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Research Article

RASSF1A Mediates p21$^{Cip1/Waf1}$-Dependent Cell Cycle Arrest and Senescence through Modulation of the Raf-MEK-ERK Pathway and Inhibition of Akt

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Abstract

Promoter hypermethylation preventing expression of the RAS association domain family 1 isoform A (RASSF1A) gene product is among the most abundant epigenetic deregulations in human cancer. Restoration of RASSF1A inhibits tumor cell growth *in vitro* and in murine xenograft models. *Rassf1a*-deficient mice feature increased spontaneous and carcinogen-induced tumor formation. Mechanistically, RASSF1A affects several cellular functions, such as microtubule dynamics, migration, proliferation, and apoptosis; however, its tumor-suppressive mechanism is incompletely understood. To study the functional consequences of RASSF1A expression in human cancer cells, we made use of a doxycycline-inducible expression system and a RASSF1A-deficient lung cancer cell line. We observed that RASSF1A induces cell cycle arrest in G_1 phase and senescence *in vitro* and in tumors established in immunodeficient mice. RASSF1A-mediated growth inhibition was accompanied by the up-regulation of the cyclin-dependent kinase inhibitor p21$^{Cip1/Waf1}$ and proceeded independently of p53, p14^{Arf}, and p16^{Ink4a}. Loss of p21$^{Cip1/Waf1}$ or coexpression of the human papilloma virus 16 oncoprotein E7 was found to override RASSF1A-induced cell cycle arrest and senescence. Conditional RASSF1A affected mitogen-activated protein kinase and protein kinase B/Akt signaling to up-regulate p21$^{Cip1/Waf1}$ and to facilitate its nuclear localization. In summary, RASSF1A can mediate cell cycle arrest and senescence in human cancer cells by p53-independent regulation of p21$^{Cip1/Waf1}$. [Cancer Res 2009;69(5):1748–57]

Introduction

Loss of heterozygosity of chromosomal region 3p21.3 is an early and common event in the development of lung cancer and other human cancers. *RASSF1* is one of the genes encoded at this locus, and the expression of the RASSF1A gene product is frequently lost in cancers of the lung and other organs (1). Mechanistically, RASSF1A deficiency results from gene silencing through hypermethylation of a CpG island upstream of exon 1α of *RASSF1*, which is encountered in a wide range of human cancers (2). Accordingly, RASSF1A has been implied as putative tumor suppressor. In support, restoration of RASSF1A expression by gene transfer technology inhibits cancer cell growth *in vitro*, as well as in murine xenograft models (1). Moreover, *Rassf1a* gene–targeted mice exhibit increased incidence of spontaneously arising and carcinogen-induced tumors (3). Against this background, the molecular mechanism of RASSF1A-mediated tumor suppression is subject to extensive studies. Depending on the experimental system, RASSF1A was shown to affect multiple cellular activities, such as apoptosis, cell cycle progression, and migration. More recently, RASSF1A was reported to interact with mammalian orthologues of the Hippo/Salvator/Lats tumor suppressor pathway during mitosis regulation (4).

The main cell autonomous effector mechanisms counteracting malignant transformation are apoptosis, cell cycle arrest, and senescence (5, 6). Depending on the cellular context, these pathways are triggered by activated oncogenes, DNA damage, and additional cellular stresses. Whereas apoptosis is viewed as an important tumor suppressive mechanism, senescence has only recently been established as a barrier for cancer development (7, 8). In fibroblasts and murine lymphoma cells, oncogene-induced senescence is signaled via p14/p19^{Arf}, p16^{Ink4a}, and the DNA damage response pathways involving checkpoint kinases, such as Ataxia teleangiectasia mutated (ATM) and checkpoint kinase 2 (9–12). Studies in naevi and colon adenomas, which are preneoplastic lesions, have implied p16^{Ink4a} and DNA damage response signaling as major senescence pathways interfering with the development of human cancers (8, 11, 12). Despite their implication in the initiation of senescence, p16^{Ink4a} and p14/p19^{Arf} are not associated with the final end point of senescence. Overexpression of p16^{Ink4a} or p14/p19^{Arf} are inconsequential if the cells lack the retinoblastoma protein (pRb; refs. 13, 14), indicating the requirement of pRb for the execution of senescence. The irreversibility of the proliferative arrest that characterizes senescence seems to be based on a pRb-initiated global alteration of the chromatin. This results in a permanent and stable repression of genes with crucial roles in proliferation, such as those regulated by the E2F family of transcription factors. Such genomic alterations are microscopically visualized by the appearance of clusters of 4′,6-diamidino-2-phenylindole–stained heterochromatic regions known as senescence-associated heterochromatic foci (SAHF; ref. 15). Recently, we have reported that RASSF1A is progressively silenced in normal epithelial cells during stress-associated senescence. Moreover, RASSF1A was found epigenetically inactivated in cells overriding senescence (16).

Increased proliferation associated with transformation of primary cells usually requires the cooperation of at least two oncogenic mutations, whereas expression of individual oncogenes can promote cell cycle arrest, senescence, and apoptosis (17, 18),

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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suggesting the presence of cellular safeguards to oncogenic transformation. This is illustrated by the distinct cellular responses to hyperactivation of the Ras/Raf signaling cascade. Stimulation of oncogenic Ras or Raf can induce cell cycle arrest and senescence in various primary cell types (19). However, genetic lesions resulting in the activation of cooperating cellular oncogenes or loss of tumor suppressors (e.g., p53) disable the growth-inhibitory effects of Ras/Raf and potentiate their mitogenic activity (20). The cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/Waf1}$ is an important determinant in the cellular response to Ras/Raf activation and was independently isolated as an inducer of cellular senescence, a transcriptional target of the p53 tumor suppressor and a direct inhibitor of CDK2 (21–23). $p21^{Cip1/Waf1}$ is regulated at the transcriptional and posttranscriptional level, with transcription of $p21^{Cip1/Waf1}$ being activated by p53-dependent and p53-independent mechanisms (23). Expression of $p21^{Cip1/Waf1}$ is induced during senescence, and Ras or Raf induces a $p21^{Cip1/Waf1}$-dependent cell cycle arrest in primary fibroblasts and keratinocytes (24–27). Furthermore, genetic loss of $p21^{Cip1/Waf1}$ confers a proliferative advantage to Ras-expressing embryonic fibroblasts and promotes Ras-induced epithelial tumorigenesis (28), thus implicating $p21^{Cip1/Waf1}$ as a target of transforming oncogenes. This correlates with the observation that oncogenes and genetic lesions cooperating with Ras in cellular transformation frequently abolish $p21^{Cip1/Waf1}$ function (21, 29). Ras transduces extracellular information through a multitude of signaling cascades. Raf and Akt are components of two parallel Ras signaling pathways, which have been shown to interact (30). Maintenance of Akt activity bypasses Ras/Raf-induced cell cycle arrest, suggesting that loss of Akt activity is necessary for growth arrest elicited by Raf (31).

In the present study, we have focused on the molecular dissection of the antiproliferative activity of RASSF1A using conditional expression and the RASSF1A-inactivated human lung cancer cell line A549. We find that RASSF1A induces a senescence response *in vitro* and *in vivo*. This is imposed by the activation of the Ras-MEK-ERK pathway and inhibition of Akt signaling, leading to up-regulation of the CDK inhibitor $p21^{Cip1/Waf1}$. In addition, we find that reexpression of RASSF1A enhances nuclear accumulation of $p21^{Cip1/Waf1}$ by negative regulation of Akt.

Materials and Methods

Plasmids and reagents. A FLAG-tagged human RASSF1A cDNA was cloned into the retroviral vector pRevTRE (Clontech) to allow conditional transgene expression controlled by doxycycline in the presence of rt-TA (32). A cDNA encoding human papilloma virus (HPV) E6 and/or E7 or a constitutively active mutant of Akt was cloned into the vector pMxIG provided by T. Kitamura. All inserts were verified by sequencing. Inhibitors U0126 and LY294,002 were purchased from Calbiochem.

Cell culture, retroviral transduction, and cell cycle analysis. *Rassf1a*$^{-/-}$ and control murine embryonic fibroblasts (MEF) were generously provided by S. Tommasi and G. Pfeifer; genotypes and *Rassf1a* expression were confirmed as published (3). Gene-targeted HCT116 colorectal cancer cells were generously provided by B. Vogelstein and T. Waldman. Unless otherwise indicated, conditional cell lines were maintained in full DMEM supplemented with tetracycline-free fetal bovine serum (FBS; Clontech). Conditional RASSF1A expression was achieved by addition of doxycycline (1 μg/mL, Sigma). The Phoenix packaging cell line was used for generation of retroviruses following a standard calcium phosphate transfection protocol. Transduction efficacies were monitored by flow cytometric detection of enhanced green fluorescent protein (eGFP) expression. For cell cycle analysis, synchronized cells were released from a double thymidine block (2 mmol/L, 19 and 17 h) by the addition of full medium. Dual-color flow cytometric measurement of DNA content and S-phase fraction was performed after labeling with FITC-conjugated BrdUrd (Clontech) and propidium iodide (Sigma).

Immunoblotting and antibodies. For Western blot analysis, 40 μg of total protein lysates (NP40 lysis buffer) were diluted in reducing sample buffer and subjected to SDS-PAGE, followed by electrotransfer to nitrocellulose membranes (Hybond ECL). The following primary antibodies were used for immunoblotting: actin (C4) from ICN; p21 (C-19) and p27 (F-8) from Santa Cruz; p53 (Do-7) from BD Pharmingen; FLAG (M2) from Sigma; RASSF1A (clone[3F3]) from Abcam; cyclins D1 (H-295), E (M-20), and A (H-432), Akt1/2 (H-136), ERK1 (C-16), histone H3 (FL-136), α-tubulin (TU-02), and Raf-1 (C-12) from Santa Cruz; all phosphoepitope-specific antisera were purchased from Cell Signaling Technology.

Immunohistochemistry and antibodies. Tumor cryosections were stained for mitotic and apoptotic cells using anti-Ki-67 (MIB1, DAKO) and TUNEL (*In situ* Cell Death Detection kit, Roche), respectively. Immunohistochemical detection of RASSF1A and phosphorylated H2AX Ser^{139} (γ-H2AX) was performed in paraffin-embedded sections of s.c. xenograft tumors. Tumor samples were fixed overnight by immersion in PBS buffered 4% formaldehyde. Treatment and immunohistochemical staining of the deparaffinized and rehydrated sections was performed as described (12). The sections were incubated overnight at 4°C with mouse monoclonal RASSF1A antibody (clone[3F3], Abcam, diluted 1:200) or mouse monoclonal anti γ-H2AX Ser^{139} (clone JBW301, Millipore, diluted 1:100). Secondary antibody reactions were carried out following standard protocols (33).

Senescence-associated β-galactosidase activity. Cells growing on tissue culture dishes or tumor cryosections mounted on glass slides were fixed with 2% formaldehyde and 0.2% glutaraldehyde. Senescence-associated β-galactosidase (SAβ-gal) activity was detected following the original protocol (34).

Animal model. Irradiated (150 rad) NOD/SCID mice received single s.c. flank injections of 5 or 10 × 10^6 transgenic A549 cells diluted in 200 μL saline. Tumor growth was monitored by bidimensional measurements using a caliper. For induction of conditional RASSF1A expression, the drinking water was supplemented with doxycycline (1 mg/mL). All animal studies were conducted in compliance with institutional guidelines and German Animal Protection Law.

Statistical analysis. Differences between various experimental groups were calculated using Student's *t* test (BMDP Statistical Software, Inc.). *P* values of <0.05 were considered significant.

Results and Discussion

RASSF1A induces cell cycle arrest and senescence. To study the functional consequences of RASSF1A expression in human cells, we made use of a tetracycline-inducible expression system (32) and the A549 lung cancer cell line, which is deficient in endogenous RASSF1A, $p14^{Arf}$, and $p16^{Ink4a}$ expression (Supplementary Fig. S1*A*; ref. 35). In this model, addition of doxycycline induced transgenic expression of RASSF1A, which inhibited growth of lung cancer cells *in vitro* (Fig. 1*A*) and *in vivo* after s.c. transplantation into immunodeficient NOD/SCID mice (Fig. 1*B*). Conditional RASSF1A expression arrested A549 cells in the G_1 phase of the cell cycle (Fig. 1*A*). Titration experiments revealed that doxycycline concentrations of <0.1 μg/mL were sufficient to induce maximal transgenic RASSF1A expression, which resulted to growth inhibition in the present system. We observed an antiproliferative activity of conditionally expressed RASSF1A starting at doxycycline concentrations of as low as 0.01 μg/mL (Supplementary Fig. S1*B*). In contrast, doxycycline treatment itself had no toxic or unspecific growth inhibitory effects on parental A549 cells neither *in vitro* nor *in vivo* (Supplementary Fig. S1*C*).

To assess the antiproliferative activity of RASSF1A expressed in its physiologic context, MEFs were prepared from mice homozygously (*Rassf1a*$^{-/-}$) and heterozygously (*Rassf1a*$^{+/-}$)

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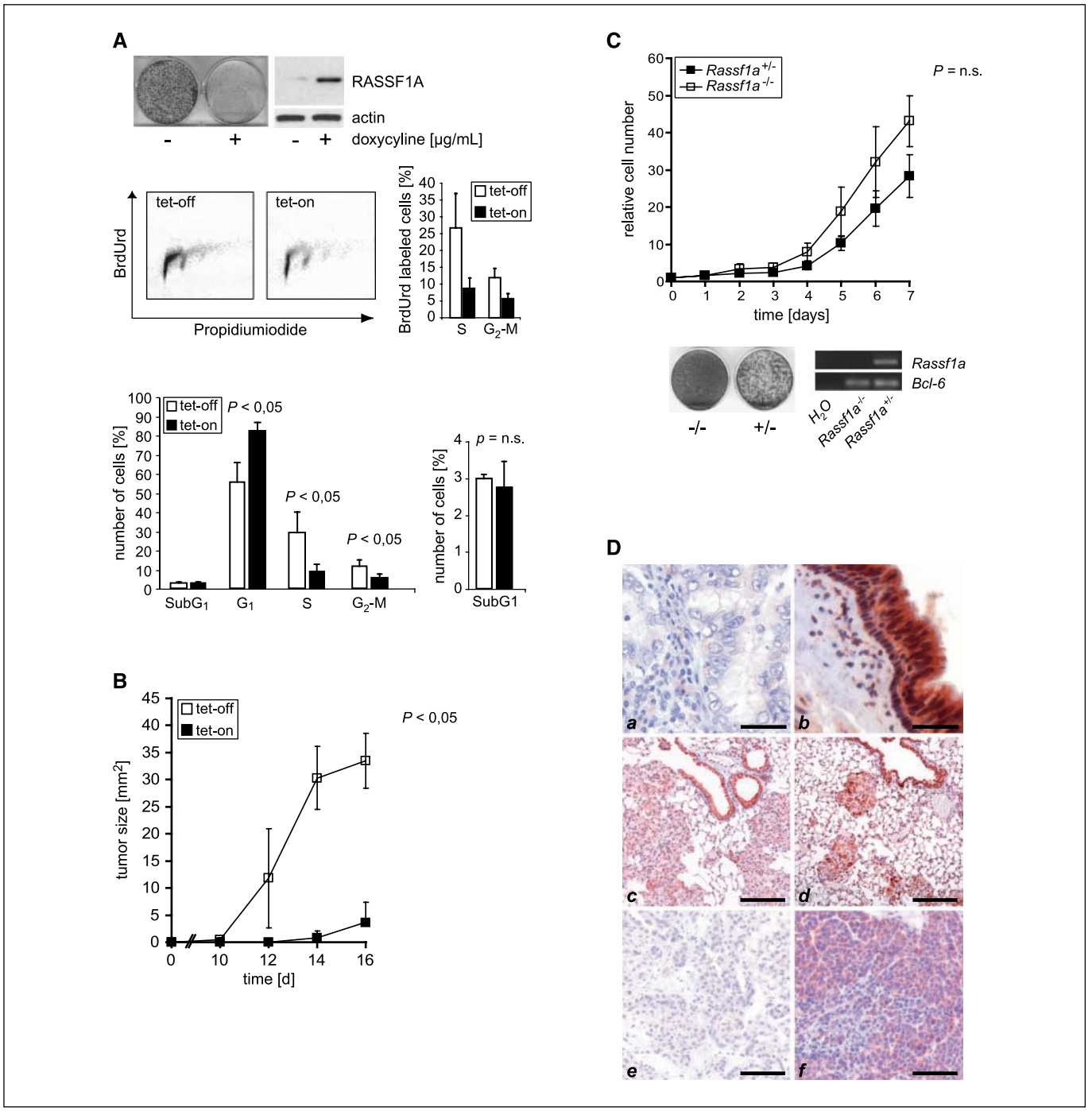

Figure 1. RASSF1A inhibits cell proliferation *in vitro* and *in vivo*. *A,* conditional A549 cells were plated at equal densities, and RASSF1A was induced by doxycycline. Clonogenic survival in relation to transgenic RASSF1A expression and confirmation of transgene expression by immunoblotting (*top*). Conditional A549 cells with (*tet-on*) or without (*tet-off*) RASSF1A expression were released from a double thymidine block by adding full medium plus BrdUrd for 90 min. Dual-color flow cytometry (*center*) to detect cellular DNA content (propidium iodide) and cells in S phase (BrdUrd incorporation). Cells in G_2-M or S phase of the cell cycle were quantified (mean percentages ± SD). Cells in sub-G_1, G_1, S, and G_2-M-phases of the cell cycle were quantified in four independent experiments (mean percentages ± SD and *P* values; *bottom*). *B,* conditional A549 cells were s.c. injected in the flanks of NOD/SCID mice. Mice were fed with drinking water supplemented with (*tet-on*) or without (*tet-off*) doxycycline, and tumor growth was monitored by bidimensional measurements (mean values ± SE and *P* value for day 16). *C, Rassf1a*$^{-/-}$ (*open boxes*) and *Rassf1a*$^{+/-}$ MEF (*closed boxes*) were arrested in G_1 phase by serum withdrawal. After the addition of FBS, cells were enumerated for 7 consecutive days (mean values ± SD of three independent experiments and *P* value for day 7). *C, Rassf1a*$^{-/-}$ (−/−) and *Rassf1a*$^{+/-}$ MEF (+/−) plated at low densities were fixed and stained after a culture period of 7 d (*left*, representative photograph). Reverse transcription and PCR to detect endogenous RASSF1A expression by the respective MEF; Bcl-6 expression was determined for quality control (*right*). *D,* paraffin-embedded sections from a human lung adenocarcinoma (*a*) and normal bronchial epithelium (*b*) were immunohistochemically analyzed for RASSF1A expression (magnification, 40×; *bars,* 50 μm). Conditional A549 cells were i.v. injected in NOD/SCID mice to establish xenograft tumors in the lung. After 4 wks, one group of mice was fed with doxycycline (*tet-on*) for 4 d to induce RASSF1A expression *in vivo* (*d*), whereas the other group received vehicle (*c*). Paraffin-embedded sections from explanted lungs were immunohistochemically stained for RASSF1A (magnification, 10×; *bars,* 200 μm). 1 × 10^7 parental A549 (*e*) and HeLa cells (*f*) were s.c. injected in NOD/SCID mice. After 28 d, xenograft tumors were removed and immunohistochemically analyzed for endogenous RASSF1A expression (magnification, 20×; *bars,* 100 μm).

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deleted in *Rassf1a* (Supplementary Fig. S1*D*; ref. 3). *Rassf1a*$^{-/-}$ MEF exhibited an increased proliferative capacity after release from cell cycle arrest in G_0 phase as induced by serum withdrawal or plating at low densities (Fig. 1*C*). Thus, conditionally, as well as endogenously, expressed RASSF1A negatively affects cell proliferation *in vitro* and *in vivo*. To compare the expression levels of transgenic RASSF1A in A549 cells to those of endogenous RASSF1A expression in human lung epithelium, we performed immunohistochemical analyses of sections from paraffin-embedded human lung adenocarcinoma and normal lung tissue (Fig. 1*D*). Normal bronchial epithelium exhibited a strong specific staining for RASSF1A (Fig. 1*D, b*), whereas endogenous RASSF1A was lost in the primary lung adenocarcinoma (Fig. 1*D, a*). For comparison, we studied lung sections from

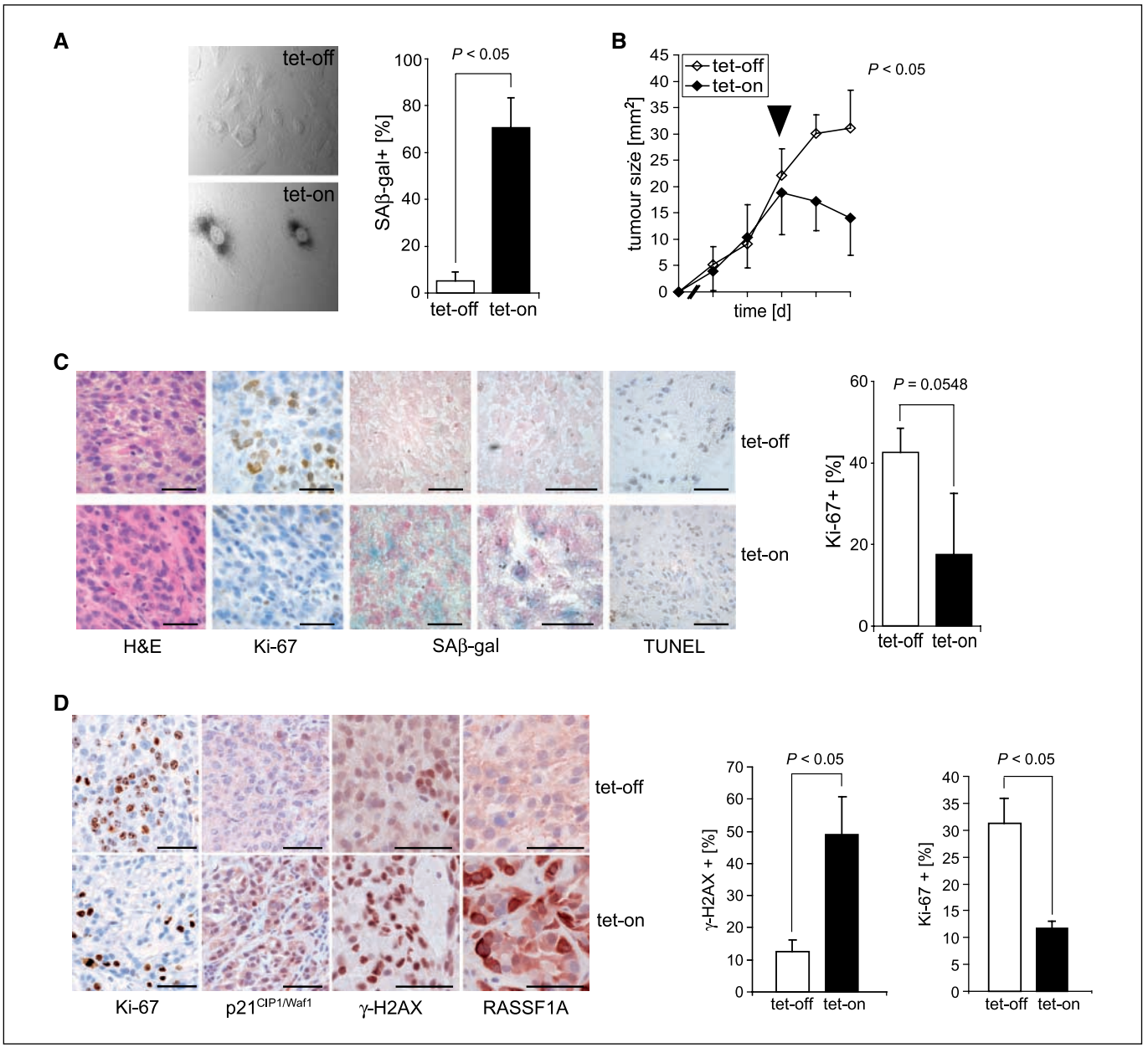

Figure 2. Conditional RASSF1A expression induces senescence in human cancer cells *in vitro* and *in vivo*. *A,* conditional A549 cells were grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline. RASSF1A induced a senescent morphology (*left*) and SAβ-gal activity (*left* and *right*; mean values ± SD of four experiments). *B,* conditional A549 cells were s.c. injected in NOD/SCID mice to establish tumors. After 14 d, one group of mice was fed with doxycycline (*tet-on*, time point indicated by the arrowhead) to induce RASSF1A expression (mean tumor sizes ± SE). *C,* frozen tumor sections obtained in a parallel experiment were stained for the proliferation marker Ki-67 (magnification, 40×; *bars,* 50 μm), SAβ-gal activity (magnification, 40× and 60×; *bars,* 50 μm), and TUNEL-positive apoptotic cells (*left*; magnification, 40×; *bars,* 50 μm). The fraction of proliferating cells with Ki-67–positive nuclei was enumerated in relation to transgenic expression (*tet-on*) of RASSF1A (*right*; mean percentage ± SD and *P* value). *D,* conditional A549 cells were s.c. injected in NOD/SCID mice to establish s.c. tumors. After 21 d, one group was fed with doxycycline for 4 d to induce RASSF1A expression *in vivo*, whereas the other group received vehicle. Paraffin-embedded sections from explanted tumors were stained for Ki-67, $p21^{Cip1/Waf1}$, RASSF1A, and γ-H2AX (*left*; magnification, 40×; *bars,* 50 μm). The percentage of γ-H2AX–positive cells was enumerated in five random visual fields per tumor section, and cells with visual dots in their nuclei were scored positive (mean values ± SD and *P* value of five random fields from two different tumors from each group). The percentage of cells with Ki-67–positive nuclei was enumerated in relation to transgenic expression (*tet-on*) of RASSF1A (mean values ± SD and *P* value).

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NOD/SCID mice bearing conditional A549 xenografts, which were established by i.v. injection of tumor cells. Sections from doxycycline-fed mice revealed transgenic RASSF1A expression in the conditional A549 xenografts, which was comparable with endogenous Rassf1a expression levels in the adjacent murine bronchial epithelium (Fig. 1*D, d*). In contrast, no RASSF1A staining was detected in tumor cell deposits within lung sections from mice kept on doxycycline-free diet (Fig. 1*D, c*). As additional controls, we analyzed tumor sections from s.c. grafted parental A549 cells (RASSF1A-negative; *e*) and HeLa cervical carcinoma cells (RASSF1A-positive; *f*). Taken together, these studies show that RASSF1A levels achieved in conditional A549 cells were in the range of those of endogenously expressed RASSF1A in normal and malignant human, as well as normal, murine tissues.

RASSF1A-mediated cell cycle arrest in A549 human lung cancer cells was accompanied by morphologic and biochemical features of sensescence (Fig. 2*A*; ref. 34). To study the effect of RASSF1A on established tumors *in vivo*, conditional A549 cells were implanted s.c. in NOD/SCID mice. After reaching linear tumor growth, RASSF1A expression was induced by feeding doxycycline to one group of mice, resulting in an instantaneous halt of tumor growth (Fig. 2*B*). Explanted tumors from doxycycline-fed mice displayed a reduced proliferative index (Fig. 2*C* and *D*). Moreover, tumors with conditionally expressed RASSF1A exhibited SAβ-gal activity (Fig. 2*C*), which serves as an indicator for senescence occurring *in vivo* (8, 9), as well as enhanced $p21^{Cip1/Waf1}$ expression (Fig. 2*D*).

In human cells, senescence is characterized by a specific cellular and molecular phenotype comprising large flat morphology, expression of SAβ-gal activity, resistance to mitogenic stimulation and formation of punctuate highly condensed domains of facultative heterochromatin, described as SAHF (15). Histone H2AX is part of SAHF, and phosphorylation of H2AX at Ser^{139} (γ-H2AX) indicates the activation of a DNA double-strand breaks (DSB) damage response by ATM, which has been observed after oncogene-induced replicative stress (36). Interestingly, we detected an increase in γ-H2AX–positive cells in tumors conditionally expressing RASSF1A (Fig. 2*D*). Whereas being an established marker of DSB, γ-H2AX may also indicate apoptosis. However, conditional RASSF1A expression did not lead to an increase in apoptosis *in vitro* (Fig. 1*A*) or in xenograft tumors *in vivo* (Fig. 2*C*). These findings suggest that RASSF1A induces γ-H2AX positivity by an alternative mechanism, such as senescence.

Recently, it was shown that several oncogenic forms of Ras, such as K-Ras, can induce growth arrest and apoptosis (37). This suggests that oncogenic Ras triggers tumor-suppressive effector proteins, which have to be inactivated during Ras-induced transformation. RASSF1A harbors a Ras association domain, which can directly bind to Ras *in vitro* (37). Moreover, RASSF1A forms a complex with activated K-Ras when overexpressed in cells, suggesting that it could mediate some of the proapoptotic and tumor-suppressive effects of the oncogene (37). As A549 cells have several defects in apoptosis (38), their endogenously mutated K-ras might rather trigger cell cycle arrest and senescence. Apparently, restoration of RASSF1A expression is required for this process. Cells with preserved apoptosis signaling and/or wild-type K-Ras will display different outcomes after RASSF1A expression. Collectively, these findings indicate that RASSF1A exerts its tumor-suppressive activity in the present cancer model mainly by induction of cell cycle arrest and senescence, but not through apoptosis.

RASSF1A-mediated cell cycle arrest and senescence requires $p21^{Cip1/Waf1}$. Cell cycle progression from G_1-S phase is orchestrated by the tightly regulated interplay between cyclins D, E, and A, CDK4, CDK6, and CDK2, and their inhibitors. Examining the expression of these regulators in relation to conditionally expressed RASSF1A, we observed reduced expression of cyclin A and increased levels of $p21^{Cip1/Waf1}$ (Fig. 3*A*). This was consistent with an arrest later in G_1 phase, which could be executed by the CDK

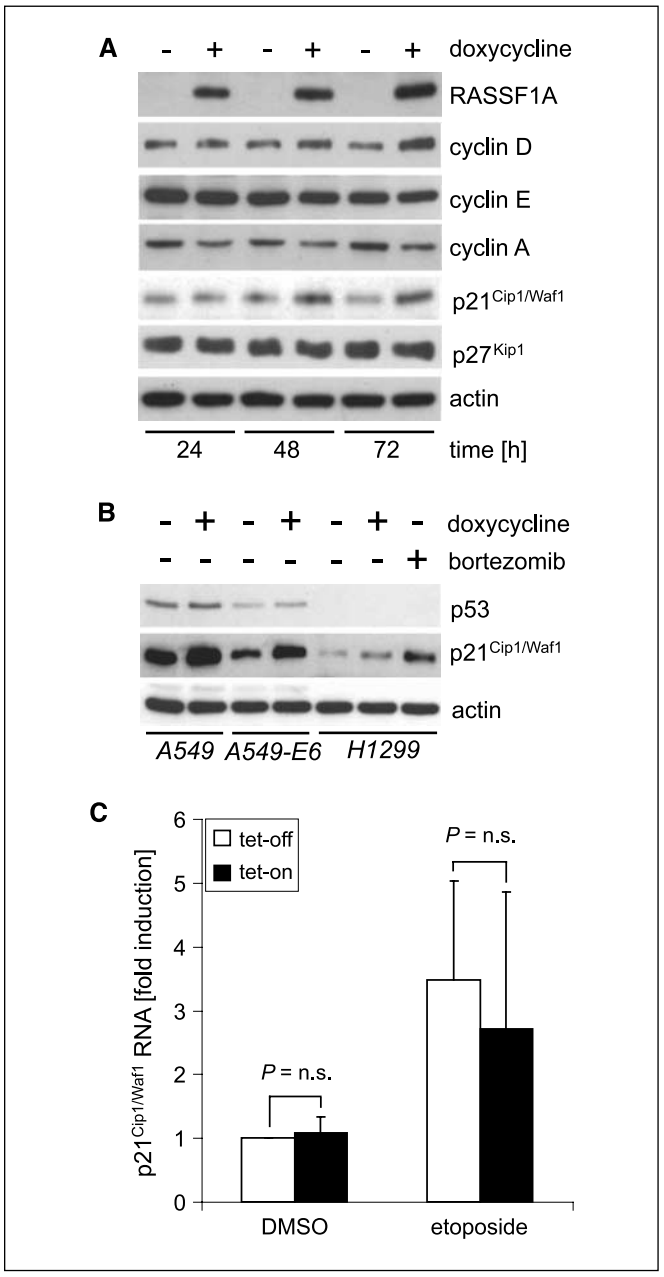

Figure 3. RASSF1A regulates $p21^{Cip1/Waf1}$ independently of p53. *A* and *B,* asynchronous conditional A549 or NCI-H1299 cells were grown in the presence or absence of doxycycline to induce RASSF1A expression. Cell extracts were obtained at several time points and analyzed by immunoblotting using the indicated primary antibodies. The HPV16 E6 oncogene was expressed to destabilize p53, and the proteasome inhibitor bortezomib was used to achieve high endogenous $p21^{Cip1/Waf1}$ levels in p53-deficient NCI-H1299 cells. *C,* conditional A549 cells growing in the absence (*tet-off*) or presence (*tet-on*) of doxycycline to induce RASSF1A were treated with the DNA-damaging agent etoposide (25 μmol/L, 6 h). RNA was extracted, and the transcriptional induction of $p21^{Cip1/Waf1}$ in relation to a housekeeping gene was assessed by reverse transcription and quantitative PCR (LightCycler, Roche) analysis (mean values ± SD and *P* values).

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inhibitor $p21^{Cip1/Waf1}$, a transcriptional target of the p53 tumor suppressor (21, 22). Oncogene-induced cell cycle arrest and senescence are generally regulated via p53 and pRb (5). As A549 cells have maintained functional p53, we sought to study whether RASSF1A induced $p21^{Cip1/Waf1}$ in a p53-dependent pathway. RASSF1A still up-regulated $p21^{Cip1/Waf1}$ in A549 cells that display reduced p53 levels through expressing the HPV16 oncogene E6, which destabilizes p53, as well as in NCI-H1299 lung cancer cells, which are homozygously deleted for p53 (Fig. 3*B*). p53-dependent induction of $p21^{Cip1/Waf1}$ RNA expression, in response to DNA damage, was not altered by RASSF1A (Fig. 3*C*). Collectively, these findings ruled out an essential involvement of p53 in the regulation of $p21^{Cip1/Waf1}$ by RASSF1A. The CDK inhibitor $p16^{Ink4a}$ is another p53-independent regulator of oncogene-induced cell cycle arrest and senescence (5, 13). As A549 cells are deficient in $p16^{Ink4a}$ (Supplementary Fig. S1*A*), in the present cancer model, RASSF1A apparently mediates cell cycle arrest via a distinct pathway affecting $p21^{Cip1/Waf1}$ and pRb.

Inhibition of CDK4 and CDK2 by $p21^{Cip1/Waf1}$ prevents phosphorylation of pRb and progression to S phase of the cell cycle (21, 22). The HPV16 oncogene E7 inactivates and destabilizes hypophosphorylated pRb to bypass the requirement of CDK activity for S-phase entry (39). Furthermore, HPV16 E7 can transform primary fibroblasts in cooperation with oncogenic Ras (40) and abolishes $p21^{Cip1/Waf1}$-mediated growth arrest caused by activation of the Raf-MEK-ERK pathway (27). Activation of the Raf-MEK-ERK pathway leads to nuclear accumulation of $p21^{Cip1/Waf1}$ whereas the HPV16 E7 protein prevents nuclear accumulation of $p21^{Cip1/Waf1}$, resulting in decreased association of $p21^{Cip1/Waf1}$ with cyclin E–CDK2 (31). Thus, HPV16 E7 abrogates $p21^{Cip1/Waf1}$-mediated inhibition of cyclin E–CDK2 activity (31, 41).

In A549 cells devoid of RASSF1A, heterologous expression of HPV16 E7 conferred no selective advantage. However, when RASSF1A was induced by doxycycline, A549 cells expressing HPV16 E7 outgrew their HPV16 E7-deficient counterparts (Fig. 4*A*). Despite the fact that HPV16 E7 increased basal expression of $p21^{Cip1/Waf1}$, its up-regulation by RASSF1A was preserved in the presence of the viral oncogene (Fig. 4*C*). Accordingly, RASSF1A-induced senescence was prevented by coexpression of HPV E7 oncogene (Fig. 4*B*). Thus, RASSF1A-dependent tumor suppression and senescence proceed probably via $p21^{Cip1/Waf1}$-dependent regulation of pRb. To further confirm that $p21^{Cip1/Waf1}$ is necessary for the tumor-suppressive activity of RASSF1A, we made use of HCT116 colorectal carcinoma cells with targeted genetic ablation of $p21^{Cip1/Waf1}$ ($p21)^{-/-}$ and their $p21^{Cip1/Waf1}$-proficient counterparts (42). Whereas expression of RASSF1A dramatically reduced clonogenic survival of $p21^{+/+}$ HCT116 cells, their $p21^{-/-}$ counterparts in fact displayed a significant increase in clonogenic survival compared with cells expressing a GFP control vector (Fig. 4*D*). In agreement with our findings in A549 cells, RASSF1A expression increased endogenous $p21^{Cip1/Waf1}$ levels in $p21^{Cip1/Waf1}$-proficient HCT116 cells (Fig. 4*D*). These results confirm that RASSF1A-mediated tumor suppression is strictly dependent on the CDK inhibitor $p21^{Cip1/Waf1}$. The surprising observation that in the absence of $p21^{Cip1/Waf1}$ RASSF1A

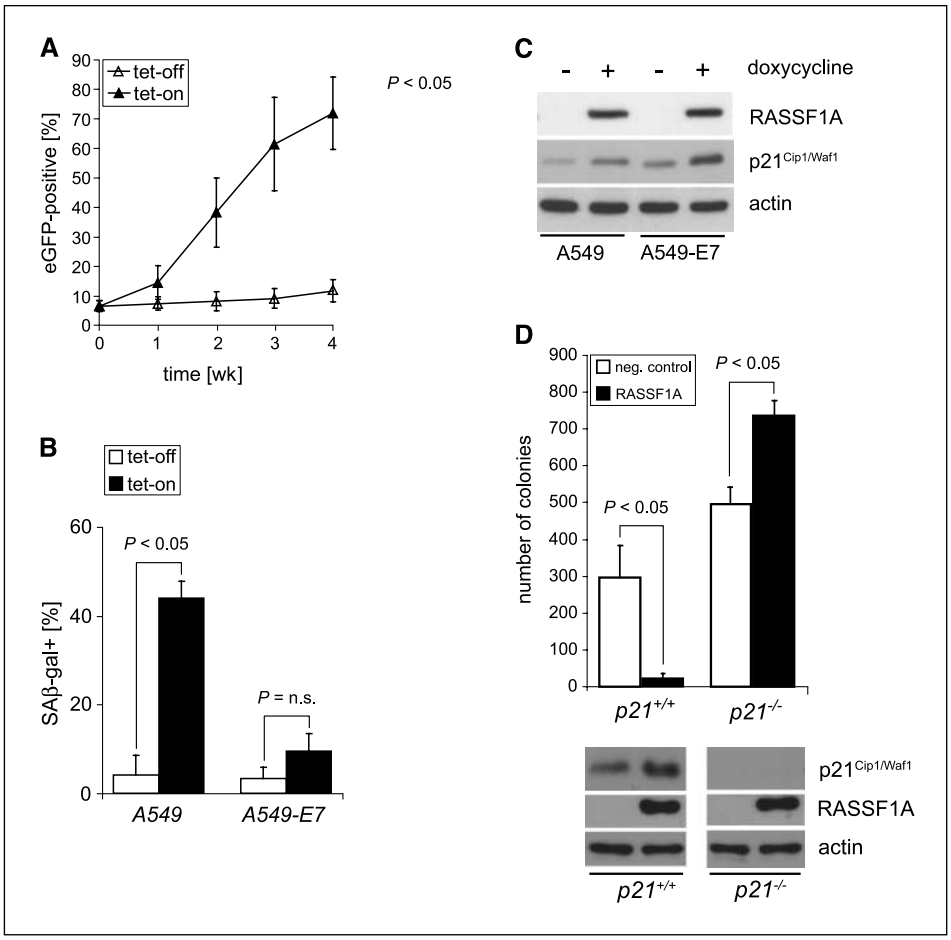

Figure 4. RASSF1A-mediated cell cycle arrest and senescence require $p21^{Cip1/Waf1}$. *A,* conditional A549 cells were mixed with conditional A549 cells expressing HPV16 E7 plus eGFP from a bicistronic cassette and were grown in the presence (*tet-on*) or absence (*tet-off*) of doxycycline. The E7/eGFP-expressing population (mean value ± SD from four experiments) outgrew the parental cells only when RASSF1A was induced. *B,* induction of senescence by conditionally expressed RASSF1A was compared in A549 cells expressing the HPV16 oncogene E7 or control vector. *C,* HPV16 E7 increased basal $p21^{Cip1/Waf1}$ expression but did not prevent the induction of $p21^{Cip1/Waf1}$ by conditionally expressed RASSF1A. *D,* HCT116 cells ($p21^{+/+}$) and HCT116 cells with targeted ablation of $p21^{Cip1/Waf1}$ ($p21^{-/-}$) were retrovirally transduced to express RASSF1A or eGFP (as control) plus a puromycin resistance gene. Clones arising under selection with puromycin were enumerated (*left*; mean values ± SD and *P* values of three experiments). The expression of $p21^{Cip1/Waf1}$ and RASSF1A was confirmed by immunoblotting.

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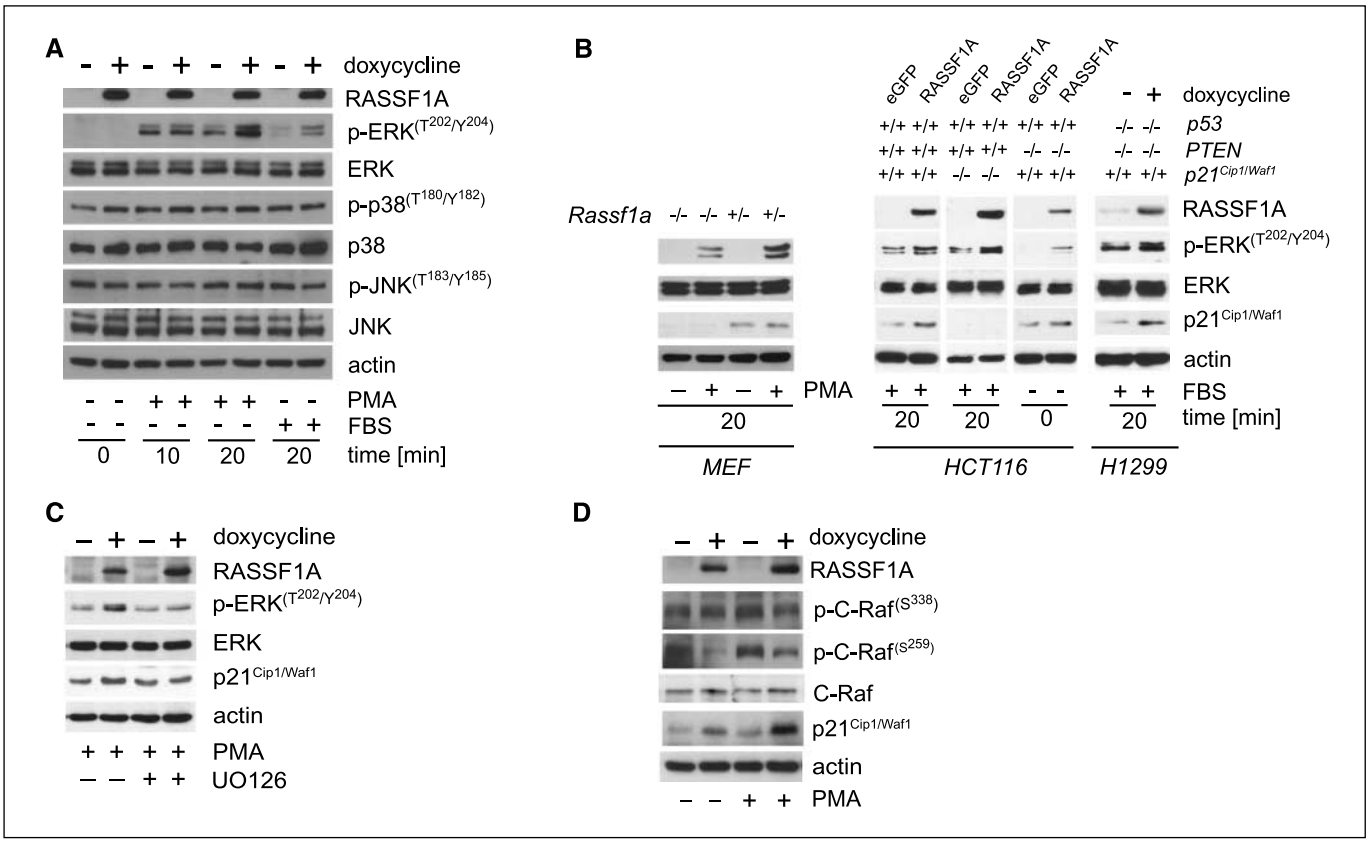

Figure 5. Exogenous and endogenous RASSF1A increases $p21^{Cip1/Waf1}$ expression by modulating ERK activation. *A,* asynchronous conditional A549 cells were grown in the presence (*tet-on*) or absence (*tet-off*) of doxycycline to induce RASSF1A expression. To study phosphorylation events, serum-starved cells were stimulated by the addition of PMA (1 μg/mL, 10 and 20 min) or FBS (10%, 20 min). Cell extracts were obtained at the above-indicated time points and analyzed by immunoblotting. *B,* murine embryonic fibroblasts from *Rassf1a*-deficient (*Rassf1a*$^{-/-}$) and *Rassf1a*-proficient (*Rassf1a*$^{+/-}$) mice were cultured under serum-free conditions for 16 h followed by treatment with PMA (1 μg/mL, 20 min, *left*). Parental HCT116 colorectal cancer cells, as well as HCT116 cells, with targeted ablation of $p21^{Cip1/Waf1}$ (*p21*$^{-/-}$) or PTEN (*PTEN*$^{-/-}$) were retrovirally transduced to express RASSF1A or eGFP (as control) plus a puromycin resistance gene. Cells were serum-starved overnight, followed by FBS stimulation (10%, 20 min, *center*). Asynchronous conditional NCI-H1299 cells were grown in the presence (*tet-on*) or absence (*tet-off*) of doxycycline to induce RASSF1A expression. To study phosphorylation events, cells were serum-starved overnight, followed by FBS stimulation (10%, 20 min, *right*). In all experiments, cell extracts were obtained at the indicated time points, and ERK phosphorylation or $p21^{Cip1/Waf1}$ expression was analyzed by immunoblotting. *C,* immunoblot analysis of cell extracts prepared from conditional A549 cells grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline to induce RASSF1A expression. Cells were serum-starved overnight, followed by stimulation with PMA (1 μg/mL, 20 min) in the absence or presence of the MEK inhibitor UO126 (20 μmol/L). *D,* conditional A549 cells grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline to induce RASSF1A expression were serum-starved overnight, followed by treatment with PMA (1 μg/mL, 20 min). Constitutive and PMA-induced phosphorylation of C-Raf and $p21^{Cip1/Waf1}$ induction were detected by immunoblotting.

seems to promote clonogenicity of HCT116 cells suggests that RASSF1A activates intracellular signaling cascades, which may lead to enhanced proliferation once the antagonism by the CDK inhibitor $p21^{Cip1/Waf1}$ is lost. In keeping, genetic loss of $p21^{Cip1/Waf1}$ confers a proliferative advantage to Ras-expressing embryonic fibroblasts and promotes Ras-induced epithelial tumorigenesis (28).

RASSF1A induces $p21^{Cip1}$ by modulating mitogen-activated protein kinase signaling. HPV16 E7 abolishes $p21^{Cip1/Waf1}$-mediated growth arrest caused by activation of Raf. Coexpression of HPV16 E7 overrides RASSF1A-mediated cell cycle arrest and senescence in RASSF1A expressing A549 cells (Fig. 4*A* and *B*) without altering RASSF1A-mediated up-regulation of $p21^{Cip1/Waf1}$ (Fig. 4*C*). These findings either point at a direct effect of RASSF1A on $p21^{Cip1/Waf1}$ or suggest an indirect mode of action via modulation of the Ras-MEF-ERK signaling pathway. Activating mutations of the Ras oncogene are found in approximately one third of all human cancers, and A549 cells harbor the *K*-ras mutation. Active Ras can induce $p21^{Cip1/Waf1}$ expression and cell cycle arrest via the $p14^{Arf}$-p53 pathway, which is defective in A549 cells (Supplementary Fig. S1*A*) and via mitogen-activated protein kinase (MAPK) signaling (43). Interestingly, HPV16 E7 abolishes $p21^{Cip1/Waf1}$-mediated growth arrest caused by the activation of the Raf-MEK-ERK pathway (31). This observation parallels our finding that coexpression of HPV16 E7 overrides RASSF1A-mediated cell cycle arrest and senescence in A549 cells (Fig. 4*A* and *B*) without altering RASSF1A-mediated up-regulation of $p21^{Cip1/Waf1}$ (Fig. 4*C*). Against this background, we reasoned whether RASSF1A-mediated up-regulation of $p21^{Cip1/Waf1}$ proceeds via activation of the Raf-MEK-ERK pathway.

After stimulation with the mitogen phorbol 12-myristate 13-acetate (PMA) or serum, conditionally expressed RASSF1A specifically enhanced the activating phosphorylation of ERK, but not of p38 kinase or c-Jun NH_2 kinase (Fig. 5*A*). In agreement, $p21^{Cip1/Waf1}$ expression and PMA-induced ERK phosphorylation were markedly reduced in *Rassf1a*$^{-/-}$ MEF (Fig. 5*B*). Serum stimulation of p53-deficient NCI-H1299 lung cancer cells conditionally expressing RASSF1A equally led to enhanced ERK phosphorylation and up-regulation of $p21^{Cip1/Waf1}$ (Fig. 5*B*). To

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further support the role of $p21^{Cip1/Waf1}$ as a main effector of the tumor-suppressive activity of RASSF1A, we again turned to the HCT116 model. RASSF1A equally modulated ERK phosphorylation in $p21^{-/-}$ and $p21^{+/+}$ HCT116 cells, suggesting that up-regulation of $p21^{Cip1/Waf1}$ by RASSF1A occurs downstream of ERK (Fig. 5*B*). This was further corroborated using conditional A549 cells grown in the presence or absence of the MEK inhibitor UO126. Pharmacologic inhibition of MEK activity clearly prevented RASSF1A-dependent up-regulation of $p21^{Cip1/Waf1}$ by PMA (Fig. 5*C*). Taken together, these data support a model of RASSF1A-mediated up-regulation of $p21^{Cip1/Waf1}$ in cancer cells through enhancement of oncogene-induced activation of Raf-MEK-ERK signaling. These findings help to explain our observation that RASSF1A expressing $p21^{-/-}$ HCT116 cells displayed increased colony formation (Fig. 4*D*).

RASSF1A modulates MAPK signaling and negatively regulates Akt. Raf is the upstream regulator of MEK, which is activated by phosphorylation at Ser^{338}. Raf itself is negatively regulated by

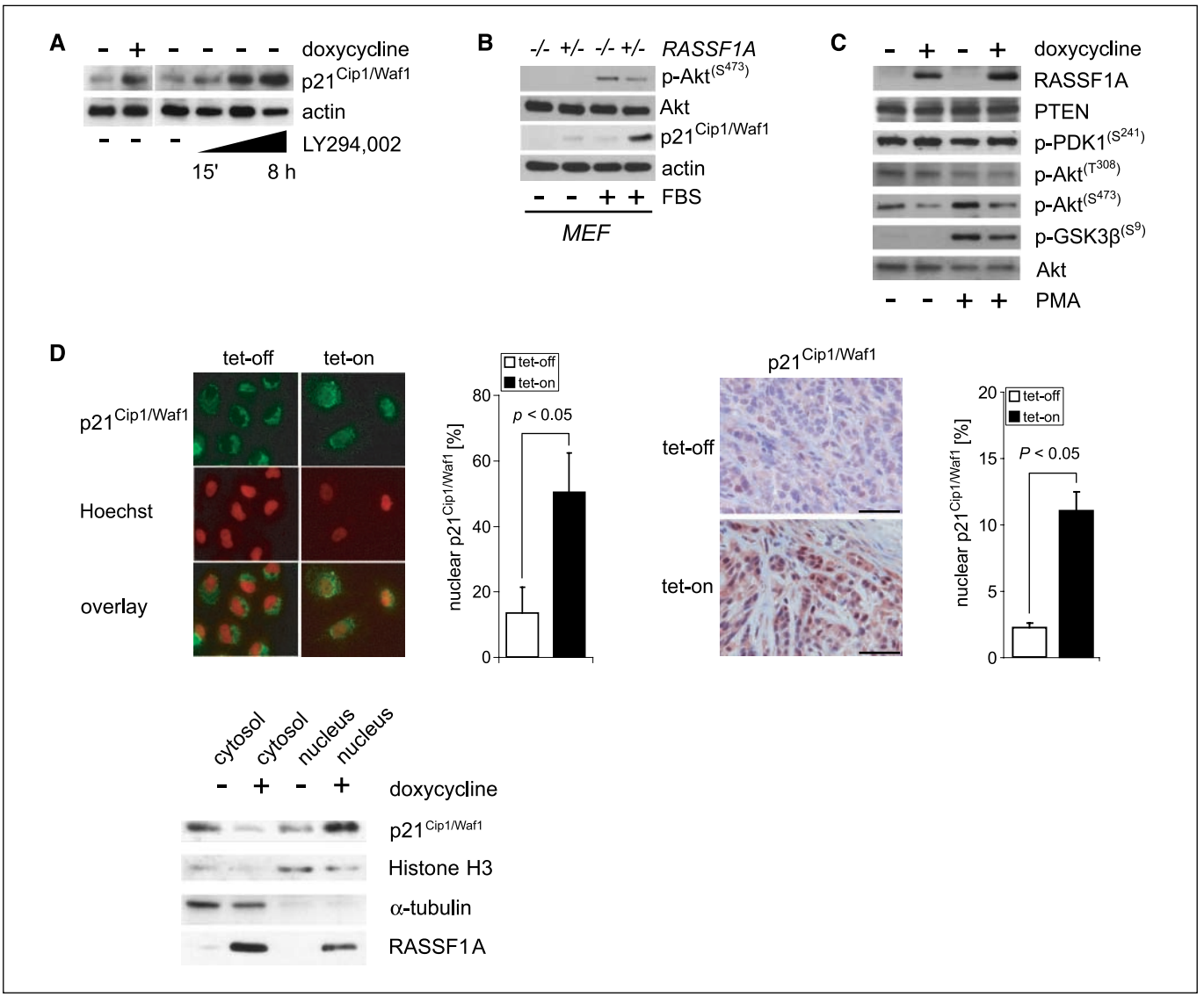

Figure 6. RASSF1A influences $p21^{Cip1/Waf1}$ translocation to the nucleus. *A,* conditional A549 cells were grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline to induce RASSF1A expression (*lanes 1 and 2*). Conditional A549 cells grown in the absence of doxycycline (*tet-off*) were treated with the PI3K inhibitor LY294,002 (30 μmol/L, for 15 min, 4 h, and 8 h; *lanes 3–6*). Note that both conditional expression of RASSF1A and PI3K inhibition result in increased expression of endogenous $p21^{Cip1/Waf1}$. *B,* embryonic fibroblasts derived from *Rassf1a*-deficient ($Rassf1a^{-/-}$) and *Rassf1a*-proficient ($Rassf1a^{+/-}$) mice were cultured under serum-free conditions for 16 h, followed by FBS stimulation (10%, 20 min). Constitutive and FBS-induced phosphorylation of Akt and $p21^{Cip1/Waf1}$ expression were analyzed by immunoblotting. *C,* serum-starved conditional A549 cells were grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline, followed by treatment with PMA (1 μg/mL, 20 min). Constitutive and PMA-induced phosphorylation of PDK1, Akt, and GSK-3β was detected by immunoblotting. *D,* cytosolic and nuclear extracts were prepared from conditional A549 cells grown in the absence (*tet-off*) or presence (*tet-on*) of doxycycline. Subcellular localization of $p21^{Cip1/Waf1}$, histone H3, α-tubulin, and RASSF1A were detected by immunoblotting, as indicated (*bottom*). Conditional A549 cells were grown on chamber slides in the absence (*tet-off*) or presence (*tet-on*) of doxycycline to induce RASSF1A. Endogenous $p21^{Cip1/Waf1}$ (*green*) was detected by indirect immunofluorescence, and nuclear DNA was counterstained with Hoechst 33342 (*red, left*; magnification, 100×). The fraction of cells with $p21^{Cip1/Waf1}$ localized to the nucleus was enumerated (*right*). Mean values ± SD of four experiments (*left*). Conditional A549 cells were s.c. injected in NOD/SCID mice to establish xenograft tumors. After 28 d, one group was fed with doxycycline for 4 d to induce RASSF1A expression *in vivo*, whereas the other group received vehicle. Paraffin-embedded sections from explanted tumors were stained for $p21^{Cip1/Waf1}$ (magnification, 40×; *bars,* 50 μm). The fraction of cells with nuclear $p21^{Cip1/Waf1}$ staining was enumerated in three random visual fields per tumor section (mean values ± SD of three random fields from two different tumors of each group, *right*).

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phosphorylation at Ser^{259} through Akt, an important effector of phosphoinositide 3-kinase (PI3K) signaling (30, 44). Maintaining activation of Akt bypasses Raf-induced cell cycle arrest, indicating that loss of Akt activity is necessary for growth arrest mediated by the Raf-MEK-ERK pathway (31). Against this background, we reasoned whether RASSF1A might enhance Raf-MEK-ERK signaling by functional inhibition of Akt. To this end, Raf phosphorylation was studied in conditional A549 cells after treatment with PMA. Indeed, RASSF1A expression decreased both constitutive and mitogen-induced phosphorylation of Raf at Ser^{259}, which closely correlated with up-regulation of $p21^{Cip1/Waf1}$ (Fig. 5*D*). To confirm Akt-dependent regulation of $p21^{Cip1/Waf1}$ expression in our experimental system, conditional A549 cells were treated with LY294,002, a pharmacologic PI3K inhibitor. Increasing concentrations of LY 294,002 resulted to up-regulation of endogenous $p21^{Cip1/Waf1}$, thus indicating that PI3K/Akt signaling negatively regulates $p21^{Cip1/Waf1}$ expression (Fig. 6*A*). In support, *Rassf1a*$^{-/-}$ MEF exhibited enhanced phosphorylation of endogenous Akt and reduced expression of $p21^{Cip1/Waf1}$ compared with *Rassf1a*-proficient control MEF (Fig. 6*B*). Thus, conditional RASSF1A, as well as endogenously expressed Rassf1a, seem to modulate ERK phosphorylation and $p21^{Cip1/Waf1}$ activity at the level of Akt.

To further study how RASSF1A acts as inhibitor of Akt, we assessed the effect of conditional RASSF1A expression on activating phosphorylation of Akt. After recruitment to plasma membrane, Akt is activated through phosphorylation at Thr^{308} by PI3K-dependent kinase 1 (PDK1), which itself is negatively regulated by the tumor suppressor phosphatase and tensin homologue deleted in chromosome 10 (PTEN). Another activating Akt phosphorylation occurs at Ser^{473} by the mTOR-Rictor complex, which acts downstream of Akt itself (45). In A549 lung cancer cells, conditionally expressed RASSF1A specifically inhibited phosphorylation of Akt at Ser^{473} but not at Thr^{308} (Fig. 6*C*). Moreover, expressing RASSF1A in NCI-H1299 cells, which are homozygously deleted in PTEN and HCT116 colorectal carcinoma cells with targeted ablation of *PTEN* (*PTEN*$^{-/-}$), left RASSF1A-mediated enhancement of MAPK signaling unaltered (Fig. 5*B*). These findings suggest that inhibition of Akt by RASSF1A proceeds independently of PTEN and downstream of PDK1.

Ras transduces extracellular signals through a multitude of intracellular cascades. Raf and Akt are components of two parallel Ras-triggered signaling pathways, which functionally antagonize each other. Activated Raf itself probably down-regulates Akt function, indicating the existence of a potential bidirectional cross-talk between these two Ras effector pathways (31). This antagonism between Raf and Akt suggests that the outcome of simultaneous stimulation of both pathways is determined by the extent and duration of the respective stimulus. In this context, the tumor suppressor RASSF1A may shift the balance toward the predominance of oncogene-triggered MAPK activation, resulting in $p21^{Cip1/Waf1}$-dependent cell cycle arrest, whereas Akt-mediated survival and proliferation signals are suppressed. This model is consistent with our observation that RASSF1A-mediated cell cycle arrest and senescence are overcome by HPV16 E7 (Fig. 4*A* and *B*). Recently, it has been shown that the HPV16 E7 can interact with PP2A, thus interfering with the ability of PP2A to dephosphorylate and inhibit Akt (46). In addition, it has been suggested that the ability of HPV16 E7 to up-regulate Akt activity is linked to its capacity to bind and inactivate pRb (47). To this point, the exact mechanism of RASSF1A-mediated modulation of the MAPK and Akt pathways remains unclear. As RASSF1A itself lacks enzymatic activity, the protein may serve as a scaffold for the integration of multiple Ras-induced signaling pathways. However, a direct physical interaction between Ras and RASSF1A under physiologic conditions has yet to be confirmed (37). Interestingly, the phosphorylation of glycogen synthase kinase 3-β (GSK3-β), a downstream target of Akt, was also reduced by RASSF1A (Fig. 6*C*). These results suggest that RASSF1A may affect additional Akt-regulated pathways involving downstream targets other than Raf and $p21^{Cip1/Waf1}$. Recently, it was shown that caspase cleavage products of MST1 and MST2 are direct inhibitors of Akt1 (48). RASSF1A was shown to induce apoptosis through the disruption of the inhibitory Raf1-MST2 complex (49). In this context, it is conceivable that RASSF1A may lead to inactivation of Akt1 by the liberation of MST2 from the inhibitory Raf-MST2 complex, which is followed by caspase-mediated cleavage of MST2 and inhibition of Akt1 by the MST2 cleavage products.

RASSF1A controls nuclear localization and expression of $p21^{Cip1/Waf1}$ via negative regulation of Akt. The activity of $p21^{Cip1/Waf1}$ as CDK inhibitor requires its nuclear localization. Akt was shown to directly phosphorylate $p21^{Cip1/Waf1}$ resulting in its cytoplasmic sequestration and inactivation (50). Using subcellular fractionation and immunoblot analysis, as well as indirect immunocytochemistry (Fig. 6*D*), we could show that conditionally expressed RASSF1A increased the fraction of A549 lung cancer cells with nuclear localization of endogenous $p21^{Cip1/Waf1}$ *in vitro* (Fig. 6*D*). In addition, immunohistochemical analysis of conditional A549 xenograft tumors from doxycycline-fed mice revealed enhanced expression and nuclear accumulation of $p21^{Cip1/Waf1}$ (Figs. 2*D* and 6*D*). Accordingly, modulation of Akt signaling by RASSF1A enhances the activity of the CDK inhibitor $p21^{Cip1/Waf1}$ at two levels, MAPK-mediated up-regulation and Akt-regulated nuclear localization.

Taken together, our findings show that RASSF1A can confer its tumor-suppressive activity via $p21^{Cip1/Waf1}$-dependent inhibition of S-phase progression. As A549 cells are also defective in $p14^{Arf}$ and $p16^{Ink4a}$ (Supplementary Fig. S1*A*) and p53 is not involved in RASSF1A-mediated growth arrest (Fig. 3*B*), RASSF1A induces cell cycle arrest and senescence in A549 human cancer cells through a distinct $p21^{Cip1/Waf1}$-dependent pathway. Conditional, as well as endogenous, expression of RASSF1A led to enhanced ERK phosphorylation correlating with increased $p21^{Cip1/Waf1}$ levels in all cellular systems studied here. Expression of the HPV16 oncoprotein E7, pharmacologic inhibition of MEK, or absence of $p21^{Cip1/Waf1}$ abolished RASSF1A-mediated downstream activities. These findings suggest a central role for the RASSF1A tumor suppressor in the integration and inhibition of oncogene-induced proliferation and survival signals in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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RASSF1A Mediates p21$^{Cip1/Waf1}$-Dependent Cell Cycle Arrest and Senescence through Modulation of the Raf-MEK-ERK Pathway and Inhibition of Akt

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