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The Molecular Scaffold Kinase Suppressor of Ras 1 Is a Modifier of Ras^{V12}-Induced and Replicative Senescence

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In primary mouse embryo fibroblasts (MEFs), oncogenic Ras induces growth arrest via Raf/MEK/extracellular signal-regulated kinase (ERK)-mediated activation of the p19^{ARF}/p53 and INK4/Rb tumor suppressor pathways. Ablation of these same pathways causes spontaneous immortalization in MEFs, and oncogenic transformation by Ras requires ablation of one or both of these pathways. We show that Kinase Suppressor of Ras 1 (KSR1), a molecular scaffold for the Raf/MEK/ERK cascade, is necessary for Ras^{V12}-induced senescence, and its disruption enhances primary MEF immortalization. Ras^{V12} failed to induce p53, p19^{ARF}, p16^{INK4a}, and p15^{INK4b} expression in KSR1^{-/-} MEFs and increased proliferation instead of causing growth arrest. Reintroduction of wild-type KSR1, but not a mutated KSR1 construct unable to bind activated ERK, rescued Ras^{V12}-induced senescence. On continuous culture, deletion of KSR1 accelerated the establishment of spontaneously immortalized cultures and increased the proportion of cultures escaping replicative crisis. Despite enhancing escape from both Ras^{V12}-induced and replicative senescence, however, both primary and immortalized KSR1^{-/-} MEFs are completely resistant to Ras^{V12}-induced transformation. These data show that escape from senescence is not necessarily a precursor for oncogenic transformation. Furthermore, these data indicate that KSR1 is a member of a unique class of proteins whose deletion blocks both senescence and transformation.

The Raf/MEK/extracellular signal-regulated kinase (ERK) cascade is one of several growth-regulatory pathways directly downstream of the small G-protein Ras. Ras was first identified as the transforming agent in Harvey murine sarcoma virus (63). Subsequent studies showed that mutations in Ras family members can lead to their constitutive activation, and these activated Ras proteins could be found in a variety of human cancers (3, 14, 51). In mammalian fibroblasts, activated Ras can cause either transformation or irreversible growth arrest, depending on the cellular context and the expression of cooperating oncogenes (11, 39, 59).

In primary murine fibroblasts, mutationally activated Ras (Ras^{V12}) leads to an irreversible growth arrest characterized by up-regulation of the tumor suppressor proteins p53 and p19^{ARF} and the cyclin-dependent kinase inhibitor p16^{INK4a} (50, 59). Primary cells arrested by activated Ras are phenotypically indistinguishable from those arrested by continuous culture (9, 27, 59). These cells have an enlarged, flattened morphology, stop proliferating at subconfluent cell densities, and express several markers of replicative senescence (59). In Ras^{V12}-expressing cells, inactivation of the p19^{ARF}/p53 pathway, either genetically or by introduction of viral oncogenes that inactivate p53-mediated growth arrest, bypasses cellular senescence and leads to transformation (22, 34, 39, 50, 58, 59). While deletion of p16^{INK4a} does not bypass Ras^{V12}-induced senescence in murine cells, disruption of p16^{INK4a} in human

cells allows for a bypass of Ras^{V12}-induced senescence (4, 6, 28, 29, 70). This could be due to both differences in regulation of the *INK4a/ARF* locus between mouse and human cells and the relative importance of different INK4 family members in mouse cells.

Ras^{V12} induces expression of both p16^{INK4a} and p19^{ARF} in primary mouse fibroblasts (50, 59), but Ras^{V12} cannot induce p14^{ARF} expression in human fibroblasts (21, 69). Differences in regulation of the *INK4a/ARF* locus in human and mouse cells are not completely understood. p15^{INK4b}, a related INK4 family member, is also regulated by Ras^{V12} in mouse fibroblasts. p15^{INK4b} is up-regulated by Ras^{V12}, and deletion of p15^{INK4b} abrogated Ras^{V12}-induced senescence in primary mouse embryo fibroblasts (MEFs) (40, 43). These data indicate that members of the INK4 family of proteins are important regulators of Ras^{V12}-induced senescence in both mouse and human fibroblasts.

Expression of constitutively activated Raf, constitutively activated MEK, or Ras effector loop mutants that activate Raf has shown that both the senescence-promoting (20, 41, 76) and oncogenic (12, 24, 44, 54, 68, 71) properties of Ras^{V12} can be replicated by activation of the Raf/MEK/ERK cascade. Expression of constitutively activated Raf or MEK in primary mouse or human fibroblasts causes cell cycle arrest, induction of p53 and p16^{INK4a}, and expression of senescence-associated (SA) β -galactosidase activity (20, 41, 76). Growth arrest induced by the Raf/MEK/ERK cascade relies primarily upon p53 signaling. The ability of constitutively activated Raf to cause growth arrest is dependent upon its ability to induce expression of p21^{CIP1} (60, 72). Furthermore, Raf/MEK/ERK signaling

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stabilizes p53 expression and can regulate whether p53 activation produces senescence or apoptosis (20).

Kinase Suppressor of Ras 1 (KSR1) is a molecular scaffold for the Raf/MEK/ERK cascade (37, 46, 49). KSR1 expression regulates the intensity and duration of growth factor-induced ERK activation to modulate a cell's proliferative, oncogenic, and adipogenic potential (36, 37, 55). Importantly, immortalized KSR1^{-/-} MEFs are resistant to oncogenic transformation by Ras^{V12}, which can be rescued by ectopic expression of KSR1 (37). Deletion of KSR1 blocks Ras^{V12}-induced ERK activation but not activation of other Ras effector pathways, indicating that KSR1-scaffolded ERK activation is necessary for Ras^{V12}-induced transformation (37).

Given the necessity of KSR1 expression in Ras^{V12}-induced transformation in immortalized MEFs, we assessed whether KSR1 was necessary for Ras^{V12}-induced senescence in primary MEFs. Ras^{V12}-induced senescence and replicative senescence are phenotypically similar and require many of the same pathways. Therefore, we also assessed whether KSR1 could play a role in cellular immortalization. We show that KSR1 is necessary for Ras^{V12}-induced senescence and that its deletion accelerates 3T9-mediated immortalization of primary MEFs. KSR1^{-/-} MEFs were resistant to Ras^{V12}-induced senescence and instead showed increased proliferation. Furthermore, Ras^{V12} failed to induce p53, p19^{ARF}, p16^{INK4a}, and p15^{INK4b} expression in primary KSR1^{-/-} MEFs. Reintroduction of wild-type KSR1, but not a mutated KSR1 construct unable to bind activated ERK, restored Ras^{V12}-induced senescence. On continuous culture, deletion of KSR1 accelerated spontaneous immortalization and increased the number of primary cultures escaping replicative crisis. Coupled to the observation that immortalized KSR1^{-/-} MEFs are resistant to Ras^{V12}-induced transformation (37, 42), these data demonstrate that KSR1-scaffolded ERK activity is necessary for Ras^{V12}-induced and replicative senescence and for transformation.

MATERIALS AND METHODS

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM minimal essential medium nonessential amino acids, 1% penicillin-streptomycin, and 55 μ M β -mercaptoethanol. Cells were incubated at 37°C in 5% CO₂.

Construction and production of recombinant retroviruses. KSR1-FLAG, KSR1.C809Y-FLAG, and KSR1.FSFP/AAAP-FLAG were subcloned from the EcoRI and SalI sites of pCMV5 (33) into the EcoRI and XhoI sites of MSCV-IRES-GFP. MSCV-IRES-GFP, MSCV-KSR1-IRES-GFP, MSCV-KSR1.C809Y-IRES-GFP, MSCV-KSR1.FSFP/AAAP-IRES-GFP, pBabePuroRas^{V12}, pBabePuroMEKEE, or pBabePuro retroviral vectors were cotransfected with an ectopic packaging vector into 293T cells. At 48 to 72 h posttransfection, viral supernatants were collected and filtered. Viral supernatants were then either stored at -80°C or used immediately to infect cells. Puromycin-resistant cells were selected with 4 μ g/ml puromycin (Sigma).

Generation of cell lines. Nonimmortalized MEFs were generated from day 13.5 KSR1^{-/-} and KSR1^{+/+} embryos as previously described (49). To assess cellular immortalization, cells were maintained in culture under a 3T9 protocol (67) until immortalized populations of cells emerged. To assess immortalization of MEFs after plating at low density, 2 \times 10⁴ passage 9 KSR1^{-/-}, KSR1^{+/-}, or KSR1^{+/+} MEFs were seeded in a 60-mm dish and fed for 4 weeks until immortalized colonies emerged. To produce KSR1^{-/-} MEFs expressing ectopic KSR1 constructs, KSR1^{-/-} MEFs were infected with MSCV-KSR1-IRES-GFP, MSCV-KSR1.C809Y-IRES-GFP, MSCV-KSR1.FSFP/AAAP-IRES-GFP, or MSCV-IRES-GFP control vector. Fluorescence was detected by flow cytometry, and cells were separated for low-positive levels of fluorescence. Cells were excited at 488 nm and separated at 530/20 nm, with the baseline fluorescence of uninfected cells having a mean intensity of 6 (range, 0 to 15), and KSR1-expressing cells having

a mean intensity of 63 (range, 21 to 101). Postsorted cells were assessed for purity by fluorescence-activated cell sorter (FACS) analysis. Collected pools of cells were grown in culture and assessed for KSR1 expression level by Western blotting.

Proliferation studies. To assess cellular senescence, cells were seeded at 1 \times 10⁴ cells per well in 24-well plates or 5 \times 10⁴ cells per 35-mm dish. Triplicate dishes were counted 3 h after seeding to account for plating discrepancies and were then assessed every 48 to 72 h for total cell number on a Beckman Coulter Counter or by trypan blue exclusion. To assess low-density growth during immortalization, cells were seeded at 2 \times 10⁴ cells per 35-mm dish. Triplicate dishes from three independent KSR1^{-/-} and KSR1^{+/+} cultures were counted 3 h after seeding to account for plating discrepancies and were then assessed every 24 h (passages 5, 10, and 15) or every 72 h (passage 18) for total cell number on a Beckman Coulter Counter.

SA β -galactosidase activity. SA β -galactosidase activity was measured as previously described (16). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and fixed for 5 min in 2% formaldehyde-0.2% glutaraldehyde, washed twice with PBS, and stained with SA β -galactosidase staining solution (1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl β -D-galactosidase] [stock of 20 mg/ml in dimethylformamide], 40 mM citric acid-sodium phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂). Cells were incubated overnight at 37°C in the absence of CO₂. Cells were visualized the following day and quantified (minimum of 50 cells per trial) for the presence or absence of staining.

Radiation response. Passage 4 KSR1^{-/-} or KSR1^{+/+} MEFs (1 \times 10⁶) were plated in complete media 24 h prior to analysis. Cells were irradiated with 8 Gy and then incubated at 37°C for the indicated times prior to lysis and Western blot analysis.

Transformation assays. MEFs stably expressing Ras^{V12} and KSR1 or control vectors were seeded in 0.32% Nobel agar at 1 \times 10⁴ cells per 35-mm dish to assess anchorage-independent growth or seeded at 1 \times 10⁵ cells per 10-cm dish to assess loss of contact inhibition. Colonies were counted, photomicrographs were taken, and dishes were stained with Wright-Giemsa 14 to 28 days after seeding.

In situ ERK activation assay. Cells were seeded at 1.5 \times 10⁴ cells/well in a 96-well plate 24 h prior to analysis and subjected to an in situ plate assay using a Li-Cor Odyssey infrared imaging system to quantify ERK activation. Cells at 70% confluence were deprived of serum for 4 h and treated with 25 ng/ml platelet-derived growth factor (PDGF) in Dulbecco's modified Eagle's medium-1% bovine serum albumin for 5 min. Anti-phospho-ERK1/2 (Cell Signaling) (1:100) and anti-ERK1 (Santa Cruz, 1:100) primary antibodies and anti-mouse Alexa Fluor 680-conjugated (Molecular Probes) (1:100) and anti-rabbit IRDye800-conjugated (Rockland) (1:100) secondary antibodies were used to detect and quantify phosphorylated and total ERK protein levels.

Lysate preparation and Western blotting. Cells were treated with trypsin and pelleted. Pellets were washed twice with PBS and frozen at -80°C. Frozen pellets were sonicated (2 \times 7 s) in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.4 U/ml aprotinin, 1 mM NaF, 10 mM β -glycerophosphate, 0.1 mM sodium orthovanadate). Following sedimentation of undissolved cellular material by centrifugation (Sorvall Biofuge) (4°C, 7 min, 14,000 rpm), lysates were assayed for protein concentration by use of a DC protein assay (Bio-Rad). A 50- μ g volume of total protein was loaded per well, and lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Western blot analysis was developed using the following primary and secondary antibodies (antibodies were from Santa Cruz unless otherwise noted): anti-p53 (Ab-7; Calbiochem) (1:2,500), anti-phospho-p53 (Ser¹⁸) (Cell Signaling) (1:1,000), anti-p19^{ARF} (Abcam) (1:400), anti-p15^{INK4b} (Biosource) (1:400), anti-MEK1/2 (Cell Signaling) (1:1,000), anti-phosphoMEK (Cell Signaling) (1:1,000), anti-phospho-ERK1/2 (Cell Signaling) (1:1,000), anti-Ras (Oncogene) (1:1,000), anti-PML (Upstate) (1:800), or antitubulin (Sigma) (1:1,000). Anti-mouse, anti-rabbit, anti-sheep, and anti-goat secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes) (1:3,000) and IRDye800 (Rockland) (1:3,000) were used to probe primary antibodies. Protein bands were detected and quantified on a Li-Cor Odyssey infrared imaging system.

RESULTS

KSR1 is necessary for Ras^{V12}-induced senescence. Expression of oncogenic Ras causes cellular senescence in primary MEFs via activation of the Raf/MEK/ERK cascade (9, 20, 41,

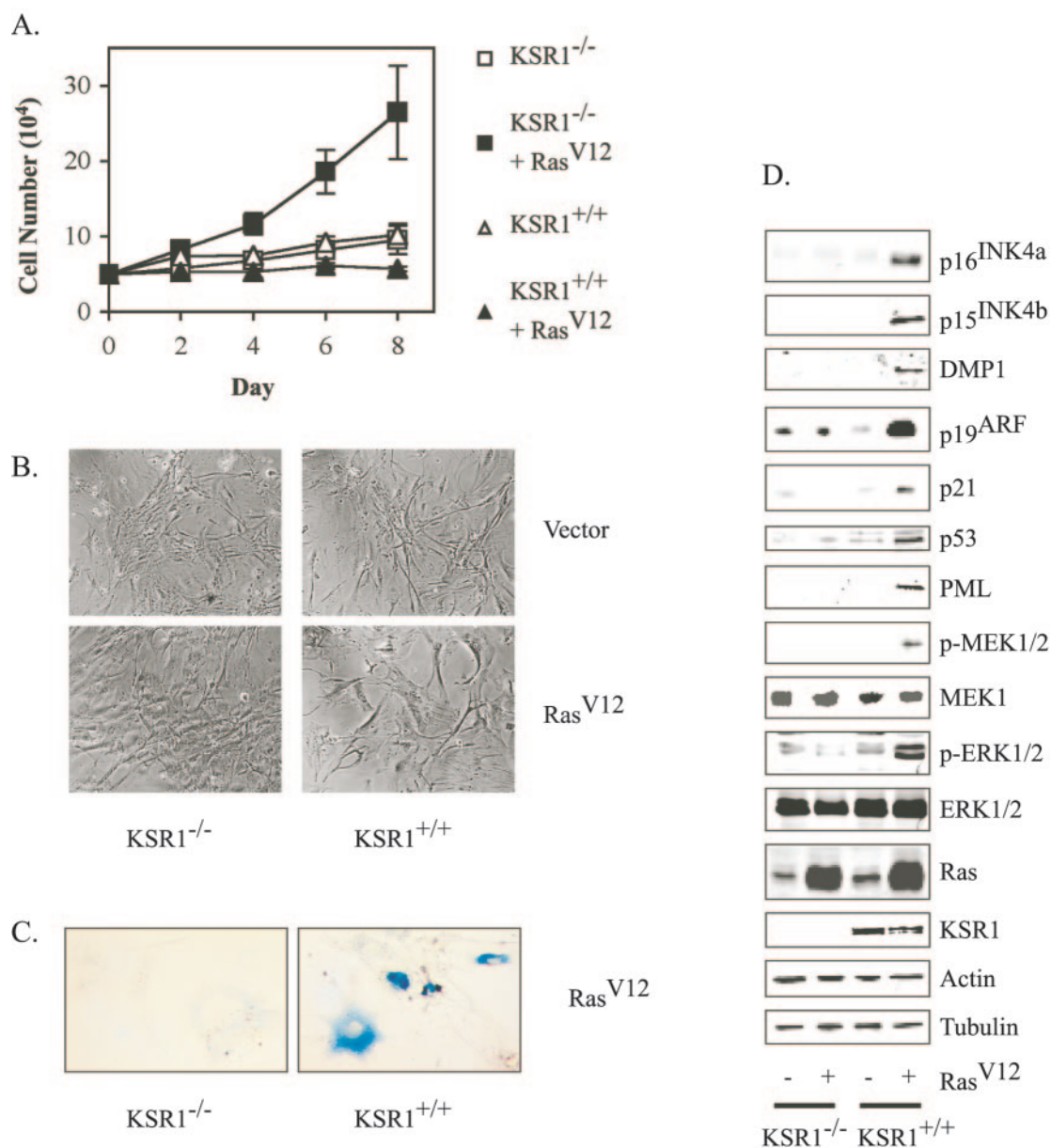


FIG. 1. KSR1 is necessary for Ras^{V12}-induced senescence in primary MEFs. (A) Passage 4 to 6 KSR1^{-/-} (squares) or KSR1^{+/+} (triangles) MEFs expressing Ras^{V12} (closed) or control (open) vectors were seeded at 5×10^4 cells per 35-mm dish. Separate duplicate dishes were assessed for cell number every 48 h on a Beckman Coulter Counter. Data are expressed as means \pm standard deviations (SD) of three independent experiments. (B) Photomicrographs (10 \times) of cells analyzed in panel A. (C) Photomicrographs (10 \times) of Ras^{V12}-expressing cells analyzed in panel A and stained to visualize SA β -galactosidase activity as described in Materials and Methods. (D) Western blot analysis of whole-cell extracts prepared from passage 4 to 6 KSR1^{-/-} and KSR1^{+/+} MEFs expressing Ras^{V12} or control vector. Lysates were probed with the indicated antibodies to detect induction and activation of each protein by Ras^{V12}. Actin and tubulin were used to demonstrate equal loading of each sample, and Ras and KSR1 expression results are shown as controls. Data are representative of four independent experiments.

59, 76). KSR1 is a molecular scaffold of the Raf/MEK/ERK cascade (37, 46, 49). Previous data indicated that KSR1 was necessary for Ras^{V12} to transform immortalized MEFs (37). To assess whether KSR1 was required for Ras^{V12}-induced senescence, early-passage KSR1^{-/-} and KSR1^{+/+} MEFs were infected with a recombinant retrovirus expressing Ras^{V12} or with control virus and were assessed for characteristics of senescence. As expected, expression of Ras^{V12} in KSR1^{+/+} MEFs induced cellular senescence. KSR1^{+/+} MEFs expressing

Ras^{V12} failed to proliferate in culture (Fig. 1A), had a characteristic enlarged and flattened morphology (Fig. 1B), and demonstrated SA β -galactosidase activity (Fig. 1C). In contrast, Ras^{V12} did not induce growth arrest in KSR1^{-/-} MEFs but instead induced proliferation. KSR1^{-/-} MEFs expressing Ras^{V12} proliferated at a much higher rate than control MEFs in culture (Fig. 1A), were morphologically similar to control fibroblasts (Fig. 1B), and lacked β -galactosidase activity (Fig. 1C). Previous data demonstrate that, although Ras activa-

tion of the Raf/MEK/ERK effector pathway is defective in immortalized $KSR1^{-/-}$ MEFs, activation of other Ras effector pathways is unaltered (37). These data indicate that the KSR1-scaffolded Raf/MEK/ERK effector pathway is critical in determining whether Ras^{V12} induces senescence or cell proliferation in primary MEFs.

Ras^{V12} is reported to induce senescence in primary cells via the $p19^{ARF}/p53$ and $INK4/Rb$ pathways (1, 13, 15, 20, 21, 30, 41, 43, 50, 52, 57, 59, 64, 76). To understand which of these pathways failed to be induced by Ras^{V12} in $KSR1^{-/-}$ MEFs, Western blotting was performed for p53, PML, $p21^{CIP1}$, $p19^{ARF}$, DMP1, $p16^{INK4a}$, and $p15^{INK4b}$ in $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs expressing Ras^{V12} and in control cells. Western blot analyses for Ras, KSR1, tubulin, and actin were performed as controls. Retroviral introduction of Ras^{V12} induced the phosphorylation of MEK and ERK and the expression of p53, PML, $p21^{CIP1}$, $p19^{ARF}$, DMP1, $p16^{INK4a}$, and $p15^{INK4b}$ in $KSR1^{+/+}$ MEFs. In contrast, expression of Ras^{V12} failed to stimulate the phosphorylation of MEK or ERK or to induce p53, PML, $p21^{CIP1}$, $p19^{ARF}$, DMP1, $p16^{INK4a}$, or $p15^{INK4b}$ expression in $KSR1^{-/-}$ MEFs (Fig. 1D). Interestingly, although $p19^{ARF}$ was not induced by Ras^{V12} in $KSR1^{-/-}$ MEFs, we routinely saw a two- to fourfold elevation in basal $p19^{ARF}$ in $KSR1^{-/-}$ MEFs compared to $KSR1^{+/+}$ MEF results (Fig. 1D, vector controls). Elevated $p19^{ARF}$ levels can indicate a lack of p53 function (34, 53). To assess whether $KSR1^{-/-}$ MEFs lacked functional p53, p53 induction and p53 activity in response to γ irradiation were assessed in early-passage $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs.

p53 was induced rapidly and transiently in both $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs (Fig. 2). To assess whether the induced p53 was transcriptionally active, Western blot analysis was performed for two transcriptional targets of p53, *mdm2* and $p21^{CIP1}$ (2, 18, 73). Both *mdm2* and $p21^{CIP1}$ were induced 2 to 4 h after exposure of $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs to γ irradiation (Fig. 2), indicating that p53 can be activated by ATM following DNA damage in $KSR1^{-/-}$ MEFs. These data demonstrate that $KSR1^{-/-}$ MEFs express functional p53 but that p53 activity cannot be induced by activated Ras^{V12} to trigger cellular senescence in the absence of KSR1.

To address whether KSR1 was necessary for Ras^{V12} to cause senescence in primary MEFs, KSR1 was reintroduced into $KSR1^{-/-}$ MEFs by use of a bicistronic retrovirus encoding KSR1 and green fluorescent protein (GFP). The level of KSR1 expression has significant effects on Ras^{V12} -mediated transformation and Raf/MEK/ERK signaling (37). Reintroduction of physiologic levels of KSR1 rescues Ras^{V12} -mediated transformation and ERK activation, whereas supraphysiologic levels of KSR1 further enhance transformation and ERK activation to a maximum at 14 times the level of KSR1 expression found in $KSR1^{+/+}$ MEFs (37). Higher levels of KSR1 expression inhibit transformation and ERK activation, consistent with the role of KSR1 as a molecular scaffold (8, 37). Therefore, cells expressing low levels of ectopic KSR1 were selected by flow cytometry to ensure that physiologically relevant levels of KSR1 expression, shown previously not to affect the rate of proliferation, were used in our analysis (Fig. 3D). Reintroduction of KSR1 restored Ras^{V12} -induced senescence in $KSR1^{-/-}$ MEFs (Fig. 3), as assessed by proliferative arrest (Fig. 3A) and SA β -galactosidase activity (Fig. 3B and C). KSR1 also restored Ras^{V12} -mediated

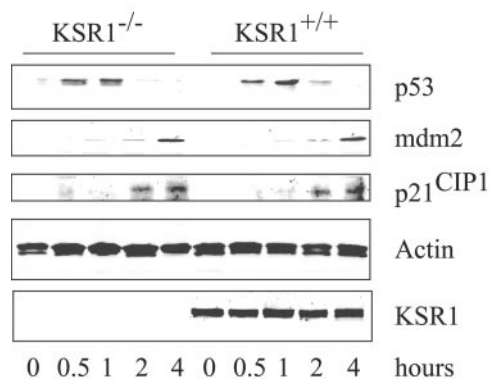


FIG. 2. Induction of p53 activity by γ irradiation is independent of KSR1. Passage 4 $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs were irradiated with 8 Gy, and whole-cell extracts were prepared at the indicated times after radiation exposure. Lysates were probed with the indicated antibodies to assess the induction of p53 activity. Actin was used to demonstrate equal loading of each sample. Data are representative of two independent experiments.

induction of p53, PML, $p21^{CIP1}$, $p19^{ARF}$, DMP1, $p16^{INK4a}$, and $p15^{INK4b}$, lowered basal levels of $p19^{ARF}$, and restored the responsiveness of $p19^{ARF}$ to Ras^{V12} (Fig. 3D).

Ras^{V12} -induced senescence requires KSR1-ERK interaction. While the site(s) of association between KSR1 and Raf has not been well defined, sites of interaction for MEK and activated ERK have been identified on KSR1. Furthermore, site-directed mutagenesis of KSR1 can abrogate the KSR1-MEK or KSR1-ERK interactions. Mutated forms of KSR1 can be used to assess the relative role each KSR1-effector interaction plays in KSR1-mediated biological functions (5, 19, 31, 47, 65, 75). To assess the effects of KSR1-scaffolded ERK activation on Ras^{V12} -induced senescence, mutated KSR1 constructs lacking the ability to interact with MEK ($KSR1.C809Y$) (47, 65, 75) or activated ERK ($KSR1.FSFP/AAAP$) (19, 31) were introduced into $KSR1^{-/-}$ MEFs expressing Ras^{V12} or control vector by use of the bicistronic retroviral system described above. Introduction of these mutated KSR1 constructs into Ras^{V12} -expressing $KSR1^{-/-}$ MEFs demonstrated that the KSR1-ERK interaction, but not the KSR1-MEK interaction, was necessary for Ras^{V12} to induce senescence. $KSR1.C809Y$, a mutated KSR1 construct unable to bind MEK (47, 65, 75), was able to induce growth arrest and SA β -galactosidase activity as well as wild-type KSR1 (Fig. 3A to C). In contrast, $KSR1.FSFP/AAAP$, a mutated form of KSR1 unable to bind activated ERK (19, 31), did not arrest growth and showed a diminished ability to promote Ras^{V12} -induced SA β -galactosidase activity. $KSR1.FSFP/AAAP$ cells expressing Ras^{V12} did not, however, proliferate at rates as high as control (GFP) cells expressing Ras^{V12} , indicating that there may be a reduction in proliferative signals from Ras^{V12} in the absence of an intact KSR1-ERK interaction (Fig. 3A-C).

Similarly to $KSR1^{-/-}$ MEFs expressing wild-type KSR1, Ras^{V12} induced expression of p53, $p19^{ARF}$, DMP1, $p16^{INK4a}$, and $p15^{INK4b}$ in $KSR1^{-/-}$ MEFs expressing $KSR1.C809Y$, providing further evidence that the KSR1-MEK interaction is dispensable during Ras^{V12} -induced senescence. In contrast, Ras^{V12} induced expression of p53 and $p19^{ARF}$, but

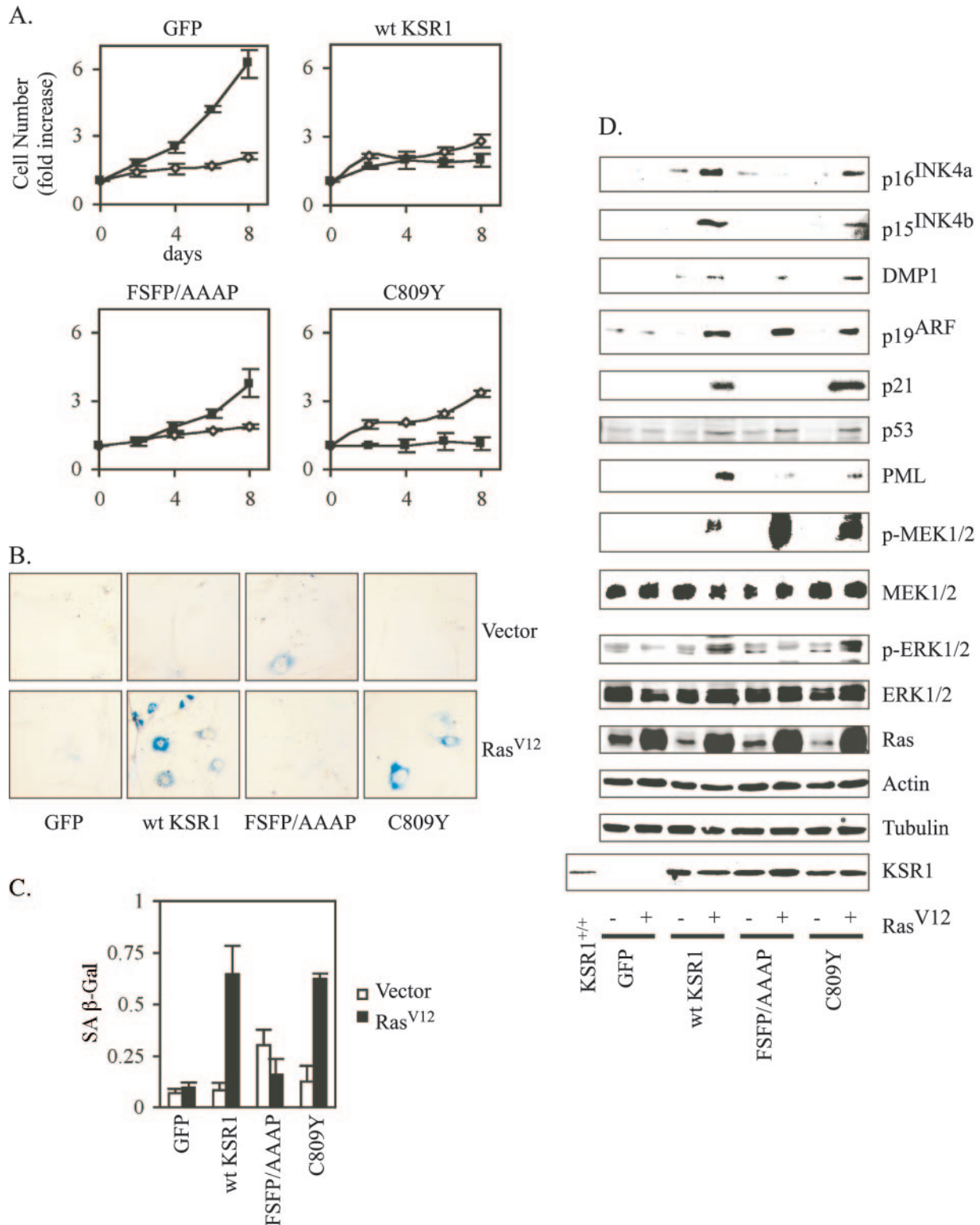


FIG. 3. The KSR1-ERK interaction is required, but the KSR1-MEK interaction is dispensable, for Ras^{V12}-induced senescence in KSR1^{-/-} MEFs. Passage 5 KSR1^{-/-} MEFs were infected with recombinant retrovirus encoding KSR1, a KSR1 construct unable to bind activated ERK (KSR1.FSFP/AAAP) or a KSR1 construct unable to bind MEK (KSR1.C809Y), and Ras^{V12} or a control virus. Low levels of KSR1 expression were selected by FACS analysis for levels of GFP expression previously shown to correlate with 1× to 5× KSR1^{+/+} levels. (A) Proliferation in passage 5 KSR1^{-/-} MEFs expressing the indicated KSR1 construct and Ras^{V12} (closed squares) or control (open diamonds) vectors. Separate duplicate dishes were assessed for cell number every 48 h on a Beckman Coulter Counter. Data are expressed as means ± SD of three independent experiments. (B) Photomicrographs (10×) of cells analyzed in panel A and stained to visualize SA β-galactosidase activity as described in Materials and Methods. (C) Quantification of cells analyzed in panel A and stained to visualize SA β-galactosidase (β-Gal) activity as described in Materials

not p16^{INK4a} or p15^{INK4b}, in KSR1^{-/-} MEFs expressing KSR1.FSFP/AAAP (Fig. 3D). Induction of p21^{CIP1} also required the interaction of KSR1 with ERK (Fig. 3D). This observation appears consistent with the p53-independent induction of p21^{CIP1} by Raf (72). Ras^{V12}-induced MEK phosphorylation is rescued by the expression of either KSR1.FSFP/AAAP or KSR1.C809Y. However, ERK activation is not rescued by KSR1.FSFP/AAAP (Fig. 3D). These data indicate that KSR1 is necessary for Ras^{V12}-induced senescence and that the effects of KSR1 require its interaction with activated ERK.

The effects of the FSFP/AAAP and C809Y mutations in KSR1 on Ras^{V12}-induced senescence led us to assess whether the KSR1-MEK or KSR1-ERK interactions were required for Ras^{V12}-mediated transformation in immortal MEFs. As observed previously (37), KSR1 expression was necessary for Ras^{V12}-induced transformation in immortalized KSR1^{-/-} MEFs (Fig. 4A). The KSR1-MEK interaction was dispensable for transformation, as KSR1.C809Y cells expressing Ras^{V12} showed enhanced colony formation in comparison to wild-type KSR1 cells. KSR1^{-/-} MEFs expressing KSR1.FSFP/AAAP were also transformed. However, disruption of the interaction of KSR1 with activated ERK diminished the transforming potential of Ras^{V12}, as these cells showed only 30% of the colonies seen with cells expressing wild-type KSR1 (Fig. 4A).

Given the contrasting results observed upon disruption of KSR1-MEK and KSR1-ERK interactions, we sought to further understand the signaling properties of cells expressing these mutated KSR1 constructs. We examined the kinetics of PDGF-induced ERK activation in immortal KSR1^{-/-} MEFs expressing KSR1, KSR1.C809Y, or KSR1.FSFP/AAAP (Fig. 4B). Cells expressing wild-type KSR1 showed rapid induction of ERK activation at 5 min, followed by a lower level of sustained signaling out to 2 h. When the KSR1-MEK interaction was disrupted by the C809Y mutation in KSR1, there was no decrease in peak ERK activation, and ERK phosphorylation was sustained in comparison to that of cells expressing wild-type KSR1. In contrast, disruption of the interaction of KSR1 with activated ERK (FSFP/AAAP) blunted peak ERK activation (Fig. 4B). These results suggest that the role of KSR1 is not only to facilitate activation of the Raf/MEK/ERK cascade but that KSR1 is also required to maintain the fidelity of Ras^{V12}-induced ERK activity necessary for senescence (Fig. 3) and transformation (Fig. 4).

KSR1 enhances the cellular response to activated MEK. Whereas previous studies have shown a role for KSR1 in facilitating the activation of Raf by Ras (45) and MEK by Raf (48, 49, 56), our data suggest that KSR1 promotes signal propagation downstream of MEK. To explore the role of KSR1 in facilitating signaling downstream of MEK, we examined cell transformation (Fig. 5) and senescence (Fig. 6) induced by a

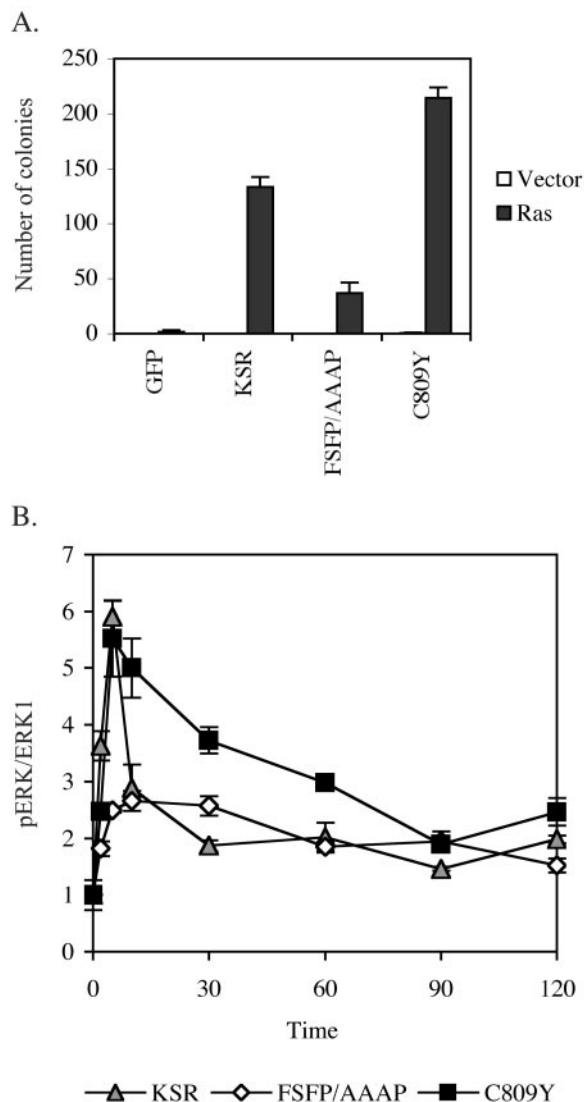


FIG. 4. The KSR1-ERK interaction is required, but the KSR1-MEK interaction is dispensable, for Ras^{V12}-induced transformation and PDGF-induced ERK activation in immortal KSR1^{-/-} MEFs. Immortal KSR1^{-/-} MEFs were infected with recombinant retrovirus encoding KSR1, KSR1.FSFP/AAAP, or KSR1.C809Y and Ras^{V12} or a control virus. Low levels of KSR1 expression were selected by FACS analysis for levels of GFP expression previously shown to correlate with 1× to 5× KSR1^{+/+} levels. (A) Cells were assessed for transformation by growth on soft agar as described in Materials and Methods. Data are expressed as means ± SD from two independent experiments. (B) Triplicate wells expressing the indicated KSR1 construct were treated with 25 ng of PDGF/ml for the indicated times, and ERK1/2 phosphorylation levels were determined by in-cell Western blotting for ERK1 and pERK1/2 with a Li-Cor Odyssey system. Data are expressed as ratios of pERK1/2 to ERK1. Data are expressed as means ± SD.

and Methods. Separate triplicate dishes were quantified for each cell line, with a minimum of 50 cells quantified per well. Data are expressed as mean percentages of cells ± SD from three independent experiments. (D) Western blot analysis of whole-cell extracts prepared from passage 5 KSR1^{-/-} MEFs expressing the indicated KSR1 constructs and Ras^{V12} or control vector. Lysates were probed with the indicated antibodies to detect induction and activation of each protein by Ras^{V12}. Actin and tubulin were used to demonstrate equal loading of each sample, and Ras and KSR1 expression results are shown as controls. Data are representative of four independent experiments.

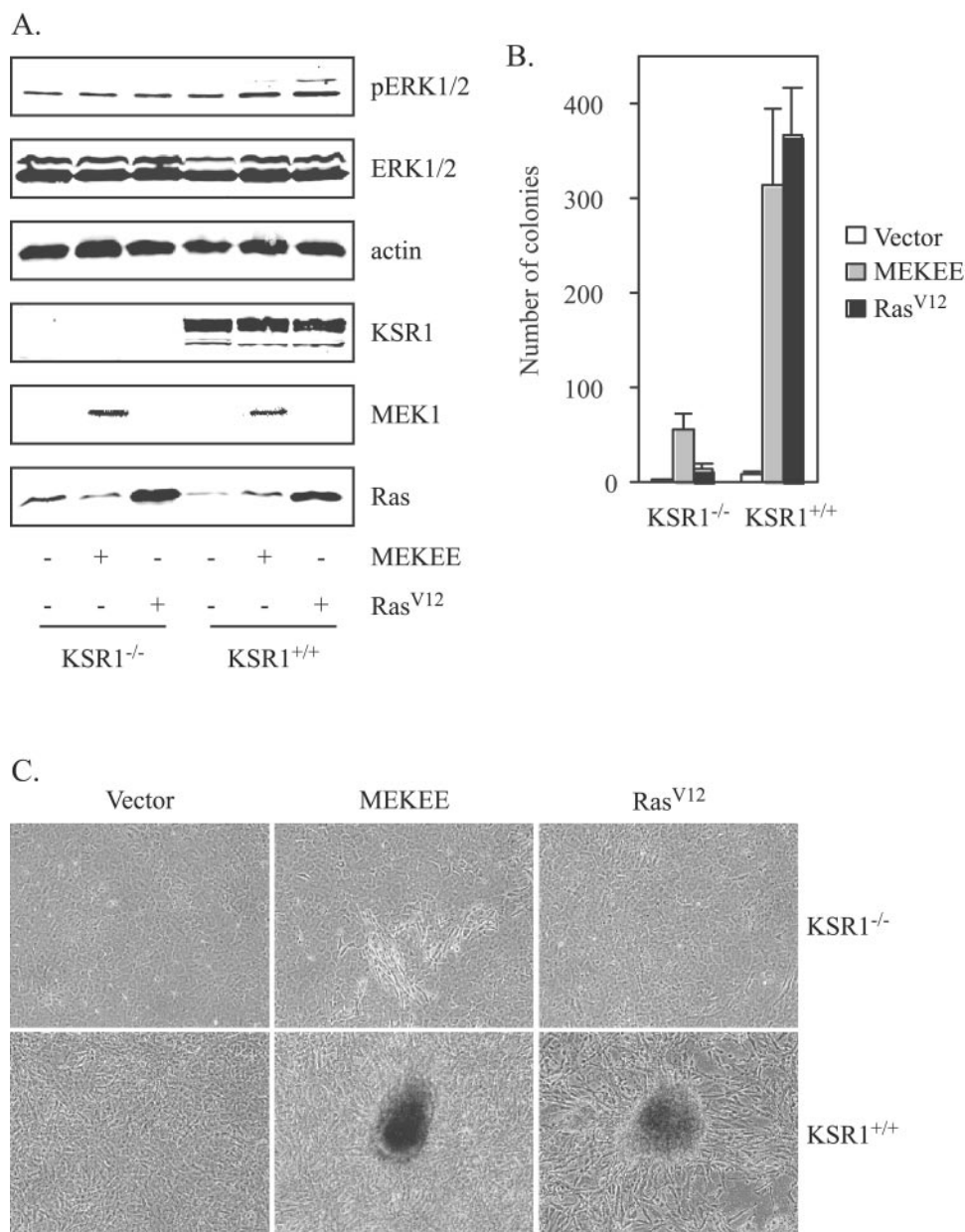


FIG. 5. KSR1 is not required for, but enhances, MEKKEE-induced transformation in immortal KSR1^{-/-} MEFs. Immortal KSR1^{-/-} and KSR1^{+/+} MEFs were infected with recombinant retrovirus encoding MEKKEE, Ras^{V12}, or a control virus. (A) Western blot analysis of whole-cell extracts prepared from immortal KSR1^{-/-} and KSR1^{+/+} MEFs expressing MEKKEE, Ras^{V12}, or control vector. Lysates were probed with antibodies against pERK1/2 and ERK1/2 to detect activation of ERK by MEKKEE or Ras^{V12}. Actin was used to demonstrate equal loading of each sample, and expression blots of Ras, MEK1, and KSR1 are shown as controls. For MEK1 blots, low exposures are shown to indicate cells that have received the MEKKEE transgene and not expression of endogenous MEK1. Data are representative of four independent experiments. (B and C) Cells were assessed for transformation by growth on soft agar (B) and focus formation (C) as described in Materials and Methods. Data are expressed as means \pm SD from two independent experiments.

constitutively active MEK construct (MEKKEE) containing glutamic acid substitutions for Ser218/222 (7, 12, 41, 44) in the presence and absence of KSR1. To study transformation, immortal KSR1^{-/-} and KSR1^{+/+} MEFs were infected with retroviruses encoding Ras^{V12} or MEKKEE and were assessed for ERK activation and transformation by examining anchorage-independent growth and loss of contact inhibition (Fig. 5). In immortal KSR1^{+/+} MEFs, Ras^{V12} or MEKKEE expression in-

creased ERK activation (Fig. 5A), caused colony formation on soft agar (Fig. 5B), and induced focus formation (Fig. 5C). As reported previously (37), Ras^{V12} was unable to increase ERK activation or transform immortal KSR1^{-/-} MEFs. MEKKEE expression in KSR1^{-/-} MEFs led to an intermediate phenotype. While there was no increase in ERK activation (Fig. 5A), the cells exhibited anchorage independence (Fig. 5B) and a loss of contact inhibition (Fig. 5C), although to a much lesser

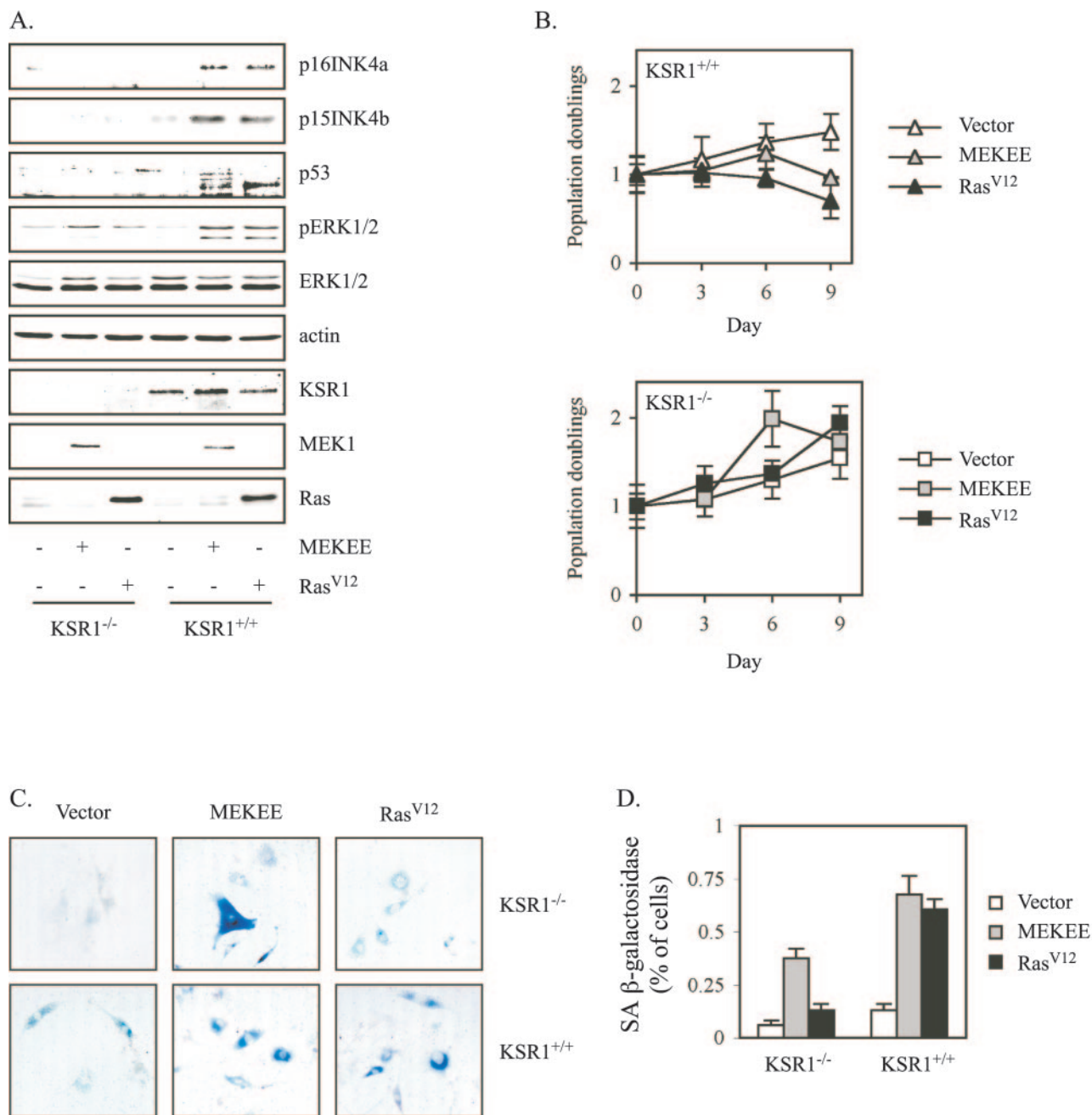


FIG. 6. KSR1 is necessary for MEKKEE-induced senescence in primary MEFs. (A) Western blot analysis of whole-cell extracts prepared from passage 4 to 6 KSR1^{-/-} and KSR1^{+/+} MEFs expressing MEKKEE, Ras^{V12}, or control vector. Lysates were probed with the indicated antibodies to detect induction and activation of each protein by MEKKEE or Ras^{V12}. Actin was used to demonstrate equal loading of each sample, and expression blots of Ras, MEK1, and KSR1 are shown as controls. For MEK1 blots, low exposures are shown to indicate cells that have received the MEKKEE transgene and not expression of endogenous MEK1. (B) Passage 4 to 6 KSR1^{-/-} (squares) or KSR1^{+/+} (triangles) MEFs expressing Ras^{V12} (closed), MEKKEE (gray), or control (open) vectors were seeded at 1 × 10⁴ cells per well in a 24-well plate. Separate duplicate dishes were assessed for cell number every 72 h by trypan blue exclusion. Data are expressed as means ± SD from two independent experiments. (C) Photomicrographs (10×) of cells analyzed in panel A and stained to visualize SA β-galactosidase activity as described in Materials and Methods. (D) Quantification of cells analyzed in panel A and stained to visualize SA β-galactosidase activity as described in Materials and Methods. Separate triplicate wells were quantified for each cell line, with a minimum of 100 cells quantified per well. Data are expressed as means ± SD from four independent experiments.

extent than seen in KSR1^{+/+} MEFs. When grown on a semi-solid medium, KSR1^{-/-} MEFs expressing MEKKEE formed approximately 25% of the colonies seen in KSR1^{+/+} MEFs and when contact inhibition was assessed required 5 to 6 weeks

to form colonies equal in size to those observed 10 days after plating KSR1^{+/+} MEFs expressing MEKKEE.

MEKKEE also caused cellular senescence in primary KSR1^{+/+} MEFs (Fig. 6). However, as observed with trans-

formation, MEKKEE caused an intermediate phenotype in primary $KSR1^{-/-}$ MEFs. Expression of MEKKEE caused a modest increase in ERK activation but failed to induce expression of p53 or the INK4 proteins (Fig. 6A). Furthermore, primary $KSR1^{-/-}$ MEFs expressing MEKKEE did not undergo growth arrest, instead proliferating as well as or better than control cells (Fig. 6B). When stained for β -galactosidase activity, primary $KSR1^{-/-}$ MEFs expressing MEKKEE consistently showed 35 to 40% positive staining (versus 75 to 80% for $KSR1^{+/+}$ MEFs expressing MEKKEE or Ras^{V12}) (Fig. 6B and C). These data demonstrate that, similarly to its effects on transformation (Fig. 5), MEKKEE cannot fully promote senescence in the absence of KSR1. These data suggest that KSR1 promotes not only the activation of MEK (48) but also transmission of the activated MEK signal to its downstream target.

Deletion of KSR1 enhances spontaneous immortalization of fibroblasts. Ras^{V12} -induced senescence and replicative senescence are both mediated, in part, by activation of the $p19^{ARF}/p53$ and $p16^{INK4a}/Rb$ pathways (50, 59). Since $KSR1^{-/-}$ MEFs were defective in Ras^{V12} -mediated induction of p53, p16^{INK4a}, and p15^{INK4b} and in Ras^{V12} -induced senescence, we assessed whether KSR1^{-/-} MEFs would exhibit a propensity to escape replicative senescence. To assess whether KSR1 was involved in spontaneous immortalization of fibroblasts, $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs were passaged by a 3T9 protocol (10, 67) until either cell death or spontaneous immortalization occurred. $KSR1^{+/+}$ MEFs showed a rapid decline in cellular proliferation beyond passage 5 and exhibited marked amounts of cell death around passages 15 to 20, consistent with previous reports (Fig. 7A) (42). $KSR1^{-/-}$ MEFs also exhibited a rapid decline in cellular proliferation after passage 5 and were similar to $KSR1^{+/+}$ MEFs in both appearance and number until approximately passage 15. At this point, $KSR1^{-/-}$ MEFs showed a brief proliferative burst (2 to 4 passages) followed by a second period of growth arrest which lasted 5 to 7 more passages. Between passages 23 and 26, $KSR1^{-/-}$ MEFs began to proliferate, and 10 out of 11 (91%) $KSR1^{-/-}$ cultures had immortalized by passage 30. In contrast, $KSR1^{+/+}$ MEFs showed no signs of proliferation until passage 35 or later, after which 4 out of 11 (36%) $KSR1^{+/+}$ cultures began to proliferate and eventually became immortal (Fig. 7A).

To assess the growth characteristics of $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs during the immortalization process, cells from three independent $KSR1^{-/-}$ and $KSR1^{+/+}$ cultures at passages 5, 10, 15, and 18 were assessed for proliferation rate at low density (Fig. 7B). Consistent with previously published data, early-passage $KSR1^{-/-}$ MEFs proliferated at a slightly slower rate than $KSR1^{+/+}$ MEFs (42). However, we did not see the marked differences in proliferative rate between $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs observed by Lozano et al. (42). The discrepancy might be explained by differences in passage number between the two experiments (see Discussion). When plated at passage 10, there was no difference in proliferative rate between $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs for the first 6 days of the experiment. We observed increased cell proliferation in $KSR1^{-/-}$ MEFs between days 7 and 10 of this experiment, however, indicating that immortalized cells may already exist within the $KSR1^{-/-}$ cultures at this time. $KSR1^{-/-}$ MEFs plated at passages 15 and 18 also showed enhanced cellular

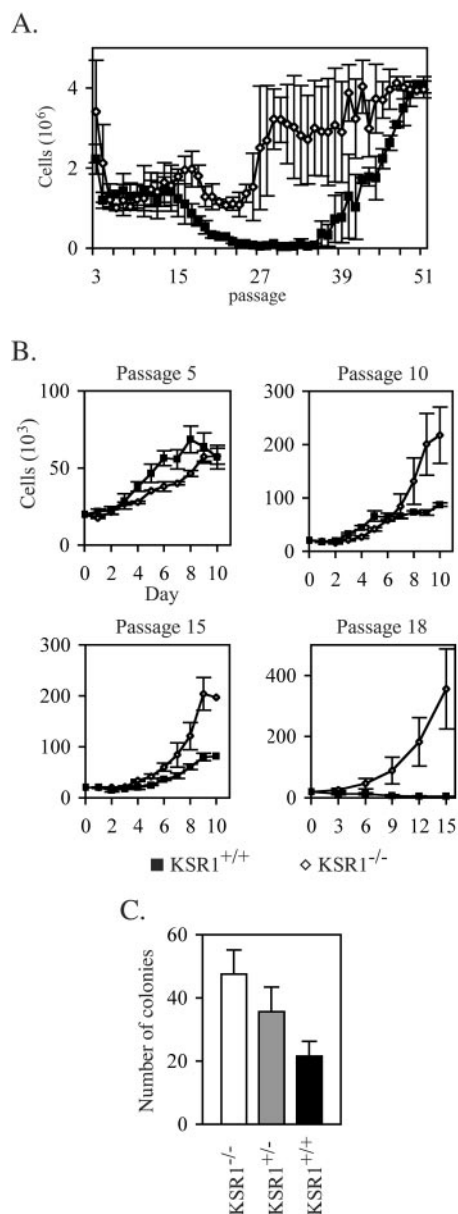


FIG. 7. Loss of KSR1 allows for early immortalization of primary mouse embryo fibroblasts. (A) Passage 3 $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs were passaged by a 3T9 protocol until immortalized populations were obtained. Data are means \pm SD of four embryos of each genotype. (B) At passages 5, 10, 15, and 18, 2×10^4 MEFs were plated in 35-mm dishes and counted either daily or every 72 h to assess low-density growth. Data are means \pm SD of three embryos of each genotype. (C) $KSR1^{+/+}$, $KSR1^{+/+}$, and $KSR1^{-/-}$ MEFs at passage 9 were seeded at 2×10^4 cells/60-mm dish and fed twice weekly for 4 weeks. Colonies consisting of at least 16 cells that arose from MEFs of each genotype were counted. Data are expressed as means \pm SD from two embryos of each genotype.

proliferation compared to $KSR1^{+/+}$ MEFs, with similar lag times of 6 to 8 days before the enhanced cell proliferation was observed (Fig. 7B). These data indicate that compared to $KSR1^{+/+}$ MEFs, $KSR1^{-/-}$ MEFs are predisposed to spontaneous immortalization. Furthermore, these data show that im-

mortalized KSR1^{-/-} MEFs exist in cultured cell populations as early as passage 10.

The effect of KSR1 on the potential of MEFs to immortalize was tested further by plating KSR1^{+/+}, KSR1^{+/-}, and KSR1^{-/-} MEFs at passage 9 at low density and allowing the cells to grow for 4 weeks. Analysis of the colonies formed from cells of each genotype (Fig. 7C) revealed that twice as many colonies arose from KSR1^{-/-} MEFs at passage 9 as from KSR1^{+/+} MEFs. An intermediate number of colonies arose from MEFs heterozygous for KSR1. These data suggest that KSR1 serves as a barrier against immortalization.

DISCUSSION

Here we show that the molecular scaffold KSR1 is a potent modifier of replicative senescence. Deletion of KSR1 enhanced spontaneous immortalization of primary MEFs, increasing the frequency of immortalization and decreasing the time necessary for primary cultures to escape from replicative crisis. KSR1 was also required for senescence induced by activated Ras^{V12}. In the absence of KSR1, Ras^{V12} stimulated proliferation instead of senescence. Expression of mutated forms of KSR1 revealed that the KSR1-ERK interaction was necessary for Ras^{V12} to cause growth arrest of primary cells. Though the interaction of KSR1 with MEK was not required for Ras^{V12}-induced senescence, KSR1 expression amplified the effect of an activated MEK construct on senescence in primary MEFs and transformation in immortal MEFs. Signaling from Ras^{V12} to both the p19^{ARF}/p53 and INK4/Rb pathways was defective in KSR1^{-/-} MEFs, which likely underlies both the escape from Ras^{V12}-induced senescence and the enhanced spontaneous immortalization observed in KSR1^{-/-} MEFs.

Ras^{V12} causes senescence by engaging the p19^{ARF}/p53 and p16^{INK4a}/Rb pathways, although the relative contributions of these pathways differ between mouse and human cells. p19^{ARF} plays a more important role in senescence in mouse cells, whereas p16^{INK4a} is more important in senescence in human cells (9, 28, 29, 50, 58, 59, 69). In murine fibroblasts, ablation of these pathways allows Ras^{V12} to enhance cell proliferation instead of growth arrest (13, 34, 50, 52, 57, 59). KSR1 was necessary for Ras^{V12} to increase phosphorylation of ERK and for the expression of p53, PML, p19^{ARF}, DMP1, p16^{INK4a}, and p15^{INK4b} (Fig. 1D). In the absence of these antiproliferative signals, Ras^{V12} caused proliferation instead of growth arrest in primary MEFs. Ras^{V12} signals to the proliferative machinery via multiple effector pathways, including the PI 3' kinase/Akt, RalGEF/Ral, and Raf/MEK/ERK cascades (23, 24). In primary murine cultures, activation of the Raf/MEK/ERK cascade, but not the other effector pathways, engages the p53/ARF and INK4/Rb pathways, causing senescence instead of proliferation (41, 76). Deletion of the molecular scaffold KSR1 blocks the ability of Ras^{V12} to signal through the Raf/MEK/ERK effector pathway to these antiproliferative pathways (Fig. 1) but leaves signaling to other Ras effectors intact (37). Thus, in primary MEFs lacking KSR1, Ras^{V12} signaling through the Raf/MEK/ERK signaling cassette to the antiproliferative p53/ARF and INK4/Rb pathways is limited, allowing proliferation instead of growth arrest.

The interaction of KSR1 with activated ERK appears nec-

essary for Ras^{V12}-induced senescence (Fig. 5) and for maximal Ras^{V12}-induced transformation (Fig. 6). While the activation of MEK is intact in cells expressing KSR1.FSFP/AAAP, maximal ERK activation is lost; indicating that the interaction of KSR1 with activated ERK is necessary for signal fidelity. Interestingly, MEK activation is increased in KSR1^{-/-} MEFs expressing KSR1.FSFP/AAAP, raising the possibility that a molecular brake on Raf activity is lost by disruption of KSR1 interaction with ERK. Dougherty et al. (17) recently reported feedback inhibition of Raf by ERK. Since MEK phosphorylation (and therefore Raf activity) is increased in KSR1.FSFP/AAAP cells, these data suggest that KSR1 may be required not only to facilitate but also to moderate signaling through the Raf/MEK/ERK cascade.

To assess whether KSR1 facilitates the activation of ERK by MEK, we examined the role of MEKEE (activated MEK) in senescence and transformation in KSR1^{-/-} MEFs. We observed that the introduction of MEKEE into KSR1^{-/-} MEFs yields a phenotype intermediate between those observed after introduction of Ras^{V12} in KSR1^{+/+} and in KSR1^{-/-} MEFs. While MEKEE transforms KSR1^{-/-} MEFs, these cells have only 20% of the transformed foci compared to the results seen with KSR1^{+/+} MEFs expressing MEKEE (Fig. 5). Additionally, while MEKEE does not cause growth arrest in primary KSR1^{-/-} MEFs, there are an increased number of β -galactosidase-positive cells (Fig. 6). These data show that activated MEK is not sufficient to compensate for the lack of KSR1, indicating that KSR1 facilitates MEK function. These data reveal a previously unappreciated role of KSR1 in the maintenance of the fidelity of signaling from MEK to ERK. Furthermore, in conjunction with the intermediate phenotype seen with disruption of the interaction between KSR1 and activated ERK (FSFP/AAAP), these data show that specific scaffold-effector interactions are necessary to maintain signaling fidelity and assure the correct cellular response to pathway activation.

Expression of KSR1.C809Y, a form of KSR1 that cannot bind MEK (47, 65), still allowed Ras^{V12} to induce MEK and ERK activation, senescence in primary MEFs, and transformation in immortal MEFs (Fig. 3 and 4). The C809Y mutation is orthologous to a loss-of-function mutation in KSR1 detected in genetic screenings using *Caenorhabditis elegans* (66). While others have shown that this mutation precludes MEK activation (56), we found that MEK and ERK phosphorylation are enhanced (Fig. 3). These differences may be due, in part, to differences in experimental design or in signaling between mammalian and invertebrate systems. The KSR1-MEK interaction is necessary for many KSR1 functions, including its ability to translocate to the nucleus (5) and promote PC12 cell differentiation (47). In these studies, KSR1.C809Y exhibited a clear loss of function, which validates the biological importance of the KSR1-MEK interaction. Others have also reported a lack of biological activity for KSR1.C809Y (32, 65), but these studies used a KSR1 overexpression system where the readout was pathway inactivation via combinatorial inhibition. We also found a lack of combinatorial inhibition by use of the KSR1.C809Y construct (unpublished data). However, loss of the KSR1-MEK interaction enhances pathway activity at physiologic levels (Fig. 3 and 4). The disruption of KSR1's interaction with MEK may impair function in certain biological

contexts (5, 47, 66) but increase function in others (Fig. 3 and 4) due to its ability to moderate signal output through the Raf/MEK/ERK signaling cassette. Elevating KSR1 expression promoted maximal activation of the Raf/MEK/ERK cascade, cell proliferation, and Ras^{V12}-induced transformation (37). However, ERK activation must be constrained within a narrow range by physiological levels of KSR1 to limit proliferative signals and promote adipogenesis (36, 37). Therefore, KSR1 may be required to interact with MEK and moderate its activity when a biological effect requires restrained ERK activation (e.g., during differentiation). These data indicate that specific KSR1-effector interactions are key regulators of a cell's replicative potential.

KSR1^{-/-} MEFs immortalize more readily than KSR1^{+/+} MEFs (Fig. 7). These data are reminiscent of the targeted disruption of p16^{INK4a}. While disruption of p16^{INK4a} does not immediately immortalize MEFs, knockdown of p16^{INK4a} levels using antisense RNA constructs accelerates the establishment of immortalized cell lines (10). Furthermore, a greater proportion of p16^{INK4a}^{-/-} cultures immortalize compared to wild-type cell results (61, 62). We observed that disruption of KSR1 prevented p16^{INK4a} expression by Ras^{V12}. Thus, a defect in p16^{INK4a}/Rb pathway regulation may be a central contributor to the accelerated immortalization of KSR1^{-/-} MEFs.

While we observed only a slight difference in the low-density proliferative rate between KSR1^{-/-} and KSR1^{+/+} MEFs at passage 5 (Fig. 7B), Lozano et al. reported a 50% reduction in the proliferative rate of early-passage KSR1^{-/-} MEFs (42). Their studies, however, were conducted at a population doubling of <6, which corresponds to our MEFs at passages 1 to 3. Although we have not conducted low-density proliferative studies at these early passages, our data are consistent with low-passage-number KSR1^{-/-} MEFs showing decreased cell proliferation compared to KSR1^{+/+} MEFs. When low-density proliferative studies were seeded at passage 10, 15, or 18, however, we observed increased proliferation in KSR1^{-/-} MEFs beginning around day 7 (Fig. 7B). These data would indicate that KSR1^{-/-} MEF cultures at passages 10, 15, and 18 already contain immortal cells, which are detectable when the MEFs were allowed to grow at a low density.

Premature senescence has been proposed as a tumor-suppressive mechanism, and escape from cellular senescence is one of several events which predispose cells toward oncogenic transformation (9). These data suggest that KSR1 might function to limit tumor susceptibility, since its deletion impedes cellular senescence. Furthermore, an inefficient senescence response resulting from disruption of KSR1 might facilitate tumor progression induced by oncogenic mutations that are not dependent upon Ras regulation of the Raf/MEK/ERK cascade. However, when assessing the transforming potential of Ras^{V12} in KSR1^{-/-} MEFs, we and others have found that KSR1 is necessary for Ras^{V12}-mediated transformation in both primary (42) and immortal (37) MEFs, indicating that KSR1 is a positive modifier of tumorigenesis. This ostensible dual role for KSR1 may result from its function as a molecular scaffold for the Raf/MEK/ERK cascade. Signaling through the Raf/MEK/ERK cascade moderates cell fate decisions depending upon cellular context. Control of signal output through molecular scaffolds has been proposed as one of several nodes directing cell fate (35, 36). In both primary and immortal MEFs,

KSR1 expression moderates ERK activity to affect a cell fate (Fig. 1 and references 36 and 37), with the biologic outcome dependent upon cellular context and the overall activation of downstream effectors.

These data suggest that, in some circumstances, escape from premature senescence may not be an inextricable step toward cell transformation and tumorigenesis. Senescent fibroblasts, when seeded as tumor stroma, can promote epithelial cell transformation in situ and tumorigenesis in vivo (9, 38). Senescent fibroblasts secrete both soluble factors and extracellular matrices that promote the growth of premalignant, but not normal, epithelial cells. Furthermore, this ability to promote tumorigenesis occurs irrespective of whether the senescence was induced by replicative exhaustion or oncogenic Ras^{V12} (9, 38). Since KSR1^{-/-} MEFs are resistant to Ras^{V12}-induced senescence (Fig. 1) and less susceptible to replicative senescence (Fig. 7), it is conceivable that targeted disruption of KSR1 function could not only block the transforming capacity of cancer cells but also diminish the ability of stromal cells to promote tumorigenesis. Xing et al. (74) recently reported that treatment of EGFR-A431 or PANC-1 cells with antisense oligonucleotides against KSR1 blocked their proliferation in situ and in immunodeficient mice. Furthermore, the authors showed that treatment of mice with antisense oligonucleotides against KSR1 reduced the volume of established PANC-1 tumors. Based on these findings, Xing et al. (74) proposed phase I clinical trials to assess inactivation of KSR1 in pancreatic cancers. Our data suggest that the targeted disruption of KSR1 by use of antisense oligonucleotides could have therapeutic efficacy against Ras^{V12}-driven tumors at multiple levels. Within the tumor cells, reduction of KSR1 expression should reduce proliferative signals. In surrounding tissues, reduction of KSR1 expression could decrease the formation of senescent cells, thereby reducing the tumor-promoting properties of the surrounding stroma.

Based on these data, we propose that KSR1 expression is a barrier that cells must overcome during immortalization. While spontaneous deletion of KSR1 to escape senescence has not been reported, disruption of KSR1-regulated pathways would have the same effect. KSR1 allows Ras^{V12} to engage the antiproliferative p19^{ARF}/p53 and INK4/Rb pathways. Spontaneous mutations within these pathways promote immortalization (25, 34). Furthermore, genetic inactivation of either of these pathways blocks both Ras^{V12}-induced and replicative senescence (1, 10, 13, 20, 26, 30, 34, 50, 52, 57–59). The inability of Ras^{V12} to induce senescence in KSR1^{-/-} MEFs, combined with the enhanced propensity of KSR1^{-/-} MEFs to immortalize, indicates that KSR1 is a potent modifier of replicative potential.

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REFERENCES

1. Alani, R. M., A. Z. Young, and C. B. Shiflett. 2001. Id1 regulation of cellular senescence through transcriptional repression of p16/INK4a. *Proc. Natl. Acad. Sci. USA* **98**:7812–7816.
2. Barak, Y., T. Juven, R. Haffner, and M. Oren. 1993. *mdm2* expression is induced by wild type p53 activity. *EMBO J.* **12**:461–468.
3. Barbacid, M. 1987. *ras* genes. *Annu. Rev. Biochem.* **56**:779–827.
4. Beausejour, C. M., A. Krtolica, F. Galimi, M. Narita, S. W. Lowe, P. Yaswen, and J. Campisi. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* **22**:4212–4222.
5. Brennan, J. A., D. J. Volle, O. V. Chaika, and R. E. Lewis. 2002. Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of Ras. *J. Biol. Chem.* **277**:5369–5377.
6. Brookes, S., J. Rowe, M. Ruas, S. Llanos, P. A. Clark, M. Lomax, M. C. James, R. Vatcheva, S. Bates, K. H. Vousden, D. Parry, N. Gruis, N. Smit, W. Bergman, and G. Peters. 2002. INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO J.* **21**:2936–2945.
7. Brunet, A., G. Pages, and J. Pouyssegur. 1994. Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. *Oncogene* **9**:3379–3387.
8. Burack, W. R., and A. S. Shaw. 2000. Signal transduction: hanging on a scaffold. *Curr. Opin. Cell Biol.* **12**:211–216.
9. Campisi, J. 2001. Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol.* **11**:S27–31.
10. Carnero, A., J. D. Hudson, C. M. Price, and D. H. Beach. 2000. p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. *Nat. Cell Biol.* **2**:148–155.
11. Chang, E. H., M. E. Furth, E. M. Scolnick, and D. R. Lowy. 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature* **297**:479–483.
12. Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall. 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**:841–852.
13. Dannenberg, J. H., A. van Rossum, L. Schuijff, and H. te Riele. 2000. Ablation of the retinoblastoma gene family deregulates G₁ control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.* **14**:3051–3064.
14. Der, C. J., T. G. Krontiris, and G. M. Cooper. 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and Kirsten sarcoma viruses. *Proc. Natl. Acad. Sci. USA* **79**:3637–3640.
15. de Stanchina, E., E. Querido, M. Narita, R. V. Davuluri, P. P. Pandolfi, G. Ferbeyre, and S. W. Lowe. 2004. PML is a direct p53 target that modulates p53 effector functions. *Mol. Cell* **13**:523–535.
16. Dimiri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **92**:9363–9367.
17. Dougherty, M. K., J. Muller, D. A. Ritt, M. Zhou, X. Z. Zhou, T. D. Copeland, T. P. Conrads, T. D. Veenstra, K. P. Lu, and D. K. Morrison. 2005. Regulation of Raf-1 by direct feedback phosphorylation. *Mol. Cell* **17**:215–224.
18. el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817–825.
19. Fantz, D. A., D. Jacobs, D. Glossip, and K. Kornfeld. 2001. Docking sites on substrate proteins direct extracellular signal-regulated kinase to phosphorylate specific residues. *J. Biol. Chem.* **276**:27256–27265.
20. Ferbeyre, G., E. de Stanchina, A. W. Lin, E. Querido, M. E. McCurrach, G. J. Hannon, and S. W. Lowe. 2002. Oncogenic *ras* and p53 cooperate to induce cellular senescence. *Mol. Cell Biol.* **22**:3497–3508.
21. Ferbeyre, G., E. de Stanchina, E. Querido, N. Baptiste, C. Prives, and S. W. Lowe. 2000. PML is induced by oncogenic *ras* and promotes premature senescence. *Genes Dev.* **14**:2015–2027.
22. Franza, B. R., Jr., K. Maruyama, J. I. Garrels, and H. E. Ruley. 1986. In vitro establishment is not a sufficient prerequisite for transformation by activated *ras* oncogenes. *Cell* **44**:409–418.
23. Gille, H., and J. Downward. 1999. Multiple *ras* effector pathways contribute to G₁ cell cycle progression. *J. Biol. Chem.* **274**:22033–22040.
24. Hamad, N. M., J. H. Elconin, A. E. Karnoub, W. Bai, J. N. Rich, R. T. Abraham, C. J. Der, and C. M. Counter. 2002. Distinct requirements for Ras oncogenesis in human versus mouse cells. *Genes Dev.* **16**:2045–2057.
25. Harvey, D. M., and A. J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev.* **5**:2375–2385.
26. Harvey, M., A. T. Sands, R. S. Weiss, M. E. Hegi, R. W. Wiseman, P. Pantazis, B. C. Giovanella, M. A. Tainsky, A. Bradley, and L. A. Donehower. 1993. In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* **8**:2457–2467.
27. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585–621.
28. Huot, T. J., J. Rowe, M. Harland, S. Drayton, S. Brookes, C. Gooptu, P. Purkis, M. Fried, V. Bataille, E. Hara, J. Newton-Bishop, and G. Peters. 2002. Biallelic mutations in p16^{INK4a} confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts. *Mol. Cell Biol.* **22**:8135–8143.
29. Huschtscha, L. L., and R. R. Reddel. 1999. p16^{INK4a} and the control of cellular proliferative life span. *Carcinogenesis* **20**:921–926.
30. Inoue, K., R. Wen, J. E. Reh, M. Adachi, J. L. Cleveland, M. F. Roussel, and C. J. Sherr. 2000. Disruption of the ARF transcriptional activator DMP1 facilitates cell immortalization, Ras transformation, and tumorigenesis. *Genes Dev.* **14**:1797–1809.
31. Jacobs, D., D. Glossip, H. Xing, A. J. Muslin, and K. Kornfeld. 1999. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev.* **13**:163–175.
32. Janssen, R. A., P. N. Kim, J. W. Mier, and D. K. Morrison. 2003. Overexpression of kinase suppressor of Ras upregulates the high-molecular-weight tropomyosin isoforms in *ras*-transformed NIH 3T3 fibroblasts. *Mol. Cell Biol.* **23**:1786–1797.
33. Joneson, T., J. A. Fulton, D. J. Volle, O. V. Chaika, D. Bar-Sagi, and R. E. Lewis. 1998. Kinase suppressor of Ras inhibits the activation of extracellular ligand-regulated (ERK) mitogen-activated protein (MAP) kinase by growth factors, activated Ras, and Ras effectors. *J. Biol. Chem.* **273**:7743–7748.
34. Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld, and C. J. Sherr. 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91**:649–659.
35. Kolch, W. 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat. Rev. Mol. Cell Biol.* **6**:827–837.
36. Kortum, R. L., D. L. Costanzo, J. Haferbier, S. J. Schreiner, G. L. Razidlo, M. H. Wu, D. J. Volle, T. Mori, H. Sakaue, N. V. Chaika, O. V. Chaika, and R. E. Lewis. 2005. The molecular scaffold kinase suppressor of Ras 1 (KSR1) regulates adipogenesis. *Mol. Cell Biol.* **25**:7592–7604.
37. Kortum, R. L., and R. E. Lewis. 2004. The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. *Mol. Cell Biol.* **24**:4407–4416.
38. Krtolica, A., S. Parrinello, S. Lockett, P. Y. Desprez, and J. Campisi. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc. Natl. Acad. Sci. USA* **98**:12072–12077.
39. Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**:596–602.
40. Latres, E., M. Malumbres, R. Sotillo, J. Martin, S. Ortega, J. Martin-Caballero, J. M. Flores, C. Cordon-Cardo, and M. Barbacid. 2000. Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *EMBO J.* **19**:3496–3506.
41. Lin, A. W., M. Barradas, J. C. Stone, L. van Aelst, M. Serrano, and S. W. Lowe. 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev.* **12**:3008–3019.
42. Lozano, J., R. Xing, Z. Cai, H. L. Jensen, C. Trempus, W. Mark, R. Cannon, and R. Kolesnick. 2003. Deficiency of kinase suppressor of Ras1 prevents oncogenic *Ras* signaling in mice. *Cancer Res.* **63**:4232–4238.
43. Malumbres, M., I. Perez De Castro, M. I. Hernandez, M. Jimenez, T. Corral, and A. Pellicer. 2000. Cellular response to oncogenic *ras* involves induction of the Cdk4 and Cdk6 inhibitor p15^{INK4b}. *Mol. Cell Biol.* **20**:2915–2925.
44. Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**:966–970.
45. Michaud, N. R., M. Therrien, A. Cacace, L. C. Edsall, S. Spiegel, G. M. Rubin, and D. K. Morrison. 1997. KSR stimulates Raf-1 activity in a kinase-independent manner. *Proc. Natl. Acad. Sci. USA* **94**:12792–12796.
46. Morrison, D. K. 2001. KSR: a MAPK scaffold of the Ras pathway? *J. Cell Sci.* **114**:1609–1612.
47. Muller, J., A. M. Cacace, W. E. Lyons, C. B. McGill, and D. K. Morrison. 2000. Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. *Mol. Cell Biol.* **20**:5529–5539.
48. Muller, J., S. Ory, T. Copeland, H. Piwnicka-Worms, and D. K. Morrison. 2001. C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. *Mol. Cell* **8**:983–993.
49. Nguyen, A., W. R. Burack, J. L. Stock, R. Kortum, O. V. Chaika, M. Afkarian, W. J. Muller, K. M. Murphy, D. K. Morrison, R. E. Lewis, J. McNeish, and A. S. Shaw. 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. *Mol. Cell Biol.* **22**:3035–3045.
50. Palmero, I., C. Pantoja, and M. Serrano. 1998. p19ARF links the tumour suppressor p53 to Ras. *Nature* **395**:125–126.
51. Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg. 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene. *Nature* **297**:474–478.

52. Peeper, D. S., J. H. Dannenberg, S. Douma, H. te Riele, and R. Bernards. 2001. Escape from premature senescence is not sufficient for oncogenic transformation by Ras. *Nat. Cell Biol.* **3**:198–203.
53. Quelle, D. E., F. Zindy, R. A. Ashmun, and C. J. Sherr. 1995. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**:993–1000.
54. Rapp, U. R., M. D. Goldsborough, G. E. Mark, T. I. Bonner, J. Groffen, F. H. Reynolds, Jr., and J. R. Stephenson. 1983. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc. Natl. Acad. Sci. USA* **80**:4218–4222.
55. Razidlo, G. L., R. L. Kortum, J. L. Haferbier, and R. E. Lewis. 2004. Phosphorylation regulates KSR1 stability, ERK activation, and cell proliferation. *J. Biol. Chem.* **279**:47808–47814.
56. Roy, F., G. Laberge, M. Douziech, D. Ferland-McCollough, and M. Therrien. 2002. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev.* **16**:427–438.
57. Sage, J., G. J. Mulligan, L. D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou, and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G₁ control and immortalization. *Genes Dev.* **14**:3037–3050.
58. Serrano, M., H. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R. A. DePinho. 1996. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**:27–37.
59. Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach, and S. W. Lowe. 1997. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**:593–602.
60. Sewing, A., B. Wiseman, A. C. Lloyd, and H. Land. 1997. High-intensity Raf signal causes cell cycle arrest mediated by p21^{Cip1}. *Mol. Cell. Biol.* **17**:5588–5597.
61. Sharpless, N. E., N. Bardeesy, K. H. Lee, D. Carrasco, D. H. Castrillon, A. J. Aguirre, E. A. Wu, J. W. Horner, and R. A. DePinho. 2001. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* **413**:86–91.
62. Sharpless, N. E., M. R. Ramsey, P. Balasubramanian, D. H. Castrillon, and R. A. DePinho. 2004. The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* **23**:379–385.
63. Shih, T. Y., A. G. Papageorge, P. E. Stokes, M. O. Weeks, and E. M. Scolnick. 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the p21src protein of Harvey murine sarcoma virus. *Nature* **287**:686–691.
64. Sreeramaneni, R., A. Chaudhry, M. McMahon, C. J. Sherr, and K. Inoue. 2005. Ras-Raf-Arf signaling critically depends on the Dmp1 transcription factor. *Mol. Cell. Biol.* **25**:220–232.
65. Stewart, S., M. Sundaram, Y. Zhang, J. Lee, M. Han, and K. L. Guan. 1999. Kinase suppressor of Ras forms a multiprotein signaling complex and modulates MEK localization. *Mol. Cell. Biol.* **19**:5523–5534.
66. Sundaram, M., and M. Han. 1995. The *C. elegans ksr-1* gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83**:889–901.
67. Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17**:299–313.
68. Webb, C. P., L. Van Aelst, M. H. Wigler, and G. F. Woude. 1998. Signaling pathways in Ras-mediated tumorigenicity and metastasis. *Proc. Natl. Acad. Sci. USA* **95**:8773–8778.
69. Wei, W., R. M. Hemmer, and J. M. Sedivy. 2001. Role of p14^{ARF} in replicative and induced senescence of human fibroblasts. *Mol. Cell. Biol.* **21**:6748–6757.
70. Wei, W., U. Herbig, S. Wei, A. Dutriaux, and J. M. Sedivy. 2003. Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. *EMBO Rep.* **4**:1061–1066.
71. White, M. A., C. Nicolette, A. Minden, A. Polverino, L. Van Aelst, M. Karin, and M. H. Wigler. 1995. Multiple Ras functions can contribute to mammalian cell transformation. *Cell* **80**:533–541.
72. Woods, D., D. Parry, H. Cherwinski, E. Bosch, E. Lees, and M. McMahon. 1997. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21^{Cip1}. *Mol. Cell. Biol.* **17**:5598–5611.
73. Wu, X., J. H. Bayle, D. Olson, and A. J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7**:1126–1132.
74. Xing, H. R., C. Cordon-Cardo, X. Deng, W. Tong, L. Campodonico, Z. Fuks, and R. Kolesnick. 2003. Pharmacologic inactivation of kinase suppressor of *ras-1* abrogates Ras-mediated pancreatic cancer. *Nat. Med.* **9**:1266–1268.
75. Yu, W., W. J. Fantl, G. Harrowe, and L. T. Williams. 1998. Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK. *Curr. Biol.* **8**:56–64.
76. Zhu, J., D. Woods, M. McMahon, and J. M. Bishop. 1998. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12**:2997–3007.