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Ras-Raf-Arf Signaling Critically Depends on the Dmp1 Transcription Factor

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Dmp1 prevents tumor formation by activating the Arf-p53 pathway. In cultured primary cells, the *Dmp1* promoter was efficiently activated by oncogenic Ha-Ras^{V12}, but not by overexpressed c-Myc or E2F-1. *Dmp1* promoter activation by Ras^{V12} depended on Raf-MEK-ERK signaling. Induction of p19^{Arf} and p21^{Cip1} by oncogenic Raf was compromised in *Dmp1*-null cells, which were resistant to Raf-mediated premature senescence. A Ras^{V12}-responsive element was mapped to the 5′ leader sequence of the murine *Dmp1* promoter, where endogenous Fos and Jun family proteins bind. *Dmp1* promoter activation by Ras^{V12} was strikingly impaired in c-Jun as well as in JunB knock-down cells, suggesting the critical role of Jun proteins in the activation of the *Dmp1* promoter. A Ras^{V12}-responsive element was mapped to the unique Dmp1/Ets site on the *Arf* promoter, where endogenous Dmp1 proteins bind upon oncogenic Raf activation. Therefore, activation of *Arf* by Ras/Raf signaling is indirectly mediated by Dmp1, explaining why *Dmp1*-null primary cells are highly susceptible to Ras-induced transformation. Our data indicate the presence of the novel Jun-Dmp1 pathway that directly links oncogenic Ras-Raf signaling and p19^{Arf}, independent of the classical cyclin D1/Cdk4-Rb-E2F pathway.

The *INK4a-ARF* locus on human chromosome 9p21 is disrupted in approximately 40% of human cancers (42). This unusual locus encodes two distinct tumor suppressor proteins, p16^{INK4a} and p14^{ARF} (p19^{Arf} in the mouse), encoded in part via alternative reading frames. p16^{Ink4a} binds to cyclin-dependent kinase 4 (Cdk4) to inhibit Rb phosphorylation, whereas p19^{Arf} binds to the p53 negative regulator, Mdm2, thereby stabilizing and activating p53 (26, 47). *Arf* is induced by potentially harmful growth-promoting signals stemming from overexpression of a variety of oncoproteins, including c-Myc, E2F-1, mutated Ras, v-Abl, and β-catenin (47). This forces incipient cancer cells to undergo p53-dependent proliferative arrest or apoptosis, providing a powerful mode of tumor suppression. In turn, *Arf*-null mice are highly prone to spontaneous tumor development and die of various forms of cancer by 15 months of age (21, 22). Recently, the creation of *Arf-GFP* knock-in mice has provided direct experimental evidence that the *Arf* promoter monitors latent oncogenic signals in vivo (56).

How *Arf* responds to oncogenic Ras signaling remains unclear. Ras family proteins play crucial roles in the control of cell growth and differentiation (29). Overexpression of activated Ras initiates DNA synthesis independent of growth factor stimulation. In immortal rodent cell lines, transformation by oncogenic Ras involves its ability to bind and activate a series of effector proteins, including Raf-1, phosphoinositide 3-OH kinase [PI(3)K], and Ral-GDS (23). Each of these molecules, in turn, activates distinct downstream targets, thereby producing different aspects of the transformed phenotype. The Ras-Raf interaction initiates the mitogen-activated protein kinase (MAPK) cascade, which involves the sequential activation of a series of protein kinases that transmit mitogenic signals to nuclear transcription factors. These kinases include Raf-1, the MEKs (MEK1 and MEK2), and the ERKs (ERK1 and ERK2). On the other hand, the ability of Ras to activate PI(3)K promotes membrane ruffling (20), and the Ral-GDS proteins act as exchange factors that can activate the Ral family of small GTPases (50). Although each of these effector pathways contributes to the transforming activity of Ras in established rodent fibroblast cell lines, activation of the Raf-MEK-ERK pathway is sufficient for transformation (10). Paradoxically, sustained overexpression of oncogenic Ras and its various effectors in nonimmortalized cells has the capacity to elicit irreversible cell cycle arrest by upregulating the levels of p16^{Ink4a}, p19^{Arf}, and p53 in mice and p16^{INK4a} and p53 in humans (29, 33, 44, 48, 57). The ability of oncogenic Ras to induce premature senescence depends on the activity of the Raf-MEK-ERK pathway that mediates proliferation (25) but is nullified in primary mouse embryo fibroblasts (MEFs) lacking either *Arf* or p53 (21, 33, 48).

Among known *Arf* activators, the Dmp1 transcription factor (cyclin D-interacting Myb-like protein 1) is a bona fide tumor suppressor (18, 19). Dmp1 was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (14). The protein binds to nonameric CCCG(G/T)ATG(T/C) DNA consensus sequences, a subset of which is also bound by proteins of the Ets family. Dmp1 can physically interact with any of the three D-type cyclins, each of which can interfere, in a Cdk4-independent manner, with Dmp1's ability

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to bind to DNA (15). Overexpression of Dmp1 in mouse fibroblasts arrests cell cycle progression, an effect that can be overridden by coexpression of D-type cyclins (15). Importantly, Dmp1 directly binds to the *Arf* promoter to activate its expression, thereby inducing p53-dependent cell cycle arrest (17).

Several lines of evidence have implicated Dmp1 in the process by which Ras induces *Arf* and p53. When primary *Dmp1*-null MEFs were explanted into culture and continuously passaged, $p19^{Arf}$ and p53 levels remained uncharacteristically low and the cells exhibited a prolonged proliferative capacity, readily yielding established cell lines that retained wild-type *Arf* and *p53*. Such cells were susceptible to transformation by oncogenic Ras alone without any requirement for an immortalizing oncogene, such as Myc or adenovirus E1A. Thus, the activity of the Arf-p53 pathway is strikingly impaired in *Dmp1*-null cells (18).

Dmp1-null mice are prone to spontaneous tumor development in their second year of life, and tumor formation was accelerated when the animals were neonatally treated with ionizing radiation or dimethylbenzanthracene (18, 19), a carcinogen that induces Ras mutations in vivo (37). When crossed onto a $Dmp1^{+/-}$ or $Dmp1^{-/-}$ background, lymphomas induced by an Eμ-*Myc* transgene were greatly accelerated with no differences between cohorts lacking one or two *Dmp1* alleles. The retention and expression of the wild-type *Dmp1* allele in tumors arising in heterozygotes indicated that *Dmp1* is haploinsufficient for tumor suppression (19, 38). Interestingly, the combined frequencies of *p53* mutation and *Arf* deletion in the $Dmp1^{-/-}$ and $Dmp1^{+/-}$ lymphomas were significantly lower than those in $Dmp1^{+/+}$ tumors (~14% versus ~50%). Thus, Dmp1 is a physiological regulator of the Arf-p53 pathway in vivo (19). The present studies were undertaken in an attempt to define the mechanism(s) by which Ras induces *Arf*. Here we show that Dmp1 is a key mediator of this process.

MATERIALS AND METHODS

Cell culture and reporter assays. Wild-type and *Dmp1*-null MEFs were established from 13.5-day-old embryos as previously described (18). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg of gentamicin/ml. BALB/3T3, C33A, and IMR-90 cells were purchased from the American Type Culture Collection. For reporter assays, 1.5×10^5 cells were seeded into 60-mm diameter culture dishes 24 h before transfection. In order to study the responsiveness of the *Dmp1* and *Arf* promoters to oncogenic Ha-Ras^{V12}, 4 μg of luciferase reporter DNA was cotransfected with 0.3 to 1 μg of Ras expression vectors and 4 μg of internal control actin promoter-secreted endocrine alkaline phosphatase vector (a gift from Michael Ostrowski, Ohio State University). Genejuice (Novagen) was used in all transfections. Specific MAPK inhibitors U0126 (for MEK/ERK), SP600125 (for JNK/SAPK), (L)-JNKI1 (for JNK/SAPK), and SB203580 (for p38) were purchased from Calbiochem. All of them were used at 10 μM.

Plasmid DNA. pBabepuro-Ha-Ras^{V12}, Ras^{V12S35}, Ras^{V12G37}, and Ras^{V12C40} viral vectors were obtained from Scott Lowe, Cold Spring Harbor Laboratory, and from Christopher Counter, Duke University (13). Expression vectors for Ha-Ras^{V12}, Ras^{V12S35}, Ras^{V12G37}, and Ras^{V12C40} were created by recloning the cDNA from the pBabepuro vector into the pcDNA3 vector (Invitrogen). Retroviral expression vectors for ΔRaf:ER[DD] and empty estrogen receptor (ER) vector were described previously (28). Rc/CMV-c-Myc was obtained from John Cleveland, St. Jude Children's Research Hospital. pcDNA-E2F-1 was received from Joseph Nevins, Duke University. Expression vectors for c-Fos and c-Jun family proteins driven by the cytomegalovirus (CMV) promoter were obtained from Tom Curran, St. Jude Children's Research Hospital. Expression vectors for D-type cyclins, cyclin A, and cyclin H were described previously (15).

Molecular cloning of the murine *Dmp1* promoter. The murine *Dmp1* promoter was cloned from the Bacterial Artificial Chromosome library derived from 129/Svj mice (Mouse ES Release II; Genome Systems Inc.) with 60-bp synthetic oligonucleotides covering the 5′ end of the murine *Dmp1* cDNA (14). A 1.8-kb PstI fragment hybridizing with the probe was cloned into the artificially created PstI site of the pGL2-basic vector to generate the −1787 PstI promoter construct. In order to create deletion mutants, the plasmid DNA was digested with SmaI plus SpeI, BstXI, NsiI, or ApaI, and the gel-purified DNA fragments were filled with Klenow enzyme or T4 DNA polymerase and then ligated. The transcription initiation sites on the murine *Dmp1* promoter were determined by using the SMART rapid amplification of cDNA ends (RACE) kit (Clontech) with total RNA isolated from NIH 3T3 cells.

In vitro mutagenesis. The murine *Dmp1* promoter deletion point mutants were generated by use of an in vitro mutagenesis kit (Stratagene). In order to introduce point mutations at the Ets site (−9 to −17) on the murine *Dmp1* promoter, PCR was performed by using oligonucleotides 5′-GCCTCGCGGCTCCGTCGTAGGTGGCTGGTTGCGC-3′ and its reverse complementary sequence. The mutated Ets site is underlined. In order to delete the Ets site, oligonucleotide 5′-GCCTCGCGGCTCCGTGGTGGCTGGTTGCGC-3′ and its reverse complementary sequence were used. In order to delete the 5′ leader sequence on the *Dmp1* promoter, oligonucleotide 5′-TGGCTGGTTGCGCTGCAGGCTAGCTCGA-3′ and its reverse complementary sequence were used. In order to mutate the AP-1 and AML1 consensus-like sequences on the 5′-untranslated region of the murine *Dmp1* promoter, oligonucleotide 5′-GGCTGGTTGCGCTCGAAAACCCCAGCTGCAGGC-3′ and its reverse complementary strand and 5′-GGTTGCGCTCGCTCAATCTAGCTGCAGGCTAGCTCG-3′ and its reverse complementary strand were used, respectively. The mutated consensus sequences for AP-1 and AML1 are underlined. All the *Dmp1* promoter mutants were subjected to sequencing analysis to confirm the presence of mutation or deletion.

Retroviruses and RNAi. Ecotropic retroviruses encoding Ha-Ras^{V12}, ΔRaf:ER[DD], or empty (ER) vector were prepared by transfecting 293T cells with a helper ecotropic retrovirus plasmid defective in psi-2 packaging sequences together with pBabepuro vectors containing Ha-Ras^{V12}, or ΔRaf:ER[DD] or with empty (ER) vector. Viruses were harvested every 6 h for 24 to 72 h after transfection, pooled, filtered, and stored at −80°C until use. Ecotropic retroviruses to knock-down mouse c-Jun, JunB, JunD, c-Fos, Fra-1, or Fra-2 were prepared by using the pSuper RNA interference (RNAi) system (Oligoengine). The 19-bp target sequence was 5′-GCGCATGAGGAACCGCATT-3′ for c-Jun, 5′-GACCAGGAGCGCATCAAAG-3′ for JunB, 5′-AAGCCAGAACACCGAGCTG-3′ for JunD, 5′-GCGGAGACAGATCAACTTG-3′ for c-Fos, 5′-ATTGGAGGATGAGAAATCG-3′ for Fra-1, and 5′-TCAACGCCATCACCACCAG-3′ for Fra-2. The effectiveness of down regulation of each gene product was studied by Western blotting with specific antibodies. In order to create growth curves of MEFs with activated c-Raf, $Dmp1^{+/+}$ and $Dmp1^{-/-}$ MEFs were infected with retroviruses expressing ΔRaf:ER or empty ER. Forty-eight hours after infection, cells were selected with 2 μg of puromycin/ml for 48 h. A total of 10^5 puromycin-resistant cells were seeded in 60-mm-diameter culture dishes and then treated with 1 μM 4-hydroxytamoxifen (4-HT; Sigma). The medium was changed every 48 h with fresh 4-HT, and cells were counted.

Northern and Western blotting. Total RNA was extracted by using TRIzol (Invitrogen) from MEFs infected with retroviruses encoding Ha-Ras^{V12}, ΔRaf:ER[DD], or empty vector. Northern blotting was performed with 10 μg of total RNA by using Turboblotter (Schleicher & Schuell). The filter was hybridized with a murine *Dmp1*-specific probe (KpnI-NcoI fragment; 426 bp) and then with a mouse β-actin-specific probe. For Western blotting, proteins were extracted with EBC buffer (14) with proteinase inhibitor cocktail III and leupeptin (Calbiochem). For detection of Dmp1, affinity-purified RAF antibodies (14) were used. The following antibodies (all from Santa Cruz Biotechnology) were used for the detection of AP-1 proteins in Western blotting, an electrophoretic mobility shift assay (EMSA), and a chromatin immunoprecipitation (ChIP) assay: c-Fos (sc-52x), FosB (sc-48x), Fra-1 (sc-605x), Fra-2 (sc-171x), c-Jun (sc-1694x), phospho-c-Jun (sc-7981x), JunB (sc-8051x), JunD (sc-74x), Fos family (sc-253x), Jun family (sc-44x), ATF-1 (sc-243x), ATF-2 (sc-187x), ATF-3 (sc-188x), CREB1 (sc-58x), and CREB 2 (sc-200x). For Western blotting of other proteins, the following antibodies were used: p-ERK (sc-7383), cyclin D1 (sc-450), cyclin D2 (sc-34B1-3), cyclin D3 (sc-18B-10), cyclin A (sc-751), cyclin H (D-10), $p16^{Ink4a}$ (sc-1207), $p21^{Cip1}$ (sc-6246), and actin (sc-1615). For detection of $p19^{Arf}$, affinity-purified rabbit antibodies were used (17, 55). For detection of p53, Ab7 (Oncogene Science) was used.

EMSA. In order to detect proteins that bind to the 5′-untranslated region of the murine *Dmp1* promoter, lysates were prepared from MEFs expressing ΔRaf:ER[DD] with or without treatment with 2 μM 4-HT for 16 h. The lysate was

incubated with ^{32}P-labeled oligonucleotide probe covering the 5′ leader sequence of the murine DMP1 cDNA obtained by annealing sense oligonucleotide 5′-TGGTTGCGCTCGCTCACCCCAGCTGCAGCCA-3′ and its reverse complementary sequence (the AP-1-like sequence is underlined, and the AML1 consensus sequence is italicized). For competition assays, a 100-fold excess of unlabeled oligonucleotides was added to reaction mixtures before probe incubation. To verify the identity of the proteins in shifted complexes, reaction mixtures were incubated with control nonimmune rabbit serum or with specific antibodies to Fos, Jun, ATF, and CREB family proteins (all from Santa Cruz Biotechnology).

ChIP. ChIP were performed as described previously (8). Briefly, MEFs expressing ΔRaf:ER[DD] were either left untreated or treated with 2 μM 4-HT for 0, 8, 16, and 24 h. The lysates were precipitated with specific antibodies to Fos, Jun family proteins, or with anti-Dmp1 antibody (RAF) and incubated at 4°C overnight. The immunoprecipitated DNA was detected by PCR, including 1 μCi of [α-^{32}P]dATP (Amersham Pharmacia) separated on a 10% nondenaturing polyacrylamide gel. For detection of the endogenous Dmp1 on the *Arf* promoter, sense primer 5′-AAAGGGCGCAGCTACTGCTA-3′ and anti-sense primer 5′-TCTTTGCTCCACGCCCATCT-3′ were used. For detection of the endogenous AP-1 family transcription factors on the murine *Dmp1* promoter, sense primer 5′-CTCGCGGCTCCGTTTCCG-3′ and antisense primer 5′-CCTGAAGGTTC CATCGCACT-3′ were used. For the control amplification of 2-kb upstream sequence on the *Dmp1* promoter, sense primer 5′-TCTCCATAGCAATGCCC TTTAC-3′ and antisense primer 5′-CGAGCCATTTGGGTATGTGTA-3′ were used.

Nucleotide sequence accession number. The gene accession number for the murine *Dmp1* promoter used in this study is AY702209.

RESULTS

Molecular cloning of the murine *Dmp1* promoter and its responsiveness to oncogenes. In order to study regulation of *Dmp1* transcription, we cloned the murine *Dmp1* promoter from a commercially available murine genomic library using synthetic oligonucleotide probes covering the 60 bp of the 5′ end of the murine *Dmp1* cDNA. A 1.8-kb PstI fragment hybridizing with the probe was cloned into the pGL2-basic vector (Promega), and a series of deletion mutants were created (Fig. 1A). The *Dmp1* promoter has multiple transcription initiation sites; however, the most commonly used site was mapped to the guanine residue 2 bp upstream from the 5′ end of the *Dmp1* cDNA first isolated from CTLL-2 cells (14). We tested basal *Dmp1* promoter-reporter activity in mouse NIH 3T3 cells, BALB/3T3 cells, and also in human carcinoma C33A cells. Deletion from the −1787 PstI site to the −374 NsiI site did not influence basal promoter activity, while deletion from the −374 NsiI site to the −88 ApaI site (Fig. 1, Del 5) dramatically decreased basal *Dmp1* promoter function in the three cell lines.

The minimal *Dmp1* promoter is TATA-less, GC rich, and contains multiple transcription factor binding sequences (Fig. 1B). In addition, AP-1-like and AML1-like consensus binding sequences are present in the 5′ leader sequence. We tested whether the *Dmp1* promoter can be activated by overexpression of c-Myc, E2F-1, or by oncogenic Ha-Ras^{V12}, all of which can upregulate p19^{Arf} mRNA and protein levels (3, 33, 55). Neither c-Myc nor E2F-1 activated the murine *Dmp1* promoter, whereas oncogenic Ras^{V12} weakly stimulated the promoter in *Ink4a-Arf*-null NIH 3T3 cells (Fig. 2A). The same data were obtained in 293T cells, where both Rb and p53 were functionally inactivated (data not shown). Oncogenic Ras overexpression induces transformation in immortalized fibroblasts but causes p19^{Arf} (and/or p16^{Ink4a}) and p53-dependent cellular senescence in primary fibroblasts (33, 44). The *Dmp1* promoter was more efficiently activated by H-Ras^{V12} in primary wild-type MEFs (Fig. 2B), as well as in IMR-90 cells, primary diploid fibroblasts derived from human fetal lung (data not shown). Overexpression of c-Myc and E2F-1 had no inductive effects on the murine *Dmp1* promoter even in primary MEFs (data not shown). As for NIH 3T3 cells, Ras^{V12} did not efficiently activate the *Dmp1* promoter in immortalized *Arf*-null MEFs; however, a response to Ras^{V12} was restored by cotransfecting a p19^{Arf} expression vector (Fig. 2B). Thus, the ability of Ras^{V12} to activate *Dmp1* is maximized in nonimmortalized cells that retain functional Arf and p53 activities.

Activation of the *Dmp1* promoter by Ras^{V12} is dependent on the Ras-MEK-ERK pathway. The transforming activity of activated Ras depends on at least three downstream effectors, including Raf-1/MAPK, Ral-GDS, and PI(3)K, which mediate different aspects of oncogenic transformation. Oncogenic H-Ras^{V12} variants with mutations T35S, E37G, or Y40C bind to and primarily activate Raf/MAPK, PI(3)K, or Ral-GDS, respectively (20). The *Dmp1* promoter was most efficiently activated by Ras^{V12S35}, followed by Ras^{V12G37}, while Ras^{V12C40} had a minimal effect (Fig. 2C). Mammalian MAPKs branch into three major pathways that involve MEK/ERK, JNK/SAPK, and p38 signaling. These three pathways are mainly activated by mitogens, by inflammatory cytokines, UV, and γ-irradiation, and by osmotic stress signaling, respectively. In order to study which of these pathways is important in Ras^{V12}-mediated *Dmp1* promoter activation, reporter assays were performed with an Ha-Ras^{V12} expression vector in the presence of MAPK inhibitors (Fig. 2D). Activation of the *Dmp1* promoter by Ras^{V12} was efficiently blocked by U0126 alone (MEK/ERK pathway), whereas SP600125 (targeting JNK/SAPK) and SB203580 (targeting p38) had partial inhibitory effects. When combined, U0126 plus SP600125 synergistically blocked *Dmp1* promoter activation by Ras, whereas the combination of SP600125 and SB203580 exhibited no additive effects (Fig. 2D). The effects of the SP compound were confirmed by an independent reporter assay with another JNK inhibitor, (L)-JNKI1 (Calbiochem). Together, these data suggest that the Raf-MEK-ERK pathway plays the major role in *Dmp1* promoter activation by Ras^{V12}.

Activated Ras^{V12} and c-Raf induce *Dmp1* mRNA. Northern blotting analysis demonstrated accumulation of *Dmp1* transcripts in cells infected with retroviruses encoding Ha-Ras^{V12} (Fig. 3A, left panel). To determine if activation of the Raf-MEK-ERK pathway can lead to alterations in *Dmp1* mRNA and protein expression, wild-type MEFs were infected with a retrovirus expressing ΔRaf:ER[DD], a mutated Raf kinase whose activity is regulated by tamoxifen (28, 54). Infected cells were either left untreated or were treated with 2 μM 4-HT for 0 to 36 h. Cells were harvested at various times thereafter and were analyzed by Northern and Western blotting with specific probes and antibodies to Dmp1. As an additional control, wild-type MEFs were infected with an empty ER vector and treated with 2 μM 4-HT. Treatment of control MEFs with 4-HT did not significantly change the levels of Dmp1 mRNA or protein (Fig. 3A and B). Activation of ΔRaf:ER by 4-HT increased the *Dmp1* mRNA threefold by 8 h and eightfold by 16 to 24 h (Fig. 3A). Proportionate increases in Dmp1 protein expression were observed (Fig. 3B). Dmp1 is a phosphoprotein with different isoforms of various molecular masses (120 to 130 kDa) (14). We noticed that some additional immunoreactive

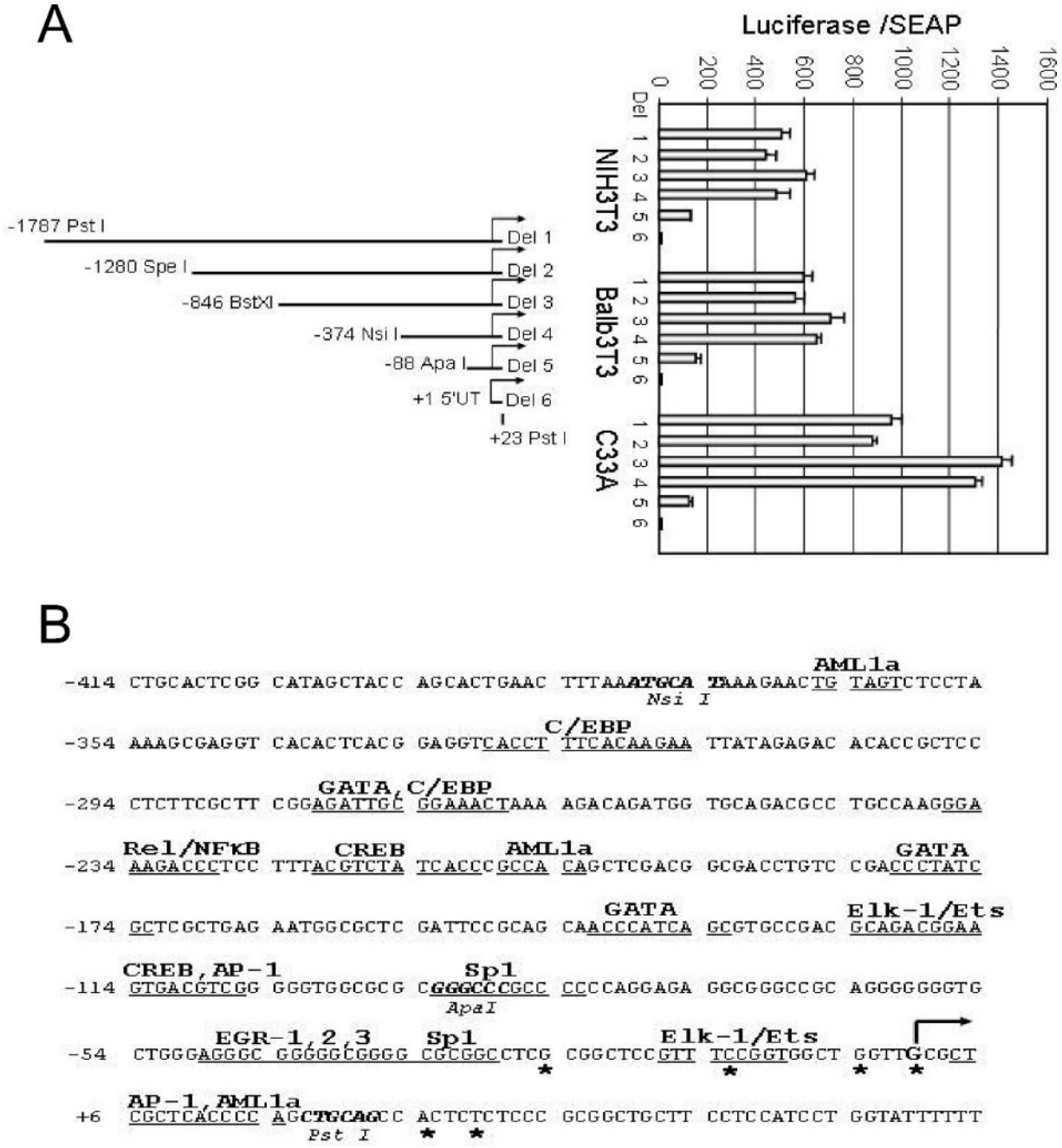

FIG. 1. Cloning of the murine *Dmp1* promoter and the sequence of the proximal region. The murine *Dmp1* promoter was cloned from the Bacterial Artificial Chromosome library derived from 129/Svj mice (Mouse ES Release II; Genome Systems Inc.) with 60-bp synthetic oligonucleotides covering the 5′ end of the murine *Dmp1* cDNA. The 1.8-kb PstI fragment hybridizing with the probe was cloned into the polylinker site of the pGL2-basic vector (Promega). (A) Deletion analysis of the Dmp1 promoter. NIH 3T3, BALB/3T3, and C33A cells were transfected with 4 μg of murine *Dmp1* promoter-luciferase constructs and 4 μg of secreted alkaline phosphatase expression vector driven by the actin promoter. Relative luciferase levels corrected by the internal control alkaline phosphatase levels are shown. Deletion of the promoter up to the NsiI site did not influence the endogenous promoter activity, while deletion of the promoter to the ApaI site resulted in a 4- to 10-fold decrease in the relative luciferase levels. (B) Nucleotide sequences of the proximal region (−414 to +65). The major transcription initiation sites determined by 5′-RACE are shown in large capital letters. Consensus sequences for possible transcription factor binding are also shown.

species of both higher (~135 kDa) and lower (~110 kDa) molecular masses were observed at 16 to 36 h after ΔRaf:ER activation, suggesting that kinases regulated by the Raf-MEK-ERK pathway could further modify the Dmp1 protein (Fig. 3B). The rate of Dmp1 protein accumulation was several hours slower than that of phospho-ERK but was very similar to that of cyclin D1 induction, which is also positively regulated by the MEK-ERK pathway (Fig. 3B).

Dmp1 is a key target in Raf-induced cellular senescence. We next infected wild-type and *Dmp1*-null MEFs with retroviruses

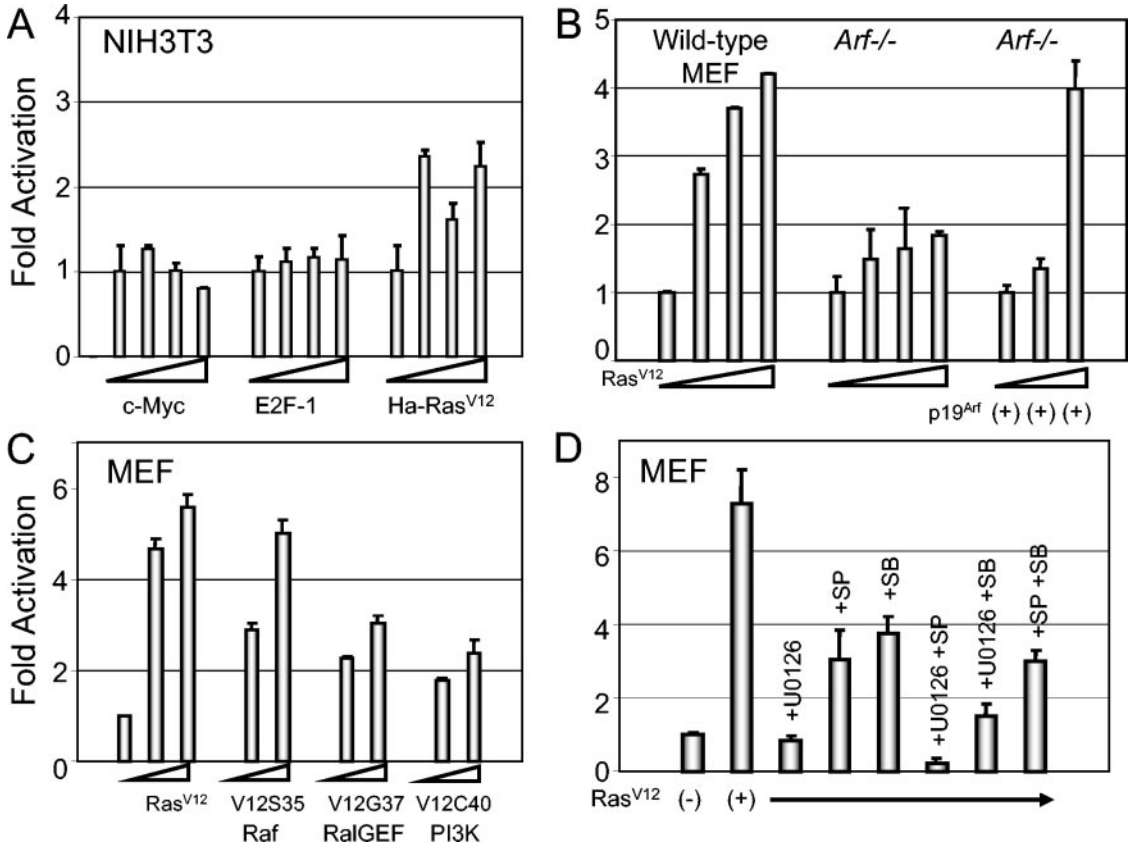

FIG. 2. Responsiveness of the *Dmp1* promoter to various oncogenic stimuli. (A) The *Dmp1* promoter is weakly activated by oncogenic Ras^{V12} but not by c-Myc or E2F-1 in 3T3 cells. NIH 3T3 cells were transfected with the luciferase reporter −374 NsiI with expression vectors for c-Myc, E2F-1, and Ha-Ras^{V12} driven by the CMV promoter. The numbers show the fold activation of the luciferase reporter corrected by the internal control secreted endocrine alkaline phosphatase levels. (B) $p19^{Arf}$ dependence of the response of the *Dmp1* promoter to Ras^{V12}. Reporter assays were performed in passage-5 wild-type MEFs (left panel), *Arf*-null MEFs (middle panel), and *Arf*-null MEFs with $p19^{Arf}$ expression vector (right panel). The *Dmp1* promoter was efficiently activated by Ras^{V12} in wild-type cells but not in *Arf*-null cells. The loss of responsiveness of the *Dmp1* promoter to Ras^{V12} in *Arf*-null cells was restored by cotransfecting the Ras^{V12} and $p19^{Arf}$ expression vectors. (C) The *Dmp1* promoter is activated by Ras^{V12S35} but not by Ras^{V12G37} or by Ras^{V12C40}. A *Dmp1* reporter assay was performed in wild-type MEFs with Ras^{V12} double mutants (Ras^{V12S35}, Ras^{V12G37}, and Ras^{V12C40}). The *Dmp1* promoter was activated most efficiently by Ras^{V12S35}, suggesting that the MAPK pathway plays the most significant role in *Dmp1* promoter activation in response to oncogenic Ras signaling. (D) Activation of the *Dmp1* promoter by MAPK pathways is inhibited by the MEK/ERK inhibitor U0126. In order to study which MAPK pathway is key to Ras^{V12}-mediated *Dmp1* promoter activation, reporter assays were performed with pCMV-Ras^{V12} in the presence of 10 μM U0126, 10 μM SP600125, 10 μM SB203580, or a combination of these compounds. Activation of the *Dmp1* promoter by Ras^{V12} was efficiently blocked by U0126.

encoding ΔRaf:ER or the control ER vector. After selection with puromycin, 10^5 cells were plated in 60-mm-diameter dishes and 1 μM 4-HT was added to activate ΔRaf:ER. The total cell number did not increase in 4HT-treated wild-type cells expressing ΔRaf:ER, since oncogenic c-Raf activation induces replicative senescence in primary MEFs (Fig. 3C, left panel). There were no significant differences in the proliferation of cells infected with the control ER vector with or without 4-HT and untreated cells infected with the ΔRaf:ER virus (Fig. 3C, left panel). In contrast, *Dmp1*-null cells expressing activated ΔRaf:ER grew exponentially after treatment with 4-HT and reached the same saturation density as control populations (Fig. 3C, right panel). When cell lysates prepared from wild-type and *Dmp1*-null cells treated with 4-HT were fractionated on denaturing gels and blotted with various antibodies, we observed accumulation of $p19^{Arf}$ (fourfold) and p53 (twofold) from 16 to 36 h in wild-type cells expressing activated ΔRaf:ER. However, the basal levels of $p19^{Arf}$ were very low in *Dmp1*-null cells (18), and neither $p19^{Arf}$ nor p53 was induced by activation of ΔRaf:ER (Fig. 3D). We also observed very rapid accumulation of $p21^{Cip1}$ in wild-type MEFs (twofold increase at 3 h and eightfold increase by 8 to 36 h), whereas the levels of $p16^{Ink4a}$ did not appreciably change upon Raf activation (Fig. 3D). Thus, Dmp1 contributes to $p19^{Arf}$ and $p21^{Cip1}$ upregulation and proliferative arrest in response to oncogenic Raf signaling.

Mapping of Ras^{V12}-responsive elements in the *Dmp1* promoter. Deletion of the *Dmp1* promoter 5′ to the −374 NsiI site did not significantly change its responsiveness to Ras^{V12} (Fig. 4A). However, when we deleted the 286-bp fragment from −374 NsiI to −88 ApaI, we observed a twofold increase in *Dmp1* promoter activity in response to oncogenic Ras (fourfold versus eightfold). Ets1/Ets2 transcription factors have been reported to play crucial roles in the regulation of the human $p16^{INK4A}$ promoter in response to Ras-Raf-MEK-ERK signaling (32). EGR-1 has also been reported to play important

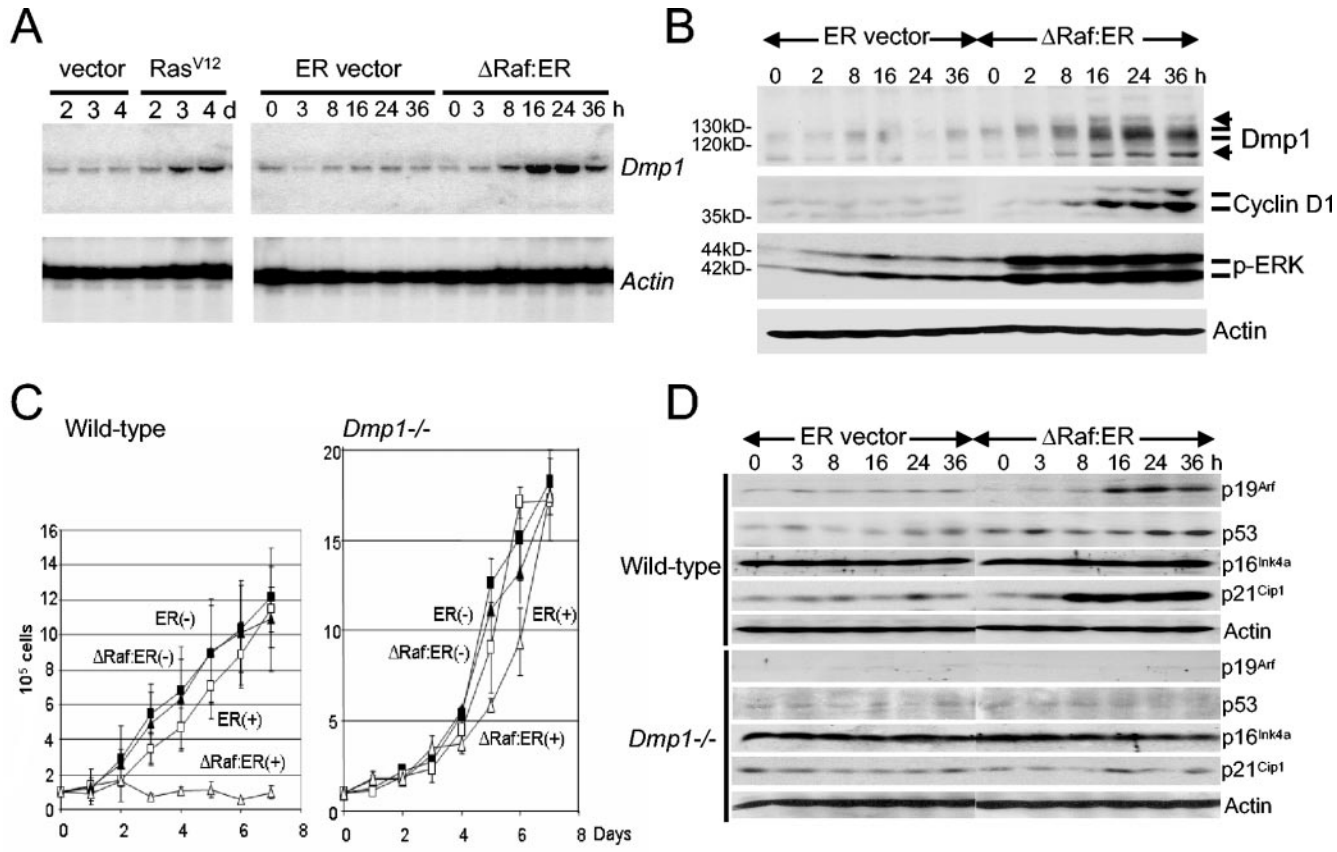

FIG. 3. Dmp1 plays a key role in c-Raf-induced senescence. (A) Ha-Ras^{V12} and activated ΔRaf:ER induce *Dmp1* mRNA. Passage-5 wild-type MEFs were infected with retroviruses expressing Ha-Ras^{V12} (left panel) or ΔRaf:ER (right panel) and selected with puromycin. Northern blotting was performed with a *Dmp1*-specific probe with β-actin as an internal control. Expression of Ha-Ras^{V12} increased *Dmp1* mRNA by threefold at day 3 to 4 (left panel). Activation of ΔRaf:ER by 2 μM 4-HT increased *Dmp1* mRNA by threefold at 8 h and by eightfold at 16 to 24 h (right panel). (B) Activated ΔRaf:ER induces the Dmp1 protein. The increase of *Dmp1* mRNA resulted in Dmp1 protein induction by threefold at 8 h and eightfold by 24 h. The kinetics was slower than for phosphor-ERK accumulation but very similar to that of cyclin D1 induction. (C) *Dmp1*-null cells are resistant to Raf-induced cell cycle arrest. In order to study the biological effects of c-Raf activation in *Dmp1*$^{-/-}$ cells, both wild-type and *Dmp1*-null MEFs were infected with retroviruses encoding ΔRaf:ER or the control ER vector. After selection with puromycin, 10^5 cells were plated in 60-mm-diameter dishes and 1 μM 4-HT was added to activate ΔRaf:ER. (Open symbols, with 4-HT; closed symbols, without 4-HT). Note that *Dmp1*-null cells expressing ΔRaf:ER (triangles) grew at the same rate as those expressing ER vector alone (rectangles), while wild-type cells expressing activated ΔRaf:ER underwent irreversible cell cycle arrest. (D) p19^{Arf} does not increase in response to ΔRaf:ER in *Dmp1*-null cells. None of p19^{Arf}, p53, and p21^{Cip1} increased in response to ΔRaf:ER in *Dmp1*-null cells, while p16^{Ink4a} levels remained constant in both wild-type and *Dmp1*-null cells.

roles in Ras signaling (2). However, mutation or deletion of these Ets/Elk, EGR-1 sites did not alter the responsiveness of the *Dmp1* promoter to oncogenic Ras^{V12}, although mutation or deletion of the former significantly lowered the basal levels (Fig. 4A; data not shown for EGR-1). In contrast, deletion of the 14-bp 5′-untranslated region (from +4 cytosine to +17 guanine) resulted in elimination of the *Dmp1* promoter response to Ras^{V12} (Fig. 4A). The presence of the Ras-responsive elements in the 5′-untranslated region was confirmed by eliminating the 14-bp fragment from the −374 NsiI promoter reporter construct (Fig. 4A).

The Ras-responsive element in the *Dmp1* promoter contains an AP-1-like sequence (CTCGCTCA) as well as an AML1-like sequence (ACCCCA) (Fig. 1B). Both AP-1 and AML1 can be activated in oncogenic Ras signaling (6, 51). The Ras response of the *Dmp1* promoter was significantly compromised by mutating the AP-1-like sequence (−88 ApaI AP-1 mut and −1787 PstI AP-1 mut), but not by mutating the AML1 consensus sequence (−88 ApaI AML1 mut), suggesting that proteins of the AP-1 family could play key roles in *Dmp1* regulation (Fig. 4A).

We therefore studied the kinetics of accumulation of Fos and Jun family members in response to ΔRaf:ER activation. We observed a rapid increase of c-Fos (3 h) and Fra-1 and also increases in the JunB and c-Jun proteins and, later, JunD (Fig. 4B). FosB was not detected in MEFs with or without Raf activation (data not shown), and we did not observe any significant change of Fra-2 levels upon Raf activation (Fig. 4B). We also searched for transcription factors that could bind to the 14-bp Ras-responsive element in an EMSA performed with nuclear extracts isolated from MEFs with activated ΔRaf:ER (16 h). A major complex (labeled A in Fig. 4C) was formed using nuclear extracts from induced cells (left panel, lane 2), and both classical AP-1 (TGACTCA) and CREB (TGACG

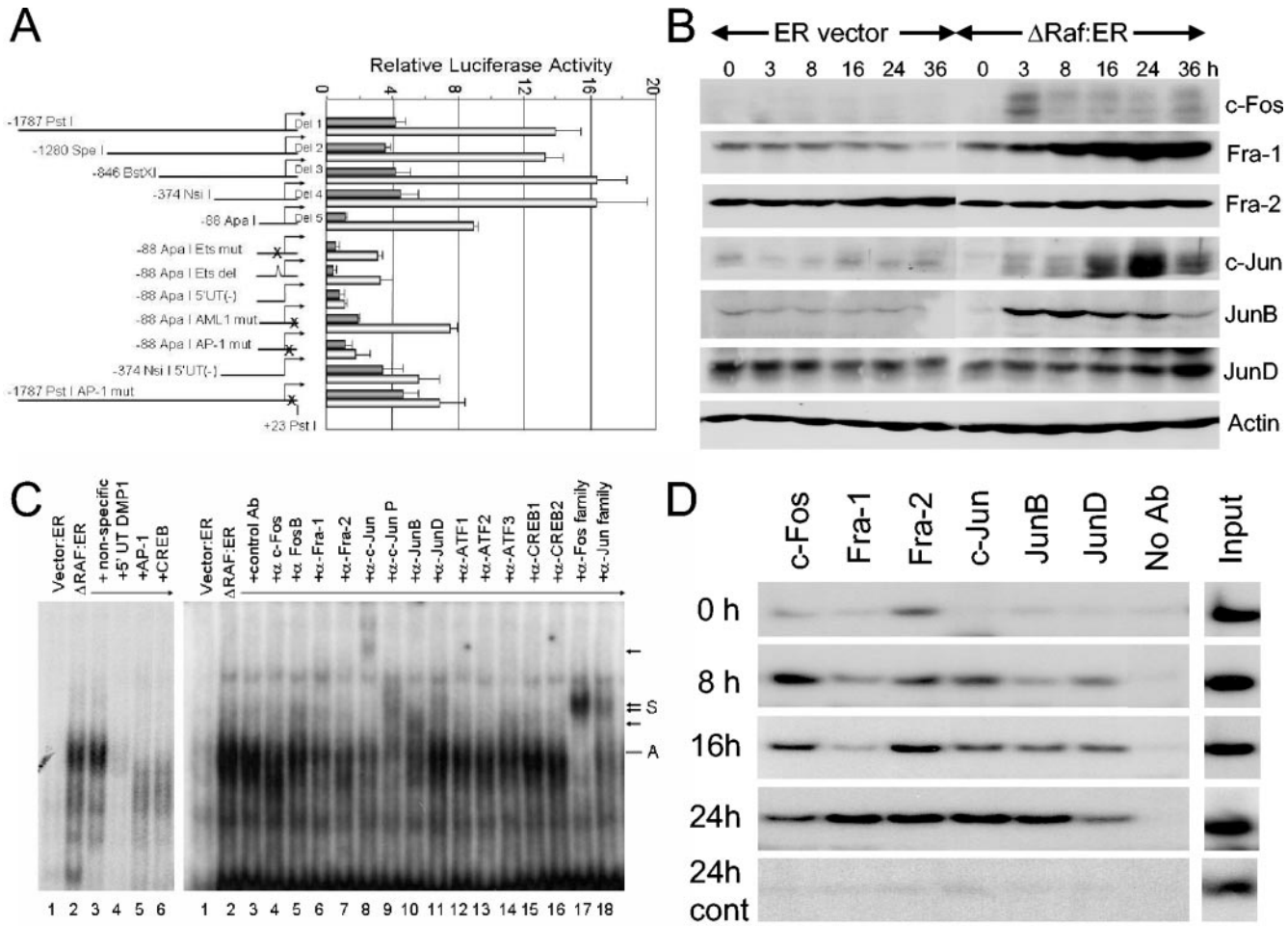

FIG. 4. Mapping of the Ras^{V12}-responsive element on the murine *Dmp1* promoter. (A) Mapping of the Ras^{V12}-responsive element on the *Dmp1* promoter based on a luciferase assay. Reporter assays were performed in IMR-90 cells with the deletion mutants described in Fig. 1A and their derivatives with (white columns) or without (black columns) Ha-Ras^{V12} expression vector. (B) Kinetics of Fos and Jun family protein accumulation in cells with activated c-Raf. Wild-type MEFs were infected with vector ER or ΔRaf:ER retroviruses and were stimulated with 4-HT. The lysates were analyzed by Western blotting with specific antibodies. (C) Identification of the transcription factors that bind to the AP-1-like sequence on the *Dmp1* promoter by EMSA. Lysates were prepared from wild-type MEFs expressing ΔRaf:ER or ER alone and stimulated with 2 μM 4-HT for 16 h. Complex A formation was antagonized by a 100-fold excess of cold oligonucleotides derived from the AP-1-like sequence on the *Dmp1* promoter, as well as classical AP-1 or CREB consensus sequences reported earlier. The complex was supershifted only with antibodies to c-Fos, Fra-1, c-Jun, phospho-c-Jun, JunB, and JunD (arrows, S). (D) ChIP assay of the *Dmp1* promoter. Significant amounts of c-Fos, Fra-1, c-Jun, JunB, and JunD were detected on the *Dmp1* promoter in response to activated Raf signaling. The specificity of the Fos/Jun signals was confirmed by control PCR amplification of the *Dmp1* promoter sequence located 2 kb upstream from the transcription initiation site (24 h cont).

TCA) consensus oligonucleotides blocked its formation (lanes 5 and 6). Antibodies to Fos family and Jun family proteins supershifted the AP-1-like complex (Fig. 4C, right panel, lanes 17 and 18). Fos-family proteins, except for FosB, were detected on the 31-bp oligonucleotides covering the Ras-responsive element (Fig. 4C, right panel, lanes 4 to 7). Among the Jun family proteins, c-Jun and especially phosphorylated c-Jun, as well as JunB, appeared to contribute to the major complexes, although a small amount of JunD was also detected (Fig. 4C, right panel, lanes 8 to 11). Although formation of complex A was completely inhibited by CREB-consensus oligonucleotides, none of the ATF (ATF-1, -2, and -3) or CREB family proteins (CREB1 and CREB2) was detected in the AP-1-like complex on the *Dmp1* promoter (Fig. 4C, right panel, lanes 12 to 16). Complex A was not supershifted with antibodies to CREM1, AML1, or c-Maf (data not shown).

Results from the EMSA were confirmed by ChIP with specific antibodies (Fig. 4D). The levels of Fos and Jun family proteins were very low on the *Dmp1* promoter before addition of 4-HT, except for Fra-2. Significantly increased levels of c-Fos (8 to 24 h), Fra-1 (24 h), c-Jun (8 to 24 h), JunB (16 to 24 h), and JunD (8 to 24 h) proteins were detected on the *Dmp1* promoter after addition of 4-HT in cells expressing ΔRaf:ER (Fig. 4D). The specificity of binding of Fos and Jun proteins to the region of interest was confirmed by PCR amplification of the sequences located 2 kb upstream of the transcription initiation site with samples harvested at 24 h (Fig. 4D, 24 h control). Thus, Fos and Jun family proteins, especially

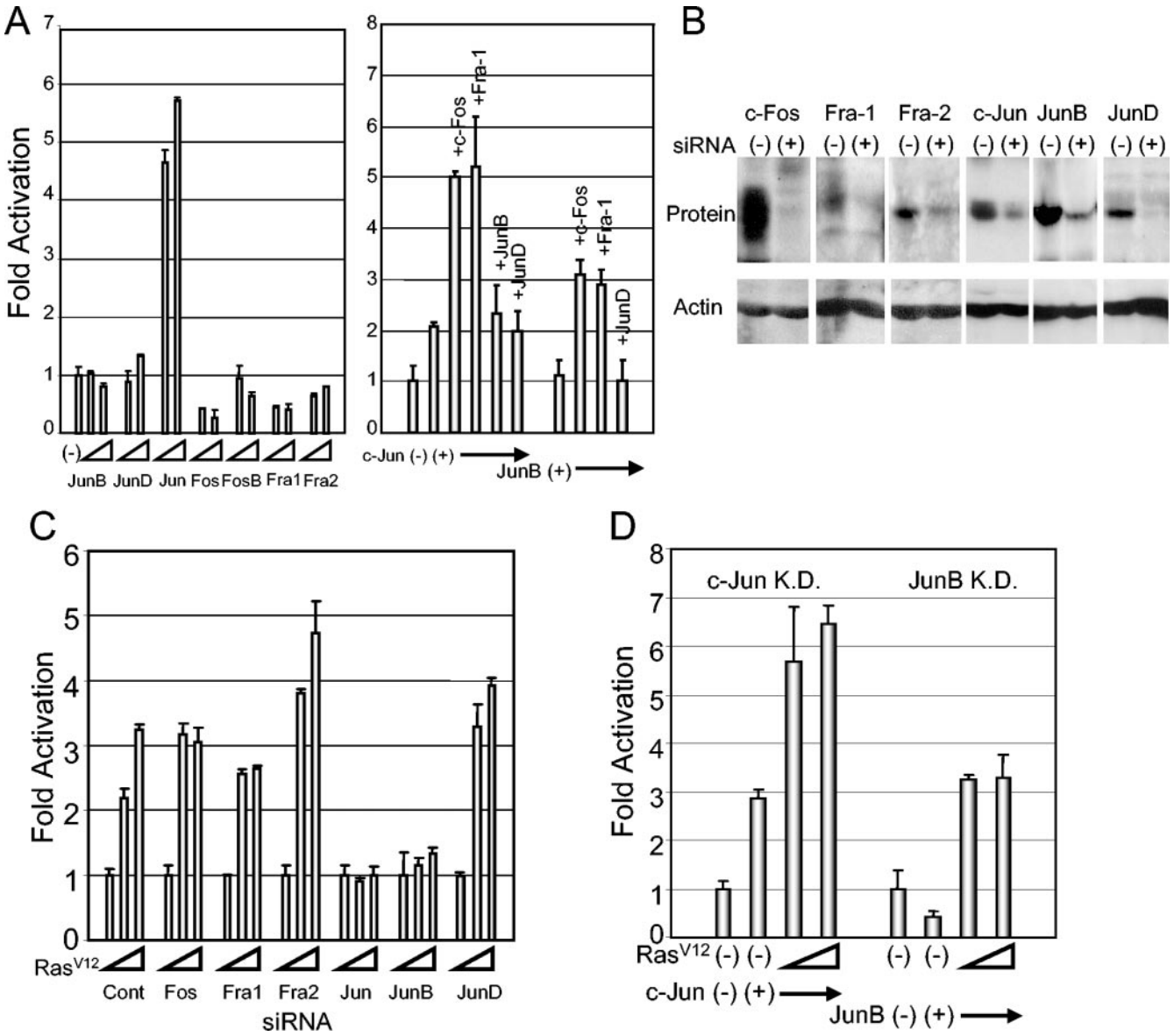

FIG. 5. Synergism of *Dmp1* promoter activation by AP-1 proteins and RNAi assay. (A) Synergistic activation of the *Dmp1* promoter by AP-1 family proteins. Synergism among Fos and Jun family proteins was tested in IMR-90 cells. For reporter assays, 0.5 to 1 μg of Fos/Jun expression vectors per dish were used for transfections in the left panel, while 0.25 μg of expression vectors were used per dish in the right panel to study synergism. Significant synergism was found between c-Jun and c-Fos or Fra-1, JunB and c-Fos or Fra-1. (B) Downregulation of Fos and Jun family proteins by siRNA. Wild-type MEFs were infected with retroviruses that produce siRNA, and puromycin-resistant cells were expanded. The lysates were studied for target protein expression with specific antibodies. (C) Ras^{V12} does not activate the *Dmp1* promoter in cells with downregulated c-Jun or JunB. Reporter assays were performed with the −88 ApaI *Dmp1* promoter in MEFs with downregulated AP-1 proteins. *Dmp1* promoter activation by Ras^{V12} was completely attenuated in cells where c-Jun or JunB proteins were knocked down, whereas c-Fos, Fra-1, Fra-2, or JunD proteins were dispensable for *Dmp1* promoter activation by Ras. (D) Restoration of the Ras responsiveness of the *Dmp1* promoter in Jun knock-down cells. Reporter assays were performed in c-Jun or JunB knock-down (K.D.) MEFs with c-Jun or JunB and Ras^{V12} expression vectors. The responsiveness of the *Dmp1* promoter to Ras^{V12} was completely restored by transfection of c-Jun or JunB knock-down cells with a c-Jun or JunB expression vector, respectively.

c-Fos, c-Jun, JunB, and JunD, bind to the endogenous *Dmp1* promoter 5′-untranslated region in response to oncogenic Ras-Raf signaling.

Oncogenic Ras does not activate the *Dmp1* promoter in c-Jun and JunB knock-down cells. Detection of Fos and Jun proteins on the *Dmp1* promoter does not necessarily mean that they are transcriptional activators of the *Dmp1* promoter. In order to test which of these proteins is physiologically relevant, we performed reporter assays with Fos and Jun family proteins alone or in combination. When tested alone, only c-Jun activated the promoter to significant levels. (Fig. 5A, left panel). All of the Jun proteins synergistically activated the *Dmp1* promoter by collaborating with c-Fos or Fra-1, whereas no synergism was observed among Jun family proteins (Fig. 5A, right panel).

We next used short interfering RNAs (siRNAs) (7) to knock

down the level of c-Fos, Fra-1, Fra-2, c-Jun, JunB, or JunD in MEFs. Figure 5B shows that the endogenous protein levels were significantly downregulated by 80 to 90% in cells that were infected with retroviruses prepared by use of the pSUPER.retro.puro RNAi system (Oligoengine). Oncogenic Ras^{V12} completely failed to activate the *Dmp1* promoter in MEFs in which c-Jun or JunB levels were reduced, whereas it activated the *Dmp1* promoter three- to fourfold in cells with downregulated c-Fos, Fra-2, or JunD (Fig. 5C). The responsiveness of the *Dmp1* promoter to Ras in c-Jun or JunB knockdown cells was completely restored by cotransfecting a c-Jun or JunB expression vector with Ras^{V12} (Fig. 5D). We confirmed by Western blotting that overexpression of c-Jun/JunB under the control of the CMV promoter was strong enough to overcome the effects of siRNAs (data not shown). These data underscore the critical roles of c-Jun and JunB transcription factors in the activation of *Dmp1* in response to oncogenic Ras signaling.

Mapping of Ras-responsive elements on the murine *Arf* promoter. Although oncogenic Ras signaling increases both $p19^{Arf}$ mRNA and protein (33), the mechanism of *Arf* induction by Ras^{V12} has not yet been clarified. In wild-type MEFs, Ras^{V12} induced a sevenfold activation of the *Arf* promoter, which was compromised in an *Arf* promoter with disrupted Dmp1/Ets binding sequence (Fig. 6A, left panel) (17). In order to demonstrate the relative importance of Dmp1 versus Ets family proteins in the *Arf* promoter activation by Ras, we repeated these assays using both wild-type and *Dmp1*-null MEFs. This revealed a dramatic reduction in the response of the *Arf* promoter to Ras^{V12} in cells lacking Dmp1 (Fig. 6A, middle panel). In order to rule out the possibility that events secondary to Dmp1 loss were responsible for the failure of the *Arf* promoter to respond to oncogenic Ras, we performed a reporter assay on the *Arf* promoter by transfecting $Dmp1^{-/-}$ MEFs with Dmp1 and Ras^{V12} expression vectors. Our data indicated that ectopic expression of Dmp1 restores the responsiveness of the *Arf* promoter to oncogenic Ras (Fig. 6A, right panel). This result is consistent with those of previous studies in which *Dmp1*-null cells exhibited only minor accumulation of $p19^{Arf}$ and p53 in response to oncogenic Ras, enabling these cells to be transformed by oncogenic Ras alone (18).

We then used a ChIP assay to determine whether endogenous Dmp1 binds to the *Arf* promoter in vivo. Dmp1 bound to the endogenous *Arf* promoter after activation of ΔRaf:ER; we saw an increased signal even though the input chromatin was 50% lower (Fig. 6B). We conclude that the Ras^{V12}-responsive element falls within the Dmp1/Ets consensus binding sequence on the *Arf* promoter and that *Dmp1* activity is required to efficiently enable *Arf* induction by oncogenic Ras.

Our present study demonstrates that ΔRaf:ER induces Dmp1 and cyclin D1 proteins almost with the same kinetics (Fig. 3B). Because gross overexpression of D-type cyclins can inhibit Dmp1-mediated transactivation in a Cdk-independent fashion (15, 16), we investigated whether D-type cyclins could influence *Arf* promoter activation by Dmp1 (Fig. 6C). The *Arf* promoter was weakly activated by overexpression of cyclin D1, D2, or D3 (~2-fold) (data not shown), consistent with the concept that the *Arf* promoter responds to hyperproliferative oncogenic signaling (47). Moreover, the D-type cyclins collaborated with Dmp1 in activating the *Arf* promoter (Fig. 6C). Importantly, this additive effect was dependent on the activation of the Rb-E2F pathway, since a cyclin D1 mutant that does not interact with Cdk4 (D1K114E) (15) did not activate the *Arf* promoter. Thus, while overexpressed D-type cyclins can inhibit Dmp1 activity in a Cdk4-independent manner, their ability to activate Cdk4 conveys an opposing signal. Interestingly, the stimulatory effects on the *Arf* promoter were limited to D-type cyclins, since none of the other cyclins (cyclin A or cyclin H) affected *Arf* promoter activation by Dmp1 (Fig. 6C).

Our model of the signaling cascades that link the oncogenic Ras-Raf-MEK-ERK pathway and the Arf-Mdm2-p53 tumor surveillance pathway is depicted in Fig. 7.

DISCUSSION

Overexpression of oncogenic Ras^{V12} acts through the Raf-MEK-ERK pathway and AP-1 signaling to activate *Dmp1*. In turn, Dmp1 activates the *Arf* promoter, ultimately leading to a p53 response that limits oncogene-induced cell proliferation and tumorigenesis. Although our investigators previously recognized that the Dmp1 protein was induced by explantation of early-passage MEFs and that its accumulation preceded that of $p19^{Arf}$ as primary cells were passaged in culture (18), we now appreciate that the *Dmp1* promoter can respond to a specific subset of Ras-dependent signals. Among the three different oncogenes that we studied (c-Myc, E2F-1, and Ha-Ras^{V12}), each of which can induce *Arf* (3, 33, 55), only Ras^{V12} activated the *Dmp1* promoter. Our findings indicate that Ras^{V12} signaling to *Arf* is mediated primarily by Dmp1, whereas E2F-1 and Myc address *Arf* via other mechanisms.

Ras transformation correlates with increased c-Jun transcriptional activity and an increase in AP-1-mediated gene expression (reviewed in references 30 and 46). In experiments employing the ΔRaf:ER system, c-Jun protein accumulation peaked at 16 to 24 h after 4-HT treatment, the timing of which was coincident with expression of Dmp1 and $p19^{Arf}$. Significantly increased levels of the c-Jun protein were detected on the *Dmp1* promoter after Raf activation, suggesting that endogenous c-Jun can activate the Dmp1-$p19^{Arf}$ axis in response to oncogenic Ras-Raf signaling. Overexpression of c-Jun alone activated the *Dmp1* promoter, and *Dmp1* promoter activation by Ras^{V12} was completely abolished in c-Jun knockdown cells. Thus, we conclude that c-Jun is the most prominent AP-1 protein that activates the *Dmp1* promoter in response to oncogenic Ras-Raf signaling. Control of cell cycle progression by c-Jun was shown to be p53 dependent (43). Indeed, c-Jun is upregulated in many carcinomas (11), and it plays a key role in chemically induced hepatocellular carcinoma in mice (11). Thus, the concept that c-Jun is an oncogene (27) fits with the observation that c-Jun activates the *Dmp1* promoter in response to an oncogenic stimulus.

The finding that activation of the *Dmp1* promoter by Ras was significantly attenuated in JunB knock-down cells was quite unexpected, since c-Jun and JunB are known to have antagonistic functions (30). JunB can suppress cell proliferation by transcriptional activation of $p16^{Ink4a}$ in MEFs (35). Since JunB alone does not activate the *Dmp1* promoter, JunB heterodimers, possibly in association with Fos family proteins, should play important roles in the *Dmp1* promoter activation response to oncogenic Ras. Transcriptionally less active JunB

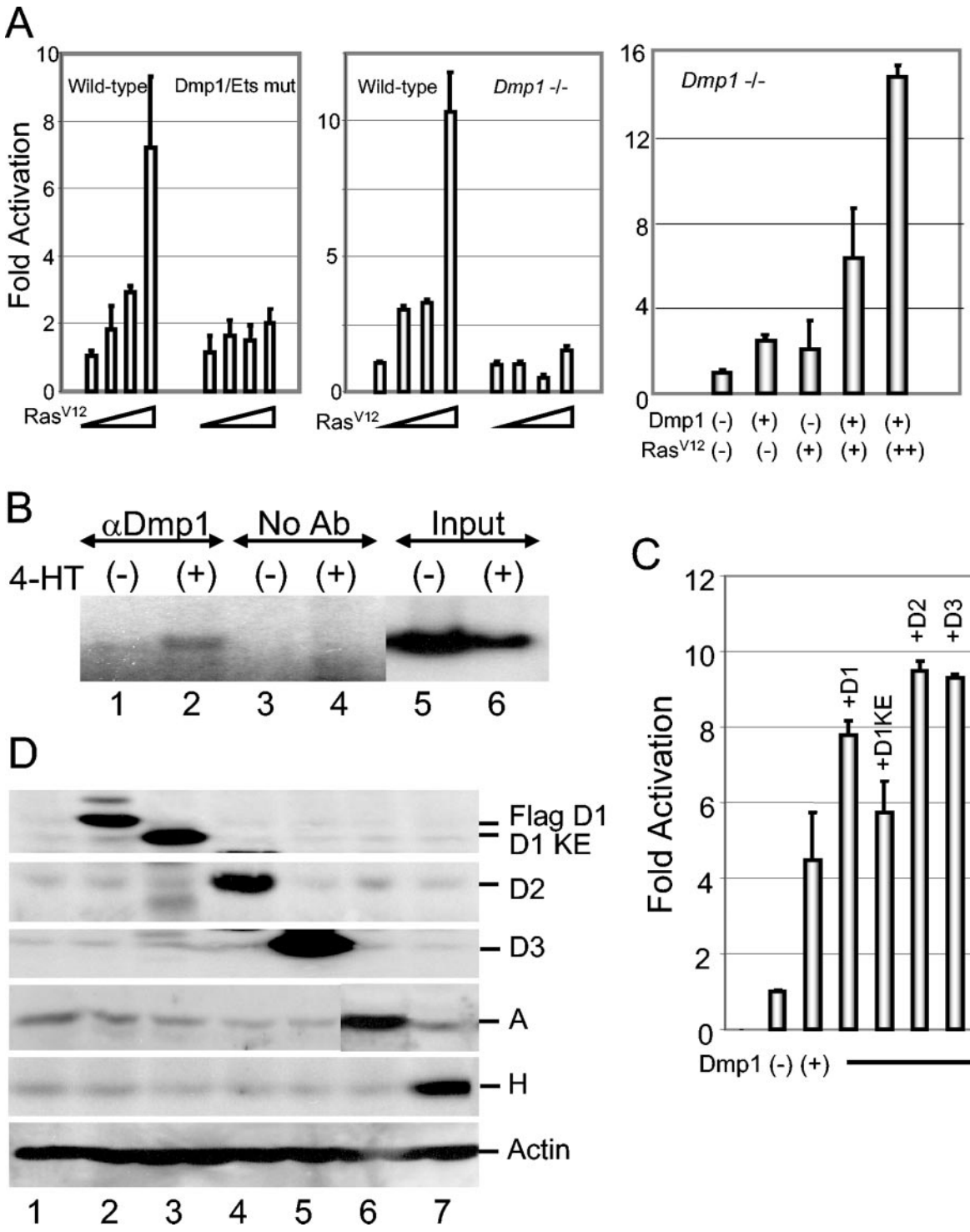

FIG. 6. Dmp1 plays a key role in *Arf* promoter activation by Ras^{V12}. (A) Mapping of the Ras^{V12}-responsive element on the *Arf* promoter. (Left panel) Reporter assay performed with either wild-type or mutant murine *Arf* promoter to study the importance of the Dmp1/Ets site in response to oncogenic Ras activation. *Arf* promoter activation was almost completely abolished by mutating the Dmp1/Ets binding site (17). (Middle panel) Reporter assay performed with wild-type *Arf* promoter in both wild-type and *Dmp1*-null MEFs. Oncogenic Ras does not activate the *Arf* promoter in *Dmp1*-null cells. (Right panel) Restoration of the Ras responsiveness of the *Arf* promoter in *Dmp1*-null cells. The responsiveness of the *Arf* promoter to Ras^{V12} was completely recovered by transfection of *Dmp1*-null cells with the Dmp1-expression vector. (B) ChIP assay on the *Arf* promoter. Endogenous Dmp1 protein was found on the *Arf* promoter in response to oncogenic Raf signaling (lanes 1 and 2). Lanes 3 and 4, no antibodies were used for the immunoprecipitation; lanes 5 and 6, signals from total chromatin samples. (C) Cyclin D1 does not inhibit the Dmp1 activity on the *Arf* promoter. A reporter assay was performed in NIH 3T3 cells with expression vectors for Dmp1 and cyclins. Dmp1 and D-type cyclins additively activated the *Arf* promoter, while other cyclins (cyclin A and cyclin H) had little effect on *Arf* promoter activation by Dmp1. The K114E cyclin D1 mutant (D1 KE) that does not interact with Cdk4 did not influence Dmp1's activity on the *Arf* promoter, suggesting that the collaborative effect was dependent on Cdk4 activation. (D) Detection of cyclins in transfected cells. The expression of cyclins was confirmed by Western blotting of lysates from the luciferase assay with specific antibodies. Lane 1, pFLEX1 vector only; lane 2, Flag-tagged cyclin D1; lane 3, cyclin D1 KE mutant; lane 4, cyclin D2; lane 5, cyclin D3; lane 6, cyclin A; lane 7, cyclin H.

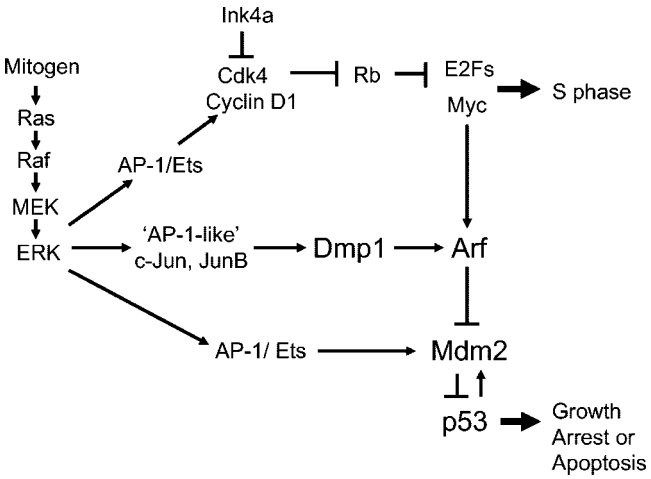

FIG. 7. The novel Jun-Dmp1 pathway. Dmp1 is a key molecule linking Ras-Raf-MEK-ERK oncogenic signaling and the Arf-Mdm2-p53 tumor suppressor pathway. The pathway mediated by Dmp1 directly links oncogenic Ras-Raf signaling and $p19^{Arf}$ through activation of c-Jun/JunB proteins. Oncogenic Ras activates both the Jun-Dmp1 pathway and the classical cyclin D1/Cdk4-Rb-E2F pathway to activate *Arf* gene expression to achieve premature senescence. Since the Ras-Raf pathway activates the *Mdm2* promoter as well, the levels of p53 are delicately regulated by the activity of the Dmp1-Arf and AP-1/Ets-Mdm2 pathways.

can substitute for c-Jun in mouse development and cell proliferation, and JunB can restore the expression of genes regulated by Jun/Fos, but not those regulated by Jun/ATF (36).

Although oncogenic Ras has been reported to induce premature senescence by upregulating $p19^{Arf}$ and p53 levels, the mechanism of $p19^{Arf}$ activation has remained poorly understood. Activated Ras induces cyclin D1 and accelerates its assembly with Cdk4 (1, 9), and it has generally been assumed that E2F family proteins play important roles in *Arf* regulation in response to Ras signaling (Fig. 7). However, results from two different groups suggest that oncogenic Ras can increase *Arf* mRNA levels in an E2F-independent manner (34, 41). The Ras^{V12}-responsive element was mapped to the Dmp1/Ets binding sequence in the murine *Arf* promoter. We also observed increased binding of the endogenous Dmp1 protein to the Dmp1/Ets site within the *Arf* promoter in response to activation of ΔRaf:ER. In contrast, activated ΔRaf:ER failed to stop the growth of *Dmp1*-null cells. These data strongly indicate that Dmp1 plays a major role in conveying oncogenic Ras-Raf signaling to the Arf-p53 pathway in rodent fibroblasts. We propose the presence of the Jun-Dmp1 pathway that directly links Ras-Raf signaling and $p19^{Arf}$. This novel pathway collaborates with the classical cyclin D1/Cdk4-Rb-E2F pathway to activate the *Arf* gene expression in response to oncogenic Ras signaling (Fig. 7).

The induction of $p21^{Cip1}$ by activated Raf was also impaired in *Dmp1*-null cells. A simple interpretation is that loss of Dmp1 attenuates Arf-p53 function, preventing the accumulation of canonical p53-responsive genes, $p21^{Cip1}$ among them. However, this is unlikely to be the only explanation, because the rate of $p21^{Cip1}$ accumulation in response to Raf expression was faster than that of $p19^{Arf}$ in wild-type MEFs, and because high-intensity B-Raf and c-Raf signaling can cause $p21^{Cip1}$-dependent cell cycle arrest in NIH 3T3 cells that lack the *Ink4a-Arf* locus (45, 54). Since both the mouse and human $p21^{Cip1}$ promoter lacks typical Dmp1-consensus sequences, it is unlikely that Dmp1 binds and activates the $p21^{Cip1}$ promoter directly. Further studies will be required to clarify the roles of Dmp1 in $p21^{Cip1}$ regulation in response to Ras-Raf signaling.

Mdm2 acts as a major regulator of the tumor suppressor p53 by targeting its destruction, inhibiting its transcriptional activation, and by accelerating nuclear-to-cytoplasmic shuttling of p53. $p19^{Arf}$ can antagonize each of these processes (49). The Mdm2 intronic promoter is regulated by oncogenic Ras signaling as well as by p53 signaling (39). Activation of the Mdm2 promoter by Ras is also dependent on AP-1 and Ets-like elements and is p53 independent (39). Interestingly, the structures of the murine Dmp1 promoter and 5′-untranslated region are very similar to that of the Mdm2 promoter, although the Ets site was dispensable for Dmp1 promoter activation by Ras^{V12}. Since oncogenic Ras activates both the Mdm2 and Dmp1-$p19^{Arf}$ pathways (Fig. 7), the levels of p53 in response to oncogenic Ras should be delicately controlled by opposing effects between Mdm2 and Dmp1-$p19^{Arf}$-regulated signaling pathways.

Overexpressed D-type cyclins antagonize Dmp1 transcriptional activity in a Cdk-independent fashion when tested using artificial promoter-reporter plasmids containing concatamerized Dmp1 consensus binding sequences or with some natural promoters, such as that derived from the *CD13/aminopeptidase N* gene (15, 16). However, the results were reversed on the *Arf* promoter, where D-type cyclins cooperated to enhance the activity of Dmp1 in a Cdk4-dependent manner. The *Arf* promoter contains both Dmp1- and E2F-binding sites, enabling Ras^{V12}-induced cyclin D1 to assemble with Cdk4, promote the release of E2Fs from Rb, and thereby collaborate with Dmp1 in activating *Arf* gene expression (17). On the other hand, the *CD13/aminopeptidase N* promoter, which lacks E2F consensus sequences, can be experimentally suppressed by D-type cyclins which, when overexpressed, can interfere with Dmp1 binding to DNA. The Dmp1/Ets consensus sequences found within these two promoters are completely identical (CCCGGA TGC) (16, 17), consistent with the idea that sequences flanking the Dmp1 binding site determine the responsiveness of the promoter to D-type cyclins. It is important to emphasize that interference with Dmp1 activity by D-type cyclins has never been demonstrated in a setting in which D-type cyclins accumulate to physiological levels. Indeed, the level of cyclin D1 achieved after Ha-Ras^{V12} expression was 10-fold lower than that generated by the cyclin D1 expression vector itself (R. Sreerameneni and K. Inoue, unpublished data).

A further complication stems from observations that efficient activation of the *Dmp1* promoter by Ras^{V12} was limited to nonimmortalized cell strains, such as primary MEFs and human IMR-90 cells, whereas *Dmp1* induction was compromised in *Arf*-null cells and other established cell lines. Oncogenic Ras has differential effects on primary cell strains versus established cell lines, inducing replicative senescence in the former but stimulating proliferation and transformation in the latter (44). When we reexpressed $p19^{Arf}$ in *Arf*-null cells, the response of the *Dmp1* promoter to Ras^{V12} was at least partially restored. We do not consider the effect of Ras in regulation of the Dmp1 expression is a consequence of growth inhibition

because (i) Dmp1 is induced by ΔRaf:ER at the same kinetics as that of cyclin D1 before the cell growth is inhibited, (ii) the magnitude of *Dmp1* promoter activation by Ras is higher in early-passage MEFs than in late-passage MEFs that are becoming senescent (Sreeramaneni and Inoue, unpublished), and because (iii) *Dmp1*$^{-/-}$ cells are morphologically transformed by Ras^{V12} (18), suggesting that the *Dmp1* promoter activation is an integral part of Ras-Arf-p53 signaling. Although we cannot yet explain why *Dmp1* activation by Ras depends upon Arf in NIH 3T3 cells and MEFs and upon p53/Rb in 293T cells, these results suggest that the presence of a functional Arf-p53 pathway and an intact p53 G_1 checkpoint may be required. Arf may modify the activities of nuclear proteins that regulate *Dmp1* transcription, possibly in a cell cycle-specific manner. The Arf status of a cell can also determine the transcriptional activity of NF-κB in response to an oncogene, although these effects are independent of p53 and Mdm2 (40). Indeed, many genes are induced or suppressed by p19^{Arf} (24), some of which might well modify the Dmp1 response. The durability of the Dmp1-Arf response to Ras might well depend upon feedback control, in which the integrity of Arf signaling, whether p53 dependent or not, appears to reinforce the ability of Ras to trigger *Dmp1* gene expression.

Recently, studies have shown that lower physiological levels of mutant Ras from a single-copy oncogenic Ras allele activate the p19^{Arf}-p53 pathway in MEFs, but not to the same extent as overexpressing oncogenic Ras driven by the retroviral promoter (12, 52). Lower levels of mutant Ras promoted cell proliferation in MEFs in culture and also in epithelial cells in vivo despite a lack of obvious cooperating events, due to the incomplete activation of the p19^{Arf}-p53 pathway (12, 52). Since human tumors that contain oncogenic Ras tend to have single mutant alleles, such studies are a good representation of early-stage human cancer. However, duplication or amplification of the mutant Ras gene should have more of a growth advantage than single Ras mutant cells, and it has been reported that both human and mouse cancers often amplify or overexpress the mutant Ras gene with progression of the disease (4, 5, 31, 53). Since the level of induction of Dmp1 and Arf by oncogenic Ras-Raf pathway is dose dependent (Fig. 2 and 6) (Sreeramaneni and Inoue, unpublished), our study presents the mechanism of prevention of tumor progression induced by overexpressed oncogenic Ras.

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