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ING2 Regulates the Onset of Replicative Senescence by Induction of p300-Dependent p53 Acetylation†

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***ING2* is a candidate tumor suppressor gene that can activate p53 by enhancing its acetylation. Here, we demonstrate that *ING2* is also involved in p53-mediated replicative senescence. ING2 protein expression increased in late-passage human primary cells, and it colocalizes with serine 15-phosphorylated p53. ING2 and p53 also complexed with the histone acetyltransferase p300. ING2 enhanced the interaction between p53 and p300 and acted as a cofactor for p300-mediated p53 acetylation. The level of ING2 expression directly modulated the onset of replicative senescence. While overexpression of ING2 induced senescence in young fibroblasts in a p53-dependent manner, expression of ING2 small interfering RNA delayed the onset of senescence. Hence, ING2 can act as a cofactor of p300 for p53 acetylation and thereby plays a positive regulatory role during p53-mediated replicative senescence.**

Cellular senescence, a process originally described by Hayflick in 1965 (28), prevents normal human fibroblasts from growing indefinitely in culture. This process, also termed replicative senescence, is driven by telomere attrition. The expression of telomerase, which maintains the telomere length, prevents cells from undergoing replicative senescence (6, 27). Stimuli having little or no impact on telomeres have also been shown to induce growth arrest with a senescence-like phenotype (generally called stress-induced senescence or premature senescence). These stimuli include DNA damage, chromatin remodeling, strong mitogenic signals, or suboptimal cell culture conditions. Thus, oncogenic *ras* can also induce senescence in cells, and promyelocytic leukemia protein (PML) has been shown to be essential for the induction of *ras*-induced senescence (17, 41).

Cells need to bypass senescence to become transformed (46). Consistent with its role in suppressing cancer development, senescence is controlled by two major tumor suppressors, the *p53* gene and the retinoblastoma gene (*Rb*) (5, 37). An increase in p53 transcriptional activity is a molecular signature for cellular senescence (3, 7, 50). The increased activity is driven by changes in p53 phosphorylation and acetylation status (41, 53).

The DNA damage sensor proteins ATM, Chk1, and Chk2 are recruited to the site of shortened telomeres in senescent cells (4, 13, 21, 29, 43, 48). Interestingly, p53 is also phosphorylated and thus activated by all the above-mentioned kinases (10), and p53 phosphorylation is a signal for subsequent acetylation (15, 33). p53 acetylation, which is required for its full activity, increases in both replicative and *ras*-induced senescence (41). During *ras*-induced senescence, PML is required for p53 acetylation by CBP in nuclear bodies (17, 41).

The ING family proteins are candidate tumor suppressors that associate with histone acetyltransferase (HAT), histone deacetylase, and factor acetyltransferase complexes (16). All the *ING* genes share a PHD finger motif, which may be implicated in chromatin-mediated transcriptional regulation (1, 40). In tumors, *ING* genes are not frequently mutated, but expression is down-regulated in several tumor types, including breast, blood, esophageal, lung, brain, bladder, stomach, and liver cancer (16, 19, 39, 49). Five members of the ING family have been identified in humans. ING1 to ING5 cooperate with the p53 tumor suppressor protein to induce cell growth arrest and apoptosis (18, 35, 36, 47). ING1, ING2, ING4, and ING5 can regulate p53 by enhancing its acetylation on lysine residues 373 or 382 (32, 35, 47). There are multiple mechanisms by which *ING* genes may modulate p53 acetylation. For example, p33ING1b (one of the three alternative splice variants of *ING1*) binds to SIRT1 and inhibits its deacetylation activity on p53, resulting in an increase in the level of active, acetylated p53 (32). p33ING1b, ING4, and ING5 can physically interact with the p300 protein and could thereby modulate HAT activity (47, 51). Finally, ING2 is not only critical to enhance p53 acetylation on lysine 382, but it is also up-regulated in response to etoposide and neocarzinostatin. In cells with down-regulated ING2, p53 acetylation, p21 expression, and apoptosis are reduced following etoposide treatment (23, 35).

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In this study, we investigated the functional role of ING2 in replicative senescence. We demonstrate that ING2 and serine 15-phosphorylated p53 (hereafter termed phospho-p53 [Ser15]) protein expression is elevated during replicative senescence in human fibroblasts. Under these conditions, ING2 and phospho-p53 (Ser15) colocalize (i.e., assemble at distinct nuclear foci) and physically interact with p300. This complex is located outside PML nuclear bodies. Mechanistically, ING2 physically interacts with p300 in vitro and in vivo and enhances p300-mediated p53 acetylation in vitro. ING2 also regulates the onset of replicative senescence: the overexpression of ING2 in young fibroblasts induces premature senescence in a p53-dependent manner, and down-regulation of ING2 decreases p53 acetylation and delays the onset of replicative senescence. We propose that ING2 plays a major role in regulating the p53-dependent senescence checkpoint.

MATERIALS AND METHODS

Cell culture, transfection, retroviral gene transfer, and SA-β-Gal assays. MRC5 fibroblasts, Amphopack 293 (BD Biosciences Clontech), and U2OS cells were grown in standard cell culture medium at 37°C. Cells were transfected with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. For fibroblast infection, the packaging cell line Amphopack 293 was transfected for 3 h. Thirty hours after transfection, the medium containing the virus was recovered and filtered with 0.45-μm low-binding protein filters (Pall Corporation, MI). The media containing the viral particles was used to infect the fibroblasts for 8 h in the presence of hexadimethrine bromide (Sigma) at 8 μg/ml. The medium was subsequently replaced with fresh medium, and cells were allowed to grow for 24 h. MRC5 cells were selected with G418 (800 μg/ml; Invitrogen) or Puromycine (1 μg/ml; Sigma) for 5 to 7 days. Selection was continued until all mock-infected cells were dead; subsequently, the cells were grown in medium without any selection. Senescence-associated β-galactosidase (SA-β-Gal) activity was detected in fibroblasts as previously described (14, 45).

Plasmids and primers. (i) Plasmids. ING2 was cloned in pCLXSN (38) vector and pFLAG-CMV-6c vector (Sigma). p53 interfering RNA (RNAi), ING2 RNAi, and control RNAi were all cloned in pSuperRetro (Oligoengine). The respective sequences have all been previously described (12, 23). A previously described retrovirus vector, pBabe-Puro *ras* (H-*Ras*V12) (45), was used to induce senescence. For reverse transcription-PCR (RT-PCR), the sequences of the primers used were CGAGATCCCTCCAAAATCAA and ATCCACAGTCTTC TGGGTGG for *gapdh* and AAAATCGGGCAAGACAAATG and GAAGCT TCCCTTTCCTGCTT for *ING2*.

(ii) Antibodies. The following antibodies were used: for anti-p53, monoclonal antibodies DO-1 and 1801 (Santa Cruz) and polyclonal antibody CM1 (Signet Laboratories); for anti-phospho-p53 (Ser15), rabbit polyclonal and monoclonal 16G8 (Cell Signaling); for anti-acetyl-p53 (Lys382), polyclonal (Oncogene Research); for anti-ING2, goat polyclonal ab2643 (Abcam) and rabbit polyclonal Ping2 (35); for anti-p300, rabbit polyclonal N15 (Santa Cruz) and monoclonal RW128 (Upstate); for anti-PML, monoclonal PGM-3 (Santa Cruz); for anti-Flag, monoclonal M2 (Sigma); for anti-β-actin, mouse monoclonal AC-15 (Sigma). Secondary antibodies were purchased from Jackson Laboratories, Southern Biotechnology Associates, and Santa Cruz.

Western blot analysis. Subconfluent cultures of cells were harvested and lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP-40, 5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche). Equal amounts of cell lysates (20 μg) were resuspended in 2× Tris-glycine sodium dodecyl sulfate (SDS) sample buffer, electrophoresed on 10% SDS-polyacrylamide gels, and electrophoretically transferred to Immobilon-P membranes (Millipore).

Immunoprecipitation. Whole-cell lysates were prepared from MRC5 cells or transfected U2OS cells, using previously described immunoprecipitation buffers and conditions (44). For each immunoprecipitation, 2 μg of the antibody was incubated with 1 mg of the lysate for 1 h. After being washed, the samples were analyzed by Western blotting for detection of the coimmunoprecipitated proteins.

Immunofluorescence. Cells were grown on glass slides for 48 h (to minimize stress that may arise during passaging), treated with a hypotonic lysis buffer, and fixed in 100% cold ethanol. The entire immunofluorescence protocol was carried out as previously described (44). The slides were visualized with a Zeiss Axioskop fluorescence microscope equipped with a high-performance charge-coupled device imaging system (IP Lab spectrum). Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optishot microscope with a 60× objective. Sequential excitation at 488 nm, 568 nm, and 647 nm was provided by a krypton-argon gas laser. Emission filters of 522/32, 598/40, and 680/32 were used for collecting green, red, and far-red fluorescence in channels one, two, and three, respectively. After sequential excitation, green, red, and far-red fluorescent images of the same cell were saved with Laser Sharp software. Images were analyzed with both Bio-Rad and Zeiss software. The term colocalization refers to the coincidence of green and red fluorescence, as measured by the confocal microscope. Colocalization in quantification graphs indicates the percentage of the proteins that colocalize with each other as measured with the Bio-Rad and/or Zeiss software. At least 100 cells were analyzed for each colocalization experiment, and typically five to ten cells per experiment were quantified using the Bio-Rad LaserSharp software. Statistical analysis with the software determined the Pearson's correlation (PC), which compares the colocalization of two different types of fluorescence and their intensity; the higher the fluorescence intensities are at the site of colocalization, the higher the PC will be, with a maximum of 1. The experiments were repeated at least twice.

Expression and purification of ING2 protein. ING2 was cloned in the pFASTBAC plasmid (Invitrogen). Recombinant pFASTBAC plasmid was used to transform competent DH10BAC cells for transposition to the bacmid shuttle vector. Bacmid recombinants were identified by LacZ selection on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) plates, and composite bacmid DNA was isolated from selected colonies. Cellfectin (Invitrogen) was used to transfect recombinant DNA in monolayers of Sf9 insect cells. Viral supernatant was harvested after 4 days and used for further infections. To produce ING2protein, Sf9 cells growing in suspension were infected with the virus (multiplicity of infection, 3) and harvested 72 h after infection. ING2 protein was purified with Probond resin (ProBond Purification System, Invitrogen), under native conditions as recommended by the manufacturer. The protein was dialyzed twice at 4°C for at least 6 h in a Tris buffer (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 20 μM $ZnCl_2$, 1 mM DTT, and 25% glycerol). The ING2 recombinant protein was >90% homogenous as assessed by Coomassie blue staining.

In vitro p53 acetylation. Assays were performed in buffer A (50 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 10 mM sodium butyrate). The amounts of purified proteins used for assays were as follows: p53, 1 μg; p300, 5 to 20 ng; and ING2, 5 to 40 ng. Baculovirus-purified p300 and p53 proteins were purchased from Proteinone (College Park, MD). Glutathione *S*-transferase–p53 protein was purchased from Santa Cruz. Substrate concentrations were 0.1 to 0.2 mg/ml for [1-^{14}C]acetyl coenzyme A ([1-^{14}C]acetyl-CoA) (Amersham) or 10 μM for cold acetyl-CoA (Sigma). Assays were incubated at 30°C for 30 min and initiated by addition of the protein substrate (p53) to a mixture containing p300 and acetyl-CoA in buffer A. The incorporation of [1-^{14}C]acetyl-CoA was quantified after SDS-polyacrylamide gel electrophoresis with a PhosphorImager (Fuji). Cold acetylation was detected by using the anti-acetyl-p53 (Lys382) antibody, and the signal intensity was determined using Aida software.

RESULTS

ING2 expression during replicative and premature senescence. *ING1* has been previously shown to play a role in replicative senescence (20). In addition, p53 acetylation on lysine 382 increases during replicative senescence (41), and acetylation enhancement on this residue can be mediated by ING2 (35). Therefore, we investigated the involvement of *ING2* in p53-mediated replicative senescence. We carried out the studies with young primary MRC5 fibroblasts at a population-doubling value of 28 (PDL28) and senescent fibroblasts at PDL63 (Fig. 1A and B). The p53 profile of expression was similar to what has been previously reported with no marked increase in the p53 protein expression (3, 7, 53). However, in contrast to the PDL28 fibroblasts, the p53 protein was transcriptionally active in PDL63 MRC5 cells, leading to the induction of p21$^{WAF1/SDI1}$ (hereafter referred as p21) (Fig. 1C and D).

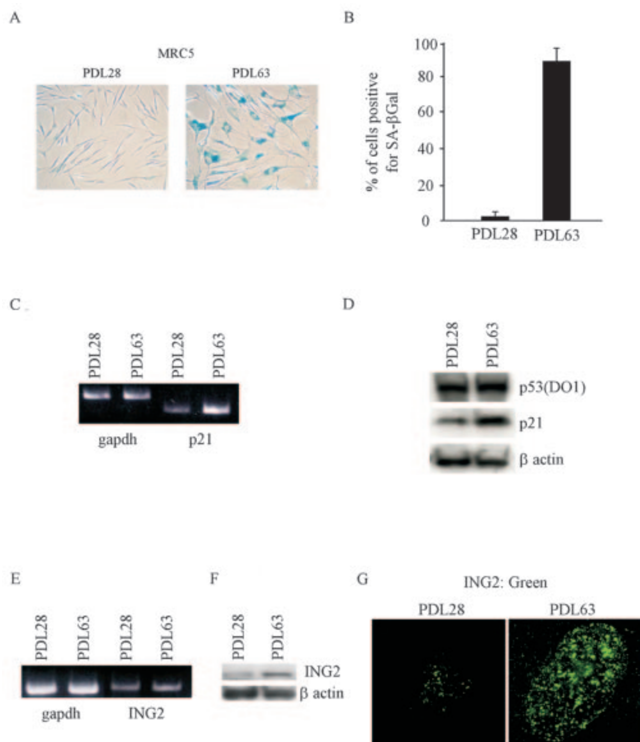

FIG. 1. Expression of ING2 in normal young (PDL28) and replicative senescent (PDL63) MRC5 human fibroblasts. Typically, senescent cells were larger, with larger nuclei and a decreased density of confluence. More than 90% of the senescent cells were positive by the SA-β-Gal assay, whereas with PDL28 fibroblasts, very few cells were positive. Though the amount of detectable p53 was similar between PDL28 and PDL63, a strong increase in $p21^{WAF1/SDI1}$ mRNA and protein was observed in the senescent fibroblasts, thereby indicating the presence of transcriptionally active p53. Other known p53 target genes were also transcriptionally activated, e.g., WIG1 and PERP (data not shown). (A and B) Replicative senescence induced SA-β-Gal expression. MRC fibroblasts at PDL28 and PDL63 were stained for SA-β-Gal expression (A) and quantitated (B). The graphs represent means ± standard deviation (SD). (C and D) p53 and $p21^{WAF1/SDI1}$ protein expression during replicative senescence. Lysates (C) or mRNA (D) was analyzed for the expression of p53 and $p21^{WAF1/SDI1}$ (for protein in panel C) or only $p21^{WAF1/SDI1}$ (for mRNA in panel D). β-Actin and *gapdh* expression were used as controls in the experiments shown in panels C and D, respectively. (E) *ING2* mRNA expression is unaltered in young and replicative senescent fibroblasts. RNA was extracted from young and senescent fibroblasts. RT-PCR was carried out with specific primers against *gapdh* or *ING2* primers. (F) ING2 protein expression increases during replicative senescence. Cell extracts from young and senescent fibroblasts were subjected to Western blotting and probed with ING2 (rabbit) or β-actin antibody. (G) ING2 immunofluorescence increased during replicative senescence. Cells from young and senescent fibroblasts were subjected to immunofluorescence with ING2 (goat) antibodies. Nuclei were typically larger in senescent cells.

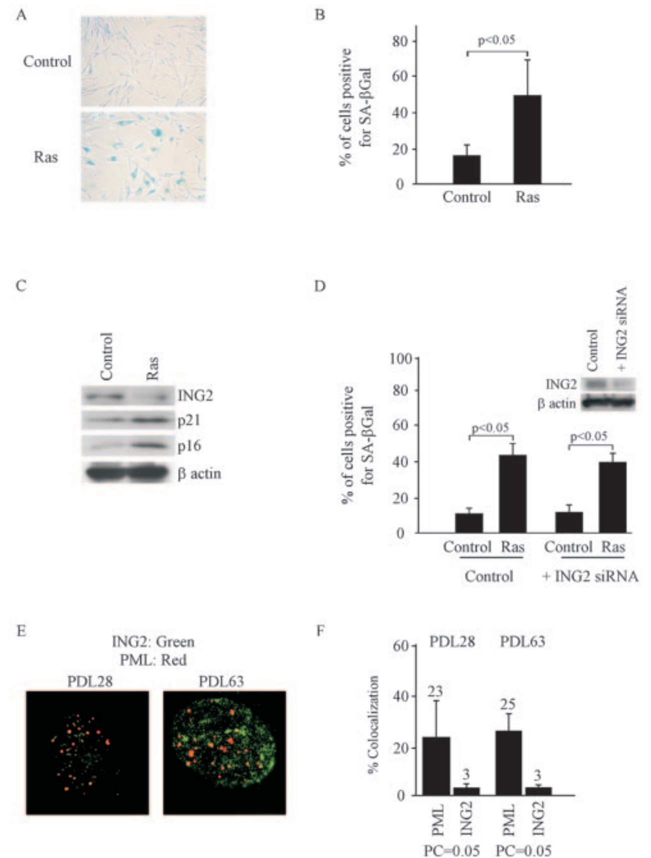

FIG. 2. ING2 is not involved in *ras*-induced and PML-mediated senescence. (A) *ras*-induced senescence showed the expression of SA-β-Gal. SA-β-Gal was assayed in young MRC5 fibroblasts infected with an empty (control) or *ras*-containing retrovirus. (B) Quantification of the percentage of cells positive for SA-β-Gal staining. The graphs represent means ± SD. At least 300 cells were counted from the experiment shown in panel A. (C) ING2 protein expression in *ras*-induced senescent fibroblasts. Extracts were prepared from cells infected with empty (control) or *ras*-containing retrovirus and Western blotted with antibodies against ING2, $p21^{WAF1/SDI1}$, p16, and β-actin. (D) *ras*-induced senescence in MRC5 fibroblasts in which ING2 was knocked down. Quantification of the percentage of cells positive for the SA-β-Gal staining was performed; the graphs represent means ± SD. At least 300 cells were counted. (Top) Western blot detection of ING2 (rabbit) and β-actin in MRC5 fibroblasts containing scrambled or ING2 siRNA retroviral constructs. (E) Immunofluorescence detection of ING2 and PML in young and senescent fibroblasts. Cells from young and senescent fibroblasts were subjected to immunofluorescence with ING2 (goat)/PML antibodies. (F) Quantification of PML/ING2 colocalization. The graphs represent means ± SD. Statistical significance of the colocalization was performed using PC.

To determine whether *ING2* mRNA and protein expression was altered during senescence, we carried out RT-PCR and Western blot analysis of PDL28 and PDL63 fibroblast lysates. While the *ING2* mRNA level remained unchanged (Fig. 1E), ING2 protein level was four- to sixfold higher in senescent fibroblasts (Fig. 1F). A similar increase of ING2 expression was also observed with senescent WI38 human fibroblasts when compared with young fibroblasts (data not shown). ING2 was localized in detergent nonextractable nuclear foci in both young and senescent fibroblasts. In contrast to Western analysis of total ING2 content, prelysis of the cells prior to fixation allowed the elimination of most of the soluble proteins and detection of mainly insoluble proteins. Both the intensity and the extent of the focal accumulation of ING2 increased in senescent cells (Fig. 1G).

To determine whether the enhanced ING2 expression was specific for replicative senescence, we induced premature senescence in PDL28 fibroblasts with a *ras*-expressing retrovirus. Premature senescence was observed in at least 50% of the

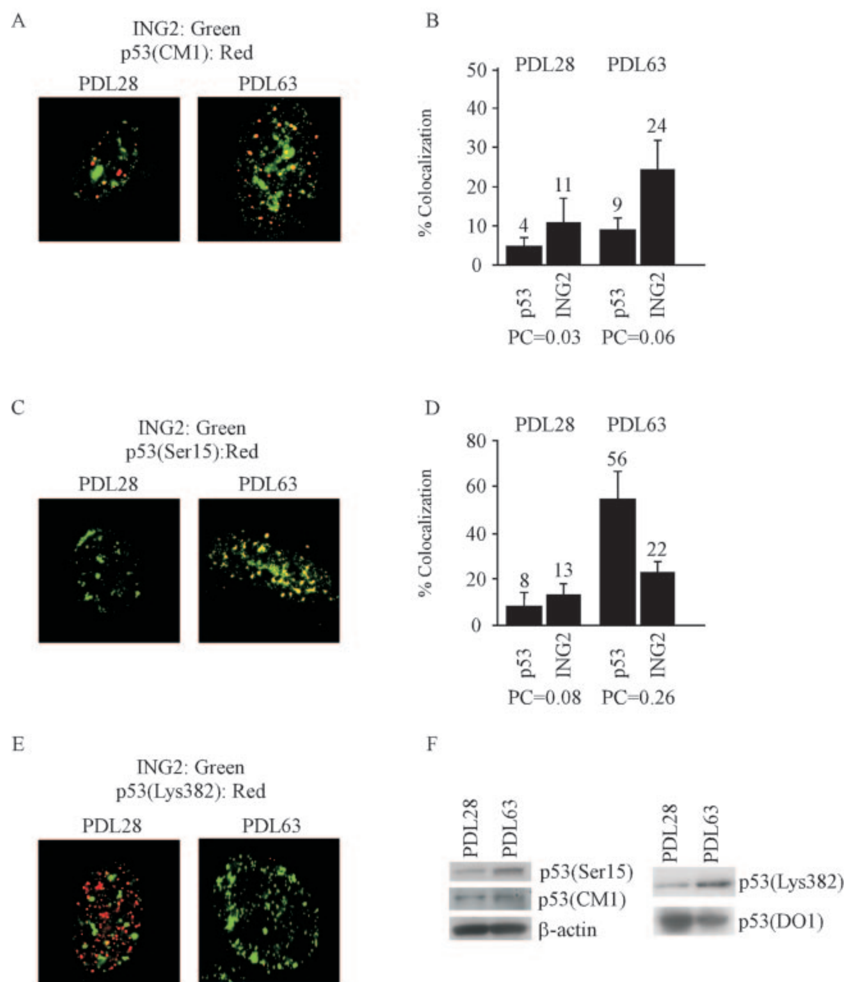

FIG. 3. ING2 and translationally modified p53 colocalize in replicative senescent fibroblasts. (A) ING2 colocalized with p53 in both young and senescent fibroblasts. Immunofluorescence was carried out in young and senescent fibroblasts with antibodies against ING2 (goat) and p53 (CM1). (B) Quantification of ING2/p53 (CM1) colocalization. The graphs represent means ± SD. Statistical significance of the colocalization was performed using PC. (C) ING2 colocalized with p53(Ser15) in senescent fibroblasts. The experiment was the same as that shown in panel A, except that immunofluorescence was done with ING2 (goat)/p53 (Ser15) (rabbit) antibodies. (D) Quantification of ING2/p53 (Ser15) colocalization. The graphs represent means ± SD. Statistical significance of the colocalization was performed using PC. (E) Colocalization pattern of ING2 and p53 (Lys382). The experiment was the same as that shown in panel A, except that immunofluorescence was done with ING2 (goat)/p53 (Lys382) antibodies. (F) Expression of posttranslationally modified p53 in young and senescent fibroblasts. Extracts from young and senescent fibroblasts were subjected to Western blotting and probed with antibodies against p53 (Ser15), p53 (CM1), and β-actin antibodies. For the detection of acetylated p53, p53 was immunoprecipitated with DO-1 and 1801 antibodies in young or senescent fibroblasts, and probed with p53 (Lys382) antibodies. The amount of p53 protein immunoprecipitated was detected with p53 (DO-1).

cells, as detectable by an SA-β-Gal assay (Fig. 2A and B). In contrast to late-passage fibroblasts, ING2 protein levels decreased during *ras*-induced senescence (Fig. 2C), while the amount of p21 and p16 protein increased. Furthermore, *ras* induced premature senescence in fibroblasts in which ING2 was knocked down (Fig. 2D). These data indicate that ING2 may not play a significant role in *ras*-induced senescence.

PML plays a pivotal role in *ras*-induced senescence and may also play a role in replicative senescence (17, 41). To determine whether the role of ING2 in replicative senescence could also be mediated via PML, we examined ING2/PML immunocolocalization in PDL28 and PDL63 fibroblasts. We did not observe any statistically significant differences in the probability (as designated by PC) or the percentage of colocalization between ING2 and PML in young and senescent fibroblasts (Fig. 2E and F). These results indicate that ING2 may have a unique role in replicative senescence, which is likely to be independent of PML.

ING2 colocalizes with posttranslationally modified p53. p53 transcriptional activity is regulated by ING2 (35), which increased during replicative senescence (Fig. 1); therefore, we investigated the relationship between ING2 and p53 in late-passage versus early-passage cells. First, we wanted to determine whether p53 and ING2 could physically interact (directly or as part of a complex) and colocalize, especially in senescent fibroblasts. The level of total p53 did not change between PDL28 and PDL63 when tested with different anti-p53 antibodies (Fig. 1D and 3F). However, p53 (detected by the C-

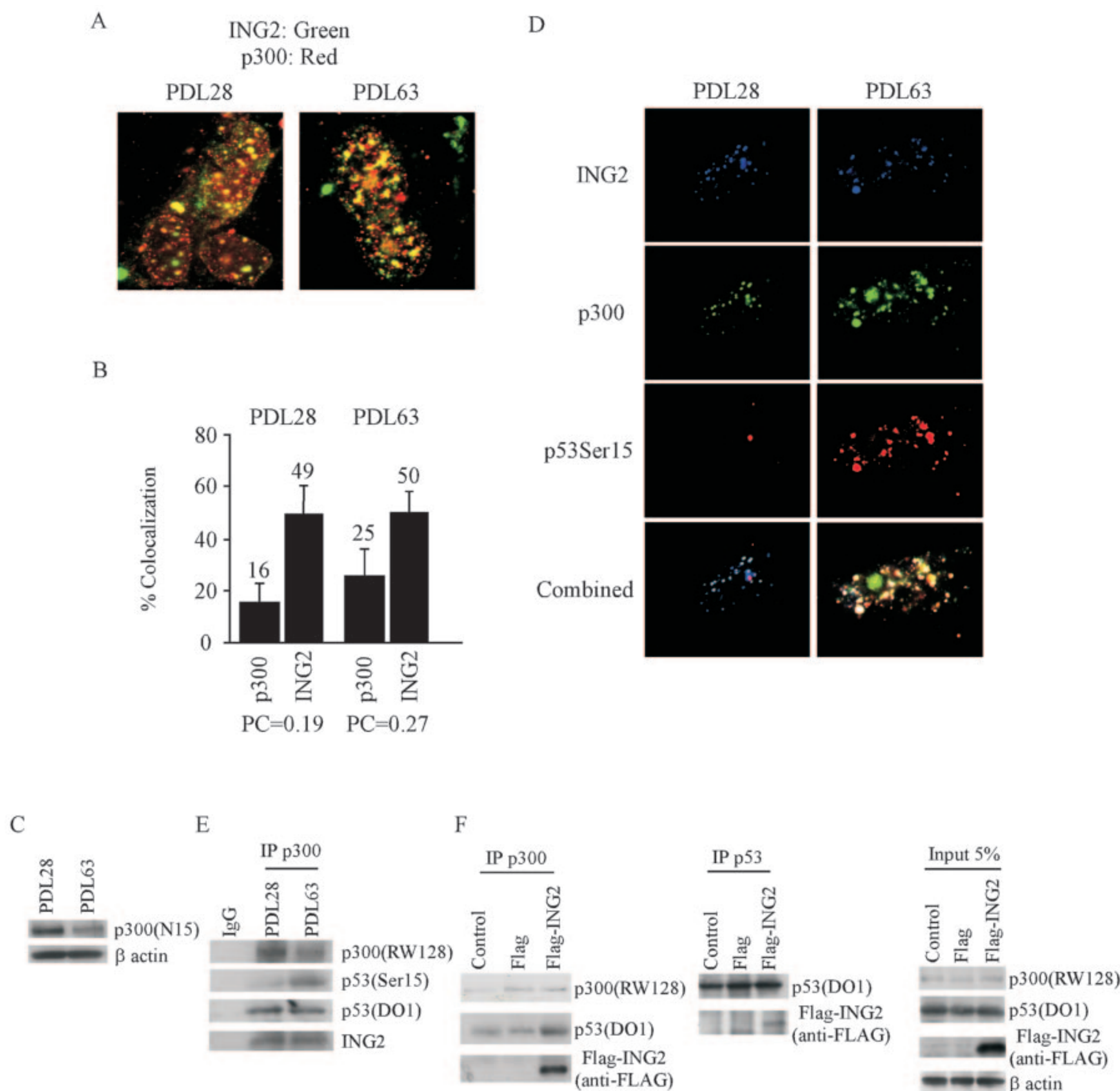

FIG. 4. ING2 and p300 colocalizes and may form a triple complex with p53. (A) ING2 protein and p300 colocalized in young and senescent fibroblasts. Immunofluorescence was carried out in young and senescent fibroblasts with antibodies against ING2 (goat) and p300 (N15). (B) Quantification of ING2/p300 colocalization. The graphs represent means ± SD. Statistical significance of the colocalization was performed using PC. (C) Levels of p300 in young and senescent fibroblasts. Lysates were made from young and senescent fibroblasts, Western blotted, and probed with antibodies against p300 (N15) and β-actin antibodies. (D) ING2, p300, and p53 (Ser15) colocalized together in senescent fibroblasts. The experiment was the same as that shown in panel A, except that immunofluorescence was done with antibodies against ING2 (goat), p300 (N15)/p53 (Ser15) (rabbit) antibodies. White foci represent triple colocalization. (E) ING2, p53 (Ser15), and p300 may form a triple complex in senescent fibroblasts. The experiment was the same as that shown in panel C, except that lysates were immunoprecipitated with p300 (N15) antibody. The immunoprecipitates were self probed with antibodies against p300 (N15) antibody. Coprecipitating proteins were detected with antibodies against p53 (Ser15) (16G8) and ING2 (rabbit). Lysates used were the same as in panel C and Fig. 1B and 3F. (F) ING2 enhances the complex between p53 and p300. U2OS cells were transfected with a Flag-ING2 expression vector. Lysates were immunoprecipitated with antibodies against p53 (DO-1 and 1801) or p300 (N15), and immunoprecipitation was verified with self antibody p53 (DO-1) or p300 (RW128). Coimmunoprecipitated proteins were detected with antibodies against p53 (DO-1) and Flag (M2) antibodies. A total of 5% of the amount of whole-cell lysates used for immunoprecipitation was probed with p300 (RW128), p53 (DO-1), Flag (M2), and β-actin antibody.

terminal-specific antibody CM1) partly colocalized with ING2 (Fig. 3A), and we observed an increase in colocalization during senescence that was statistically significant (Fig. 3B).

p53 is phosphorylated at serine 15 and acetylated at lysine 382 during replicative senescence (29, 41, 53). We observed that the protein level of p53-serine 15 phosphorylation and p53-lysine 382 acetylation increased in PDL63 fibroblasts (Fig. 3F). However, while the colocalization of ING2 with p53 (Ser15) was dramatically enhanced in senescent fibroblasts (Fig. 3C and D), we reproducibly detected a drastic decrease in the number of foci containing p53 lysine 382 during senescence (Fig. 3E). We hypothesized that the p53-lysine 382 epitope could be masked by a protein complex during senescence, which prevented its subsequent detection by immunofluorescence. To determine whether such masking of the p53-lysine 382 epitope was also possible in young fibroblasts, we treated

PDL28 fibroblasts with etoposide and adriamycin. As reported earlier (35), etoposide treatment increases the ING2 protein level. However, lysine 382-acetylated p53 was not observed by immunofluorescence in cells treated with etoposide (see Fig. S1A and S1B in the supplemental material), thereby indicating that masking of p53-lysine 382 was possible under certain conditions when both p53 and ING2 were stabilized. Overall, these results demonstrate that ING2 colocalized with, at the very least, serine 15-phosphorylated p53 during replicative senescence, suggesting that ING2 functions in complexes containing activated, modified p53 molecules.

Serine 15-phosphorylated p53 and ING2 complex with p300 in senescent fibroblasts. The acetylation of p53 occurs during senescence (41); therefore, we wanted to examine whether the ING2/p53 complex has any physical or functional interactions with two members of the HAT family, p300 and CBP, both known to acetylate p53 on lysine 382 (26). The level of CBP was the same in PDL28 and PDL63 (see Fig. S2C in the supplemental material). ING2 and CBP foci did not show any appreciable colocalization in either the young or senescent fibroblasts (see Fig. S2A and S2B in the supplemental material). The total amount of cellular p300 protein level, detected with three antibodies (N15, C20, and RW128), decreased in senescent fibroblasts (Fig. 2C and data not shown). A similar decrease in p300 levels has also been observed after UV irradiation (54). With the same antibodies, numerous endogenous p300 foci were detected by immunofluorescence. These detergent nonextractable foci possibly accounted for the chromatin-bound p300 and remained at approximately equal levels in both young and senescent fibroblasts. More significantly, the p300 foci showed a small but statistically significant colocalization with ING2 in both young and senescent fibroblasts (Fig. 4A and B) with the PC for ING2/p300 colocalization increased by 50% in senescent cells.

To determine whether the ING2/p300 colocalization and serine 15-phosphorylated p53/ING2 colocalization might be one and the same, triple colocalization was performed. Indeed, with PDL63 cells, ING2, p300, and p53-serine 15 colocalized extensively with each other, as visualized by white foci (Fig. 4D). The extremely low level of serine 15-phosphorylated p53 in young fibroblasts may have prevented its detection or colocalization with the ING2/p300 complex in young fibroblasts. Moreover, immunoprecipitation of lysates from early- and late-passage cells with the p300 antibody indicated that p300 physically interacted with ING2 and p53, preferably in senescent cells (Fig. 4E). The complex formation between p53, ING2, and p300 was further corroborated in transient transfections in wild-type p53-containing U2OS cells, where immunoprecipitation of endogenous p300 also contained p53 and transfected Flag-ING2 (Fig. 4F). More p53 was detected with immunoprecipitated p300 when ING2 was also cotransfected, thereby indicating that ING2 may enhance the interaction between p53 and p300. Furthermore, immunoprecipitation of endogenous p53 contained transfected Flag-ING2. Collectively, these results indicate that ING2, p300, and serine 15-phosphorylated p53 are complexed together preferentially during replicative senescence in human cells.

ING2 increases p300-mediated p53 acetylation in vitro. The above results, which indicate that ING2 may enhance colocalization and physical interaction between p53 and p300 in senescent cells, led us to examine whether ING2 can increase in vitro p300-dependent p53 acetylation. As previously reported (26), increasing amounts of p300 progressively enhanced p53 acetylation, as detected either by an acetylation-specific p53 antibody (Fig. 5A) or by the incorporation of ^{14}C-acetyl-CoA into p53 (Fig. 5B). With a constant amount of p300, increasing the amounts of purified ING2 protein led to a further two- to fourfold increase in the acetylation of lysine 382 on p53, as determined by the two detection methods (Fig. 5C and D). No p53 acetylation was detected with ING2 protein alone. Therefore, ING2 enhances p300-induced p53 acetylation in vitro.

ING2 expression level regulates the switch between senescence and proliferation. To investigate how ING2 has a direct effect on replicative senescence, we took two approaches to altering endogenous ING2 protein levels. First, we overexpressed ING2 in PDL28 MRC5 fibroblasts, using an ING2-expressing retrovirus, and analyzed the occurrence of premature senescence by the SA-β-Gal assay. ING2 overexpression induced premature senescence in PDL28 fibroblasts (Fig. 6A to C). However, ING2 overexpression-mediated induction of senescence was not observed in isogenic MRC5 cells in which p53 had been inactivated by a small interfering RNA (siRNA) retroviral construct (Fig. 6B and C). These results confirmed and extended our earlier observation that the presence of functional p53 is required for the growth-suppressive functions of ING2 (35).

Based on the above results, we hypothesized that if overexpression of ING2 promoted senescence, expression knockdown of ING2 could induce cell growth in senescent cells. Presenescent late-passage MRC5 fibroblasts (PDL59) were infected with retroviral constructs containing p53 siRNA or ING2 siRNA and cultured for 3 weeks. The number of resultant colonies was then counted. Immunofluorescence and Western analysis indicated that ING2 expression was effectively down-regulated by the ING2 siRNAs tested (Fig. 6D and E). Moreover, ING2 knockdown led to a decrease in p53 acetylation on lysine 382 (Fig. 6E), consistent with the possibility that ING2 may play a role in the induction of p53 acetylation during replicative senescence. As expected, the down-regulation of p53 expression resulted in an enhancement of colony formation (Fig. 6F). The number of both small (10 to 50 cells, about four to five doublings) and large (>50 cells; >5 doublings) colonies was increased. Interestingly, the down-regulation of ING2 resulted in a statistically significant increase only in the number of small colonies (containing 10 to 50 cells). Thus, suppression of ING2 expression results in an extension of the life span of human fibroblasts for a limited number of divisions.

DISCUSSION

We have previously demonstrated that *ING2* is a DNA damage-inducible gene that negatively regulates cell proliferation through the activation of p53 by enhancing its acetylation (35). In this communication, using a combination of biochemical and cell biological assays, we have demonstrated that *ING2* is also a component of the p53-mediated replicative senescence pathway. ING2 expression levels modulate the onset of p53-dependent replicative senescence in primary normal human fibroblasts (Fig. 6). This role of ING2 is possibly specific to

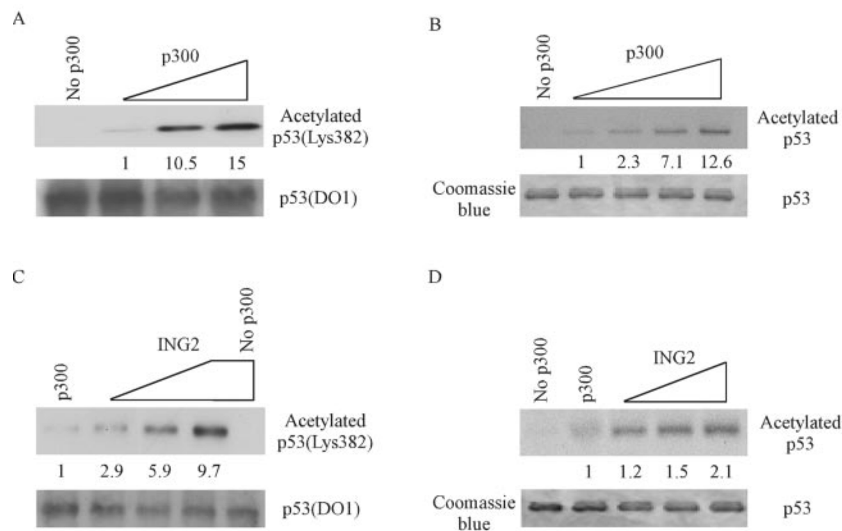

FIG. 5. ING2 enhances p300-induced p53 acetylation. (A and B) p300 acetylated p53 on lysine 382 in vitro. p53 was incubated with increasing amounts of p300 (5 to 20 ng) either in a cold reaction (A) or in the presence of ^{14}C-labeled acetyl-CoA (B). The p53 acetylation (Lys382) was detected either by probing with p53 (Lys382) antibody (A) or by autoradiography (B). The overall amount of p53 protein was monitored by probing with p53 (DO-1) (A) or by Coomassie blue staining (B). The quantification of ^{14}C incorporation was assessed with a PhosphorImager. (C and D) ING2 enhances p300-induced p53 acetylation on lysine 382 in vitro. p53 acetylation on lysine 382 was detected by Western blotting. Increasing amounts of ING2 (10, 20, and 40 ng) were incubated with a constant amount of p300 (5 ng) either in a cold reaction (C) or in the presence of ^{14}C-labeled acetyl-CoA (D). p53 acetylation (Lys382) was detected either by probing with p53 (Lys382) antibody (C) or by autoradiography (D). The overall amount of p53 protein was monitored by probing with p53 (DO-1) (C) or by Comassie blue staining (D). The quantification of ^{14}C incorporation was assessed using a PhosphorImager.

replicative senescence and not PML-mediated *ras*-induced senescence, because ING2 levels are not altered after *ras* expression and *ras* can induce premature senescence in fibroblasts in which ING2 has been knocked down (Fig. 2). We demonstrate that during replicative senescence, ING2 colocalized with posttranslationally modified p53 and p300 (Fig. 3). ING2 also enhanced the binding of p53 to p300, and acted as a cofactor for p300-mediated p53 (lysine 382) acetylation (Fig. 4F and 5). While the interaction between p53 and ING2 may be either direct or indirect, our results indicate that both the above proteins interacted with p300 in an in vivo physiological process, replicative senescence. Hence, these results implicate ING2 as a member of the p53-driven replicative senescence pathway and suggest that ING2, p300, and posttranslationally modified p53 play a major role in regulating senescence. However, compared with cells in which p53 is knocked down, the delay of senescence, due to ING2 knockdown, is limited to only a few additional population doublings (three to five). This may be due to redundancy between the ING proteins. Indeed, ING1 has also been implicated in replicative senescence (20).

Replicative senescence can be bypassed by p53 inactivation, which can occur by antibody microinjection and by the overexpression of p53 dominant-negative mutants, viral oncoproteins, antisense oligonucleotides, or siRNAs (5, 8, 22, 52). p53 inactivation results in an additional 10 to 20 cell divisions before reaching a crisis caused by telomere attrition (9). During replicative senescence, the transcriptional activity of p53 is increased, which in turn results in the enhanced expression of p21 and ultimately leads to the cellular senescence phenotype (3, 7, 50). p53 activation occurs through changes in its phosphorylation and acetylation status (2, 11, 24, 42). When p53 is activated during replicative senescence, phosphorylation on residues serine 15 and threonine 18 increases, whereas it decreases on serine 392 (53). Similarly, p53 acetylation on lysines 320 and 382 increases the p53 transactivation function, possibly as a result of acetylation-induced conformational changes (31, 34, 42). p53 phosphorylation on serine 15 has been directly correlated with p21 transcription in replicative senescent fibroblasts (29). Incidentally, we found that other known p53 target genes (like the WIG1 and PERP genes) were transcriptionally activated during replicative senescence (data not shown). Confirming these previous results, we observed that p53-serine 15 phosphorylation and p53-lysine 382 acetylation were increased during replicative senescence in MRC5 fibroblasts (Fig. 3). The mechanistic role of p53 acetylation in replicative senescence remains to be determined.

We have previously reported that ING2 overexpression enhances p53 acetylation on lysine 382, thereby activating p53 and allowing the cells to undergo apoptosis (35). ING2 can enhance p53 acetylation by positively modulating the activity of acetyltransferase complexes. PCAF, CBP, and p300 are the known coactivators of p53 and potentiate its transcriptional activity, as well as its biological function, in vivo by recruiting and acetylating the p53 C-terminal domain (24). Hence, p300-deficient cells fail to fully acetylate p53 and do not transactivate p21 or undergo G_1/S arrest after UV irradiation (30). It must be noted that for immunofluorescence studies, because of the prelysis used prior to the fixation of cells, the pattern of expression of insoluble proteins does not necessarily reflect the overall amount of protein contained in cells as observed by Western blot analysis. Here, we have demonstrated that p300 (but not CBP) played an essential role with ING2 during replicative senescence (Fig. 4; see Fig. S2 in the supplemental material). Mechanistically, we show that the acetylation func-

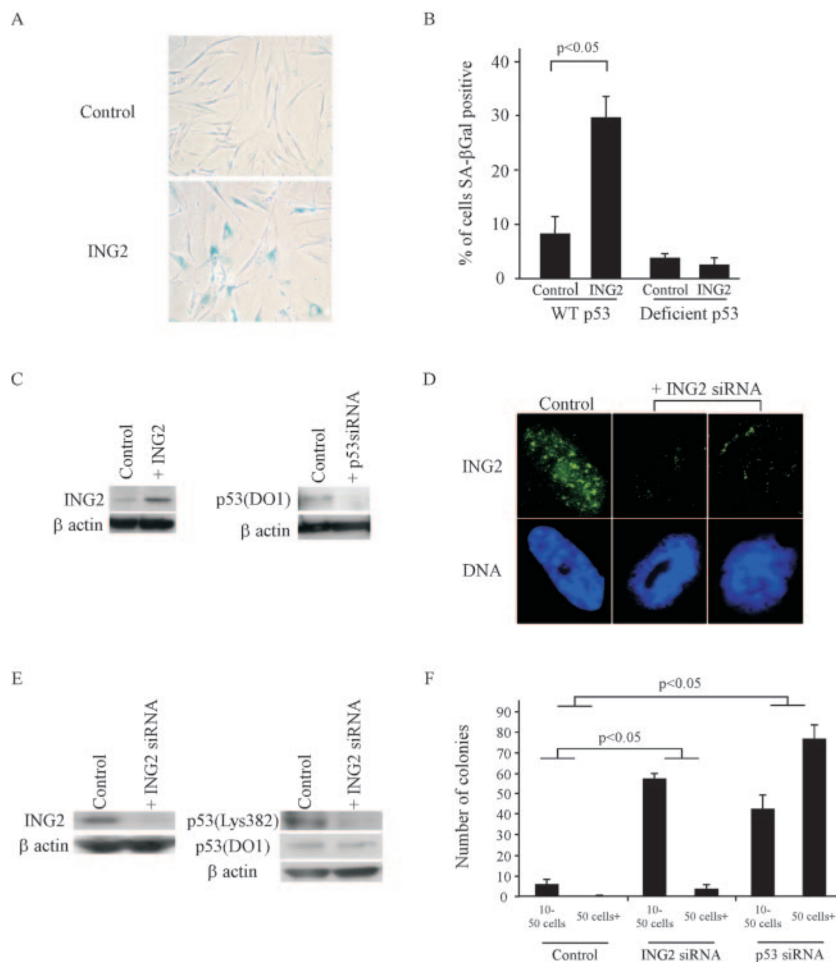

FIG. 6. ING2 levels modulate replicative senescence in fibroblasts. (A) ING2 overexpression in young fibroblasts induced premature senescence. Young fibroblasts were infected with an empty retrovirus (control) or a retrovirus containing ING2. Senescence was detected by SA-β-Gal. (B) Effect of ING2 on replicative senescence depended on p53. The quantitation of the results shown in panel A and a parallel experiment done with MRC5 p53 siRNA was carried out. The graphs represent means ± SD. At least 300 cells were counted for each experiment. (C) Levels of ING2 and p53 during induction of senescence in normal human and p53-deficient fibroblasts. ING2 expression in young fibroblasts infected with empty retrovirus or retrovirus containing ING2 (left) or p53 protein expression in fibroblasts infected with a retrovirus containing a scrambled siRNA or p53 siRNA (right). Cell lysates were probed with antibodies against ING2 (rabbit), p53 (DO-1), and β-actin. (D) Loss of ING2 in near-senescence fibroblasts leads to its reduced detection by immunofluorescence. MRC5 fibroblasts were infected with retrovirus containing two different ING2 siRNAs and selected. After infection, fibroblasts were fixed, and immunofluorescence was carried out with antibodies against ING2 (goat). DNA was stained by DAPI (4′,6′-diamidino-2-phenylindole). (E) The expression knockdown of ING2 leads to a decrease in p53 (Lys382) acetylation but not p53 detected with DO-1 in MRC5 fibroblasts infected with a retrovirus containing ING2 siRNA. After selection, cells were lysed, and lysates were probed with antibodies against ING2 (rabbit), p53 (Lys382), and β-actin. (F) The down-regulation of ING2 stimulates cell proliferation. ING2 or p53 siRNAs were retrovirally infected in MRC5 fibroblasts. After selection, cells were trypsinized and counted, and the same number of cells was seeded in flasks for each experiment. Three weeks later, cells were fixed with formaldehyde and stained with crystal violet. Colonies with 10 to 50 cells or with >50 cells were counted. The experiments were done in triplicate. The graphs represent means ± SD.

tion of p300 on p53 was enhanced by ING2 in vitro (Fig. 5). Therefore, we propose that the cofactor function of ING2 on p300-dependent p53 acetylation may have a major impact during a physiological process such as senescence.

In a previous study using transfected p53 and ING2 alleles in cancer cell lines, p53 did not interact with ING2 (35). Here, we conclusively demonstrate that during senescence, ING2 and p53 were part of the same complex with p300 (Fig. 4). We, therefore, speculate that the interaction between p53 and ING2 may not only depend on physiological context, but may also be governed by different posttranslational modifications that are not necessarily recapitulated during a transient transfection experiment.

So, how does ING2 modulate p53 function during replicative senescence? The increase in ING2-p53-p300 colocalization during senescence probably results in ING2-mediated enhanced physical interaction between p53 and p300 (Fig. 4F), leading to the increased acetylation of the tumor suppressor. Other mutually nonexclusive possibilities regarding the effect of ING2 on p300 also exist. For example, direct interaction between ING2 and p300 can alter p300 conformation and facilitate p53 acetylation. ING2 may also be involved in regu-

lating deacetylase complexes and may prevent the access of deacetylases to p300 and/or p53. Indeed, ING1b can directly interact with SIRT1 and thus enhance p53 acetylation (32). It is also possible that ING2 has some effect on the previously reported E3 ubiquitination activity of p300 (25). The p300-ING2-p53 complex may bind to promoters of genes, because p53 DNA binding activity increases in senescent cells (3). This complex may also be localized on telomeres or in a novel senescence-specific subnuclear body.

p53 has been previously shown to be involved in *ras*-induced senescence (41). During *ras*-induced senescence, an increased amount of p53 was detected within PML nuclear bodies (NBs), which colocalized with CBP. In the absence of PML, p53 acetylation on lysine 382 was reduced and cells did not undergo senescence (41). By contrast, in replicative senescence, only a small fraction of ING2 colocalized in PML NBs in both young and replicative senescent fibroblasts (Fig. 2). Moreover, very little ING2 colocalization was observed with CBP. Instead, enhanced colocalization was observed with the CBP homolog, p300 (Fig. 4; see Fig. S2 in the supplemental material). The complex formed by ING2, p300, and phospho-p53 (Ser15) colocalized outside the PML NBs (data not shown). Interestingly, in contrast to replicative senescence, during *ras*-induced senescence the level of ING2 decreased and overexpression of *ras* was able to induce premature senescence with the same efficiency in cells in which ING2 was knocked-down (Fig. 2). Thus, *ras*-induced senescence and replicative senescence may have divergent cellular signatures, and ING2 does not play a significant role in *ras*-induced senescence. Notably, *ras*-induced senescence relied on p14/p19ARF-dependent activation of p53, whereas replicative senescence relies on the activation of stress and checkpoint kinases. ING2, thus, appears to play a specific role in augmenting one signaling pathway and not the other, possibly due to its specific role in promoting p53 acetylation that occurs after ATM and Chk2 phosphorylation.

Though p53 and p16 are two major tumor suppressors that govern the senescence process, other auxiliary factors such as ING2 may also help to fine-tune the process and allow it to proceed to its logical end point. Hence, the loss of factors such as ING2 does not have as dramatic an effect as the loss of p53 on cellular proliferation. The challenge that remains is deciphering how signal transduction is processed from the telomeres to factors like ING2, so that they can function during replicative senescence. It has been demonstrated that p53 phosphorylation is modulated by pathways involving ATM, ATR, Chk1, and Chk2 proteins—all of which are activated by uncapped telomeres (13, 29, 43, 48). Apart from their role in signal transduction, Chk1 and Chk2 have also been implicated recently in replicative senescence (13, 21). It is tempting to speculate that these DNA perturbation-sensing proteins may functionally interact with ING2 and that such interactions may affect their mutual functions in a temporal manner. The relative effects of these auxiliary factors may become more apparent with the availability of ING2 gene knockout mice and mice generated from an intercross with p53 knockout mice.

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