activating the p38 MAP kinases. Knockdown of MKK3/6 completely abolished the ability of activated Ras to induce phosphorylation of p38 MAPK, increase $p21^{WAF1/CIP1}$ expression, and decrease cyclin A levels (Figure 5C). Furthermore, in cells where MKK3/6 expression had been knocked down, we observed markedly reduced inhibition of BrdU incorporation into DNA after 3 days of dox treatment compared to control cells transfected with GFP siRNA (Figure 5D).

It has been shown that activation of p38 MAPK by a 4HT inducible form of the MAP kinase kinase kinase MEKK3, ΔMEKK3:ER*, induces cell cycle arrest in CCl39 cells and Rat-1 fibroblasts (Garner et al., 2002). We therefore tested the effect of MEKK3 activation in HOSE cells: activation of ΔMEKK3:ER* by 4HT also induced phosphorylation of p38 MAPK that was accompanied by induction of $p21^{WAF1/CIP1}$ expression and decrease of cyclin A protein levels (Figure S2A). We also detected a more than 90% inhibition of BrdU incorporation in HOSE ΔMEKK3:ER* cells after 48 hr of 100 nM 4HT treatment compared to no effect on cell cycle progression of 4HT on HOSE cells expressing a kinase-inactive form of ΔMEKK3:ER* (Figure S2B). Furthermore, we found that activation of ΔRaf:ER* by 4HT in the HOSE ΔRaf:ER* cells induced p38 MAPK phosphorylation within 72 hr of treatment (Figure S2C). Like oncogenic Ras, activation of Raf increased $p21^{WAF1/CIP1}$ protein levels and decreased cyclin A levels in HOSE cells (Figure S2C) and induced a cell cycle arrest, measured by inhibition of BrdU incorporation, of about 50% (Figure S2D).

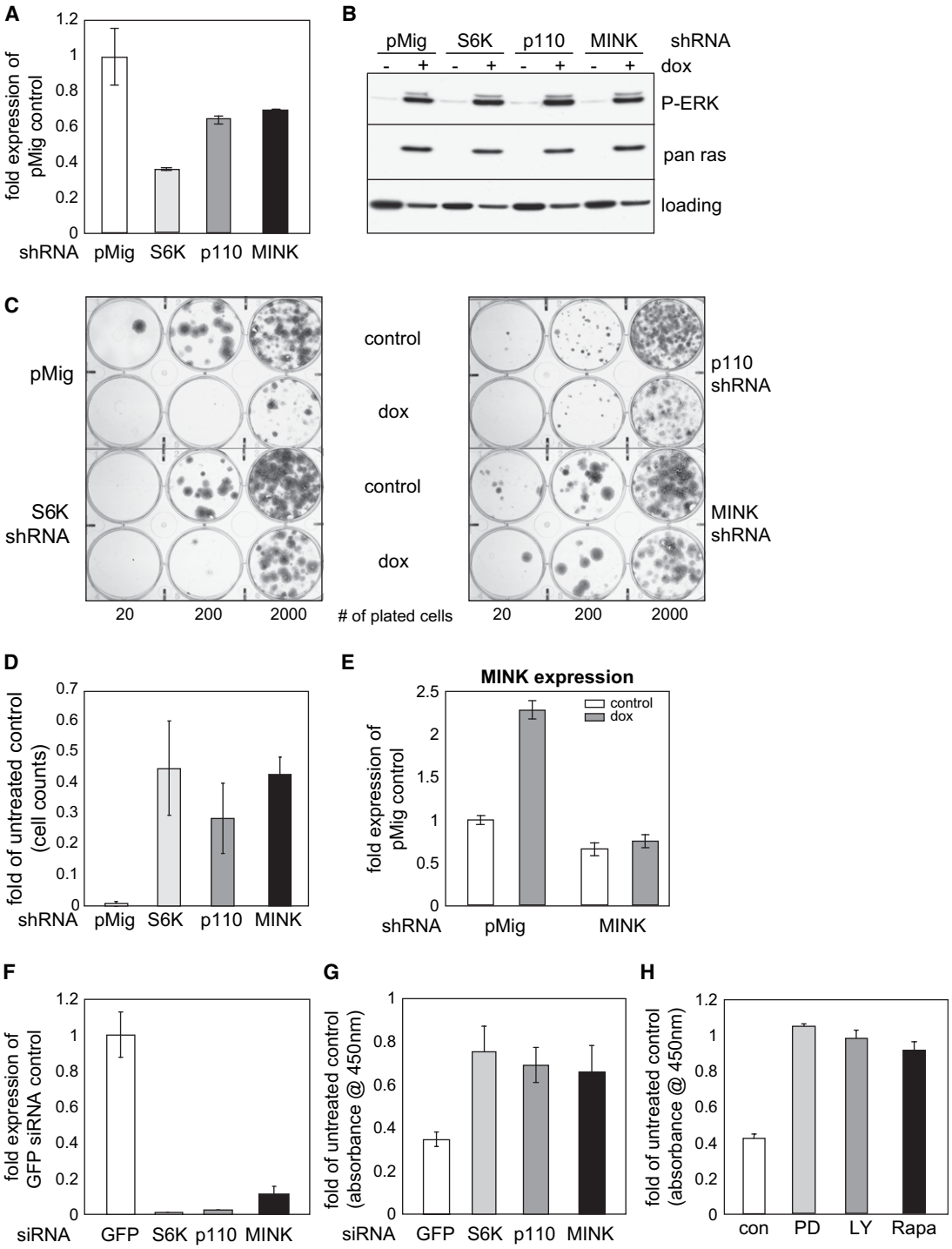

Figure 3. RNAi Library Screen for Escape from Ras-Induced Growth Arrest in HOSE Cells

(A) HOSE V12 H-Ras cells were infected with pMIG retroviruses targeting $p70^{S6K1}$, p110α, and MINK using the shRNA sequence identified in the RNAi library screen. Total RNA was used to perform quantitative RT-PCR by using specific primers for $p70^{S6K1}$, p110α, and MINK. (Data shown are representative of three independent experiments.)

(B–E) Puromycin-resistant HOSE V12 H-Ras cell populations expressing the shRNA sequences identified in the RNAi library screen were FACS sorted for GFP expression and 10^6 of these selected cells/10 cm dish were subsequently treated with 1 μg/ml dox for 14 days. Cells were then plated either at indicated cell numbers for clonogenic assays, at 200 cells/6 well for cell counting assay, or at 20,000 cells/10 cm plate for Western blot and quantitative RT-PCR analysis and treated with or without 1 ng/ml dox. After 21 days, total cell lysates were used in Western blot analysis to detect Ras and phospho-ERK (B), cells were stained with crystal violet (C), cell numbers were counted (D), or total RNA used in quantitative RT-PCR to detect MINK expression (E).

(F and G) HOSE V12 H-Ras cells were transfected with the specific siRNA oligos (62.5 nM), cultured in complete media, and retransfected after 24 hr. Forty-eight hours after the first transfection, cells were plated to be treated with or without 1 ng/ml dox for 72 hr and assayed by using

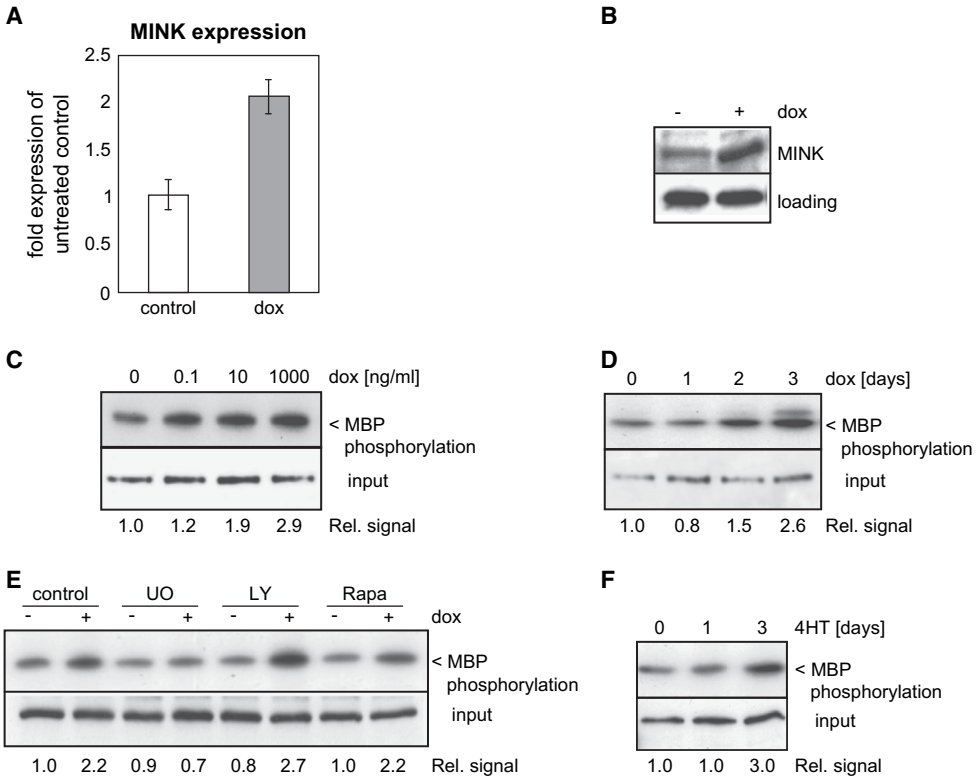

Figure 4. Activated Ras Expression Induces Expression and Kinase Activation of MINK

(A and B) HOSE V12 H-Ras cells were treated with or without 10 ng/ml dox for 72 hr. Total RNA was used in quantitative RT-PCR by using MINK-specific primers (A), or total cell lysates were used in Western blot analysis to detect MINK (B).

(C–E) HOSE V12 H-Ras cells were transfected with an HA-tagged wild-type (wt) MINK expression construct and treated with different concentrations of dox for 72 hr (C), with or without 10 ng/ml dox for the indicated times (D) or for 72 hr with or without 10 ng/ml dox after pretreatment with 10 μM of the MEK inhibitor UO126 (UO), 10 μM of the PI 3-K inhibitor LY294002 (LY), or 50 nM of the mTOR inhibitor Rapamycin (Rapa) for 30 min (E). Cells were then lysed, and in vitro kinase assays were performed by using myelin basic protein (MBP) as a substrate in the presence of [γ-^{32}P]ATP. The reaction products were subjected to SDS-PAGE and autoradiography (top panels). Bottom panels, anti-HA antibody Western blot to detect tagged MINK. The radiolabeled MBP bands were analyzed by scanning and densitometry, the signal normalized to the loading control, and the ratio of stimulated versus unstimulated sample given below lane.

(F) HOSE V12 H-Ras cells were infected with the ΔRaf:ER* inducible Raf construct. Puromycin-resistant cells were transfected with HA-tagged wt MINK and treated with or without 100 nM 4-hydroxytamoxifen (4HT) for the indicated times. In vitro kinase assay was then performed as above. Error bars show standard deviation of the population from the arithmetic mean of at least three independent experiments.

To address whether the involvement of MINK in Ras-induced growth arrest is limited to ovarian epithelial cells, we also studied human diploid fibroblasts. In Hs68 cells overexpressing hTERT, activation of Raf1 using a 4 hydroxytamoxifen (4HT)-inducible estrogen receptor fusion protein leads to a major decrease in cell proliferation that is largely reversed by knockdown of MINK using RNAi (Figure S3). Raf induction of p38 activation is also reversed by MINK RNAi in these fibroblasts. It is therefore possible that MINK plays a role in p38-mediated growth arrest downstream of Ras and Raf in human primary fibroblasts as well as ovarian epithelial cells. In a different epithelial cell system, human lung small airway epithelial cells immortalized with SV40 large T and telomerase (Lundberg et al., 2002), the induction of expression of V12 K-Ras also leads to an inhibition of cell proliferation which, although rather slower than in HOSE cells, is reversed by reduction in MINK expression (data not shown).

To analyze the pathway linking MINK and p38 MAPK, systematic RNAi analysis was carried out on the 14 known members of the MAP3K family in the human genome, MAP3K1–MAP3K14 (HUGO nomenclature). As shown in Figures S4A and S4B, knockdown of two different MAP3Ks, MAP3K8 (Cot/Tpl2) and MAP3K5 (Ask1), relieves Ras-induced growth arrest. Neither of these appears to work in the upstream part of the pathway, as their knockdown does not result in reduction of ERK activation by Ras, although it does block p38 induction (Figure S4C). Ask1 has been reported to activate both p38 and JNK stress-activated kinases and to be influenced by oxidative stress through a number of mechanisms, making it a good candidate for linking MINK with p38 activation. By contrast, Cot/Tpl2 has not

quantitative RT-PCR for expression of the target genes (F) or BrdU ELISA for proliferation (G).

(H) HOSE V12 H-Ras cells were treated with or without the indicated chemical inhibitors (PD98059- PD, LY294002- LY, Rapamycin-Rapa) for 30 min prior to start of treatment with 1 ng/ml dox for further 72 hr. Cells were assayed by using an ELISA to detect BrdU incorporation.

Error bars show standard deviation of the population from the arithmetic mean of at least three independent experiments.

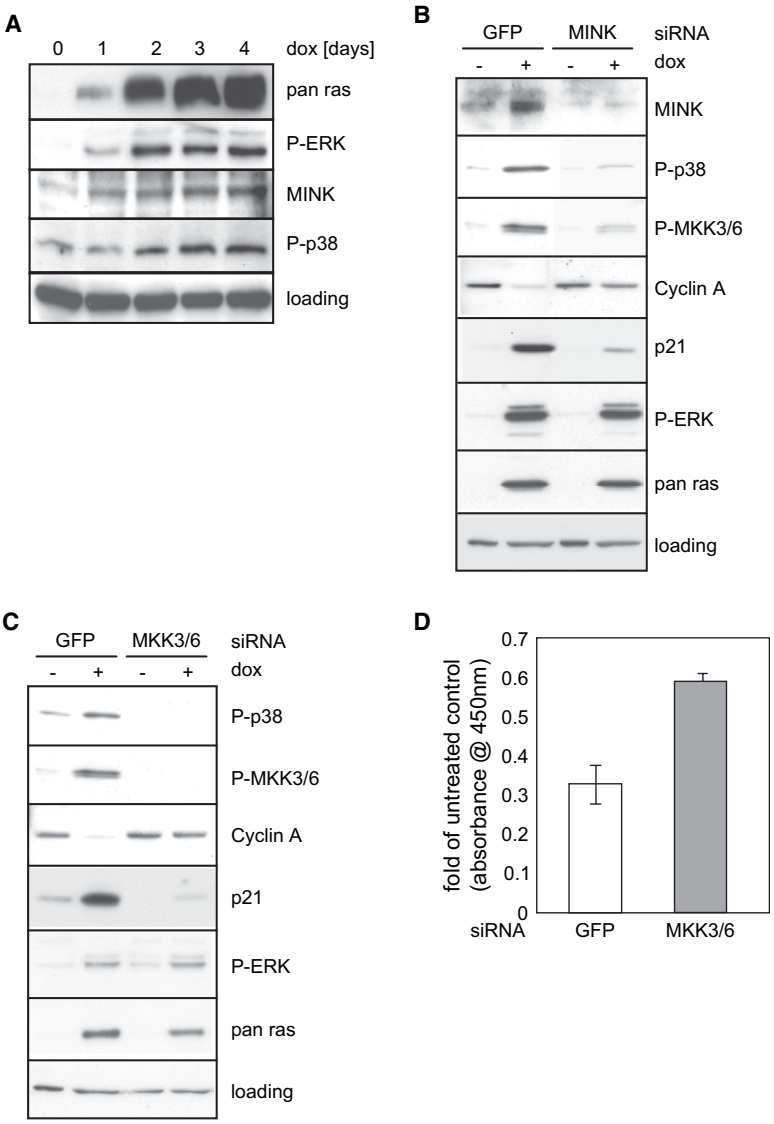

Figure 5. Ras-Induced Growth Arrest in HOSE V12 H-Ras Cells Involves MINK-Mediated Activation of p38 MAPK Downstream of the Raf/ERK Pathway

(A) HOSE V12 H-Ras cells were treated with or without 10 ng/ml dox for the indicated times, and total extracts were immunoblotted with the indicated antibodies.

(B) HOSE V12 H-Ras cells were double transfected on consecutive days with 62.5 nM siRNAs targeting MINK and GFP as a control. Forty-eight hours after the first transfection, cells were treated with or without 10 ng/ml dox for an additional 72 hr. Total cell lysates were then prepared and Western blot analysis performed.

(C and D) HOSE V12 H-Ras cells were double transfected with 62.5 nM siRNA targeting GFP as a control and MKK3 and MKK6 (with 31.25 nM each) on consecutive days. Forty-eight hours after the first transfection, cells were treated with 10 ng/ml dox for 72 hr and whole cell lysates were used to perform Western blot analysis (C) or assayed by using an ELISA to detect BrdU incorporation (D).

In (D), error bars show the standard deviation of the population from the arithmetic mean of five independent replicates.

previously been reported to have a significant role in the regulation of p38 activation, suggesting the data here could reflect a more indirect mechanism. Because knockdown efficiency was only tested for MAP3K5 and MAP3K8, it is possible that some of the other MAP3Ks could influence this pathway but are not being effectively targeted by the siRNA pools used here.

Ras-Induced Reactive Oxygen Species Promote Activation of MINK and p38 MAPK

Reactive oxygen species (ROS) are produced in response to Ras activation, with high ROS levels being associated with growth arrest and senescence (Campisi, 2005; Lee et al., 1999). We therefore analyzed the effect of Ras activation upon ROS generation, as detected by DCF-DA staining, in our cell system. V12 H-Ras HOSE cells exhibited a marked increase in the levels of cellular ROS within 24 hr of dox addition, and the increase became greater with extended treatment time (Figure 6A). Pretreatment of cells with the MEK inhibitor PD98059 completely abrogated Ras-induced ROS (Figure 6A), implying a critical role for the MEK/ERK pathway in this process. Pretreatment of cells with the antioxidant N-acetyl cysteine (NAC) also strongly inhibited Ras-induced ROS levels (Figure 6B), together with the appearance of the senescent cell morphology characteristic of HOSE cells after Ras-induced arrest (Figure 6C).

To assess further the effect of antioxidant addition upon indicators of Ras-induced growth arrest, we treated V12 H-Ras HOSE cells with NAC for 30 min prior to dox-induced Ras expression. Figures 6D and 6E illustrate that, whereas Ras expression and ERK phosphorylation are unaffected by NAC addition, MKK3/6, p38, and MINK kinase activation are all profoundly compromised by such treatment. An alternative antioxidant, pyrrolidine dithiocarbamate plus ascorbate, showed a similar effect (data not shown). Lastly, the direct addition of H_2O_2 to V12 H-Ras HOSE cells, in order to induce rapid oxidative stress, resulted in phosphorylation of p38 and MKK3/6 within 1 hr in a dose-dependent manner (Figure 6F). Consistent with this, MINK kinase activity was also rapidly induced in response to H_2O_2 treatment in both V12 H-Ras HOSE cells and COS-7 cells (Figure 6G). Taken together, these results suggest that activated Ras-induced cell

cycle arrest may be mediated through a sequential signaling pathway that involves activation of Raf/MEK/ERK, leading to ROS-mediated activation of MINK and the p38 stress kinase, resulting in $p21^{WAF1/CIP1}$ induction.

MINK Is Required for Ras-Induced Senescence, but Not Transformation

The fact that PI 3-kinase and $p70^{S6K1}$ were identified here as mediators of Ras-induced growth arrest in addition to their known roles in Ras-induced transformation raised the possibility that MINK might also function in both context-dependent outcomes of Ras signaling. However, we have failed to find any evidence that MINK function is required to maintain the transformed phenotype of several human tumor cell lines with activating mutations in their endogenous *K-Ras* gene. Stable knockdown of MINK expression by vector-based RNAi in several tumor cell lines carrying activating Ras mutations, such as A549, HCT116, HT29, and H1299, failed to compromise their transformed phenotype as measured by colony formation in soft agar (Figure S5), although knockdown of p110α and S6K1 does impair this to varying extents. Thus, reduction of MINK mRNA expression by about 80% does not affect the transformed phenotype of tumor cells with activated Ras. In addition, the establishment of the Ras transformed phenotype in immortalized cell lines is not affected by expression of either kinase inactive or wild-type (wt) MINK. Activated Ras expression constructs were cotransfected along with those encoding kinase dead or wt MINK, or control plasmids. Neither kinase dead MINK, which appears to have dominant-negative activity (data not shown) nor wt MINK had a significant impact on the level of Ras driven transformation of NIH 3T3 cells as measured in a focus formation assay. (V12 H-Ras + control plasmid, 29 ± 3 foci; V12 H-Ras + kinase dead MINK plasmid, 26 ± 3 foci; and V12 H-Ras + wt MINK plasmid, 29 ± 4 foci, n = 3.) To the extent that conclusions can be reached from negative data, these experiments suggest that MINK may not be required for Ras-induced transformation.

Discussion

Ras-Induced Growth Arrest in HOSE Cells

The ovarian epithelial cells used here expressing human papilloma virus E6 and E7 oncoproteins, the catalytic subunit of telomerase, and tetracycline-inducible activated Ras were originally made with the aim of providing an in vitro model for ovarian cancer. However, in contrast to experiences in other cell systems using immortalizing genes together with constitutive Ras expression (Hahn et al., 1999), induction of Ras activation caused growth arrest rather than transformation in this ovarian epithelial cell system. The induction of growth arrest by Ras in the presence of E6 suggests that p53 does not play a major role in the cell cycle arrest in these cells. This would be expected with HPV E6 present, which appears to be keeping p53 levels very low compared to the levels in the SV40 early region and telomerase immortalized IOSE 80 cells (data not shown). Indeed, Ras causes a slight decrease in p53 protein levels (Figure 1), perhaps through Ras induction of MDM2 via the Raf/ERK pathway (Ries et al., 2000). The use of p53 RNAi constructs also failed to impair Ras-induced growth arrest (data not shown). Ras here induces $p21^{WAF1/CIP1}$ expression in a p53-independent manner, and abrogation of this induction by RNAi alleviated Ras-induced arrest (Figures 1 and 2). In addition, there is no sign of p16 induction. It therefore appears that inducible expression of Ras leads to a growth arrest in this ovarian epithelial cell system by a mechanism that does not involve either p53 or p16, the two pathways most commonly associated with oncogene-induced growth arrest (Lloyd et al., 1997; Serrano et al., 1997; Sewing et al., 1997). A p53-independent Ras induction of $p21^{WAF1/CIP1}$-mediated cell cycle arrest has previously been reported in myeloid leukemia cells (Delgado et al., 2000).

MINK Signaling in Ras-Induced Growth Arrest

After activation of Ras and Raf, MINK expression and activity are induced relatively slowly over a few days. There are effects both on MINK transcription and on the activity of MINK protein expressed from an exogenous promoter (Figure 4). The delayed response indicates that the induction is likely to be indirect. MINK has been reported to activate both the JNK and p38 MAPK stress kinase pathways (Dan et al., 2000), and p38 MAPK has been implicated in cellular growth arrest in some systems (Haq et al., 2002; Wang et al., 2002), so it is tempting to speculate that the involvement of MINK in Ras-induced growth arrest in HOSE cells is due to its ability to control p38 MAPK, as confirmed in Figure 5. MINK appears likely to be activating p38 MAPK through downstream MAP kinase kinase kinases, such as MAP3K5 (Ask1), which in turn activate MKK3 and MKK6, which finally activate p38 MAPK.

Delayed induction of p38 MAPK downstream of the Raf pathway has been previously reported to play a role in Ras-induced premature senescence in fibroblasts (Bulavin et al., 2003; Deng et al., 2004; Wang et al., 2002). High, but not moderate, intensity prolonged activation of Ras, and Raf/ERK led to senescence in a p38 MAPK-dependent manner (Deng et al., 2004; Wang et al., 2002). p38 MAPK induction may also be important in causing cell senescence in response to a number of stresses in addition to expression of the Ras oncogene, such as oxidative stress, telomere shortening, DNA damage, and inappropriate culture conditions (Haq et al., 2002; Iwasa et al., 2003). Ras induction of p38 MAPK activation has been reported to limit the ability of Ras to transform rat intestinal epithelial cells, in contrast to Ras induction of ERK and JNK pathways, which promote transformation (Pruitt et al., 2002; Shields et al., 2002). Multiple mechanisms exist by which activation of p38 MAPK can inhibit cell proliferation (Bulavin and Fornace, 2004).

MINK may represent a missing link between prolonged Raf/ERK activation and p38 MAPK induction. Although the nature of the intermediate steps linking ERK activation and MINK induction are not known in detail at present, they are sensitive to conditions that reduce the level of reactive oxygen species produced after expression of activated Ras. Oxidative stress is necessary and sufficient for the activation of MINK downstream of Ras. Activated Ras expression has long been associated with elevation of the levels of ROS, both in the induction of cell proliferation (Irani et al., 1997) and, at higher ROS levels, cell senescence (Lee et al., 1999). Interestingly,

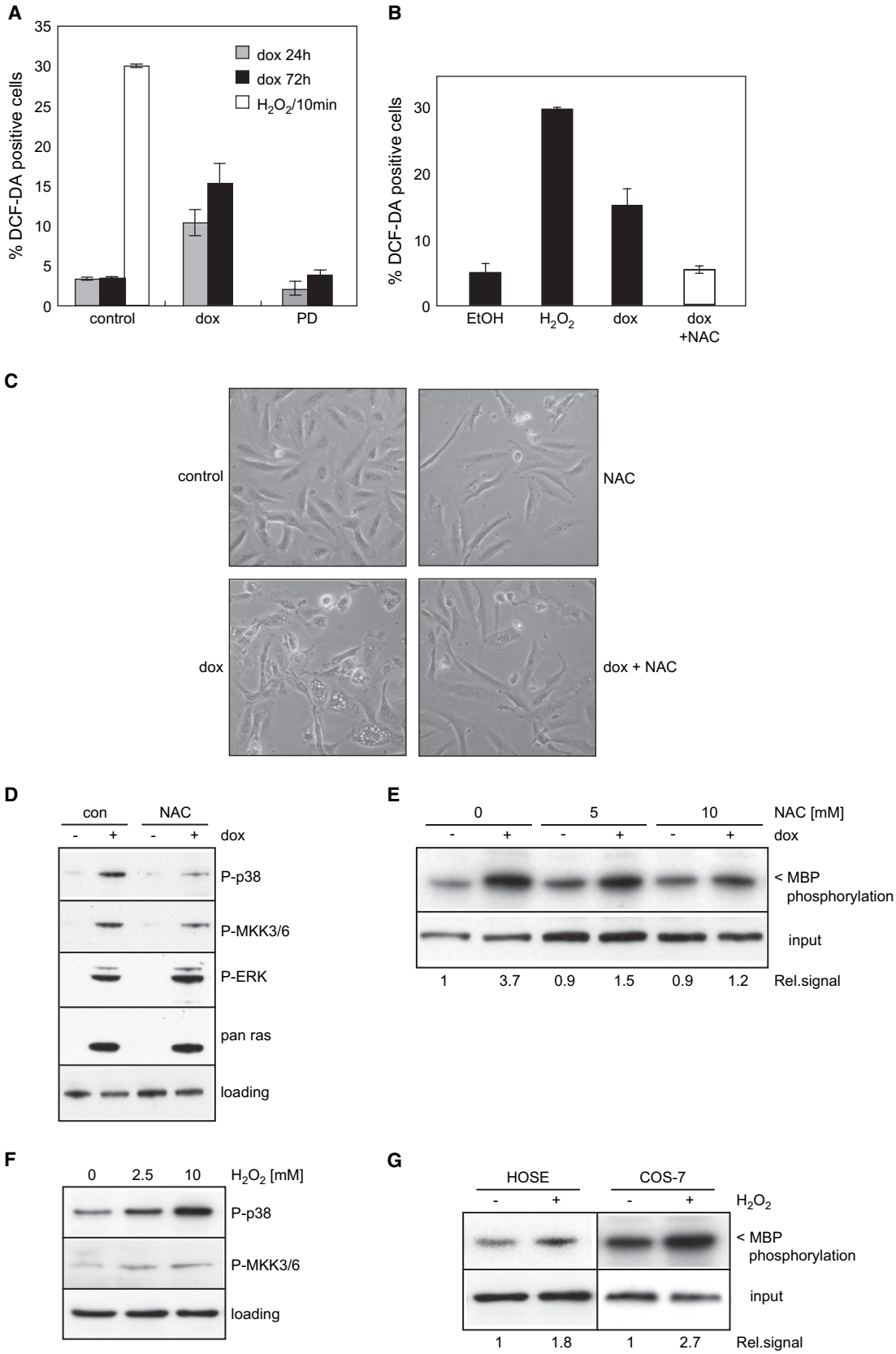

Figure 6. Induction of ROS Downstream of the Raf/ERK Pathway Is Essential for MINK and p38 MAPK Activation

(A) HOSE V12 H-Ras cells were treated with or without 1 μg/ml dox for the indicated times after pretreatment or not with 10 μM of the MEK inhibitor PD98059 (PD), and reactive oxygen species (ROS) levels were analyzed by FACS.

a recently reported genetic suppressor element screen in rat embryo fibroblasts found the oxidoreductase Seladin-1 as a necessary component of Ras-induced, p53-dependent senescence (Wu et al., 2004). The induction of MINK activity after Ras activation could perhaps result from inhibition of oxidation-sensitive phosphatases (Rhee et al., 2000) or from redox-sensitive induction of autocrine production of cytokines (Haddad et al., 2001).

Screening for Regulators of Growth Arrest and Transformation

The screen of 654 human genes, including all protein kinases, for those with an essential role in Ras-induced growth arrest in HOSE cells yielded three verified hits: PI 3-kinase p110α, ribosomal protein S6 kinase p70^{S6K1}, and MINK. Of these, p110α and p70^{S6K1} have been well characterized as playing important roles in malignant transformation, acting downstream of Ras and other oncogenes (Downward, 2003). PI 3-kinase and S6 kinase signaling have not previously been implicated in the induction of premature senescence by Ras, although the PI 3-kinase inhibitor LY294002 has previously been reported to block hydrogen peroxide-induced senescence in human diploid fibroblasts (Wang et al., 2004) and PTEN deletion has recently been associated with induction of p53-dependent senescence in mouse prostate glands (Chen et al., 2005). As early targets of Ras signaling, it is not unexpected that PI 3-kinase and p70^{S6K1} might play important roles in this response to expression of activated Ras. Although the mechanism underlying the involvement of PI 3-kinase and p70^{S6K1} in Ras-induced growth arrest was not explored in detail, it seems very likely that the initial signaling pathways regulated by Ras may be similar to those situations where transformation results. Nevertheless, the final phenotypic outcome for the cell is determined by signal integration with other inputs further down the pathways, along with effects of signal amplitude and duration.

The screen failed to identify components of the Raf/MEK/ERK pathway, despite the ability of PD98059 to block short-term Ras-induced growth arrest (Figure 3G). This could reflect redundancy in this pathway, ineffective targeting of pathway components by the shRNA vectors, or failure of inhibition of ERK to allow long-term clonogenic proliferation despite avoiding the short-term cell cycle arrest. Similarly, MKK3 and MKK6 were not picked up: use of siRNA oligos indicated that both need to be targeted simultaneously (data not shown). This failure raises the question of the success rate of the screen at picking up critical molecules in Ras-induced growth arrest signaling. Redundancy clearly has the potential to limit the success of the screen but is hard to avoid short of using high throughput screening with very small pools of RNAi vectors designed to target closely related proteins simultaneously. However, a more important issue here is likely to be the relative inefficiency of the RNAi: the use of large pools of vectors and stable selection in combination with the low number of retroviral integration events possible per cell means that the selection for the phenotype must normally be achieved by a single integrated retroviral vector Although RNAi can certainly provide gene silencing of this potency, it is likely that a proportion of the vectors in the library will be unable to provide sufficient levels of knockdown, even if they can show reasonable silencing in transient transfections of a single vector.

The fact that PI 3-kinase and p70^{S6K1} were identified here as mediators of Ras-induced growth arrest in addition to their known roles in Ras-induced transformation raised the possibility that MINK might also function in both context-dependent outcomes of Ras signaling. On the other hand, MINK is clearly acting much further downstream than PI 3-kinase or p70^{S6K1}, so it might act in pathways that are only involved in growth arrest and not in other aspects of Ras biology. We have failed to find any evidence that MINK function is required to maintain the transformed phenotype of several human tumor cell lines with activating mutations in their endogenous *K-Ras* gene. It seems, therefore, that MINK functions in Ras signaling below any point of commonality between transformation and growth arrest, or premature senescence, signaling. MINK is therefore likely to act only to mediate the growth inhibitory effects of Ras signaling and not the transformation promoting effects and is thus a potential tumor suppressor that could limit the potential of activated mutant Ras proteins to promote malignancy. We are currently investigating whether there is evidence for mutation or reduced expression of MINK or its close relatives in human tumors.

Experimental Procedures

RNAi Screen

DNAzol (Invitrogen) was used to extract genomic DNA from expanded colonies. pRS vector-specific primers were used to perform PCR amplification of the shRNA inserts, and products were subcloned by using the TA-cloning system (Invitrogen) followed by DNA

(B) ROS levels were assessed in cells treated for 72 hr with 10 ng/ml dox and with or without 10 mM of the antioxidant NAC for 1 hr.
In (A) and (B), as a positive control for ROS production, HOSE V12 H-Ras cells were treated with H_2O_2 for 10 min.
(C) The phase contrast micrographs of HOSE V12 H-Ras cells after 48 hr treatment with 10 ng/ml dox and with or without 10 mM of the antioxidant NAC.
(D) HOSE V12 H-Ras cells were treated with or without 10 mM of NAC for 30 min prior treatment with 10 ng/ml dox for further 72 hr. Whole-cell lysates were used to perform Western blot analysis.
(E) HOSE V12 H-Ras cells were transfected with an HA-tagged wt MINK expression construct and treated with or without 10 ng/ml dox for 48 hr after pretreatment with 5 or 10 mM of NAC for 30 minutes. In vitro kinase assays were performed with MBP as a substrate in the presence of [γ-^{32}P]ATP. The reaction products were subjected to SDS-PAGE and autoradiography.
(F) HOSE V12 H-Ras cells were treated with indicated concentrations of H_2O_2 for 1 hr, and total cell extracts were immunoblotted with the indicated antibodies.
(G) HOSE V12 H-Ras cells and COS-7 cells were transfected with an HA-tagged wt MINK expression construct and treated with or without 5 mM of H_2O_2. In vitro kinase assays were performed by using MBP as a substrate in the presence of [γ-^{32}P]ATP. The reaction products were subjected to SDS-PAGE and autoradiography.
In (E) and (G), relative signals were calculated as described for Figure 4C.
Error bars show standard deviation of the population from the arithmetic mean of at least three independent experiments.

sequencing. For more information on primers and pRS methodologies see http://www.screeninc.nl/.

Transfection of siRNA Oligos

A pool of four (SMART pools, Dharmacon) or three (Ambion) predesigned siRNA oligos per gene of interest were tested. The sequence that induced the strongest knockdown of mRNA expression (as validated by quantitative RT-PCR) was principally used for further experiments, after confirmation that at least one other sequence against the same gene had similar biological effects (sequences can be found in Table S2). Cells were transfected with 62.5 nM siRNA (Ambion) or 70 nM SMART pool siRNA (Dharmacon) using TransMessenger transfection reagent (Qiagen). After 24 hr, cells were retransfected by using the same protocol and, after a further 24 hr, were set up and treated as appropriate.

mRNA Analysis by Real-Time RT-PCR

Total RNA was extracted by using the RNAeasy system (Qiagen), and complementary DNA was synthesized (Invitrogen), used as a template for TaqMan real-time PCR analysis using SYBR-Green (Applied Biosystems) to measure relative transcript levels of target genes, and normalized to 18S RNA levels. Sequences of PCR primers can be found in Table S3.

Measurement of Intracellular ROS Levels

Cells were harvested by trypsinization and incubated with 50 μM DCF-DA for 30 min at 37°C before analysis by FACS using propidium iodide to counterstain and exclude dead cells. Cell Quest 3.2 (BD Biosciences) software was used for analysis.

Cell Transformation Assays

Colony formation assays in soft agar were carried out as described in Rodriguez-Viciana et al. (1997) by using tumor cell lines stably expressing pRS constructs targeting MINK, $p70^{S6K1}$, and the PI 3-kinase p110α (see Table S2). Effective knockdown was checked by RT-PCR. Focus formation assay in NIH 3T3 cells cotransfected with V12 H-Ras expression construct and dominant-negative or wt MINK were carried out as described in Rodriguez-Viciana et al. (1997).

For further detail of experimental methods, see the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://www.molecule.org/cgi/content/full/20/5/673/DC1/.

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