Dysfunction of Nucleus Accumbens-1 Activates Cellular Senescence and Inhibits Tumor Cell Proliferation and Oncogenesis

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Abstract

Nucleus accumbens-1 (NAC1), a nuclear factor belonging to the BTB/POZ gene family, has emerging roles in cancer. We report here that NAC1 acts as a negative regulator of cellular senescence in transformed and nontransformed cells, and dysfunction of NAC1 induces senescence and inhibits its oncogenic potential. We show that NAC1 deficiency markedly activates senescence and inhibits proliferation in tumor cells treated with sublethal doses of γ-irradiation. In mouse embryonic fibroblasts from NAC1 knockout mice, following infection with a Ras virus, NAC1−/− cells undergo significantly more senescence and are either nontransformed or less transformed in vitro and less tumorigenic in vivo when compared with NAC1+/+ cells. Furthermore, we show that the NAC1-caused senescence blunting is mediated by ΔNp63, which exerts its effect on senescence through p21, and that NAC1 activates transcription of ΔNp63 under stressful conditions. Our results not only reveal a previously unrecognized function of NAC1, the molecular pathway involved and its impact on pathogenesis of tumor initiation and development, but also identify a novel senescence regulator that may be exploited as a potential target for cancer prevention and treatment. Cancer Res; 1–14. ©2012 AACR.

Introduction

Cellular senescence is a state in which cells lose the capacity to divide and are irreversibly arrested, and are usually characterized by flattened and enlarged morphology, increased cytoplasmic granularity, and up-regulated activity of senescence-associated β-galactosidase (SA-β-gal; 1, 2). Senescence can occur after a number of cell divisions or be induced by some stimuli such as oncogenes, radiation, and so on. It is now becoming increasingly clear that oncogene-induced senescence (OIS) is one of the critical mechanisms that prevent tumor initiation, development and progression (3), and evasion of cellular senescence constitutes one of the major contributors to tumorigenesis. Cancer cells can also be forced to undergo senescence by therapeutic interventions such as chemotherapeutic agents and radiotherapy. Therapy-induced senescence (TIS) can influence the outcome of treatments (4–6). It is now generally appreciated that evasion of senescence is an important event in tumorigenesis, cancer recurrence, and treatment failure (7–9). Yet, the molecular determinants and mechanisms underlying senescence bypass remain poorly defined.

Nucleus accumbens-1 (NAC1) is a transcription factor repressor that belongs to the BTB/POZ gene family. The highly conserved BTB/POZ domain is required for NAC1 homodimerization, and the homodimer complex participates in regulating a variety of biologic functions (10). Overexpression of NAC1 is found in several types of human carcinomas including ovarian cancer, cervical cancer, breast cancer, and colon cancer (11–13). It was observed in patients with ovarian cancer that the expression level of NAC1 is significantly higher in recurrent posttreatment tumors than pretreated primary tumors (11, 12, 14). Also, NAC1, which encodes NAC1, is amplified in many ovarian high-grade serous carcinomas (15). There are studies reporting that upregulation of NAC1 promotes tumor cell growth and survival, migration, and invasion, and resistance to chemotherapeutic drugs (12, 16–18). We recently reported that NAC1 promotes a prosurvival autophagy through the HMGB1-mediated pathway and contributes to cisplatin resistance (19). These studies suggest that expression of NAC1 not only bestows oncogenic potential, but may also undermine therapeutic outcomes. Nevertheless, the precise functions of NAC1 in tumor initiation, development, and progression are still not well elucidated.

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In this study, we have uncovered a novel function of NAC1, which may serve as an important mechanism contributing to its oncogenic potential. We found that NAC1 acts as a negative regulator of cellular senescence, blunting radiation, or oncogene-induced cellular senescence through modulation of ΔNp63 expression. NAC1-mediated blunting of senescence enhances tumor cell proliferation, bolsters Ras-mediated transformation of mouse embryonic fibroblasts (MEF), and promotes tumor formation. Our study has not only revealed a previously unrecognized function of NAC1 in cancer and its impact on pathogenesis of tumor development and progression, but also identified a new senescence regulator that may be exploited as a potential target for cancer prevention and treatment.

Materials and Methods

Cell lines and cell culture

Human ovarian cancer cell lines SKOV3 and A2780, and human cervical cancer cell line HeLa, were purchased from American Type Culture Collection (Manassas, VA). SKOV3/N130 and HeLa/N130 lines were generated by introduction of an inducible (Tet-Off) expression construct of a NAC1 deletion mutant (N130). SKOV3/N130 and HeLa/N130 cells were cultured in RPMI-1640 medium supplemented with 10% FBS; primary wild-type, NAC1+/−, and NAC1−/− MEFs were derived from NAC1 knockout mouse embryo and the wild-type littermate, and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS. A2780 cells were also cultured in DMEM supplemented with 10% FBS. All of the cell culture media contain 100 U/mL penicillin and 100 mg/mL streptomycin.

siRNA and plasmid transfection

siRNAs targeting NAC1, ΔNp63, p53, p21, and the nontargeting siRNA were synthesized by Qiagen or Cell Signaling. Transfection of siRNA was conducted according to the manufacturer’s protocol. Briefly, cells in exponential phase of growth were plated in 6-well cell culture plates at 1 × 10^5 cells/well, grown for 24 hours, and then transfected with siRNA using oligofectamine and OPTI-MEM I–reduced serum medium (Invitrogen). Concentrations of siRNA were chosen based on dose–response studies. pCDNA3.1-FLAG-ΔNp63 plasmid was a gift from Dr. Edward Ratovitski (Department of Dermatology, Johns Hopkins University School of Medicine, MD). Transfection of the plasmid was carried out using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

SA-β-gal assay

Activity of SA-β-gal was measured as described (20). Briefly, cells were fixed with 0.2% glutaraldehyde for 15 minutes at room temperature, washed thrice with PBS, and incubated at 37°C overnight in SA-β-gal solution (1 mg/mL X-gal, 5 mmol/L K₃Fe(CN)₆, 5 mmol/L K₄Fe(CN)₉, 150 mmol/L NaCl, and 2 mmol/L MgCl₂ in PBS at pH 6.0). Blue stained senescent cells were counted under a light microscope.

Cell proliferation assay

Cell proliferation was measured using a BrdUrd Cell Proliferation Assay Kit from Millipore, according to the manufacturer’s instruction.

Clonogenic assay

Cells subjected to different treatments were plated in 35-mm tissue culture dishes (numbers of cells, varying with different cell lines, was determined experimentally to generate single colonies). Following incubation at 37°C in a humidified atmosphere containing 5% CO₂/95% air for 10 days, cells were stained with 1% methylene blue in 50% methanol and colonies (>50 cells) were counted.

Cell-cycle analysis

Cell cycle was analyzed using the method of propidium iodide staining. Briefly, cells were plated in 96-well round bottom plates at a density 2 × 10^5 cells per well, centrifuged at 450 × g for 5 minutes, and fixed with ice-cold 70% ethanol for 12 hours at 4°C. Cells were then washed with PBS and incubated with 200 μL of Guava Cell Cycle Reagent (Millipore) for 30 minutes at room temperature in the dark. Samples were analyzed on Guava EasyCyte Plus Flow Cytometry System (Millipore).

Soft agar assay

Cells were suspended in complete culture medium containing 0.3% soft agar, seeded (5 × 10^4 cells/well) in 6-well plates precoated with 0.6% agar in complete culture medium, and then incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air for 12 to 14 days. At the end of incubation, colonies were counted and photographed.

Apoptosis assay

Apoptosis was determined by flow cytometric analysis of Annexin V and 7-amoactinomycin D staining. Briefly, 100 μL of Guava Nexin reagent (Millipore) was added to 1 × 10^5 cells in 100 μL, and the cells were incubated with the reagent for 20 minutes at room temperature in the dark. At the end of incubation, the cells were analyzed by a Guava EasyCyte Plus Flow Cytometry System (Millipore).

Western blotting and antibodies

Cells were lysed in M-PER mammalian protein extraction reagent (Roche) supplemented with a cocktail of protease inhibitors (Roche), followed by centrifugation at 14,000 × g for 10 minutes. After centrifugation, cell lysates were collected and protein concentrations were measured. Protein (10–20 μg) were resolved by SDS-PAGE, and then transferred to PVDF membrane (Bio-Rad). The membranes were incubated with primary antibodies in 3% BSA/TBST for overnight, followed by incubation with secondary antibodies at room temperature for 1 hour. The protein signals were detected by ECL method. Antibodies to Ras were purchased from BD Bioscience, antibodies to p21, ΔNp63, TAp63, and p53 were purchased from Santa Cruz Biotechnology Inc.; α-tubulin antibody was purchased from Cell Signaling Technology; and anti-NAC1 antibody was obtained from Abcam Inc.
RNA isolation and quantitative real-time PCR

Total RNA was prepared using TRIzol reagent (Roche). First-strand cDNA was synthesized using Omniscript reverse transcription kit (Qiagen) with random primers, according to the manufacturer’s instruction. Real-time PCR (RT-PCR) was conducted on a Stratagene Mx3005P using Brilliant II SYBR Green QPCR master mix (Stratagene) and the following primer sets: β-actin, 5′-GCCAACAGTCTGCTTG-3′ (forward) and 5′-GCTCAGGAGGACAGTCTTG-3′ (reverse); ΔNp63, 5′-CAGTCCAGGACCGCAAGC-3′ (reverse), 5′-ATCCGGGCCCTCAAAGCAG-3′ (reverse). After 40 cycles, data were collected and analyzed by MxPro software (Stratagene).

Luciferase reporter assay

SKOV3/N130 or HeLa/N130 cells cultured in the presence or absence of doxycycline (3 × 10⁵ cells), or A2780 cells (3 × 10⁵ cells) with or without silencing of NAC1 expression, were transfected with 1 μg of ΔNp63 reporter construct, pGL3-823-Luc vector (a generous gift from Dr. Jan-Kan Chen, Chang Gung University, Taiwan), and 0.025 μg of PRL vector as an internal control, using FuGENE 6 transfection reagent. Twenty-four hours later, the cells were γ-irradiated. Cells were then rinsed with PBS and lysed in 300 μL 1 × reporter lysis buffer (Promega). For NAC1+/−, NAC1−/− MEFs, 3 × 10⁵ NAC1+/−, and NAC1−/− MEFs were cotransfected with 1 μg of ΔNp63 reporter construct, 0.025 μg PRL control vector, Ras expression construct. Forty-eight hours after transfection, the cells were rinsed with PBS and lysed in 300 μL 1 × reporter passive lysis buffer. Lysates were used directly for luciferase activity assay by using the Dural-Luciferase Reporter Assay System (Promega) and luminometer (Perkin Elmer).

Production and infection retrovirus constructs

A retrovirus carrying the NAC1-V5 expression vector was constructed by cloning the entire coding sequence of NAC1 into the pWZL-Hygro retroviral vector. pBabe-RasV12 was constructed by subcloning a cDNA encoding human H-Ras 12V mutant into pBABE vector. pDCR-vector and pDCR-RasV12 were generous gifts from Dr. Channing Der (University of North Carolina School of Medicine, NC). Packaging cell line 293T was used for retrovirus production. MEFs were infected with viruses for 48 hours, followed by selection with antibiotics. For infection with pWZL or pWZL-NAC1, pBABE or pBabe-RasV12, transduced cells were selected with hygromycin (for immortal cells, 7.5 μg/mL for 2 weeks; for primary cells, 5 μg/mL for 3 days); for infection with pDCR or pDCR-RasV12, transduced cells were selected with G418 (500 μg/mL).

Tumorigenesis study

Female, 5-week-old athymic (nu/nu) mice were purchased from the National Cancer Institute (Frederick, MD) and were housed in sterile filter-capped cages. The animal studies were approved by our Institutional Animal Care and Use Committee. In these experiments, 2 × 10⁶ immortalized or 4 × 10⁶ primary MEFs at exponentially growing phase were harvested, washed, resuspended in 200 μL of culture medium, and injected s.c. into the flank of athymic mice. Tumor sizes were determined by measuring the length (l) and the width (w) with calipers. Tumor volume was calculated using the formula: \( V = lw^2/2 \). At the end of the experiment, the mice were euthanized and tumors were surgically dissected. The tumor specimens were either fixed in 4% paraformaldehyde or frozen in optimal cutting temperature compound (Tissue-Tek). Samples were then processed for histopathologic examination. For mice-bearing HeLa/N130 tumors, they were either given vehicle or doxycycline (125 mg/kg) i.p. daily to suppress expression of N130 mutant.

Statistical analysis

Student t test was used to analyze the significance of difference. Results with \( P < 0.05 \) were considered statistically significant.

Results

Evidence for NAC1 as a negative regulator of senescence in tumor cells

In culturing and growing SKOV3/N130 and HeLa/N130 cells harboring an inducible (Tet-Off) expression construct of a NAC1 deletion mutant (N130; ref. 12), we observed that during activation of this NAC1 deletion mutant, the cells showed a decreased proliferation rate upon continuous passaging as compared with the cells without activation of this NAC1-dominant negative mutant (Supplementary Fig. S1A). We further found that the decreased proliferation was accompanied by increases in the numbers of SA-β-gal–positive cells and in the numbers of cells with altered cellular morphology such as flattening and enlargement (Supplementary Fig. S1B), both of which are considered features of senescent cells. p21, a cyclin-dependent kinase inhibitor and an inducer of cellular senescence (21), was also increased (Supplementary Fig. S1C). These observations, which suggest a possible role for NAC1 in reducing replicative senescence, prompted us to investigate whether the function or expression of NAC1 indeed impacts induction of cellular senescence. Because activation of DNA damage response is a proven cause for induction of senescence (22), we used a sublethal dose of γ-irradiation (6 Gy) to induce senescence. As shown in Fig. 1A, when NAC1 was inactivated upon removal of doxycycline, the morphology of SKOV3/N130 and HeLa/N130 cells subjected to γ-irradiation became spread and flattened, and these cells had a significant increase in the populations with SA-β-gal staining (Fig. 1A); in contrast, these alterations were not observed in the cells without expression of the NAC1-dominant negative mutant (Fig. 1A). In the cells with a dysfunctional NAC1, there was also an increase in the amount of p21 (Fig. 1B). Inactivation of NAC1 also significantly enhanced the proliferation-inhibitory (Fig. 1C) and the colony formation–inhibitory effects (Fig. 1D), and caused a G2–M cell-cycle arrest (Fig. 1E), suggesting that senescence occurs in G2 phase. To ascertain the inhibitory effect of NAC1 on senescence, we knocked down the expression of this protein in A2780 cells, followed by treatment with 4 Gy of γ-irradiation. Multiple NAC1-targeted siRNA sequences were tested to confirm the specificity of the effect of NAC1. Figure 1B shows that 2 NAC1-targeted siRNA sequences selected from several active sequences had similar effects on p21 expression. Silencing of
NAC1 expression in A2780 cells showed similar effects to the dominant mutant on cellular morphology, SA-β-gal activity, p21, cell proliferation, colony formation, and cell-cycle progression (Fig. 1A–D). The enforced proliferative arrest seen in the NAC1-deficient cells did not seem to be a consequence of activation of apoptosis, as there was no significant Annexin V staining in those cells (Supplementary Fig. S2). The role of p21 in senescence induction mediated by NAC1 deficiency was further shown when p21 expression was silenced (Supplementary Fig. S3). Consistently, the effects of NAC1 function on senescence induction were recapitulated in the tumor xenografts of HeLa/N130 cells grown in mice (Fig. 1F). Taken together, all of the above evidence point to a role for NAC1 as a negative regulator of cellular senescence.

**Induction of senescence by inhibiting NAC1 is independent of p53 status**

Figure 1B shows that deficiency of NAC1 resulted in upregulation of p21, a transcriptional target of the p53 family; however, SKOV3 and HeLa cells are either p53-null or have a transcriptionally inactive p53 (23), suggesting that p53 is not necessary for senescence caused by NAC1 dysfunction. To test this hypothesis, we compared SA-β-gal activity, cell proliferation, and p21 level in the wild-type p53-containing A2780 cells when NAC1 was knocked down or both of NAC1 and p53 were knocked down, followed by γ-irradiation treatment. Figure 2 shows that silencing of p53 did not abrogate the effects of NAC1 depletion on senescence (Fig. 2A–C), implying that induction of senescence caused by NAC1 inhibition does not depend on p53 function.
**ΔNp63 is required for the NAC1-mediated senescence evasion**

As p53 was not required for upregulation of p21 caused by NAC1 deficiency, we next examined whether p63, belonging to the p53 protein family, was involved. ΔNp63, a predominant p63 isoform known to antagonize p53 (24), can bind to the promoter of p21, inhibiting its transcription (25). We found that the expression of ΔNp63 protein was markedly downregulated in cells with deficiency of NAC1 after treatment with γ-irradiation (Fig. 3A). In contrast, there were no changes in the expression of TAp63 protein, another isoform of p63 that has similar functions to p53 (Fig. 3A), suggesting that the effect of NAC1 on ΔNp63 expression is specific. To determine whether ΔNp63 is indeed required for the NAC1-mediated suppression of senescence, we silenced the expression of ΔNp63 in cancer cells with active NAC1 function, followed by γ-irradiation. Silencing of ΔNp63 expression either in SKOV3/N130 or HeLa/N130 cells cultured in the presence of doxycycline, or in A2780 cells, led to induction of cellular senescence (Fig. 3B–D). The role of ΔNp63 in NAC1-mediated evasion of senescence was further verified by overexpressing ΔNp63 in NAC1-deficient cells. As shown in Fig. 3E and F, ectopic expression of ΔNp63 in SKOV3/N130 or HeLa/N130 cells with inactivation of NAC1 or in A2780 cells with silencing of NAC1 significantly blunted the activation of senescence caused by γ-irradiation (Fig. 3E and F). These results indicate that ΔNp63 is a necessary mediator of NAC1-mediated bypass of senescence, and that upregulation of p21 may be responsible for induction of senescence resulting from dysfunction of NAC1. Expression of p15, p16, and p18, which are also known to play roles in senescence, did not show differences between the NAC1-activated and -inactivated cells after irradiation treatment (Supplementary Fig. S4).

**Transcription of ΔNp63 is modulated by NAC1**

Because NAC1 was shown to control cellular senescence through ΔNp63 (Fig. 3), next we sought to address whether the functional status of NAC1 plays a role in modulating transcription of ΔNp63 under stressful conditions, as NAC1 is a transcription cofactor. Quantitative RT-PCR (qRT-PCR) analysis showed that inactivation or silencing of NAC1 did not affect the expression of ΔNp63 mRNA in cells without exposure to γ-irradiation; however, NAC1-deficient cells had ~3-fold decreases in ΔNp63 mRNA after treatment with γ-irradiation, as compared with the cells with an intact NAC1 (Fig. 4A). To obtain more direct evidence for transcriptional regulation of ΔNp63 by NAC1, we conducted a reporter gene assay. Cells with or without an intact NAC1 were transiently transfected with a ΔNp63 promoter-luciferase construct, and the activity of the reporter gene (luciferase activity) was measured 48 hours later. Figure 4B shows that as compared with the cells with an intact NAC1 function, the cells lacking NAC1 function showed a 5- to 7-fold reduction in the ΔNp63 promoter activity.
Consistently, the expressions of Dn63 mRNA, the promoter activity, and protein level were similarly downregulated in NAC1⁻/⁻ MEFs transduced with RasV12, as compared with NAC1⁺/⁺ MEFs (Supplementary Fig. S5 and Fig. 5D). These results indicate that NAC1 plays a critical role in promoting the transcription of Dn63 under stress conditions.
NAC1 plays an essential role in blunting Ras-induced senescence in primary MEFs

To explore the role of NAC1-mediated reduction of senescence in oncogenesis, we assessed the effect of NAC1 on oncogene-induced senescence in our genetically engineered primary MEFs that are NAC1<sup>−/−</sup>, NAC1+/−, or NAC1<sup>+</sup>/+. The knockout cells were confirmed by genotyping (Supplementary Fig. S6). Unexpectedly, we observed a low level of senescence in the H-Ras<sup>V12</sup>–infected NAC1<sup>+</sup>/+ MEFs (Fig. 5C and D), likely because of a low activity of the Ras variant used, or because of spontaneous immortalization of the MEFs, which would then lead to sensitivity to transformation by Ras. In contrast, when H-Ras<sup>V12</sup> was transduced into NAC1<sup>−/−</sup> MEFs, the growth of NAC1<sup>−/−</sup> cells was arrested (Fig. 5A), colony formation of NAC1<sup>−/−</sup> cells was inhibited (Fig. 5B), and 80% of the NAC1<sup>−/−</sup> cells were SA-β-gal positive (Fig. 5C), p21 levels were upregulated and ΔNp63 was downregulated (Fig. 5D), indicating that these NAC1<sup>−/−</sup> MEFs were undergoing robust senescence. Similar to NAC1<sup>+/−</sup> MEFs, these H-Ras<sup>V12</sup>-activated senescence responses were also significantly attenuated in NAC1<sup>+/−</sup> MEFs (Fig. 5A–D). To further address the role of NAC1 in weakening the Ras-induced senescence, we tested whether reexpression of NAC1 in NAC1<sup>−/−</sup> MEFs would abrogate cellular senescence. Figure 5E shows that expression of NAC1 in NAC1<sup>−/−</sup> MEFs decreased the senescence response induced by Ras transduction, as evidenced by increased cellular proliferation and decreased SA-β-gal staining. These observations indicate that NAC1 plays an essential role in blunting Ras-induced cellular senescence.

Loss of NAC1 activates cellular senescence and suppresses Ras-induced transformation and tumorigenesis

Finally, to evaluate whether blunting of senescence driven by NAC1 contributes to tumorigenesis, we compared the...
colony-forming ability of NAC1<sup>+/+</sup>/Ras<sup>v12</sup> and NAC1<sup>-/-</sup>/Ras<sup>v12</sup> MEFs. Figure 6A shows that NAC1<sup>+/+</sup> MEFs infected with the control virus did not form any colonies in soft agar, but NAC1<sup>+/+</sup> MEFs infected with Ras<sup>v12</sup> virus were able to form colonies. In contrast, NAC1<sup>-/-</sup> MEFs infected with Ras<sup>v12</sup> virus did not produce any colonies (Fig. 6A). In athymic nude mice, NAC1<sup>+/+</sup>/Ras<sup>v12</sup> MEFs developed tumors with high frequency (5/5; Fig. 6B). Contrastingly, NAC1<sup>-/-</sup>/Ras<sup>v12</sup> MEFs were not tumorigenic in athymic nude mice (Fig. 6B). These results suggest that expression of NAC1 promotes acquisition of transformed phenotype in primary MEFs.

To further show the role of NAC1 in promoting cellular transformation and decreasing senescence, we used the SV40-immortalized NAC1<sup>+/+</sup> and NAC1<sup>-/-</sup> MEFs, as immortalized rodent cell lines can readily be transformed by Ras<sup>v12</sup> alone. Like primary MEFs, after H-Ras<sup>v12</sup> transduction, NAC1<sup>-/-</sup>/SV40 MEFs proliferated slower and formed less colonies than NAC1<sup>+/+</sup>/SV40 (Supplementary Fig. S7). Although expression of Ras<sup>v12</sup> conferred on both NAC1<sup>+/+</sup>/SV40 and NAC1<sup>-/-</sup>/SV40 MEFs, the ability to grow under anchorage-independent conditions, the Ras<sup>v12</sup>-expressing NAC1<sup>+/+</sup>/SV40 cells flourished, forming significantly higher numbers of colonies in soft agar, as compared with Ras<sup>v12</sup>-expressing NAC1<sup>-/-</sup>/SV40 cells (Fig. 6C). NAC1<sup>+/+</sup>/SV40 and NAC1<sup>-/-</sup>/SV40 MEFs infected with the control virus did not form colonies in soft agar (Fig. 6C), indicating that these MEF lines are immortalized but not transformed. Consistently, the Ras<sup>v12</sup>-expressing NAC1<sup>+/+</sup>/SV40 MEFs developed larger tumors more rapidly than the Ras<sup>v12</sup>-expressing NAC1<sup>-/-</sup>/SV40 MEFs in nude mice (Fig. 6D). Most of the tumors derived from the Ras<sup>v12</sup>-expressing NAC1<sup>+/+</sup>/SV40 cells reached a size of ~0.8 cm<sup>3</sup> within 2 weeks after inoculation, whereas the tumors derived from the Ras<sup>v12</sup>-expressing NAC1<sup>-/-</sup>/SV40 cells.
-expressing NAC1−/−/SV40 cells hardly grew to 0.3 cm³ in volume (Fig. 6D). Moreover, HE staining of the tumor specimens showed that the morphology of the RasV12-expressing NAC1+/+ and the RasV12-expressing NAC1−/−/SV40 cells were distinctive: the cells from the RasV12-expressing NAC1+/+/SV40 tumors were densely arranged and elongated, whereas the cells from the RasV12-expressing NAC1−/−/SV40 tumors were round and loosely organized.

Figure 5. A functional NAC1 suppresses Ras-induced senescence in primary MEFs. A, growth curves of NAC1+/+, NAC1+/−, NAC1−/− primary MEFs transduced with a H-RasV12 virus or a control virus. B and C, NAC1+/+, NAC1+/−, NAC1−/− primary MEFs were transduced with a H-RasV12 virus or a control virus. B, forty-eight hours later, cells were plated and incubated for 10 days at 37 °C. Cells were stained and colonies were counted. C, 6 days after transduction, SA-β-gal–positive cells were examined. **, P < 0.01 vs. NAC1−/− + Ras, t test.
that activating cellular senescence by repressing NAC1 can suppress the oncogenic potential of this nuclear factor.

Discussion

This study uncovers NAC1 as a new regulator of cellular senescence. We reveal that the tumor recurrence–associated gene NAC1 negatively regulates senescence, and that blunting of senescence driven by NAC1 may represent an essential molecular function contributing to the oncogenic potential of this nuclear factor. Our results show that under stressful conditions, loss of NAC1 function restores senescence response in tumor cells (Fig. 1) and MEFs (Figs. 5 and 6). More importantly, we show both in vitro and in vivo that blunting of cellular senescence by NAC1 cooperates with Ras to promote cellular transformation and tumor development (Figs. 5 and 6). Although it has been shown that overexpression of NAC1 is linked to cancer development, recurrence, and resistance to therapy, the exact roles that this nuclear factor may play are largely unknown. Thus, our findings provide new insights into the functions of NAC1 in tumor pathogenesis.

Senescence response is often accompanied by activation of tumor-suppressor network such as p53 and pRB pathways, which prevent aberrant and unlimited proliferation of tumor cells (26). Therefore, OIS is believed to serve as an initial barrier in tumor development. p53 has been shown to be involved in activation of cellular senescence under various circumstances (27); however, in this study, induction of senescence caused by loss of NAC1 seems to be independent of p53, as the cells without a functional p53 retain senescence response when NAC1 is inactivated or depleted (Fig. 2). p53-independent induction of senescence was also observed by others (28). NAC1-mediated suppression of senescence, however, requires ΔNp63, a p53 homologue, as even when NAC1 is intact, knockdown of ΔNp63 abolishes bypass of senescence, and when NAC1 is defective, ectopic expression of ΔNp63 blocks induction of the senescence (Fig. 3). ΔNp63 plays a critical role in bypassing cellular senescence (29, 30), and is frequently overexpressed in various types of tumor cells (31–33). Indeed, when senescence is activated in the NAC1-deficient cells subjected to radiation or oncogenic stress, ΔNp63 protein is downregulated (Fig. 3), suggesting that suppression of senescence by NAC1 is mediated via ΔNp63, which inhibits the expression of p21. We further show that NAC1 modulates transcription of ΔNp63 in stressed cells (Supplementary Fig. S5 and Fig. 4). However, in ChIP assays we failed to enrich significant promoter sequences including p63. There are at least 2 explanations for this finding. First, it is likely that the NAC1 antibodies available are not suitable for ChIP. Alternatively, NAC1 does not directly bind to DNA and thus NAC1 regulates transcriptional activity through binding to and modulating the transcription functions of its cofactor(s) that directly interact with specific gene promoters or enhancers. Therefore, the precise mechanism by which NAC1 regulates transcription of ΔNp63 remains to be delineated. Our experiments show that the changes in ΔNp63 protein (Fig. 3) were larger than the changes in ΔNp63 mRNA (Fig. 4), suggesting that posttranscriptional regulation of ΔNp63 may also be
involved in changes of the level of this protein. Although it was reported that degradation of DNp63 protein is promoted by stress such as DNA damage and oncogene activation (29, 34), in our pulse-chase experiments we found that loss of NAC1 barely affects the turnover of DNp63 in the stressed cells (data not shown). Thus, it seems that NAC1 promotes bypass of cellular senescence mainly via modulating transcription of DNp63 under stressful conditions.

In our experiments, the senescence induction was low in the Ras-transduced wild-type MEFs (Fig. 5), which could be...
because of these MEFs, which had undergone spontaneous immortalization, or the activity level of the Ras variant used in this study was low, and these may actually lead to sensitivity to transformation by Ras in NAC1+/C0 MEFs. We found that blunting of cellular senescence mediated by NAC1 plays a crucial role in tumorigenesis, and that NAC1-deficient cells are much less transformed and tumorigenic in the presence of oncogene Ras. However, absence of NAC1 does not seem to completely reverse the effects of Ras, suggesting that the NAC1-mediated bypass of senescence, although impactful, is only one of the components that drive tumor initiation and development. Our observation raises the possibility that targeting NAC1 to restore senescence response may be explored as a potential new strategy for cancer preventive. In addition, it is likely that the NAC1-mediated blunting of senescence may also influence other facets of cancer, such as tumor dormancy, response to therapeutic intervention, metastasis, and so on. Exploring the effects of NAC1-mediated senescence on these features of cancer might shed significant new light on the importance of NAC1 and senescence in cancer prevention and treatment. In addition, NAC1 has been shown to be associated with Nanog in a protein complex that is necessary for maintaining the stemness of mouse embryonic stem cells (35, 36). Nanog is capable of preventing terminal differentiation of embryonic stem cells and sustaining their pluripotency through a protein network involving NAC1 (37). The validated interaction of NAC1 with Nanog (37), as part of a multimember family necessary for maintaining the stemness of mouse embryonic stem cells, implies that NAC1 function may play a role in preventing or determining the terminal differentiation of cells. It would be interesting to investigate whether or not this function of NAC1 in stem cells is associated with its effect on cellular senescence.

In summary, the main finding in this study is that we identify NAC1 as a negative regulator of cellular senescence, and blunting of senescence caused by NAC1, which is mediated through DНР63, plays an important role in promoting tumorigenesis. The identification of NAC1 as a senescence regulator, along with the pathways involved, should help further understand the molecular and cellular functions of this nuclear factor in cancer, and may provide a potential target for cancer prevention and treatment.

Figure 6. (Continued) D, nude mice were inoculated s.c. at the right or left flank with immortalized NAC1+/SV40 or NAC1+/+/SV40 MEFs (2 x 10⁶ cells in 100 μL) infected with a H-RasV12 virus. Tumor size was measured twice a week. Hematoxylin and eosin stainings of the tumors. Scale bar, 50 μm. E, SA-β-gal activity in the fresh-frozen sections of the tumors. F, expression of the cell proliferation marker, Ki67, in paraffin sections of the tumors. Ki67-positive cells were counted. The bars are mean ± SD of triplicate determinations; results shown are the representative of 3 identical experiments. Average tumor sizes of each group were shown. The photograph was taken 15 days after inoculation. *, * P < 0.05; **, ** P < 0.01, t test. Scale bar, 100 μm.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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