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

Yi Zhang1,3, Yan Cheng3, Xincong Ren3, Tsukasa Hori3, Kathryn J Huber-Keener3, Li Zhang1, Kai Lee Yap2, David Liu4, Lisa Shantz5, Zheng-Hong Qin1, Suping Zhang1, Jianrong Wang1, Hong-Gang Wang3, Ie-Ming Shih2, and Jin-Ming Yang1,3‡

1Department of Pharmacology, College of Pharmaceutical Sciences, Cyrus Tang Hematology Center, Soochow University, China; 2Department of Pathology, John Hopkins University School of Medicine, Baltimore, MD 21231; Departments of 3Pharmacology, 4Neural & Behavioral Science, 5Cellular and Molecular Physiology, and The Penn State Hershey Cancer Institute, The Pennsylvania State University College of Medicine, Hershey, PA 17033;

Running Title: Regulation of senescence by NAC1

Key words: NAC1, senescence, ΔNp63, oncogene, tumorigenesis

Precis: This study offers new insights into mechanisms of senescence and how its bypass is important for tumor development and progression.

*Supported by grants from US Public Health Service (R01CA135038 and RO1CA103937), Department of Defense (BC103654), Elsa Pardee Foundation, National Natural Sciences Foundation of China (81072146; 81101913), and by a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Disclosure of Potential Conflict of Interest: No potential conflicts of interest were disclosed.

‡Address correspondence to: Jin-Ming Yang, Penn State College of Medicine, Department of Pharmacology and The Penn State Hershey Cancer Institute, CH74, 500 University Drive, P.O. Box 850, Hershey, PA 17033-0850; E-mail address: juy16@psu.edu; Tel: 717-531-1630; FAX: 717-531-0011
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 non-transformed 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 sub-lethal doses of γ-irradiation. In mouse embryonic fibroblasts (MEFs) from NAC1 knockout mice, following infection with a Ras virus, NAC1−/− cells undergo significantly more senescence and are either non- 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.
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, etc. 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 biological 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 ovarian cancer patients that the expression level of NAC1 is significantly higher in recurrent post-treatment tumors than pre-treated primary tumors (11, 12, 14). Also, NACC1, which encodes NAC1, is amplified in many ovarian high-grade serous carcinomas (15). There are studies reporting that up-regulation 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 pro-survival 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.

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 MEFs, 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, USA). 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% fetal bovine serum; 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 supplemented with 10% fetal bovine serum. A2780 cells were also cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. 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 non-targeting siRNA, were synthesized by QIAGEN (Valencia, CA, USA) or Cell Signaling (Beverly, MA, USA). Transfection of siRNA was performed according to the manufacturer’s protocol. Briefly, cells in exponential phase of growth were plated in six-well cell culture plates at $1 \times 10^5$ cells/well, grown for 24h, and then transfected with siRNA using Oligofectamine and OPTI-MEM I-reduced serum medium (Invitrogen, Carlsbad, CA, USA). 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). 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 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 150 mM NaCl, and 2 mM 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 BrdU 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 2×10⁵ cells per well, centrifuged at 450 × g for 5 minutes, and fixed with ice-cold 70% ethanol for 12 hour 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 FlowCytometry System (Millipore).

**Soft-agar assay.** Cells were suspended in complete culture medium containing 0.3% soft agar, seeded (5×10⁴ cells/well) in 6-well plates pre-coated with 0.6% agar in complete culture medium, and then
incubated at 37 °C in a humidified atmosphere containing 5% CO2/95% air for 12-14 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-aminoactinomycin D staining. Briefly, 100 μl of Guava Nexin reagent (Millipore, Bedford, MA, USA) was added to 1 × 10^5 cells in 100 μl, and the cells were incubated with the reagent for 20 min at room temperature in the dark. At the end of incubation, the cells were analyzed by a Guava EasyCyte Plus FlowCytometry System (Millipore, Bedford, MA).

**Western blotting and antibodies.** Cells were lysed in M-PER mammalian protein extraction reagent (Roche) supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN), followed by centrifugation at 14,000× g for 10 minutes. After centrifugation, cell lysates were collected and protein concentrations were measured. Protein (10-20μg) were resolved by SDS-PAGE, and then transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibodies in 3% BSA/TBST at 4°C for overnight, followed by incubation with secondary antibodies at room temperature for 1 h. The protein signals were detected by ECL method. Antibodies to Ras was purchased from BD Bioscience (BD, NJ, USA), antibodies to p21, ΔNp63, TAp63, and p53 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA); α-tubulin antibody was purchased from Cell Signaling Technology (Danvers, MA, USA); anti-NAC1 antibody was obtained from Abcam Inc (Cambridge, MA, USA).
**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 was performed on a Stratagene Mx3005P using Brilliant II SYBR Green QPCR master mix (Stratagene) and the following primer sets: \( \beta\)-actin, 5’-GCCAACACAGTGCTGTCTGG-3’ (forward) and 5’-GCTCAGGAGGAGCAATGATCTTG-3’ (reverse); \( \Delta Np63 \), 5’-CAGTCGAGCACCGCCAAG-3’ (forward), 5’-ATCCGGGCCTCAAAGCAG-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\( \times \)10^5 cells), or A2780 cells (3\( \times \)10^5 cells) with or without silencing of NAC1 expression, were transfected with 1\( \mu \)g of \( \Delta Np63 \) reporter construct, pGL3-823-Luc vector (a generous gift from Dr Jan-Kan Chen, Chang Gung University), and 0.025\( \mu \)g of PRL vector as an internal control, using FuGENE 6 transfection reagent. Twenty-four hour later, the cells were \( \gamma\)-irradiated. Cells were then rinsed with PBS and lysed in 300 \( \mu \)l 1\( \times \) reporter lysis buffer (Promega, Madison, USA). For NAC1\(^{+/+}\), NAC1\(^{-/-}\) MEFs, 3\( \times \)10^5 NAC1\(^{+/+}\), NAC1\(^{-/-}\) MEFs were cotransfected with 1\( \mu \)g of \( \Delta Np63 \) reporter construct, 0.025\( \mu \)g PRL control vector, Ras expression construct. 48 h after transfection, the cells were rinsed with PBS and lysed in 300 \( \mu \)l 1\( \times \) reporter passive lysis buffer. Lysates were used directly for luciferase activity assay by using the Dural-Luciferase Reporter Assay System (Promega, Madison, USA) and luminometer (Pekin 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 sub-cloning 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). 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, 7.5μg/ml for 2 weeks; for primary cells, 5μg/ml for 3 days); for infection with pDCR or pDCR- RasV12, transduced cells were selected with G418 (500μg/ml).

**Tumorigenesis Study.** Female, five-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, re-suspended 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: \( V = \frac{lw^2}{2} \). 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 histo-pathologic examination. For mice bearing Hela/N130 tumors, they were either given vehicle or doxycycline (125mg/kg) i.p. daily to suppress expression of N130 mutant.

**Statistical analysis.** Student’s 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) (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 (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 (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 (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 sub-lethal 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); by 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/M 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. Fig. 1B shows that two 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 appear to be a consequence of activation of apoptosis, as there was no significant Annexin V staining in those cells (Fig. S2). The role of p21 in senescence induction mediated by NAC1 deficiency was further demonstrated when p21 expression was silenced (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

Fig. 1B demonstrates that deficiency of NAC1 resulted in up-regulation 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. Fig. 2 shows that silencing of p53 did not abrogate the effects of NAC1 depletion on senescence (Fig. 2A-Fig. 2C), 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 up-regulation 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 down-regulated in cells with deficiency of NAC1 following treatment with γ-irradiation (Fig. 3A). By 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 - Fig. 3D). The role of ΔNp63 in NAC1-mediated evasion of senescence was further verified by over-expressing ΔNp63 in NAC1-deficient cells. As shown in Fig. 3E and Fig. 3F, 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 Fig. 3F). These results indicate that ΔNp63 is a necessary mediator of NAC1-mediated bypass of senescence, and that up-regulation 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 following irradiation treatment (Fig. S4)

Transcription of ΔNp63 is modulated by NAC1

Since 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 co-factor. 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 following 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 performed 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. Fig. 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 ΔNp63 mRNA, the promoter activity, and protein level were similarly down-regulated in NAC1−/− mouse embryonic fibroblasts (MEFs) transduced with RasV12, as compared with NAC1+/+ MEFs (Fig. S5 and Fig. 5D). These results indicate that NAC1 plays a critical role in promoting the transcription of ΔNp63 under stress conditions.

**NAC1 plays an essential 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−/−, NAC1+/− or NAC1+/+. The knockout cells were confirmed by genotyping (Fig. S6). Unexpectedly, we observed a low level of senescence in the H-RasV12-infected NAC1+/+ MEFs (Fig. 5C and Fig. 5D), likely due to a low activity of the Ras variant used, or due to spontaneous immortalization of the MEFs, which would then lead to sensitivity to transformation by Ras. By contrast, when H-RasV12 was transduced into NAC1−/− MEFs, the growth of NAC1−/− cells was arrested (Fig. 5A), colony formation of NAC1−/− cells was inhibited (Fig. 5B), 80% of the NAC1−/− cells were SA-β-gal positive (Fig. 5C), p21 levels were up-regulated and ΔNp63 was down-regulated (Fig. 5D), indicating that these NAC1−/− MEFs were undergoing robust senescence. Similar to NAC1+/+ MEFs, these H-RasV12-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 re-expression of NAC1 in NAC1<sup>+/−</sup> MEFs would revoke cellular senescence. Fig. 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 suppress 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. Fig. 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. By 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 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 demonstrate the role of NAC1 in promoting cellular transformation and decreasing senescence, we utilized 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 (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$^{v12}$-expressing NAC1$^{-/-}$/SV40 cells (Fig. 6C). NAC1$^{+/+}$/SV40 and NAC1$^{-/-}$/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$^{v12}$-expressing NAC1$^{+/+}$/SV40 MEFs developed larger tumors more rapidly than the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 MEFs in nude mice (Fig. 6D). Most of the tumors derived from the Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 cells reached a size of ~0.8 cm$^3$ within 2 weeks following inoculation, whereas the tumors derived from the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 cells hardly grew to 0.3 cm$^3$ in volume (Fig. 6D). Moreover, HE staining of the tumor specimens showed that the morphology of the Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 and the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 cells were distinctive: the cells from the Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 tumors were densely arranged and elongated, while the cells from the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 tumors were round and loosely organized.

To determine whether activation of cellular senescence could suppress the transformed phenotype seen in the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 MEFs, we examined and compared SA-β-gal staining and Ki67 staining in fresh sections of dissected tumors formed by Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 or Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 cells. Fig. 6E shows that the numbers of cells positive for SA-β-gal staining were significantly higher in the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 tumors than that in the Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 tumors ($p<0.01$). In contrast, the immunohistochemistry staining for Ki67, a marker of cell proliferation, was significantly higher in the tumor xenografts of the Ras$^{v12}$-expressing NAC1$^{+/+}$/SV40 cells than that in the tumor xenografts of the Ras$^{v12}$-expressing NAC1$^{-/-}$/SV40 cells (Fig. 6F). These results provide further support for the conclusion that activating cellular senescence by repressing NAC1 can suppress the oncogenic potential of this nuclear factor.
The current 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 (Fig. 5 and Fig. 6). More importantly, we demonstrate both in vitro and in vivo that blunting of cellular senescence by NAC1 cooperates with Ras to promote cellular transformation and tumor development (Fig. 5 and Fig. 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 appears 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 homolog, 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 down-regulated (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 (Fig. 4 and Fig. S5). However, in ChIP assays we failed to enrich significant promoter sequences including p63. There are at least two 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 co-factor(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 post-transcriptional regulation of ΔNp63 may also be involved in changes of the level of this protein. Although it was reported that degradation of ΔNp63 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 ΔNp63 in the stressed cells (data not shown). Thus, it appears that NAC1 promotes bypass of cellular senescence mainly via modulating transcription of ΔNp63 under stressful conditions.

In our experiments, the senescence induction was low in the Ras-transduced wild-type MEFs (Fig. 5), which could be due to that these MEFs 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−/− 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, etc. 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 demonstrated 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 multi-member 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 ΔNp63, 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.
References

Legends to Figures

Figure 1. NAC1 status impacts γ-irradiation-induced senescence in tumor cells. SKOV3/N130 and Hela/N130 cells cultured in the presence or absence of doxycycline were treated or untreated with 6 Gy of γ-irradiation, or A2780 cells with or without silencing of NAC1 were treated or untreated with 4 Gy of γ-irradiation. Three days later, (A) Cell morphology and SA-β-gal positive cells were examined by phase contrast microscopy; (B) p21 was determined by western blot. Tubulin was used as a loading control; (C) BrdU incorporation was determined using a BrdU Cell Proliferation Assay Kit; (D) SKOV3/N130, Hela/N130 and A2780 cells treated as described above were plated and incubated for 6 days at 37 °C. Cells were stained, and colonies were counted under a light microscope; (E) SKOV3/N130, Hela/N130 and A2780 cells were subjected as described above. Three days later, cell cycle was analyzed by the method of propidium iodide staining; (F) mice were inoculated s.c. with Hela/N130 cells (2 x 10^6 cells in 100 μl), and then were given doxycycline (120 mg/kg, daily) or vehicle. When tumors reached 100 mm^3, the mice were treated daily with 2 Gy of γ-irradiation for 3 days. Five days later, tumors were removed and examined for SA-β-gal activity. The bars are the mean ± s.d of quadruplicate determinations. **p < 0.01, t-test. Results shown are the representative of three identical experiments. Bar: 100 μm

Figure 2. Depletion of p53 does not affect senescence induction in NAC1-deficient cells. A2780 cells were transfected with a non-targeting RNA, a siNAC1, sip53, or both siNAC1 and sip53, followed by γ-irradiation treatment (4 Gy). Three days later, (A) SA-β-gal positive cells were counted; (B) NAC1, p53 and p21 were examined by western blot; (C) BrdU incorporation was determined using a BrdU Cell Proliferation Assay Kit. The bars are mean ± s.d. of triplicate determination; results shown are the representative of three identical experiments. ** p < 0.01 versus non-targeting, t-test. Bar: 100 μm
Figure 3. **ΔNp63 is involved in senescence blunting mediated by NAC1.** (A) SKOV3/N130 and Hela/N130 cells cultured in the presence or absence of doxycycline were treated or untreated with 6 Gy γ-irradiation, or A2780 cells with or without silencing of NAC1 were treated or untreated with 4 Gy γ-irradiation. Three days later, (A) TAp63 and ΔNp63 were examined by western blot; (B, C, D) SKOV3/N130 and Hela/N130 cells cultured in the presence of doxycycline, or A2780 cells, were transfected with a non-targeting RNA or siΔNp63, followed by treatment with γ-irradiation. Three days later, (B) SA-β-gal positive cells were examined; (C) ΔNp63 and p21 were examined by western blot; (D) BrdU incorporation was determined using a BrdU Cell Proliferation Assay Kit; (E, F) SKOV3/N130 and Hela/N130 cells cultured in the absence of doxycycline, or A2780 cells with silencing of NAC1 expression, were transfected with an empty control vector or a ΔNp63 expressing plasmid, followed by γ-irradiation treatment. Three days later, (E) SA-β-gal positive cells were examined. (F) ΔNp63 and p21 were determined by western blot. The bars are mean ± s.d. of triplicate determinations; results shown are the representative of three identical experiments. **p < 0.01, t-test; Bar: 100 μm

Figure 4. **NAC1 modulates the transcription of ΔNp63.** (A) SKOV3/N130 and Hela/N130 cells cultured in the presence or absence of doxycycline, or A2780 cells with or without silencing of NAC1, were treated with γ-irradiation. Twenty-four hours later, ΔNp63 mRNA was analyzed by qRT-PCR. The bars are mean ± s.d. of triplicate determinations; results shown are the representative of three identical experiments. **p < 0.01, t-test; (B) SKOV3/N130, Hela/N130 and A2780 cells subjected to same treatment as above were transfected with a ΔNp63 promoter construct, followed by γ-irradiation
treatment. Twenty-four hours later, luciferase activity was measured. Luciferase activity of the $\Delta Np63$ promoter was normalized to the activity of a co-transfected Renilla luciferase expression vector. $\Delta Np63$ promoter activity in the absence of NAC1 function following $\gamma$-irradiation was arbitrarily set at 1. Each value represents the mean ± s.d. of triplicate determinations from one of three identical experiments. **$p < 0.01$, $t$-test.

**Figure 5. A functional NAC1 suppresses Ras-induced senescence in primary MEFs.** (A) Growth curves of NAC1$^{+/+}$, NAC1$^{+/c}$, NAC1$^{c/-}$ primary MEFs transduced with a H-Ras$^{{V12}}$ virus or a control virus; (B, C) NAC1$^{+/+}$, NAC1$^{+/c}$, NAC1$^{c/-}$ primary MEFs were transduced with a H-Ras$^{{V12}}$ virus or a control virus. (B) Forty-eight h later, cells were plated and incubated for 10 days at 37 °C. Cells were stained and colonies were counted; (C) Six days after transduction, SA-β-gal-positive cells were examined. **$p < 0.01$ versus NAC1$^{c/-}$ + Ras, $t$-test; (D) Ras, p21, and $\Delta Np63$ were analyzed by western blot; (E) growth curves (up left) and SA-β-gal activity (up right and low right) of NAC1$^{c/-}$ primary MEFs co-transduced with H-Ras$^{{V12}}$ and NAC1 or an empty control vector. Low left is western blots of NAC1 and Ras. The bars are mean ± s.d. of triplicate determinations; results shown are the representative of three identical experiments. **$p < 0.01$, $t$-test. Bar: 100 $\mu$m

**Figure 6. A functional NAC1 promotes Ras-induced transformation and tumor formation.** (A) Primary MEFs infected with a H-Ras$^{{V12}}$ virus vector or a control vector was plated on soft agar ($5 \times 10^4$ cells/plate). Ten days later, photomicrographs were taken. **$p < 0.01$ versus NAC1$^{c/-}$/Ras, $t$-test; (B) Nude mice were inoculated s.c. at the right or left flank with primary NAC1$^{+/+}$ or NAC1$^{c/-}$ MEFs ($4 \times 10^6$ cells in 100 $\mu$l) infected with a H-Ras$^{{V12}}$ virus. Tumor size was measured twice a week; (C) Immortalized MEF cells infected with a H-Ras$^{{V12}}$ virus or a control vector were plated on soft agar ($5 \times 10^4$ cells/plate). Ten days later, photomicrographs were taken; (D) Nude mice were inoculated s.c. at
the right or left flank with immortalized NAC1<sup>+/+</sup>/SV40 or NAC1<sup>-/-</sup>/SV40 MEFs (2×10<sup>6</sup> cells in 100 μl) infected with a H-Ras<sup>V12</sup> virus. Tumor size was measured twice a week. Hematoxylin and eosin (HE) stainings of the tumors; 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 ± s.d. of triplicate determinations; results shown are the representative of three 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. Bar: 100 μm
Dysfunction of Nucleus Accumbens-1 Activates Cellular Senescence and Inhibits Tumor Cell Proliferation and Oncogenesis

Yi Zhang, Yan Cheng, Xingcong Ren, et al.

Cancer Res Published OnlineFirst June 4, 2012.