

# The tumor suppressor ING1 contributes to epigenetic control of cellular senescence

María Abad,<sup>1,\*</sup> Alberto Moreno,<sup>1</sup> Alicia Palacios,<sup>2</sup> Masako Narita,<sup>4</sup> Francisco Blanco,<sup>2,3</sup> Gema Moreno-Bueno,<sup>1</sup> Masashi Narita<sup>4</sup> and Ignacio Palmero<sup>1</sup>

<sup>1</sup>Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM, E-28029 Madrid, Spain

<sup>2</sup>CIC bioGUNE, E-48160 Derio, Spain

<sup>3</sup>IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

<sup>4</sup>Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, CB2 0RE Cambridge, UK

## Summary

Cellular senescence is an effective tumor-suppressive mechanism that causes a stable proliferative arrest in cells with potentially oncogenic alterations. Here, we have investigated the role of the p33ING1 tumor suppressor in the regulation of cellular senescence in human primary fibroblasts. We show that p33ING1 triggers a senescent phenotype in a p53-dependent fashion. Also, endogenous p33ING1 protein accumulates in chromatin in oncogene-senescent fibroblasts and its silencing by RNA interference impairs senescence triggered by oncogenes. Notably, the ability to induce senescence is lost in a mutant version of p33ING1 present in human tumors. Using specific point mutants, we further show that recognition of the chromatin mark H3K4me3 is essential for induction of senescence by p33ING1. Finally, we demonstrate that ING1-induced senescence is associated to a specific genetic signature with a strong representation of chemokine and cytokine signaling factors, which significantly overlaps with that of oncogene-induced senescence. In summary, our results identify ING1 as a critical epigenetic regulator of cellular senescence in human fibroblasts and highlight its role in control of gene expression in the context of this tumor-protective response.

**Key words:** cellular senescence; chromatin; ING1; p53; histone marks.

## Introduction

Higher organisms possess defense mechanisms that restrain the ability of cells with potentially oncogenic alterations to progress to form tumors (Lowe *et al.*, 2004). In this context, cellular senescence has emerged recently as an antiproliferative tumor-suppressive mechanism, at a similar level with apoptosis (Prieur & Peeper, 2008; Collado & Serrano, 2010). Cellular senescence can be elicited by a series of alterations in normal cellular homeostasis, including telomere dysfunction, DNA damage, oxidative stress, or aberrant promitogenic signals (Campisi & d’Adda di Fagagna, 2007; Courtois-Cox *et al.*, 2008). The senescent phenotype is defined by several cellular and molecular markers, best characterized in fibroblasts, including flat extended morphology, increased heterochromatinization, and a pH-specific Beta-Galactosidase activity (Senescence-Associated Beta Galactosidase, SA-BetaGal; Dimri *et al.*, 1995; Collado & Serrano, 2006). Senescence is characterized by a specific gene expression program, where epigenetic regulation plays an essential role (Adams, 2007; Funayama & Ishikawa, 2007; Narita, 2007). The relevance of oncogene-induced senescence as a tumor-protective barrier *in vivo* is now firmly established (Braig *et al.*, 2005; Chen *et al.*, 2005; Collado *et al.*, 2005; Michaloglou *et al.*, 2005; Courtois-Cox *et al.*, 2006; Dankort *et al.*, 2007). Senescence is activated in premalignant lesions, as a result of aberrant mitogenic signals caused by activated oncogenes, blocking progression to malignant lesions (reviewed in Mooi & Peeper, 2006; Collado *et al.*, 2007; Courtois-Cox *et al.*, 2008; Prieur & Peeper, 2008). Given the importance of senescence as an effective tumor-suppressive mechanism, there is an obvious interest in elucidating the regulatory mechanisms of senescence. The ING proteins are a family of sequence-conserved proteins, frequently inactivated in human tumors, which control vital cellular processes such as apoptosis, proliferation, or cell migration (reviewed in Soliman & Riabowol, 2007; Ythier *et al.*, 2008; Coles & Jones, 2009). ING proteins participate in transcriptional control via the reading and establishment of chromatin marks (Champagne & Kutateladze, 2009). In particular, ING proteins act as specific readers of trimethylated Lysine 4 in histone H3 (H3K4me3), a distinctive mark of active promoters and also take part in complexes with histone acetyl-transferase (HAT) or deacetylase (HDAC) activities (Soliman & Riabowol, 2007). ING proteins have also been connected to the p53 pathway, as regulators of p53 protein stability and posttranslational modification, or as transcriptional cofactors, although there is also evidence for p53-independent functions (Coles & Jones, 2009). Given the relevance of cellular senescence as an antitumor barrier, we sought to dissect the mechanisms whereby ING1, the founding member of the ING family, regulates senescence. Our

Correspondence

Ignacio Palmero, Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM, Arturo Duperier 4, E-28029 Madrid, Spain. Tel.: +34 915854491; fax: +34 915854401; e-mail: ipalmero@iib.uam.es

\*Present address: Spanish National Cancer Research Center, CNIO, Madrid, Spain.

Accepted for publication 5 November 2010

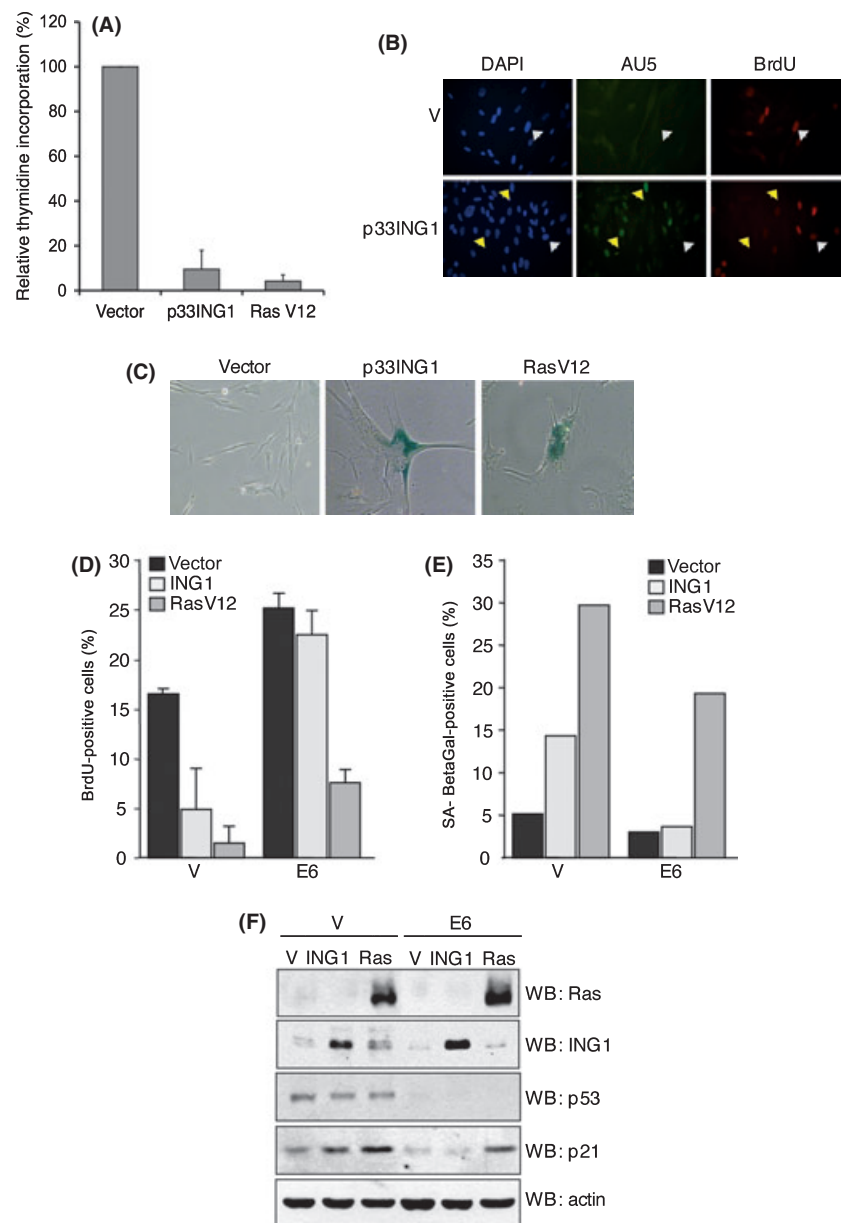
data support a critical role for this protein in the circuitry responsible for implementation of senescence, in a p53-dependent manner, and highlight its role in chromatin control and regulation of gene expression in this context.

## Results

### p33ING1 induces senescence in human fibroblasts in a p53-dependent manner

To investigate the role of the ING1 locus in cellular senescence, we ectopically expressed p33ING1, the major product of the human ING1 locus (also known as ING1b), in early passage IMR-90 human fibroblasts. Retroviral transduction was used to achieve moderate stable expression, to avoid nonphysiological

overexpression. As a control, we used the activated form of the human Ha-Ras oncogene (RasV12), an extensively characterized trigger of oncogene-induced senescence in these cells (Serrano et al., 1997). Enforced expression of ING1 caused a dramatic reduction in proliferation, as shown by a decrease in thymidine incorporation or in the number of BrdU-positive cells (Fig. 1A,D). The inverse correlation between ING1 expression and BrdU incorporation could also be observed in individual cells by immunofluorescence. Eighty-five percent of AU5-positive cells were BrdU-negative, while 65% of AU5-negative cells or vector-infected cells were BrdU-negative, confirming the antiproliferative effect of ectopic ING1 (Fig. 1B). ING1-expressing fibroblasts also displayed distinctive markers of cellular senescence, such as SA-BetaGal activity (Fig. 1C,E), and flat enlarged morphology (Fig. 1C). Thus, ectopic expression of p33ING1 in normal human



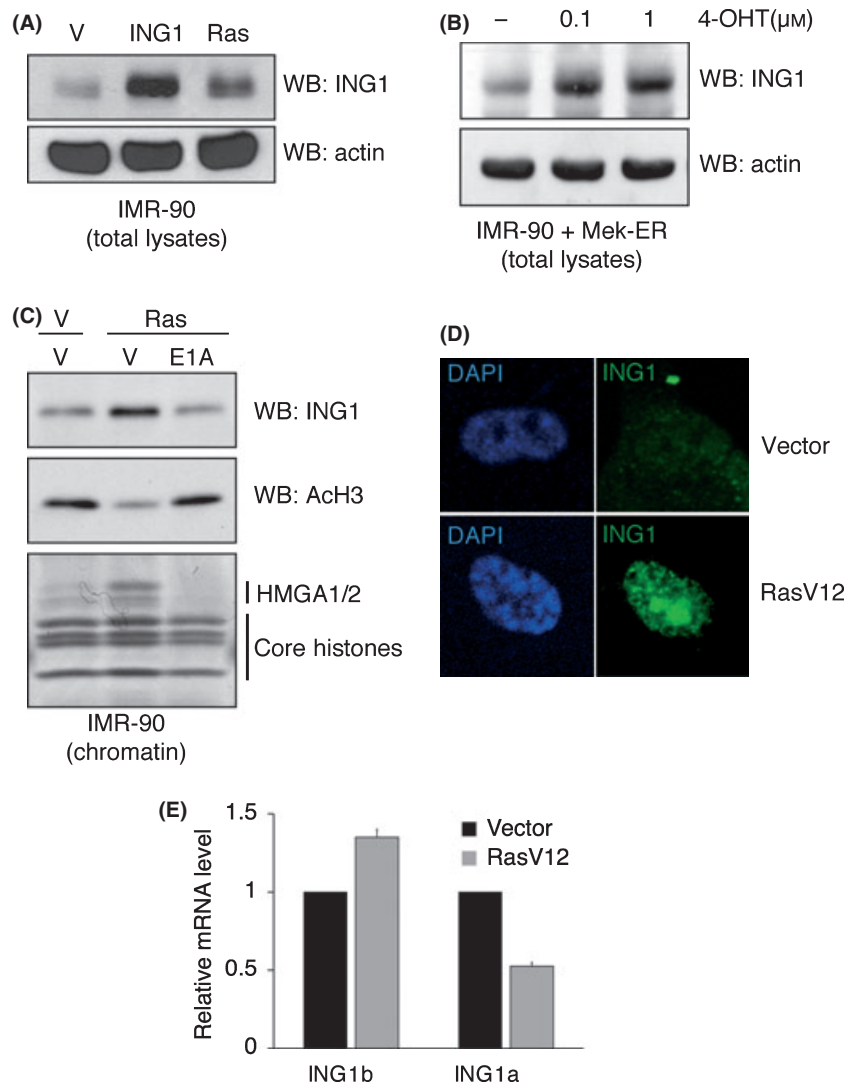
**Fig. 1** p53-dependent induction of senescence by ING1. (A) Incorporation of tritiated thymidine in early passage IMR-90 fibroblasts after retroviral transduction with vectors expressing p33ING1, RasV12, or empty vector. The values shown are relative to vector-infected cells. The average and SD from three experiments are shown. (B) Immunofluorescence microscopy showing expression of AU5-tagged ectopic p33ING1 (AU5) and BrdU incorporation (BrdU) in IMR-90 fibroblasts infected with the indicated vectors. Nuclei were counterstained with DAPI. White arrowheads indicate BrdU-positive, ING1-negative cells, and yellow arrowheads indicate BrdU-negative, ING1-positive cells. (C) Representative micrographs of fibroblasts infected with the indicated vectors, after detection of SA-BetaGal activity. (D) Rate of BrdU incorporation of IMR-90 fibroblasts serially infected with the HPV16 E6 oncoprotein (E6) or empty vector, followed by infection with p33ING1, RasV12, or empty vector, at day 6 postselection. The average and SD from three experiments is shown. (E) Percentage of SA-BetaGal-positive cells, as in panel D. A representative experiment at day 6 postselection is shown. (F) Western blot analysis of the indicated proteins in cells serially infected with E6 and p33ING1 (ING1) or RasV12 (Ras).

fibroblasts provokes a cell-cycle arrest with features of cellular senescence, supporting a causal role for ING1 in the implementation of this response (see also Goeman *et al.*, 2005). p33ING1, like other ING proteins, has been functionally linked to the p53 pathway (Coles & Jones, 2009) via mechanisms including control of p53 protein stability, posttranslational modifications or as transcriptional cofactors. In human fibroblasts, both the p53 and the p16INK4A-Rb pathway need to be inactivated to bypass oncogene-induced senescence (Collado *et al.*, 2007). To investigate the functional connection between p33ING1 and p53 in the context of senescence, we inactivated the p53 pathway in these cells, using the E6 oncoprotein from human papilloma virus (Fig. 1F). Ectopic expression of p33ING1 failed to induce a significant cell-cycle arrest in fibroblasts expressing E6 (Fig. 1D). Similarly, the induction of the senescence marker SA-BetaGal by p33ING1 was largely abolished in E6-expressing cells (Fig. 1E). In agreement with previous reports, inactivation of the p53 pathway was not sufficient to bypass senescence by RasV12 in these cells (Fig. 1E), which requires additional inactivation of the Rb pathway (Collado *et al.*, 2007). Interestingly, ING1

increased the level of p21CIP1, a p53 target associated with senescence. The induction of p21CIP1 by ING1 was lost in E6-expressing cells (Fig. 1F), in contrast to the effect of Ras on p21, which is known to be p53-independent (Kivinen *et al.*, 1999). Thus, p33ING1 induces senescence in human fibroblasts through a p53-dependent mechanism.

ING1 is essential for oncogene-induced senescence

To gain insights into the involvement of the endogenous p33ING1 protein in senescence, we analyzed the expression of ING1 in senescent fibroblasts. We observed a clear increase in p33ING1 total protein levels in Ras-senescent fibroblasts, relative to nonsenescent vector-infected fibroblasts (Fig. 2A). The induction of p33ING1 by oncogenic stress was confirmed using an inducible system for oncogene-induced senescence, where the activity of a fusion of the Ras effector MEK with the estrogen receptor (MEK-ER) can be controlled by addition of 4-hydroxy-tamoxifen (4-OHT). The levels of p33ING1 increased in 4-OHT-treated MEK-ER fibroblasts, in parallel with the onset of a

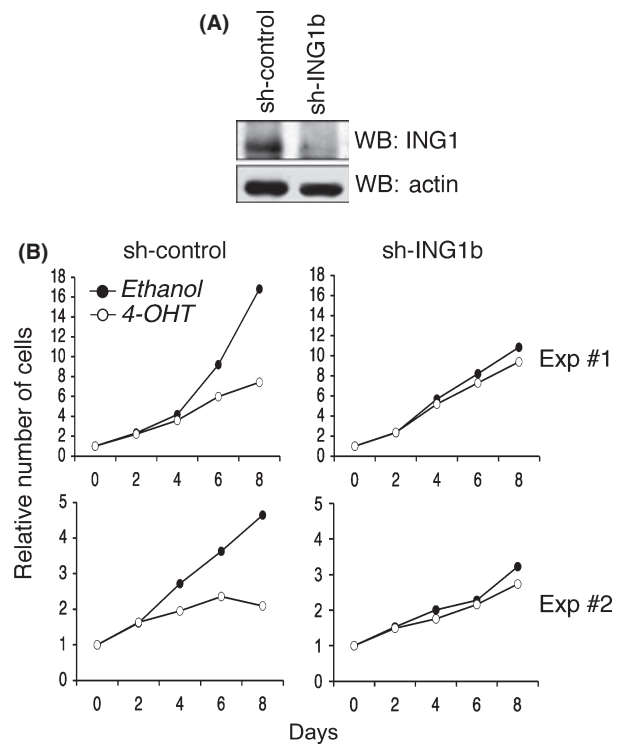


**Fig. 2** Regulation of ING1 during senescence. (A) Western blot analysis of the p33ING1 protein in total cell lysates from early passage IMR-90 fibroblasts infected with vectors expressing p33ING1 (ING1) or RasV12 (Ras), at day 6 postselection. (B) Western blot analysis of the p33ING1 protein in IMR-90 fibroblasts expressing MEK with the estrogen receptor 3 days after addition of 4-hydroxy-tamoxifen (4-OHT). (C) Western blot analysis of the indicated proteins in chromatin fractions of IMR-90 cells serially infected with vectors for E1A and Ras, and the corresponding empty vectors. Core histones were detected with Coomassie blue. (D) Immunofluorescence microscopy of Ras-senescent or control IMR-90 fibroblasts with an antibody against ING1. DAPI was used to stain the nuclei. (E) Quantitative PCR analysis of the expression of the ING1a and ING1b transcripts in fibroblasts expressing RasV12 or controls with empty vector. The average and SD from two experiments is shown.

cell-cycle arrest in these cells (data not shown). p33ING1 is a predominantly nuclear protein that participates in transcriptional control. Consistent with the results with total lysates, we observed a clear increase in p33ING1 in the chromatin fraction of Ras-senescent IMR-90 fibroblasts, in parallel with known markers of senescent chromatin, like reduced acetylated histone H3 or increased HMGA histone-like proteins (Narita *et al.*, 2003, 2006) (Fig. 2C; Fig. S1). Significantly, the accumulation of p33ING1 was abolished in fibroblasts co-expressing RasV12 and E1A, an adenoviral oncoprotein that allows bypass of oncogene-induced senescence in these cells by inactivating essential tumor-suppressive pathways (Serrano *et al.*, 1997). This observation clearly establishes a link between ING1 accumulation in chromatin and Ras-induced senescence, and not only Ras signaling. The accumulation of ING1 in nuclei during oncogene-induced senescence was further confirmed in immunofluorescence experiments, using an anti-ING1 antibody (Fig. 2D). We analyzed if the increase in p33ING1 protein levels in senescence could also be observed at the level of RNA. The human *ING1* locus encodes two protein products, p33ING1 (encoded by transcript ING1b) and p47ING1A (transcript ING1a). Quantitative PCR analysis did not show a robust increase in the transcript encoding p33ING1 (transcript ING1b) in Ras-senescent cells (< 2-fold; Fig. 2E), suggesting that the accumulation of p33ING1 is mediated by posttranscriptional mechanisms. It has been reported that p47ING1A RNA and protein increase during replicative senescence in human fibroblasts (Soliman *et al.*, 2008). In contrast to this finding, we observed a decrease in the ING1a-specific transcript in Ras-senescent human fibroblasts (Fig. 2E). We could not study the p47ING1A protein in this setting, because a specific band corresponding to p47ING1A was not detectable in our Western blot experiments (data not shown). Next, we wished to determine whether p33ING1 is a physiological mediator of oncogene-induced senescence. With this purpose, we used stable expression of a miR30-based short hairpin RNA specific for the transcript encoding p33ING1 (transcript ING1b), which led to a clear reduction in p33ING1 protein (Fig. 3A). Vectors encoding sh-ING1b or the empty miR30 backbone were introduced into IMR-90 fibroblasts, previously infected with a MEK-ER vector. Addition of 4-OHT to MEK-ER fibroblasts resulted in a dramatic reduction in their growth rate, indicative of induction of senescence (Fig. 3B). In contrast, the growth of MEK-ER fibroblasts expressing sh-RNA against p33ING1 was not significantly inhibited by 4-OHT, in comparison with control vehicle, although the shRNA itself inhibited growth to some degree. Therefore, p33ING1 accumulates in oncogene-senescent chromatin, and its suppression by RNA interference impairs oncogene-triggered senescence, supporting a critical role for this protein in the implementation of this response.

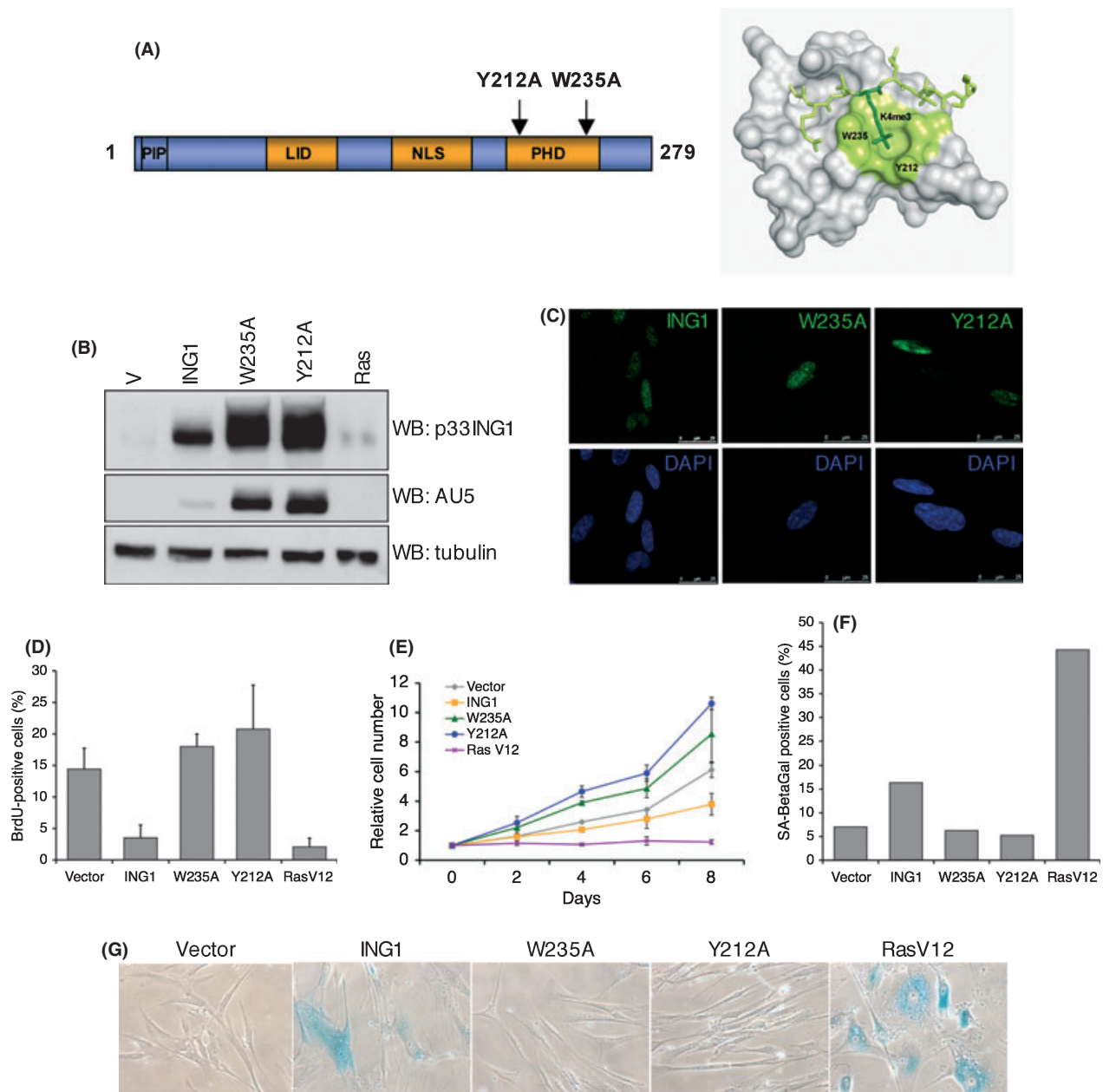
### Recognition of chromatin marks is essential for the induction of senescence by ING1

ING proteins participate in the recognition of the histone mark H3K4me3 (histone H3 trimethylated in Lysine 4), a feature of



**Fig. 3** Bypass of oncogene-induced senescence by RNA interference against p33ING1. (A) Western analysis of p33ING1 protein in IMR-90 fibroblasts infected with the empty shRNA vector LMP (sh-control) or a vector expression shRNA against p33ING1 (sh-ING1b). (B) Growth curves of IMR90-MEK with the estrogen receptor fibroblasts retrovirally infected with the indicated shRNA vectors, treated with 1  $\mu$ M 4-hydroxytamoxifen or ethanol. The number of cells at each time point is represented relative to the number of cells at day 1. Two independent experiments are shown.

transcriptionally active promoters (Shi *et al.*, 2006; Hung *et al.*, 2009; Palacios *et al.*, 2008; Pena *et al.*, 2006, 2008; reviewed in Champagne & Kutateladze, 2009). Structural studies have identified several residues in the conserved plant homeo domain (PHD) domain essential for the binding of ING proteins to H3K4me3 (Pena *et al.*, 2006; Shi *et al.*, 2006). Given the relevance of epigenetic control in gene regulation during senescence (Adams, 2007; Narita, 2007), we decided to investigate the impact of this feature of ING1 in regulation of senescence. To this end, we designed mutations in two ING1 residues equivalent to those essential for H3K4me3 binding in ING2 and ING4 (Palacios *et al.*, 2006, 2008; Pena *et al.*, 2006; Shi *et al.*, 2006), specifically Tyrosine 212 and Tryptophan 235, which were replaced with Alanines (designated Y212A and W235A, respectively, Fig. 4A). Both mutant p33ING1 proteins were easily detected after retroviral transduction of IMR-90 fibroblasts, reaching levels significantly higher than the wild-type form (Fig. 4B). Immunofluorescence studies also revealed a normal pattern of subcellular localization, showing nuclear staining with no obvious differences in the distribution within the nucleus (Fig. 4C). We investigated the ability of both histone-binding mutants to induce senescence. In contrast to the reduced proliferation observed in cells with wild-type ING1, fibroblasts



**Fig. 4** Recognition of histone marks is essential for ING1-induced senescence. (A) *Left*, Schematic representation of the p33ING1 protein, indicating the position of the residues mutated in this work. PIP, PCNA-interacting protein motif; LID, Lamin Interacting Domain; NLS, nuclear localization signal; PHD, plant homeo domain. *Right*, Three-dimensional model of the complex of ING1-PHD bound to H3K4me3. ING1 is shown in gray, with the hydrophobic pocket involved in the binding highlighted in green. The position of the two mutated residues, Y212 and W235, is shown. The N-terminus of H3K4me3 is shown in green in stick representation. (B) Western blot showing expression of ectopic AU5-p33ING1 (AU5) or total p33ING1 in IMR-90 cells expressing the indicated versions of p33ING1 or RasV12. Tubulin was used as a loading control. (C) Confocal fluorescence images showing subcellular localization of wild-type ING1 (ING1) and the Y212A and W235A mutants in retrovirally infected IMR-90 cells. Nuclei were stained with DAPI. (D) Rate of BrdU incorporation in IMR-90 cells expressing the indicated vectors, at day 6 postselection. The average and SD of three independent experiments is shown. (E) Growth curve of IMR-90 cells as in D. The average and SD of two independent experiments is shown. (F) Percentage of SA-BetaGal-positive cells at day 6 postselection, as in D. A representative experiment is shown. (G) Micrographs showing the morphology and SA-BetaGal staining in cells infected with the indicated vectors.

expressing either of the mutants showed proliferation rates similar or higher than controls, as measured by BrdU incorporation or cumulative growth over an 8-day period (Fig. 4D,E). Consistently, expression of the W235A or Y212A mutants did not cause an increase in the number of SA-BetaGal-positive cells, or

provoked a change in cell morphology, in contrast to wild-type ING1 (Fig. 4F,G). Oncogenic Ras was used in these assays as a positive control for senescence induction. These results clearly indicate that the recognition of histone marks is essential for the induction of senescence by p33ING1 in human fibroblasts.



### A tumor-associated mutation in ING1 abolishes its ability to trigger senescence

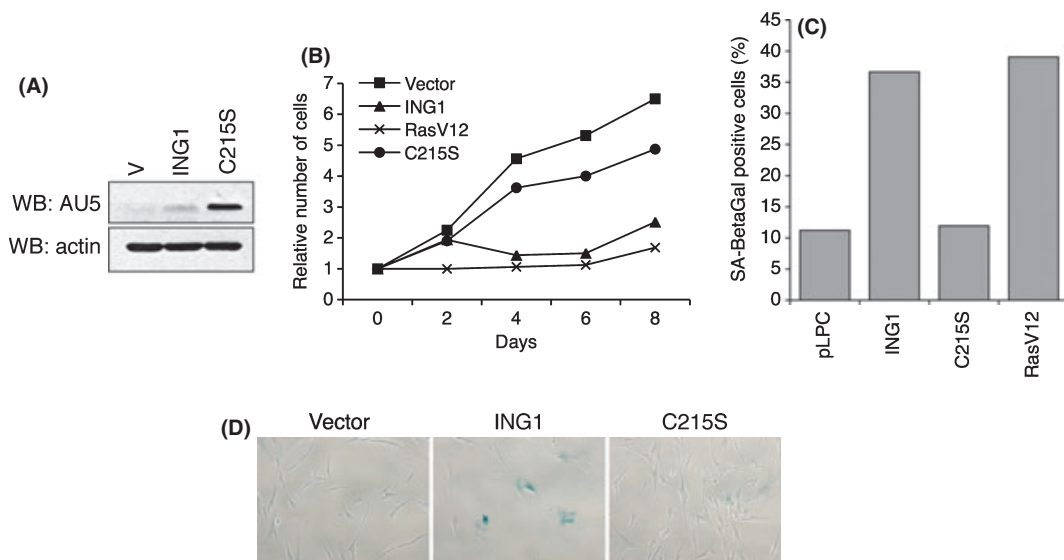
The ING1 locus is inactivated in a large number of human tumors, including squamous cell carcinoma, breast cancer, and melanoma (Nouman *et al.*, 2003; Ythier *et al.*, 2008). The most frequent alteration of ING1 in tumors is its reduced expression and/or aberrant localization. However, a small number of point missense mutations in ING1 have also been found in human tumors. To test the impact of cancer-associated mutations in ING1's ability to trigger senescence, we focused on the C215S mutation found in primary head and neck squamous cell carcinomas (HNSCC) and hepatocellular carcinomas (HCC; Gunduz *et al.*, 2000). The mutated residue (Cys 215) is predicted to play an essential role in stabilizing the structure of the conserved PHD domain. We confirmed that an ING1 PHD domain containing the C215S mutation is essentially unfolded in solution (Fig. S2; see Data S1 for Methods). To test whether this cancer-associated mutation in ING1 could impinge in its ability to trigger senescence, we infected IMR-90 early passage fibroblasts with a retrovirus expressing the C215S mutant. As with the other mutants used in this study, the C215S version was easily detected, reaching levels higher than the wild-type protein (Fig. 5A) and showed normal nuclear localization (data not shown). Ectopic expression of this mutant version of ING1 failed to trigger an antiproliferative response in human fibroblasts (Fig. 5B). Also, no obvious induction of senescence markers, such as flat morphology or SA-BetaGal activity, was observed (Fig. 5C and 5D). Thus, a mutation in ING1 present in human tumors abolishes ING1-induced senescence. This observation further strengthens the link between regulation of senescence by ING1 and its tumor-protective action.

### Connection of ING1 with SAHFs

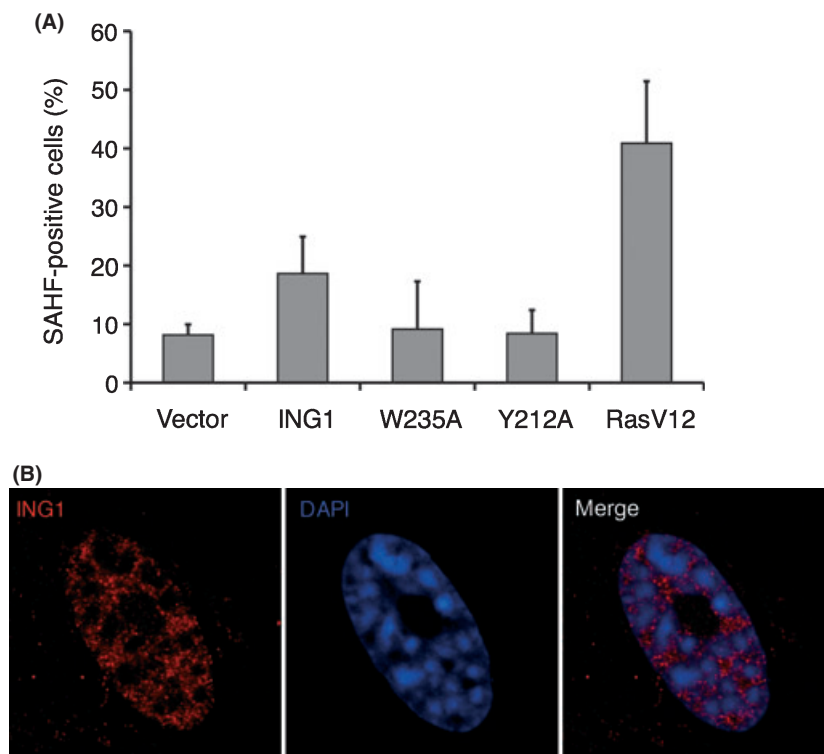
In human fibroblasts, and some other cell types, senescence is accompanied by the appearance of macroscopic regions of facultative heterochromatin, known as senescence-associated heterochromatin foci, or SAHFs (Narita *et al.*, 2003, 2006). The SAHFs are identified as spots with intense DAPI staining, they are enriched in heterochromatin markers, such as heterochromatin protein1 (HP1) and H3K9me3, and exclude euchromatic markers, like acetylated histones and H3K4me3 (Narita *et al.*, 2003, 2006). As the link to transcriptional repression and chromatin control appears to be essential for senescence induction by ING1 (Fig. 4, see also Goeman *et al.*, 2005), we set to investigate the connection between ING1 and the SAHFs. Consistent with their relative ability to trigger senescence, ectopic expression of wild-type ING1 led to an increase in SAHF-positive cells, a robust senescence marker in this cell type, but no increase was observed after expression of either histone-binding mutant (Fig. 6A). However, we did not observe a significant accumulation of ectopic or endogenous ING1 in the SAHFs, in immunofluorescence experiments. Consistent with our data showing that the pro-senescence activity of p33ING1 requires H3K4me3 association, ING1, like H3K4me3, was found excluded from SAHFs (Figs 6B and S3). Thus, these data reinforce the critical role of the association between ING1 and H3K4me3 for senescence induction.

### ING1-induced senescence is defined by a specific genetic signature

Our results with histone-binding mutants of p33ING1 strongly support the notion that induction of senescence by p33ING1



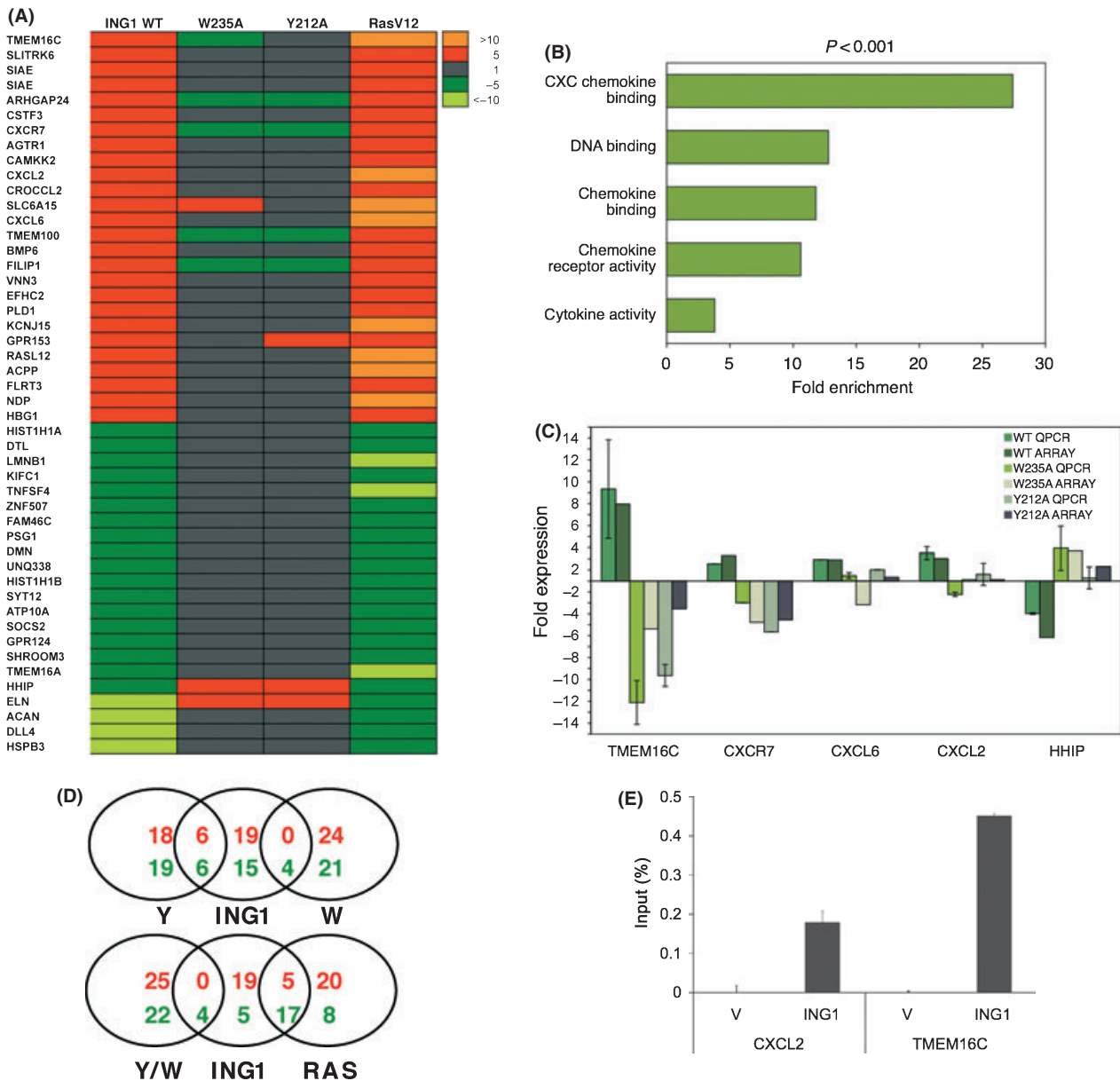
**Fig. 5** Lack of senescence induction in a cancer-associated ING1 mutant. (A) Western blot showing the expression of ectopic AU5-tagged wild-type p33ING1 (ING1) or the C215S mutant. Actin was used as a loading control. (B) Growth curves of IMR-90 fibroblasts expressing the indicated vectors. (C) Percentage of SA-BetaGal-positive cells after infection of IMR-90 fibroblasts with the indicated vectors, at day 6 postselection. A representative experiment is shown. (D) Micrographs showing the morphology and SA-BetaGal staining in IMR-90 fibroblasts infected with the indicated vectors.



**Fig. 6** Connection of ING1 with SAHFs. (A) Percentage of SAHF-positive cells in IMR-90 fibroblasts infected with the indicated vectors. The average and SD from three experiments is shown. (B) Confocal fluorescence images showing subcellular localization of endogenous ING1 in Ras-senescent IMR-90 fibroblasts. SAHFs are visualized as hyperchromatic foci in the DAPI staining.

depends on its ability to regulate gene expression through recognition and establishment of chromatin marks. To investigate the transcriptional signature associated with the induction of senescence by p33ING1, we performed a genome-wide expression profile analysis. To this end, early passage IMR-90 fibroblasts were infected with vectors encoding wild-type p33ING1 or the Y212A and W235A mutants. Ras-senescent fibroblasts were also analyzed, as a control for oncogene-induced senescence. Total RNA was prepared at a time postselection where the differential effects of each ING1 protein were evident (Fig. S4A) and used to interrogate whole genome expression arrays. A subset of 286 genes was differentially expressed at least twofold in IMR-90 fibroblasts arrested by wild-type ING1 expression, with respect to vector-infected fibroblasts (Table S1). Within this group, 200 genes (70%) were upregulated in ING1-expressing cells, while 86 genes (30%) were downregulated. Next, we compared the expression profiles caused by wild-type or histone-binding-mutant versions of p33ING1. Of note, the expression profiles caused by the two mutants were highly similar (over 69% coincidence). This pattern is in keeping with the expected structural consequences of the mutations and their impact in senescence. Most importantly, the expression patterns associated with wild-type or mutant ING1 could be easily discriminated. For 72% of the genes associated with wild-type ING1, the expression in both mutants was different to wild type, raising to 94% in at least one of the mutants (Fig. 7D, Table S1). In most cases, genes regulated with wild-type ING1 were unchanged with the mutants (151 genes, 53%), but we also found some examples

(31 genes, 11%) of opposite regulation in wild type and mutants that might reflect dominant-negative effects (see for example CXCR7, upregulated by ING1 and downregulated by the mutants; or HHIP1, with the reverse pattern (Table S1, Fig. 7C). Interestingly, although both histone-binding mutants behaved very similarly in this assay, there were statistically significant differences between them, with a higher similarity between the Y212A mutant and wild-type ING1. These results closely correlate with the binding affinities for H3K4me3 of each mutant (Pena *et al.*, 2008). The functional category analysis of ING1-regulated genes identified a significant enrichment for genes involved in chemokine and cytokine signaling. Notably, these factors have recently been identified as important regulators of senescence, as part of the senescence associated secretory phenotype (SASP) (Acosta *et al.*, 2008; Kuilman *et al.*, 2008) (Fig. S4B). Next, we compared the set of ING1-specific genes with those modified in Ras-senescent fibroblasts to identify common signatures between Ras- and ING1-induced senescence. We found a significant overlap between both signatures (24% of genes with statistically significant differential expression, with a FDR of 0.10) and selected a group of 45 genes co-regulated in Ras-senescent and ING1-senescent cells (Fig. 7A). Interestingly, although p33ING1 caused both up- and downregulation, the similarity between p33ING1 and Ras was higher within the set of genes downregulated by ING1 (Fig. 7D). As for the complete set of ING1-regulated genes, the subset overlapping with Ras also showed a significant enrichment for genes involved in chemokine and cytokine signaling (Fig. 7B; Acosta *et al.*, 2008; Kuilman *et al.*, 2008). The differential expression



**Fig. 7** Genetic signature of ING1-induced senescence. (A) Heat-map representation of gene expression profiles of genes with differential expression in ING1-infected fibroblasts, no regulation with ING1 mutants and overlapping expression with Ras-infected fibroblasts (see text for details). (B) Functional enrichment analysis of the subset of genes represented in A. The fold enrichment of the indicated set relative to total genome is represented for each category. Statistical significance was determined with a Fisher's exact test using the FatiGO application (C). Validation by real-time quantitative PCR of a selection of genes identified in the microarray analysis. The expression relative to vector-infected controls is shown. In each case, the relative expression data obtained from the array is shown for reference. The average and SD from three independent QPCR assays is shown. (D) Venn diagram of genes with differential expression in IMR-90 with wild-type p33ING1 (ING1), relative to the Y212A mutant (Y), the W235A mutant (W), both mutants together (Y/W) or RasV12 (RAS). The data corresponds to the 50 genes most up- or downregulated in each set. Red numbers indicate upregulated genes and green numbers, downregulated genes. (E) Chromatin immunoprecipitation showing binding of ectopic p33ING1 to promoter regions of CXCL2 and TMEM16C. The graph shows the percentage of input immunoprecipitated with the AU5 antibody for each locus in vector (V) or AU5-p33ING1 (ING1) infected fibroblasts, after subtraction of background (nonspecific antibody precipitation). The average and SD from triplicates are shown.

results from the microarray analysis were successfully validated for a selection of genes, using real-time quantitative PCR (Fig. 7C). To test the direct involvement of ING1 in the transcriptional regulation of these genes, we performed chromatin immunoprecipitation (ChIP) assays for two of the genes of the ING1 signature, CXCL2 and TMEM16C. ChIP assays showed

specific binding of ectopic p33ING1 to chromatin in promoter regions of both genes in ING1-senescent fibroblasts (Fig. 7E). Of note, in both cases, ING1 binding was detected within a genomic region enriched in H3K4me3 marks in IMR90 cells ([http://neomorph.salk.edu/human\\_methylome/browser.html](http://neomorph.salk.edu/human_methylome/browser.html)). Thus, induction of senescence by p33ING1 is characterized by a



specific genetic signature, which partially overlaps with that of Ras-induced senescence, with a strong presence of genes of the senescent secretory phenotype.

## Discussion

The products of the *ING1* locus participate in the control of cellular processes relevant to tumor protection, such as apoptosis, cell-cycle arrest, or DNA repair (Soliman & Riabowol, 2007; Coles & Jones, 2009; Menendez *et al.*, 2009). Alterations of the *ING1* locus are frequent in different types of human tumors (Nouman *et al.*, 2003; Ythier *et al.*, 2008), indicating an important role in tumor suppression. Likewise, mice genetically deficient in the *Ing1* locus show increased predisposition to lymphoma formation (Kichina *et al.*, 2006; Coles *et al.*, 2007). Senescence is increasingly recognized as an essential tumor-suppressive mechanism (Campisi & d'Adda di Fagagna, 2007; Prieur & Peeper, 2008; Collado & Serrano, 2010). Therefore, identifying the mechanism by which tumor suppressors engage the senescent response is highly relevant to understand how they prevent tumor formation, and the impact of their alterations in human tumors. The link between ING proteins and cellular senescence has been previously investigated (Garkavtsev & Riabowol, 1997; Goeman *et al.*, 2005; Pedoux *et al.*, 2005; Abad *et al.*, 2007; Soliman *et al.*, 2008; reviewed in Menendez *et al.*, 2009). However, these studies have frequently led to conflicting results, and the role of specific ING isoforms or the precise mechanisms involved have remained unclear. Here, we have used cell-based gain and loss of function approaches, combined with expression profiling to dissect the role of p33ING1 in senescence. Collectively, our results identify ING1 as a critical epigenetic regulator of oncogene-induced senescence in human fibroblasts. We show that p33ING1 accumulates in chromatin during oncogene-driven senescence, ectopic p33ING1 triggers a senescent phenotype, and RNA interference against p33ING1 impairs growth inhibition by the MEK oncogene. From a mechanistic point of view, one of the major conclusions of our work is that chromatin-mediated gene regulation has an essential role in senescence control by ING1. ING1 participates in gene regulation via the recognition of the chromatin mark H3K4me3 and recruitment of histone-modifying complexes (Champagne & Kutateladze, 2009). We show here that the independent mutation of two residues essential for H3K4me3 binding abolishes ING1's ability to induce senescence. Biophysical and biochemical studies with ING1 and the close homolog ING2 indicate that the Y212 and W235A mutations used in this study specifically impair binding to H3K4me3, but they do not disrupt folding or interaction with partners (Pena *et al.*, 2006; Pena *et al.*, 2008; Shi *et al.*, 2006). Supporting this notion, we find that all the ING1 mutants used in our study (Y212A, W235A, C215S) retain the ability to coprecipitate with p53, an interaction critical for ING1 tumor-suppressive action (Fig. S5). Thus, our data establish a clear correlation between H3K4me3 recognition and senescence induction by ING1. Previous reports have revealed an

important role in senescence for a number of proteins involved in the establishment or recognition of chromatin marks, like BMI (Jacobs *et al.*, 1999), CBX7 and 8 (Gil *et al.*, 2004; Dietrich *et al.*, 2007), JMJD3 (Barradas *et al.*, 2009), or Suv39H (Braig *et al.*, 2005). ING proteins are highly specific readers of H3K4me3 marks. Genetic alterations that result in aberrant addition, removal, or reading of this chromatin mark are frequent in leukemias and solid tumors (Chi *et al.*, 2010). However, evidence of direct involvement of H3K4me3 regulators in senescence is scarce. The MLL1 methylase (Kotake *et al.*, 2009) and the dual H3K4me3/H3K36me de-methylases JHDM1A and JHDM1B (He *et al.*, 2008; Pfau *et al.*, 2008) are mediators of oncogene and replicative senescence, via the regulation of the *Ink4a/Arf* locus. To our knowledge, ING proteins (this report and Pedoux *et al.*, 2005) are the only examples of specific H3K4me3 readers involved in senescence, further underlying the importance of proper regulation of this mark in the context of chromatin dynamics in senescence.

Transcriptional repression of some loci during senescence occurs in heterochromatin domains known as SAHFs (Narita *et al.*, 2003, 2006). ING1 has been linked to transcriptional repression (Kuzmichev *et al.*, 2002; Goeman *et al.*, 2005), and we show here that it accumulates in senescent chromatin. However, ING1 does not appear to be a stable component of the SAHFs. Rather, it was found consistently excluded from the SAHFs and frequently enriched in its periphery. Of note, H3K4me3 is also excluded from the SAHFs (Narita *et al.*, 2003). Two hypothesis consistent with ING1's bivalent role as reader of active chromatin marks and component of repressor complexes (Champagne & Kutateladze, 2009) could be considered. p33ING1 might contribute to define the boundaries between SAHFs and active chromatin. Alternatively, it might participate in the dynamic switch between transcriptional activation and repression in euchromatin of senescent nuclei. Further experiments are needed to identify the precise mechanism linking ING1 to chromatin domains in senescent cells.

Our expression analysis has allowed us to define a distinct genetic signature for ING1-induced senescence of human fibroblasts, which partially overlaps with that associated to Ras-induced senescence. Interestingly, this common signature is enriched in mediators of cytokine and chemokine signaling. Cytokines and chemokines play critical roles in different aspects of normal physiology and pathology, including inflammation, immune response, or tumor growth and metastasis (Mantovani *et al.*, 2008; Lazennec & Richmond, 2010; Schetter *et al.*, 2010). Cytokines and chemokines can induce proliferation of tumor or normal cells (Mantovani *et al.*, 2008), but they can also promote senescence (Acosta *et al.*, 2008; Coppe *et al.*, 2008; Kuilman *et al.*, 2008; Wajapeyee *et al.*, 2008; Rodier *et al.*, 2009) defining the so-called SASP or senescence-messaging secretome (SMS) (Kuilman & Peeper, 2009). Our data suggest that the pro-senescence action of ING1 in primary fibroblasts is largely mediated by the activation of a secretory response, similarly to other senescence triggers. ING1, like

other ING proteins, can cooperate with NF-kappaB in transcriptional control (Garkavtsev *et al.*, 2004; Gomez-Cabello *et al.*, 2010), and putative NF-kappaB sites are present in many genes of the ING1 signature (data not shown). Interestingly, NF-kappaB is an important player in gene regulation during senescence, in part as a regulator of SASP/SMS genes (Bernard *et al.*, 2004; Acosta *et al.*, 2008; Wang *et al.*, 2009). Collectively, our results are consistent with a model where ING1 contributes to SASP/SMS gene expression during senescence, in cooperation with NF-kappaB.

The ING protein ING2 has also been implicated in cellular senescence. Similarly to p33ING1, ING2 can trigger senescence in a p53-dependent manner (Pedeux *et al.*, 2005), but conflicting results have been described for the silencing of ING2 (Pedeux *et al.*, 2005; Kumamoto *et al.*, 2008). Interestingly, ING2 is the ING protein most closely related to p33ING1, and they are associated to similar transcriptional repressor complexes (Doyon *et al.*, 2006). It is feasible that regulation of senescence is a common feature of this subclass of 'repressor ING's', and it would be interesting to determine whether the role of ING2 in senescence involves a chromatin-related mechanism, as with ING1.

While chromatin-mediated regulation appears to be the essential feature for induction of senescence by ING1, other mechanisms might also play a role. DNA damage caused by hyperproliferation, telomere dysfunction, or other stimuli can activate senescence (Campisi & d'Adda di Fagagna, 2007; Collado *et al.*, 2007). We have found evidence of a DNA damage response in ING1-expressing cells but this response was very limited and, more importantly, did not correlate with the ability to induce senescence of the different ING1 versions tested (Fig. S6).

Our results also show that the implementation of senescence by p33ING1 requires an intact p53 pathway. The p53 pathway plays an essential role in senescence control in different settings. In the case of the human fibroblasts, the Rb pathway plays a similar role and concomitant inactivation of both pathways is required to bypass senescence (Campisi & d'Adda di Fagagna, 2007; Collado *et al.*, 2007). There are conflicting results about the dependence on p53 of the biologic functions of ING1 *in vivo* or *in vitro* (Coles & Jones, 2009). Most likely, the link between ING1 and p53 is context dependent. In this respect, our data clearly indicate that implementation of senescence by ING1 in this cell type is fully dependent on p53, and therefore represents an example of a p53-dependent function of ING1 linked to tumor protection.

ING1 can be altered by different mechanisms in human cancer, including missense point mutations in a subset of tumors (Ythier *et al.*, 2008). We find that a version of p33ING1 carrying a cancer-associated mutation was ineffective in induction of senescence. This is the first direct evidence of the association between senescence and tumor-specific alterations in ING1. Taken together with the independent observation of defective apoptosis in other tumor-associated ING1 mutants (Pena *et al.*, 2008), our results strongly indicate that the ability to implement

cellular senescence is a requisite for an effective tumor-protective action by ING1, further supporting the notion that apoptosis and senescence play equivalent roles as protective barriers against tumorigenesis.

## Experimental procedures

### Cell culture

IMR-90 primary human diploid fibroblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum and containing antibiotics, at 37 °C in 5% CO<sub>2</sub>. As exceptions, IMR-90 cells infected with the E6 oncoprotein were grown in medium with 20% fetal bovine serum, and IMR-90 expressing an inducible form of MEK1 (MEK1:ER) were cultured in DMEM without Phenol Red (GIBCO).

### Mutagenesis

Mutant versions of p33ING1 were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), using as a template the ORF of the human p33ING1 protein cloned in the retroviral vector pLPC (Goeman *et al.*, 2005). Specific mutations were incorporated by PCR using Pfu Turbo DNA Polymerase (Stratagene), and they were confirmed by sequencing.

### RNA interference vectors

For the generation of shRNAmirs, we used the LMP vector (Dickins *et al.*, 2005, a gift from S. Lowe, CSHL, USA) that allows the production of specific shRNAs using a miR30 microRNA backbone. The cloning of the ING1b-specific sequence into the LMP vector was performed following the recommended protocols (<http://www.openbiosystems.com>). The ING1b sequence targeted was ACCTTTCGACTTGCAGAGAAAT (nucleotides 77–99, relative to the AUG).

### Retroviral transduction

We used early passage IMR-90 with < 30 PDL (*Population Doubling Level*) in all our experiments. We performed retroviral transduction with amphotropic and ecotropic viruses. In the latter case, IMR-90 cells were previously infected with a virus driving the expression of the ecotropic receptor. Retroviral infection was performed essentially as described in Goeman *et al.*, 2005, with minor modifications. In brief, for the production of virus, 293T cells were transiently transfected with 10 µg of pCLEco or pCLAmpho vector (which express the viral genes *gag*, *pol* and *envEco* or *envAmpho*) and 10 µg of the vector of interest. Forty-eight hours later, the supernatant containing the viral particles was diluted 1 in 2 with fresh medium, filtered, and polybrene was added to a final concentration of

8  $\mu\text{g mL}^{-1}$ . This supernatant was added to IMR-90 cells, seeded the previous day at a concentration of  $8 \times 10^5$  cells per 10-cm dish. This procedure was repeated 12 h later using 4  $\mu\text{g mL}^{-1}$  of polybrene and 24 h later using 8  $\mu\text{g mL}^{-1}$ . After 24 h of recovery with fresh medium, cells were subjected to antibiotic selection (2  $\mu\text{g mL}^{-1}$  of Puromycin or 400 mg  $\text{mL}^{-1}$  of G418) for a period of 3–6 days.

### Growth curves

Infected cells were seeded in duplicate a day after the end of selection at a density of 20 000 cells per well in 24-well plates. At the indicated time points, cells were trypsinized and counted. In the case of MEK1-inducible IMR-90 cells, following infection with the shRNA-mir vectors and selection, cells were treated with 1 mM of 4-OHT (Sigma, St Louis, MO, USA), or with an equivalent amount of ethanol. Cells were trypsinized and counted at the indicated time points, after beginning of treatment.

### Senescence-associated Beta-Galactosidase activity assay

Infected and selected IMR-90 cells were seeded in 6-well plates at a density of 40 000 cells per well. The day after, cells were fixed and stained for SA-BetaGal activity as previously described (Dimri *et al.*, 1995). At least 200 cells were counted to measure the percentage of SA-BetaGal-positive cells.

### BrdU incorporation assay

IMR-90 cells were plated in 8-well glass chamber slides (LabTek, Rochester, NY, USA), at 30 000 cells per chamber. Twenty-four hours later, cells were incubated for 6 h with 10  $\mu\text{M}$  BrdU. BrdU-positive cells were detected by immunofluorescence using an anti-BrdU antibody (1:1000 dilution; Megabase Research Products, Lincoln, NE, USA). Cells were costained with DAPI to visualize nuclei. To determine the percentage of BrdU-positive cells, at least 200 nuclei were counted.

### Immunofluorescence

Cells were seeded in chamber slides as above-mentioned at a density of 30 000 cells per chamber. Twenty-four hours later, they were processed essentially as described in (Gonzalez *et al.*, 2006). We used the following antibodies: AU5 (1:500, MMS-135R Covance, Princeton, NJ, USA), ING1 (LG1, a rabbit polyclonal against the C-terminus of the human p33ING1 protein; 1:500), gamma-H2AX (1:500, JBW301; Upstate, Billerica, MA, USA).

### Western blot

Preparation of total lysates and Western blot analysis was carried out as previously described (Palmero *et al.*, 2002). Chromatin was isolated as described (Narita *et al.*, 2006). The antibodies

used were p53 (1:500, DO-1; Santa Cruz, Santa Cruz, CA, USA), ING1 (LG1, 1:1000), p21CIP1 (1:500, C19, Santa Cruz), Ras (1:2000, OP-40; Calbiochem, Darmstadt, Germany), and AU5 (1:500, MMS-135R; Covance), Acetylated histone H3 (1:1000; Upstate), Phospho-ATM (1:1000, 05-740; Upstate).

### Quantitative PCR

Total RNA was isolated from asynchronously growing IMR-90 cells using Tri Reagent (Sigma). Two micrograms of total RNA was used to synthesize cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). One microgram of reaction was used to perform standard PCR or quantitative PCRs. For quantitative PCR of ING1 transcripts, the following primers were used: *ING1 exon1b Forward*, GGACTACCTGGACTCCAT; *ING1 exon1a Forward*, TCGGAGACAGTTTCAGGC; *ING1 exon2 Reverse*, CGACTGAAGCGCTCGTA; *GAPDH Forward*, CAGAAGACTGTGGATGG; *GAPDH Reverse*, GCTTCACCACCTTCTTG. For validation of microarray results, the following primers were used, for each gene forward primer followed by reverse primer: *TMEM16C*, TCAGAGCAGAAGGCTTGATG, AACATGATA-TCGGGGGCTTG; *CXCL2*, CATCGAAAAGATGCTGAAAAATG, TTCAGGAACAGCCACCAATA; *CXCL6*, GTCCTTCGGGCTCCTTGT, CAGCACAGCAGAGACAGGAC; *CXCR7*, CGATGCCTCCA-GAGTCTCA, GGCAGATCATTTGGTGCTCT; *HHIP*, TTCACAA-ACCTGTTCAAAGTGGA, ATGCGAGGCTTAGCAGTCC. The 18S ribosomal RNA was used as internal reference. For quantitation of ChIP, the following primers were used: *CXCL2*, CAGGCGG-TTATCTCGGTATCTC, CTTTATGCATGGTTGGGGC; *TMEM16C*, TCACTTCTTTGGGTATCGGGT, CACAGGGGCTGAAAAACAC.

### Microarray gene expression profiles

Microarray experiments were performed using Human Whole Genome 4X44K array G4112F (Agilent Technologies, Santa Clara, CA, USA). Two independent retroviral infections were performed in early passage IMR-90 fibroblasts with wild-type p33ING1, W235A and Y212A ING1 mutants, RasV12 and empty vector. Total RNA from the different cell populations was extracted as previously described (Moreno-Bueno *et al.*, 2009). RNA was labeled and hybridized to arrays using the Low RNA Linear Amplification Kit and the In Situ Hybridization Kit Plus (Agilent Technologies), respectively. Competitive hybridization was performed for each sample, using as a reference a reverse-labeled sample from vector-infected cells. After hybridization and washing, the slides were scanned in an Axon GenePix Scanner (Axon Instruments Inc., Union City, CA, USA) and analyzed using Feature Extraction Software 10.0 (Agilent Technologies). Two different RNA samples obtained from each condition were labeled with Cy5-dUTP. The RNA samples from control, vector-infected cells were labeled with Cy3-dUTP (Agilent Technologies). Two additional hybridizations were performed using the reciprocal fluorochrome labeling. For the identification of genes with differential expression in IMR90 cells expressing p33ING1 with respect to control cells, we used the GEPAS gene expression

analysis package (<http://gepas3.bioinfo.cipf.es>) and selected those genes whose signal differed by a factor of at least twofold in all of the samples with a standard deviation lower than 0.5. This subset was further analyzed with respect to their expression in the rest of samples. Functional enrichment analysis was performed using the FatiGO application (<http://babelomics.bioinfo.cipf.es/>).

## Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed essentially as described in Gomez-Cabello et al., 2010. from IMR-90 fibroblast infected with AU5-tagged p33ING1 or empty vector. Immunoprecipitation was carried out with an anti-AU5 antibody or an isotype-matched nonspecific mouse IgG.

## Acknowledgments

We thank Manuel Serrano for valuable assistance with experiments in the final phase of the project and critical reading of the manuscript, Scott Lowe for reagents, Manuel Collado for comments on the manuscript, Adrián Martín and Weronika Kucharewicz for help with immunoprecipitation experiments, and Carmen L Pereira for help with the structural analysis. This work is supported by grants from the Spanish Ministry of Science and Innovation to IP (BFU2006-10882, SAF2009-09031) and FJB (CTQ2008-03115/BQU).

## References

- Abad M, Menendez C, Fuchtbauer A, Serrano M, Fuchtbauer EM, Palmero I (2007) Ing1 mediates p53 accumulation and chromatin modification in response to oncogenic stress. *J. Biol. Chem.* **282**, 31060–31067.
- Acosta JC, O’Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, Fumagalli M, Da Costa M, Brown C, Popov N, Takatsu Y, Melamed J, d’Adda di Fagagna F, Bernard D, Hernando E, Gil J (2008) Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133**, 1006–1018.
- Adams PD (2007) Remodeling chromatin for senescence. *Aging Cell* **6**, 425–427.
- Barradas M, Anderton E, Acosta JC, Li S, Banito A, Rodriguez-Niedenfuhr M, Maertens G, Banck M, Zhou MM, Walsh MJ, Peters G, Gil J (2009) Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev.* **23**, 1177–1182.
- Bernard D, Gosselin K, Monte D, Vercamer C, Bouali F, Pourtier A, Vandenbunder B, Abbadie C (2004) Involvement of Rel/nuclear factor-kappaB transcription factors in keratinocyte senescence. *Cancer Res.* **64**, 472–481.
- Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436**, 660–665.
- Campisi J, d’Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* **8**, 729–740.
- Champagne KS, Kutateladze TG (2009) Structural insight into histone recognition by the ING PHD fingers. *Curr. Drug Targets* **10**, 432–441.
- Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725–730.
- Chi P, Allis CD, Wang GG (2010) Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat. Rev. Cancer* **10**, 457–469.
- Coles AH, Jones SN (2009) The ING gene family in the regulation of cell growth and tumorigenesis. *J. Cell. Physiol.* **218**, 45–57.
- Coles AH, Liang H, Zhu Z, Marfella CG, Kang J, Imbalzano AN, Jones SN (2007) Deletion of p37Ing1 in mice reveals a p53-independent role for Ing1 in the suppression of cell proliferation, apoptosis, and tumorigenesis. *Cancer Res.* **67**, 2054–2061.
- Collado M, Serrano M (2006) The power and the promise of oncogene-induced senescence markers. *Nat. Rev. Cancer* **6**, 472–476.
- Collado M, Serrano M (2010) Senescence in tumours: evidence from mice and humans. *Nat. Rev. Cancer* **10**, 51–57.
- Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M (2005) Tumour biology: senescence in premalignant tumours. *Nature* **436**, 642.
- Collado M, Blasco MA, Serrano M (2007) Cellular senescence in cancer and aging. *Cell* **130**, 223–233.
- Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* **6**, 2853–2868.
- Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, Hollstein PE, MacCollin M, Cichowski K (2006) A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell* **10**, 459–472.
- Courtois-Cox S, Jones SL, Cichowski K (2008) Many roads lead to oncogene-induced senescence. *Oncogene* **27**, 2801–2809.
- Dankort D, Filenova E, Collado M, Serrano M, Jones K, McMahon M (2007) A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors. *Genes Dev.* **21**, 379–384.
- Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, Lowe SW (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nat. Genet.* **37**, 1289–1295.
- Dietrich N, Bracken AP, Trinh E, Schjerling CK, Koseki H, Rappsilber J, Helin K, Hansen KH (2007) Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus. *EMBO J.* **26**, 1637–1648.
- Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocket M, Campisi J (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 9363–9367.
- Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, Selleck W, Lane WS, Tan S, Yang XJ, Cote J (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Mol. Cell* **21**, 51–64.
- Funayama R, Ishikawa F (2007) Cellular senescence and chromatin structure. *Chromosoma* **116**, 431–440.
- Garkavtsev I, Riabowol K (1997) Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. *Mol. Cell. Biol.* **17**, 2014–2019.
- Garkavtsev I, Kozin SV, Chernova O, Xu L, Winkler F, Brown E, Barnett GH, Jain RK (2004) The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. *Nature* **428**, 328–332.



- Gil J, Bernard D, Martinez D, Beach D (2004) Polycomb CBX7 has a unifying role in cellular lifespan. *Nat. Cell Biol.* **6**, 67–72.
- Goeman F, Thormeyer D, Abad M, Serrano M, Schmidt O, Palmero I, Baniahmad A (2005) Growth inhibition by the tumor suppressor p33ING1 in immortalized and primary cells: involvement of two silencing domains and effect of Ras. *Mol. Cell. Biol.* **25**, 422–431.
- Gomez-Cabello D, Callejas S, Benguria A, Moreno A, Alonso J, Palmero I (2010) Regulation of the microRNA processor DGCR8 by the tumor suppressor ING1. *Cancer Res.* **70**, 1866–1874.
- Gonzalez L, Freije JM, Cal S, Lopez-Otin C, Serrano M, Palmero I (2006) A functional link between the tumour suppressors ARF and p33ING1. *Oncogene* **25**, 5173–5179.
- Gunduz M, Ouchida M, Fukushima K, Hanafusa H, Etani T, Nishioka S, Nishizaki K, Shimizu K (2000) Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. *Cancer Res.* **60**, 3143–3146.
- He J, Kallin EM, Tsukada YI, Zhang Y (2008) The H3K36 demethylase Jhdm1b/Kdm2b regulates cell proliferation and senescence through p15(Ink4b). *Nat. Struct. Mol. Biol.* **15**, 1133–1134.
- Hung T, Binda O, Champagne KS, Kuo AJ, Johnson K, Chang HY, Simon MD, Kutateladze TG, Gozani O (2009) ING4 mediates crosstalk between histone H3 K4 trimethylation and H3 acetylation to attenuate cellular transformation. *Mol. Cell* **33**, 248–256.
- Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M (1999) Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev.* **13**, 2678–2690.
- Kichina JV, Zeremski M, Aris L, Gurova KV, Walker E, Franks R, Nikitin AY, Kiyokawa H, Gudkov AV (2006) Targeted disruption of the mouse ing1 locus results in reduced body size, hypersensitivity to radiation and elevated incidence of lymphomas. *Oncogene* **25**, 857–866.
- Kivinen L, Tsubari M, Haapajarvi T, Datto MB, Wang XF, Laiho M (1999) Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites. *Oncogene* **18**, 6252–6261.
- Kotake Y, Zeng Y, Xiong Y (2009) DDB1-CUL4 and MLL1 mediate oncogene-induced p16INK4a activation. *Cancer Res.* **69**, 1809–1814.
- Kuilman T, Peeper DS (2009) Senescence-messaging secretome: SMS-ing cellular stress. *Nat. Rev. Cancer* **9**, 81–94.
- Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS (2008) Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133**, 1019–1031.
- Kumamoto K, Spillare EA, Fujita K, Horikawa I, Yamashita T, Appella E, Nagashima M, Takenoshita S, Yokota J, Harris CC (2008) Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence. *Cancer Res.* **68**, 3193–3203.
- Kuzmichev A, Zhang Y, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). *Mol. Cell. Biol.* **22**, 835–848.
- Lazennec G, Richmond A (2010) Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol. Med.* **16**, 133–144.
- Lowe SW, Cepero E, Evan G (2004) Intrinsic tumour suppression. *Nature* **432**, 307–315.
- Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. *Nature* **454**, 436–444.
- Menendez C, Abad M, Gomez-Cabello D, Moreno A, Palmero I (2009) ING proteins in cellular senescence. *Curr. Drug Targets* **10**, 406–417.
- Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720–724.
- Mooi WJ, Peeper DS (2006) Oncogene-induced cell senescence – halting on the road to cancer. *N. Engl. J. Med.* **355**, 1037–1046.
- Moreno-Bueno G, Peinado H, Molina P, Olmeda D, Cubillo E, Santos V, Palacios J, Portillo F, Cano A (2009) The morphological and molecular features of the epithelial-to-mesenchymal transition. *Nat. Protoc.* **4**, 1591–1613.
- Narita M (2007) Cellular senescence and chromatin organisation. *Br. J. Cancer* **96**, 686–691.
- Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**, 703–716.
- Narita M, Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, Myers MP, Lowe SW (2006) A novel role for high-mobility group A proteins in cellular senescence and heterochromatin formation. *Cell* **126**, 503–514.
- Nouman GS, Anderson JJ, Lunec J, Angus B (2003) The role of the tumour suppressor p33 ING1b in human neoplasia. *J. Clin. Pathol.* **56**, 491–496.
- Palacios A, Garcia P, Padro D, Lopez-Hernandez E, Martin I, Blanco FJ (2006) Solution structure and NMR characterization of the binding to methylated histone tails of the plant homeodomain finger of the tumour suppressor ING4. *FEBS Lett.* **580**, 6903–6908.
- Palacios A, Munoz IG, Pantoja-Uceda D, Marcaida MJ, Torres D, Martin-Garcia JM, Luque I, Montoya G, Blanco FJ (2008) Molecular basis of histone H3K4me3 recognition by ING4. *J. Biol. Chem.* **283**, 15956–15964.
- Palmero I, Murga M, Zubiaga A, Serrano M (2002) Activation of ARF by oncogenic stress in mouse fibroblasts is independent of E2F1 and E2F2. *Oncogene* **21**, 2939–2947.
- Pedoux R, Sengupta S, Shen JC, Demidov ON, Saito S, Onogi H, Kumamoto K, Wincovitch S, Garfield SH, McMenamin M, Nagashima M, Grossman SR, Appella E, Harris CC (2005) ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. *Mol. Cell. Biol.* **25**, 6639–6648.
- Pena PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG (2006) Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* **442**, 100–103.
- Pena PV, Hom RA, Hung T, Lin H, Kuo AJ, Wong RP, Subach OM, Champagne KS, Zhao R, Verkhusha VV, Li G, Gozani O, Kutateladze TG (2008) Histone H3K4me3 binding is required for the DNA repair and apoptotic activities of ING1 tumor suppressor. *J. Mol. Biol.* **380**, 303–312.
- Pfau R, Tzatsos A, Kampranis SC, Serebrennikova OB, Bear SE, Tschlis PN (2008) Members of a family of JmjC domain-containing oncoproteins immortalize embryonic fibroblasts via a JmjC domain-dependent process. *Proc. Natl. Acad. Sci. USA* **105**, 1907–1912.
- Prieur A, Peeper DS (2008) Cellular senescence in vivo: a barrier to tumorigenesis. *Curr. Opin. Cell Biol.* **20**, 150–155.
- Rodier F, Coppe JP, Patil CK, Hoeijmakers WA, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR, Campisi J (2009) Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat. Cell Biol.* **11**, 973–979.



- Schetter AJ, Heegaard NH, Harris CC (2010) Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* **31**, 37–49.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593–602.
- Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Pena P, Lan F, Kaadige MR, Lacoste N, Cayrou C, Davrazou F, Saha A, Cairns BR, Ayer DE, Kutateladze TG, Shi Y, Cote J, Chua KF, Gozani O (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96–99.
- Soliman MA, Riabowol K (2007) After a decade of study-ING, a PHD for a versatile family of proteins. *Trends Biochem. Sci.* **32**, 509–519.
- Soliman MA, Berardi P, Pastryeva S, Bonnefin P, Feng X, Colina A, Young D, Riabowol K (2008) ING1a expression increases during replicative senescence and induces a senescent phenotype. *Aging Cell* **7**, 783–794.
- Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR (2008) Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell* **132**, 363–374.
- Wang J, Jacob NK, Ladner KJ, Beg A, Perko JD, Tanner SM, Liyanarachchi S, Fishel R, Guttridge DC (2009) RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair. *EMBO Rep.* **10**, 1272–1278.
- Ythier D, Larrieu D, Brambilla C, Brambilla E, Pedeux R (2008) The new tumor suppressor genes ING: genomic structure and status in cancer. *Int. J. Cancer* **123**, 1483–1490.

## Supporting Information

Additional supporting information may be found in the online version of this article:

**Data S1** Methods.

**Fig. S1** Subcellular distribution of p33ING1.

**Fig. S2** Effect of the C215S mutation on protein folding.

**Fig. S3** Connection of ING1 with SAHFs.

**Fig. S4** Expression profiling.

**Fig. S5** Association to p53.

**Fig. S6** DNA damage response in ING1-expressing fibroblasts.

**Table S1** Genes with differential expression in p33ING1-infected IMR-90 fibroblasts, relative to vector-infected cells.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.