ING proteins are putative tumor suppressor proteins linked to the p53 pathway and to the chromatin modification machinery. Here we have analyzed the role of the products of the murine Ing1 locus in cellular tumor-protective responses, using mouse primary fibroblasts where the Ing1 locus has been inactivated by the integration of a βgeo cassette. We show that Ing1-deficient mouse embryonic fibroblasts display a defective senescence-like antiproliferative response against oncogenic Ras, affecting several senescence-specific markers. This phenotype is accompanied by a reduced accumulation of p53, which can be explained by the reduced basal p53 protein stability in the Ing1-deficient background. Ing1 deficiency also results in defects in the appearance of heterochromatic marks upon expression of oncogenic Ras, suggestive of impaired heterochromatin formation during oncogene-induced senescence. Our results support an important role for the Ing1 locus in protection against oncogenic stress in vivo, both as a mediator of p53 activation and as a regulator of chromatin remodeling processes.

Normal mammalian cells are endowed with defense mechanisms against potentially tumorigenic alterations, among which the pathway controlled by p53 plays a critical role (1). The ING family of proteins includes several sequence-related and evolutionarily conserved proteins, connected both with the p53 pathway and with chromatin modification processes (reviewed in Refs. 2 and 3). In humans and mice, several ING peptides have been identified, which are encoded by five different loci, named ING1 to ING5. Several ING proteins have been shown to act as upstream regulators of p53 in overexpression experiments, either increasing p53 protein stability (4) or promoting the acetylation of Lys residues at the C terminus of p53 (5, 6). The latter effect is likely mediated by the association of ING proteins to acetyltransferases such as CBP/p300 or PCAF (7) or deacetylases such as Sir2 (6). ING proteins also participate in the regulation of chromatin dynamics. They form part of large multiprotein complexes with both activating and repressing chromatin-modifying activities, in association with histone acetyltransferases or deacetylases (8–10), and also act as effector proteins recognizing specific histone marks (10–13). Enforced expression of ING proteins activates a variety of responses, such as cell cycle arrest (14), DNA repair (15), apoptosis (16), or cellular senescence (17, 18). In support of a possible role as tumor suppressors in human cancer, alterations in ING1, and other members of the ING family, have been described in different types of human tumors, including altered expression and aberrant subcellular localization and, less frequently, point mutation or homozygous deletion (19, 20). The physiological significance of the in vitro data described above and the actual role of ING proteins in tumor-protective responses in vivo remain to be unequivocally tested. In this study, we have used primary embryonic fibroblasts derived from transgenic mice where the Ing1 locus has been disrupted by a gene trap cassette to address the participation of the products of this locus in p53-mediated cellular responses to stress. We find that Ing1 deficiency results in defects in p53 activation and chromatin remodeling, in the context of the protective response against oncogenic stress in murine fibroblasts, suggesting a critical role for the Ing1 locus in cellular senescence triggered by oncogenic stress.

**EXPERIMENTAL PROCEDURES**

*Generation of Ing1 Gene Trap Mice*—The generation and phenotypical characterization of the Ing1 gene trap mice will be described in detail elsewhere. Essentially, murine embryonic stem cells (TBV-2) were electroporated with the gene trap vector pT1ATGbgeo as part of a genome-wide gene trapping screen (21). The integration site of selected clones was identified by nucleotide sequencing, using a semi-automated 5’-rapid amplification of cDNA ends-PCR protocol. One clone with a single copy insertion in the murine Ing1 locus was used to generate chimeric mice by blastocyst injection, and germ line transmitting animals were bred to generate mice with targeted Ing1 alleles.
Cell Culture—Preparation and passage of MEFs and retroviral infection experiments were carried out as described previously (22). Fibroblasts from individual embryos from over 10 different litters were used in the study. The proliferation rate was estimated by counting the number of BrdUrd-positive cells by immunofluorescence (14). For protein turnover assays, cycloheximide (Sigma) was added to the medium at a final concentration of 30 μg/ml. For growth curves, after infection and selection, cells were seeded at a density of 2 × 10⁴ per well in 24-well dishes. At the indicated time points, cells were trypsinized and counted. Senescence-associated β-galactosidase activity was detected as described previously (22).

Western Blotting Analysis—Preparation of total cell lysates, electrophoresis, and Western blot analysis were carried out as described (23). The following antibodies were used: anti-p33 ING1 (LG-1, a rabbit polyclonal against the C-terminus of the human p33 ING1 protein; 1:1000 dilution), anti-p53 (CM-5, Novocastra; 1:1000), anti-p19 ARF (54-75, a gift of David Parry, DNAX; 1:1000), anti-p16 INK4a (M156, Santa Cruz Biotechnology; 1:100), anti-p21 CIP1 (sc-397, Santa Cruz Biotechnology; 1:500), anti-acetyl-p53 (Lys-373) (06-758, Trevigen; 1:100), anti-p53 D17l (156, Santa Cruz Biotechnology; 1:100), anti-p53 (CM-5, Novocastra; 1:500), anti-p16 INK4a (M156, Santa Cruz Biotechnology; 1:100), anti-p19 ARF (54-75, a gift of David Parry, DNAX; 1:1000 dilution), anti-p53 (CM-5, Novocastra; 1:500), and antibody to DcR2 (AAP-81, Novocastra; 1:500). Detection of the fusion transcript between exon 1b of Ing1 and the gene trap cassette. Expression of Ing2 and actin was also analyzed. D, quantitation of p33 ING1 protein and RNA levels. Protein levels were quantitated from scans of Western blots. RNA levels were quantitated using real time PCR. In both cases, actin was used for normalization. wt, wild type; g/g, ing1 gene trap.

FIGURE 1. Effects of the integration of the βgeo cassette on the expression of the murine Ing1 locus. A, schematic representation of the structure of the Ing1 locus. Protein-coding regions in exon 1b and 2 are shaded. The site of integration of the βgeo cassette is indicated by an arrowhead. B, Western blot analysis of the expression of p33 ING1 in MEFs of the indicated genotypes. C, RT-PCR analysis of the expression of the alternative transcripts of the Ing1 locus in MEFs of the indicated genotypes. Exon 1b/βgeo represents the fusion transcript between exon 1b of Ing1 and the gene trap cassette. Expression of Ing2 and actin was also analyzed. D, quantitation of p33 ING1 protein and RNA levels. Protein levels were quantitated from scans of Western blots. RNA levels were quantitated using real time PCR. In both cases, actin was used for normalization. wt, wild type; g/g, ing1 gene trap.

RESULTS

Characterization of Ing1-deficient MEFs—The murine Ing1 locus contains four exons (1a, 1b, 1c, and 2), which give rise to three different transcripts through alternative splicing events (Fig. 1A). Their translation is predicted to give rise to two peptides: p33 ING1 (from an ATG codon in exon 1b) and p24 (from an internal ATG in exon 2) (24). We have mapped the site of integration of the βgeo cassette to the intron between exons 1b and 1c, 67 bp upstream of the start of exon 1c (Fig. 1A). Western blot analysis with a polyclonal antiserum against the C-terminus of p33 and p24, in wild-type MEFs, revealed a band with a relative mobility of ~37 kDa, corresponding to p33 ING1 (Fig. 1B) (24). A specific band with a mobility corresponding to p24 was never detected in our experiments. Western blot analysis in gene trap MEFs revealed a weak band of the same mobility as p33 ING1, most likely reflecting residual expression of the wild-type transcript, because of splicing around the gene trap inser-

4 The abbreviations used are: MEF, mouse embryonic fibroblast; RT, reverse transcription; BrdUrd, bromodeoxyuridine.
**Impaired Oncogenic Stress Response in Ing1-deficient Cells**

**A**

![Graph showing BrdU incorporation](image)

**B**

![Graph showing cell number](image)

**C**

![Micrograph showing morphology and senescence-associated â-galactosidase staining](image)

**D**

![Graph showing percentage of senescence-associated â-galactosidase-positive cells](image)

**FIGURE 2. Response to oncogenic Ras.** A, rate of BrdUrd incorporation of MEFs of the indicated genotypes infected with a vector expressing RasV12 (Ras) or an empty vector (V). B, growth curves of wild-type (wt) MEFs infected with vector (filled squares) or RasV12 (filled circles), and g/g MEFs infected with empty vector (empty squares) or RasV12 (empty circles). Cell number was counted at the indicated time points, and it is represented relative to the number of cells at day 1 in each case. C, micrograph showing the morphology and senescence-associated â-galactosidase staining of fibroblasts after retroviral infection with the indicated vectors (×100 magnification). D, percentage of senescence-associated â-galactosidase (SA-BetaGal)-positive MEFs of wild-type and g/g genotype, at the indicated time points after infection and selection with RasV12 (Ras) or empty vector (V). A representative experiment is shown with one wild-type and two g/g MEF preparations.

**Impact on Ras-induced Senescence**—Chronic expression of an oncogenic version of the Ha-Ras oncogene (RasV12) triggers an antiproliferative response in primary cells, reminiscent of cellular senescence (25). In MEFs, this response is mediated primarily by the ARF-p53 pathway (26, 27) and involves the stabilization and specific post-translational modifications of p53 (27, 28). Given the well-established link of p33 ING1 to p53, and its putative role as a tumor suppressor gene, we decided to test the impact of Ing1 depletion in Ras-induced cell cycle arrest, a tumor-protective response mediated by p53. First, we measured the proliferation rate of retrovirally infected MEFs of both genotypes by BrdUrd incorporation. As expected, wild-type MEFs expressing RasV12 showed a clear reduction in the number of BrdUrd-positive cells, relative to vector-infected controls. Ing1 g/g MEFs, in contrast, were largely insensitive to the effect of Ras and showed a rate of BrdUrd-positive cells similar to or even higher than their controls (Fig. 2A and supplemental Fig. 1). The different response to Ras was also evident when we measured the growth rate of infected fibroblasts of both genotypes, over a period of 9 days post-selection. Although wild-type MEFs with Ras virtually did not increase in cell number during that period, Ing1-deficient MEFs with Ras grew at a rate slightly faster than their controls (Fig. 2B). Also, although Ras-infected wild-type MEFs displayed markers of the senescent phenotype, such as the senescence-associated â-galactosidase staining and a distinctive flattened morphology, Ing1-deficient MEFs retained a normal morphology and very low number of senescence-associated â-galactosidase-positive cells (Fig. 2, C and D). We wished to determine whether the attenuation in the antiproliferative response to Ras rendered Ing1-deficient cells fully permissive to Ras-induced transformation. With this purpose, infected fibroblasts were injected in the flank of nude mice, and the appearance of visible tumors was scored. No tumors were observed in wild-type or Ing1-deficient MEFs expressing oncogenic Ras, after up to 40 days, in conditions where Ras-infected p53-null MEFs formed sizable tumors (supplemental Fig. 2). Furthermore, no evidence of oncogenic transformation of Ing1-deficient cells was either observed in soft agar colony assays or foci formation assays (data not shown). Interestingly, the impact of Ing1 deficiency appears to be specific to the response to oncogenic stress, whereas other p53-mediated phenotypes we have studied in this and other cell types were not altered. For instance, g/g MEFs enter replicative senescence and reach
immortalization at a rate indistinguishable from wild-type MEFs (supplemental Fig. 3). Also, apoptosis triggered by DNA damage or other stimuli in thymocytes is not affected by Ing1 status (supplemental Fig. 4). To understand the molecular basis of the impaired senescent response to Ras, we analyzed by Western blot the levels of several proteins involved in Ras-induced cellular senescence. The antiproliferative response elicited by Ras in mouse fibroblasts is typically accompanied by an accumulation of the p53 protein, which is mediated by the up-regulation of p19 ARF (26). Notably, the expression of RasV12 in g/g MEFs failed to provoke an increase of p53 protein levels at the same extent as wild-type cells (Fig. 3A), reflecting a defect in accumulation of p53 upon oncogenic stress. Basal p19 ARF levels were increased in g/g cells, most likely due to their higher population doubling number. Nevertheless, p19 ARF induction by Ras was similar to wild-type cells, suggesting that the signaling machinery linking oncogenic stress with ARF activation is not affected by the suppression of p33 ING1. Likewise, induction of the other product of the Ink4a/ARF locus, p16 INK4a, was observed irrespective of the presence of Ing1.

p21 CIP1 protein levels were also increased in Ras-infected wild-type cells, as described previously, and no obvious difference was observed in g/g cells (Fig. 3B). The observed induction of p21 CIP1 in both genotypes, irrespective of arrest induction, most likely reflects a p53-independent effect of Ras expression on p21 protein levels (29) and is in accordance with previous observations indicating a nonessential role for p21 CIP1 in oncogene-induced senescence (30, 31). Notably, the senescence marker and p53 target DcR2 (32) was not induced significantly by Ras in g/g fibroblasts, further supporting a defect in the implementation of senescence and in p53 activity in these cells.

Immunofluorescence experiments using antibodies against p19 ARF and p53 confirmed the results of the Western blot analysis regarding the levels of both proteins. They also showed that Ing1 status did not affect the subcellular localization of either protein in the presence or absence of oncogenic stress (Fig. 3C).

Impact on p53 Regulation—We decided to investigate the basis of the defective accumulation of p53 observed upon expression of oncogenic Ras. It has been proposed that p33 ING1 can contribute to p53 activity, both by increasing p53 protein stability (4) and by favoring the acetylation of specific lysine residues in the C terminus of the p53 protein (6). With this background, we first investigated whether the differences in p53 accumulation might reflect differences in basal protein stability. With this purpose, we measured the half-life of the p53 protein in MEFs of both genotypes by Western blot at different time points after inhibition of protein synthesis with cycloheximide. As shown in Fig. 4, A and B, the half-life of p53 protein appeared significantly reduced in g/g cells. Different members of the ING family, including p33 ING1, have been shown to increase acetylation of p53 when overexpressed (5, 6). To determine whether Ing1 could play a role in p53 acetylation in vivo, we investigated the degree of p53 acetylation after exposure to

5 M. Abad, C. Menéndez, L. González, A. Fuchtbauer, M. Serrano, E.-M. Fuchtbauer, and I. Palmero, unpublished observations.
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**FIGURE 4. p53 regulation in g/g cells.** A, turnover of p53 protein in wild-type (wt) and g/g fibroblasts was analyzed by Western blot of lysates prepared at the indicated times after addition of cycloheximide (CHX). B, quantitation of p53 turnover from scans of Western blots as shown in A. Data shown are the average and standard deviation of three independent experiments. Filled squares represent wild-type cells and filled circles g/g cells. C, acetylation of p53 after adriamycin treatment (Adr, 0.2 μg/ml, 4 h), or in untreated controls (−) was analyzed by immunoprecipitation (IP) with an antibody against acetylated p53 (Ac). The same blot was subsequently incubated with an antibody against p53. MEFS double knock-out for p53 and Mdm2 (DKO) treated with adriamycin were used as a negative control. Phosphorylated p53 was detected by Western blot on CM5 p53 immunoprecipitates using a specific antibody against p53 phosphorylated at Ser-15.

the genotoxic drug adriamycin, a stimulus known to trigger robust acetylation of p53 (33) (Fig. 4C and data not shown). Using immunoprecipitation followed by Western blot with two commercial acetylation-specific antibodies, we could detect acetylated p53 with a comparable intensity in samples both from wild-type and Ing1 g/g MEFS treated with adriamycin. Similarly, Western blot with an antibody against serine 15-phosphorylated p53 gave indistinguishable results in both genotypes (Fig. 4C). These results support a role for Ing1 in control of p53 stability, although they rule out a significant role of Ing1 products in control of p53 post-translational modification in this setting.

**Chromatin Changes during Ras-induced Senescence**—The implementation of cellular senescence in human primary fibroblasts, either triggered by oncogenic stress or by the accumulation of population doublings, involves changes in chromatin structure, with the appearance of heterochromatin domains linked to specific gene silencing (34, 35). Taking into account the participation of p33 ING1 in the control of histone modifications and chromatin structure, and prompted by the defects in Ras-induced arrest observed in Ing1-deficient MEFS, we studied the changes in chromatin structure after RasV12 infection in MEFS of normal and mutant genotypes. With this aim, we carried out immunofluorescence experiments with antibodies against histone 3 trimethylated in Lys-9 (H3K9) or HP1γ as markers of heterochromatin. The nuclear distribution of either of the heterochromatin marks studied was not overtly changed by RasV12 expression in cells of either genotype. However, when we quantitated the fluorescence intensity in individual nuclei (see Experimental Procedures), we observed a significant increase in H3K9 and HP1γ signals per nucleus in wild-type MEFS infected with RasV12 retroviruses, relative to vector-infected cells. (Fig. 5, A and B). When we analyzed both markers in Ras-infected g/g fibroblasts, we observed an increase in H3K9 signal, which was indistinguishable to wild-type cells. However, HP1γ staining in g/g cells remained unchanged after Ras infection (Fig. 5, A and B). Also, when the fluorescence signals for H3K9 and HP1γ were simultaneously scored for individual nuclei, we observed that expression of RasV12 translates in a clear correlation for both markers in wild-type cells, but this correlation was not observed in homozygous Ing1 gene trap MEFS (Fig. 5C). Taken together, these results suggest a role for Ing1 in HP1 binding to chromatin and presumably heterochromatin formation during Ras-induced senescence in mouse fibroblasts.

**DISCUSSION**

The role of the family of ING proteins in tumor protection has recently attracted a substantial amount of interest. Biochemical evidence supports their connection to the p53 pathway and their participation in multiprotein complexes with chromatin modification activities. Thus, ING proteins might represent an important link between p53-mediated responses and chromatin regulation. In this study, we have taken a genetic approach to investigate the physiological role of the murine Ing1 locus in cellular stress responses associated with tumor suppression, using primary embryonic fibroblasts genetically deficient for the products of the Ing1 locus. Our results identify a critical role of the Ing1 locus in the antiproliferative response to oncogenic stress. Ing1-deficient MEFS display a defective senescence-like phenotype in response to an activated Ras oncogene, as evidenced by their growth rate, BrdUr incorporation, cell morphology, senescence-associated β-galactosidase staining, p53 accumulation, and heterochromatin formation. The impact of Ing1 deficiency appears to be specific for the implementation of senescence against activated oncogenes, because other p53-mediated phenotypes are not affected in our
system, in agreement with observations in a different animal model of Ing1 loss of function (36). A role for ING1 as a regulator of p53 function has been suggested previously, based on overexpression experiments. Here we show that the accumulation of p53 triggered by oncogenic stress is reduced in Ing1-deficient cells. Trying to identify the molecular basis for this defect, we have observed that the basal stability of the p53 protein in stress-free conditions is significantly decreased in Ing1-deficient fibroblasts. This effect could reflect a direct role on p53 stabilization, through the disruption of the binding of Mdm2 to p53, as proposed by Leung et al. (4), although indirect effects of ING1 are also possible. On the other hand, acetylation or phosphorylation of p53 upon DNA damage do not seem to be affected by Ing1 status in our experiments. It should be noted that conflicting results have been reported so far regarding the participation of p33 ING1 in p53 acetylation, based in overexpression experiments (see for example Kataoka et al. (6) as evidence in favor but also Nagashima et al. (5) as evidence against). Other ING proteins have been shown to induce p53 acetylation in similar in vitro assays, and a possible compensatory effect could be invoked. In particular, there is strong evidence supporting a role for Ing2 in p53 acetylation in different settings (5, 18). Ing2 RNA is expressed at detectable levels both in WT and g/g MEFs, and it might compensate for Ing1 deficiency. On the other hand, it is also possible that different ING proteins participate in p53 acetylation in response to different stimuli, and we cannot formally rule out such a role for Ing1 in settings or cell types different from the ones tested here. It should be noted that we have not been able to detect p53 acetylation upon Ras expression in MEFs of either genotype (data not shown), so that we cannot exclude the existence of altered p53 modifications in this setting, in addition to the observed differences in protein levels. After submission of our manuscript, Coles et al. (37) have reported the apparently normal response to oncogenic Ras of fibroblasts from an independently generated murine model of deficiency in the Ing1 locus. Although at this stage we cannot provide an explanation for the apparent discrepancy with our results, it is worth noting that the above-mentioned model differs from ours in the expression of specific Ing1 products. Although our cells showed no expression of the Ing1c-specific transcript and reduced Ing1b expression (Fig. 1), normal expression of the Ing1c product and no Ing1b was reported by Coles et al. (37). Although other experimental differences might also be considered, it is tempting to speculate that the reported differences might reflect differential functions of Ing1 products in the response to oncogenic stress.
The participation of ING1 and other ING proteins in the establishment and recognition of specific histone marks is well established. Our observation of a defective Ras-induced arrest in g/g mouse fibroblasts, a response where chromatin changes have been reported, led us to study the possible contribution of the products of the Ing1 locus to the formation of heterochromatin in this setting. We have shown that Ras expression in wild-type MEFs causes an apparent increase in heterochromatin, as evidenced by the concomitant increase in the intensity of two heterochromatin marks, namely histone H3 methylated in Lys-9 (H3K9) and the heterochromatin protein HP1γ, in a similar scenario to human primary fibroblasts (34). Ing1 deficiency resulted in defective accumulation of HP1 and uncoupling of H3K9 and HP1 signals, which could be explained by a role for p33 ING1 in targeting HP1 proteins to H3K9 sites. The recruitment of HP1 proteins to H3K9 sites is considered an important step for heterochromatin formation and maintenance (38). Therefore, we reasoned that the uncoupling between H3K9 and HP1 observed in Ing1-deficient cells should translate in defective heterochromatinization, which paralleled a defective anti-proliferative response. Although the chromatin defects observed might reflect indirect effects of Ing1 depletion in the implementation of senescence, the solid evidence linking ING1 to chromatin regulation clearly supports the participation of Ing1 in chromatin changes as the most likely mechanism. Interestingly, it has been proposed previously that p33 ING1 could play a role in HP1 deposition during formation of nascent constitutive heterochromatin (39). One possible explanation for our observation is linked to the reported ability of p33 ING1 to modulate histone acetylation (7, 9, 39). There is clear pharmacological and genetic evidence indicating that histone deacetylation is needed for the tethering of HP1 proteins to chromatin (40–43). Although we have not measured directly histone acetylation in our cells, it is feasible that Ing1 deficiency impairs HP1 binding and heterochromatin formation during Ras-induced arrest as a consequence of its action on histone acetylation. Regardless of the precise mechanism, our results with Ing1-deficient cells show a clear correlation between defective Ras-induced arrest and impaired chromatin changes, and they highlight the importance of heterochromatin formation during Ras-induced senescence in mouse cells, in an analogous manner to the phenomenon described in human fibroblasts. In support of this notion, Brait et al. (35) have recently shown that the induction of oncogene-induced senescence in premalignant lesions in mice is impaired in animals genetically null for the histone methyltransferase Suv39h1.

To summarize, in this study we provide data that support a dual role for the Ing1 locus as a mediator of the antiproliferative response elicited by oncogenic stress, first as an upstream regulator of p53, presumably favoring p53 protein stability, and second as a mediator in chromatin regulation, contributing to heterochromatin formation. It will be of interest to determine the possible interplay between both levels of action and their relative contribution to the biological function of Ing1. Finally, our results may have important implications to understand the significance of ING1 alterations in human tumors. Although some cases of point mutations or homozygous deletion have been reported, the large majority of tumor-associated alterations of the ING1 locus involves reduced RNA and/or protein levels of p33 ING1 (19), in a situation similar to our cellular system. This study provides a possible explanation for the growth advantage for low p33 ING1-expressing cells, because it predicts that a significant reduction in p33 ING1 function should translate in a defective activation of protective responses to activated oncogenes, weakening tumor protection barriers, and contributing to tumor formation.

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