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The catalytic subunit of DNA-dependent protein kinase is downstream of ATM and feeds forward oxidative stress in the selenium-induced senescence response☆

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Abstract

Selenium induces a senescence response in cells through induction of ataxia–telangiectasia mutated (ATM) and reactive oxygen species (ROS). Although a role of the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) in DNA double-strand break repair is established, it is unclear how these proteins function in response to selenium-induced oxidative stress and senescence induction. In this study, we demonstrated that pretreating normal human diploid fibroblasts with DNA-PK kinase inhibitor NU 7026 suppressed selenium-induced senescence response. Selenium treatment induced phosphorylation of DNA-PK_{cs} on Thr-2647 and Ser-2056, the extent of which was decreased in the presence of ATM kinase inhibitor KU 55933 or the antioxidants *N*-acetylcysteine or 2,2,6,6-tetramethylpiperidine-1-oxyl. In contrast, the selenium-induced phosphorylation of ATM on Ser-1981 was not affected by NU 7026. Cells deficient in DNA-PK_{cs} or pretreated with NU 7026 or *N*-acetylcysteine were defective in selenite-induced ROS formation. Taken together, these results indicate a distinct role of DNA-PK_{cs}, in which this kinase can respond to and feed forward selenium-induced ROS formation and is placed downstream of ATM in the resultant senescence response.

Keywords: Selenium; Senescence; ATM; DNA-PK

1. Introduction

Selenium is a micronutrient essential for optimal health. A strong body of geographical, animal and clinical evidence points to a role for selenium in counteracting tumorigenesis (for details, see Ref. [1]). In particular, the Nutritional Prevention of Cancer Trial concluded that dietary supplementation of selenium three- to four-fold higher than nutritional need, in the form of selenium-enriched yeast containing 65%–80% selenomethionine, reduced mortality from all cancers and decreased the incidence of lung, colorectal and prostate cancers [2]. In contrast, the Selenium and Vitamin E Cancer Prevention Trial failed to demonstrate that selenomethionine or vitamin E, alone or in combination, prevented the incidence of prostate cancer in a population of relatively healthy men [3]. Together with results from animal studies [4,5], selenomethionine is unlikely the best active selenium compound to counteract tumorigenesis. Whatever the effective selenium speciation, the proposed mechanisms of selenium chemoprevention include induction of apoptosis by reactive oxygen species (ROS) [6–9], activation of cell cycle arrest [6,10,11], an enhancement of DNA repair [12,13], an increase in mitochondrial dysfunction [14], limiting endoplasmic reticulum stress [15], as well as decreasing angiogenesis [16–19] in cancerous cells. We have recently shown a different perspective, that selenium compounds can activate a senescence response in noncancerous, but not in cancerous, cells with doses $\leq LD_{50}$ [20]. Thus, selenium compounds, in principle, could activate early barriers of tumorigenesis and prevent the cells from progressing to the malignant stage.

DNA damage response, an early barrier of tumorigenesis [21], can be induced by the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) and ataxia–telangiectasia mutated (ATM). The only human *PRKDC* (*DNA-PK_{cs}*) mutation known to date is the L3062R missense mutation found in a severe combined immunodeficiency patient [22]; however, the human glioma cell line M059J lacks DNA-PK_{cs} protein and expresses low levels of ATM protein [23]. *ATM* is

Abbreviations: ATM, ataxia–telangiectasia mutated; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; DAPI, 4,6-diamidino-2-phenylindole; DNA-PK_{cs}, the catalytic subunit of DNA-dependent protein kinase; DSBs, double-strand breaks; MEFs, mouse embryonic fibroblasts; MSeA, methylseleninic acid; NAC, *N*-acetylcysteine; Na_2SeO_3, sodium selenite; NHEJ, nonhomologous end-joining; PBS, phosphate-buffered saline; *p*ATM Ser-1981, phospho-ATM at Ser-1981; *p*DNA-PK_{cs} Ser-2056, phospho-DNA-PK_{cs} at Ser-2056; *p*DNA-PK_{cs} Thr-2647, phospho-DNA-PK_{cs} at Thr-2647; γH2A.X, phospho-H2AX at Ser-139; ROS, reactive oxygen species; SA-β-gal, senescence-associated -β-galactosidase; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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mutated in the genome instability syndrome ataxia–telangiectasia which is characterized by neuronal degeneration, immune defects, premature aging and cancer predisposition [24]. The ATM pathway is activated by interactions with the MRE11-RAD50-NBS1 complex and by autophosphorylation on the Ser-1981 (*p*ATM Ser-1981) residue [25–28]. DNA-PK_{cs}, Ku70 and Ku80 form the holoenzyme DNA-PK that repairs DNA double-strand breaks (DSBs) by the nonhomologous end-joining (NHEJ) pathway [29,30]. The Thr-2647 residue of DNA-PK_{cs} is phosphorylated (*p*DNA-PK_{cs} Thr-2647) *in vivo* in an ATM-dependent manner after ionizing radiation [31]. DNA-PK_{cs} autophosphorylation on Ser-2056 (*p*DNA-PK_{cs} Ser-2056) is induced by DNA DSBs and is required for proper NHEJ repair [32,33].

Originally known as major kinases in the signaling and repair of DNA DSBs, emerging lines of recent evidence link DNA-PK and ATM to redox regulation. Low levels of oxidative stress can activate DNA-PK during mitochondrial respiration [34]. Moreover, ATM protein can be activated by direct oxidative stress or in selenium-treated cells [20,35,36], and neurons deficient in ATM show increased oxidative stress [37,38]. Because ATM kinase is not the only kinase attributed to H2A.X phosphorylation on Ser-139 (γH2A.X) [20] and both ATM and DNA-PK_{cs} can function redundantly in the cellular exposure to ionizing radiation [31,37,39], we hypothesized that DNA-PK_{cs} participates in the selenium-induced DNA damage and senescence responses. We show here that DNA-PK_{cs} is placed downstream of ATM, relays oxidative stress and is required for the senescence response in normal diploid fibroblasts treated with selenium compounds.

2. Method and materials

2.1. Cell culture and chemicals

The human pulmonary MRC-5 and colorectal CCD 841 CoN normal fibroblasts and wild-type and DNA-$PK_{cs}^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured as described previously [20,40]. MRC-5 and CCD 841 CoN cells were chosen to recapitulate the selenium-induced senescence response based on our previous results [20]. The DNA-$PK_{cs}^{-/-}$ and wild-type MEFs have been extensively employed for DNA damage response studies [40]. Sodium selenite (Na_2SeO_3, 2 μmol/L, 24 h), methylseleninic acid (MSeA, 2 μmol/L, 24 h), *N*-acetylcysteine (NAC, a thiol-containing derivative of L-cysteine, 5–10 mmol/L, 24 h) and 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo, a membrane permeable nitroxide compound, 0.5–1 mmol/L, 24 h) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in phosphate-buffered saline (PBS). NU 7026 and KU 55933 (10 μmol/L, 24 h) were purchased from Tocris (Ellisville, MO, USA) and were dissolved in dimethyl sulfoxide.

2.2. Detection of ROS

Intracellular ROS were detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA, USA), which is nonfluorescent until the removal of the acetate group by intracellular esterases upon increased oxidative stress. After Na_2SeO_3 treatment, the cells were rinsed once with PBS and then incubated with 10 μmol/L CM-H_2DCFDA in PBS for 45 min at 37°C. The intracellular ROS levels were detected by using the filter set: Ex., 540 nm; Em., 490 nm, which is equipped in a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech, Cary, NC, USA). The fluorescent intensity was measured and normalized to cells without any treatment. In preparation for ROS analysis by means of flow cytometry, the cells were trypsinized and washed before incubation with CM-H_2DCFDA. After the cells were spun down, the resuspended cells were analyzed by recording the mean FITC-A signals of each sample using the BD FACSCanto II flow cytometer. The data were analyzed using FlowJo version 7.6.4 (Tree Star Inc., Ashland, OR, USA). For ROS analysis by fluorescence microscope, cells were incubated with 10 μmol/L CM-H_2DCFDA in 4% paraformaldehyde for 30 min at 37°C in the dark [41]. After being rinsed gently with PBS, cells were imaged immediately under a Zeiss Axio Observer Z1m fluorescent microscope (Zeiss, Thornwood, NY, USA) using the software Axiovision. All samples were imaged using the GFP 488-nm excitation spectra setting, and corresponding bright-field pictures were taken.

2.3. Immunofluorescence

Immunofluorescence analysis was performed as described previously with modifications [20,42]. Briefly, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with ice-cold methanol for 10 min at −20°C and then with 0.3% Triton X-100 for 10 min, and blocked in 10 % normal goat serum in PBS containing glycine at 0.3 mol/L for 1 h. The coverslips were incubated with the following antibodies overnight at 4°C: *p*DNA-PK_{cs} Thr-2647 (lot 903801, 1:300, Abcam, Cambridge, MA, USA), *p*DNA-PK_{cs} Ser-2056 (lot 696143, 1:300, Abcam), DNA-PK_{cs} (lot 715104, 1:300, Abcam), ATM (lot YF-10-17-02, 1:500, Epitomics, Burlingame, CA, USA) and *p*ATM Ser-1981 (lot 20772, 1:500, Rockland, Gilbertsville, PA, USA). The slides were then washed in PBS and incubated with Alexa secondary antibodies (Alexa Fluor 488 and 594, 1:200, Invitrogen) for 1 h at room temperature in the dark. Cells were then washed in PBS and mounted onto slides containing a drop of 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). All the images were obtained using the same parameters of brightness, contrast and exposure time by using a Zeiss Axio Observer Z1m fluorescence microscope, and the images were processed using deconvolution with the software AxioVision Release 4.7.2.0. Ten nuclei were randomly chosen and outlined using the spline function of the software, and the densometric intensity of each of the proteins (DsRed and GFP) was obtained and normalized by that of DAPI as described previously [43,44].

2.4. Cell survival assay

Cells were trypsinized and counted using a hemocytometer. The number of untreated cells was set as 100%. For apoptotic analysis, cell pellets were resuspended in 500 μl binding buffer containing Annexin V-FITC and Sytox green dye according to manufacturer's instruction (Apoptosis Detection Kit Plus, K201-400; Biovision Inc., Mountain View, CA, USA). The cells were then analyzed (Ex., 488 nm; Em., 530 nm in the FL1 channel) by using a BD FACSCanto II flow cytometer.

2.5. Senescence assays

We detected expression of senescence associated-β-galactosidase (SA-β-gal) by using a Senescence Detection Kit (MBL Co. Ltd., Woburn, MA, USA) according to the manufacturer's instructions and our previous publication [20].

2.6. Statistics

All the experiments were independently performed and repeated three times. The data were analyzed using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). One-tailed Student's *t*-test was applied to determine statistical significance ($P<.05$) between the treatments.

3. Results

3.1. DNA-PK_{cs} is involved in the selenium-induced senescence response

We first tested the hypothesis that DNA-PK_{cs} is involved in the selenium-induced senescence response. Pretreatment of normal MRC-5 and CCD 841 CoN human fibroblasts with NU 7026, a DNA-PK kinase inhibitor [45–51], completely suppressed the SA-β-gal expression induced by Na_2SeO_3 (Fig. 1A and C) and MSeA (Fig. 1B and D). Consistent with our previous results [20], pretreatment of MRC-5 cells with the antioxidants NAC or Tempo significantly decreased cellular expression of SA-β-gal after the selenium treatment. Compared to MRC-5 cells, CCD 841 CoN cells showed greater SA-β-gal expression before and after selenium treatment. Representative pictures are shown in Supplemental Figs. 1 and 2. Thus, DNA-PK_{cs} is involved in selenium-induced senescence response in normal diploid fibroblasts.

3.2. DNA-PK_{cs} is phosphorylated on Ser-2056 and Thr-2647 after Na_2SeO_3 treatment and is downstream of ATM and ROS-dependent

We next determined whether and how selenium treatment activates DNA-PK_{cs} and ATM. Analyses of immunofluorescence results indicated that Na_2SeO_3 treatment in MRC-5 cells induced the formation of *p*DNA-PK_{cs} Ser-2056 (Fig. 2A and Supplemental Fig. 3) and Thr-2647 (Fig. 2B and Supplemental Fig. 4). Because selenite-treated MRC-5 cells show induction of *p*ATM Ser-1981 [20], we next determined the sequential events of DNA-PK_{cs} and ATM phosphorylation after selenium treatment. The selenite-induced *p*DNA-PK_{cs} Ser-2056 focus formation was completely prevented by pretreatment either with KU 55933 (10 μmol/L, 24 h) or NU 7026 (10 μmol/L, 24 h) (Fig. 2A). Furthermore, the selenite-induced *p*DNA-PK_{cs} Thr-2647 focus formation was significantly decreased by pretreatment with KU 55933, but not by NU

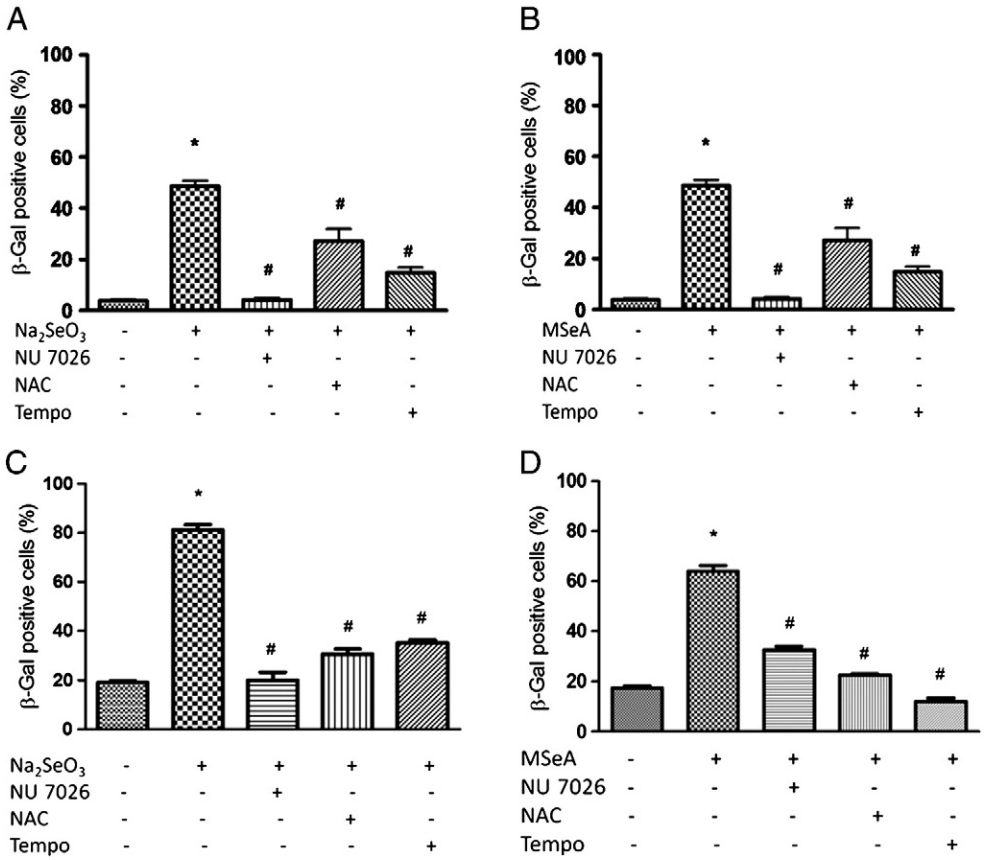

Fig. 1. Selenium-induced senescence response in normal human diploid fibroblasts is dependent on DNA-PK kinase activity and ROS. MRC-5 (A, B) and CCD 841 CoN (C, D) normal human diploid fibroblasts were pretreated with the DNA-PK kinase inhibitor NU 7026 (10 μmol/L) or the antioxidants Tempo (0.5 mmol/L) or NAC (5 mmol/L) for 24 h, followed by treatment with 2 μmol/L Na_2SeO_3 (A, C) or 2 μmol/L MSeA (B, D) for 48 h. SA-β-gal was measured 7 days after recovery from the selenium treatment. Values are means±S.E.M. ($n=3$; *$P<.05$, compared with cells without treatment; #$P<.05$, compared with the cells treated with selenium only).

7026 (Fig. 2B). In contrast, the selenite-induced *p*ATM Ser-1981 focus formation was not affected by pretreatment with NU 7026 but was significantly attenuated by pretreatment with KU 55933 (Fig. 3 and Supplemental Fig. 5). Treatment of MRC-5 cells with Na_2SeO_3, KU 55933 or NU 7026 did not affect the expression of total ATM or DNA-PK_{cs} (Supplemental Figs. 3–5). Unlike MRC-5 cells, U-2 OS osteosarcoma cells did not show any significant induction of the DNA-PK_{cs} phosphorylation events when treated with Na_2SeO_3 (2 μmol/L, 24 h) (data not shown). Because selenium-induced senescence can be inhibited by either NU 7026 or antioxidants (Fig. 1), we asked whether the selenite-induced phosphorylation of *p*DNA-PK_{cs} is associated with ROS. Pretreatment of Tempo (1 mmol/L, 24 h) completely suppressed ($P<.05$) selenite-induced focus formation of *p*DNA-PK_{cs} Ser-2056 (Fig. 2A) and *p*DNA-PK_{cs} Thr-2467 (Fig. 2B) in MRC-5 cells. In conclusion, selenite-induced formation of *p*DNA-PK_{cs} Ser-2056 and Thr-2647 focus formation is downstream of *p*ATM Ser-1981 formation and depends on ROS in MRC-5 normal diploid fibroblasts.

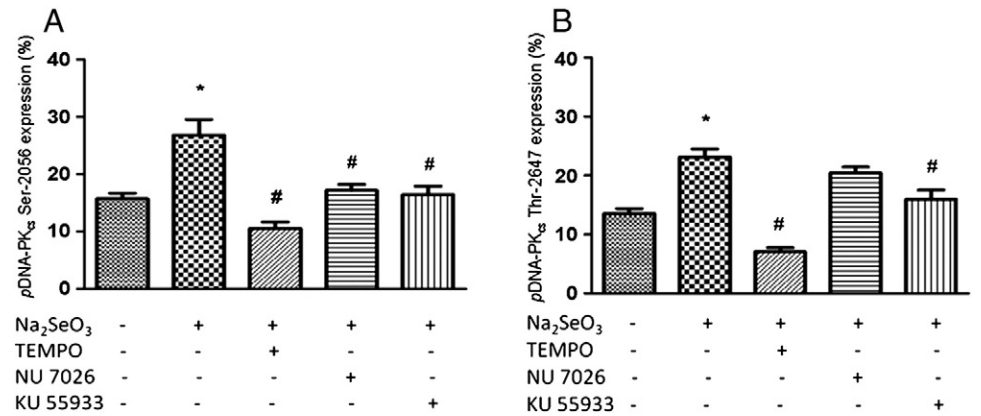

Fig. 2. The selenium-induced formation of *p*DNA-PK_{cs} Ser-2056 and Thr-2467 foci in MRC-5 cells is dependent on ROS. MRC-5 cells were sequentially treated with NU 7026 (10 μmol/L, 24 h), KU 55933 (10 μmol/L, 24 h) or the antioxidant Tempo (1 mmol/L, 24 h) then with Na_2SeO_3 (2 μmol/L, 24 h), followed by immunofluorescence analysis of phosphorylation of DNA-PK_{cs} on Ser-2056 (A) or on Thr-2647 (B). Values are means±S.E.M. ($n=3$; *$P<.05$, compared with cells without treatment; #$P<.05$, compared with the cells treated with Na_2SeO_3 only).

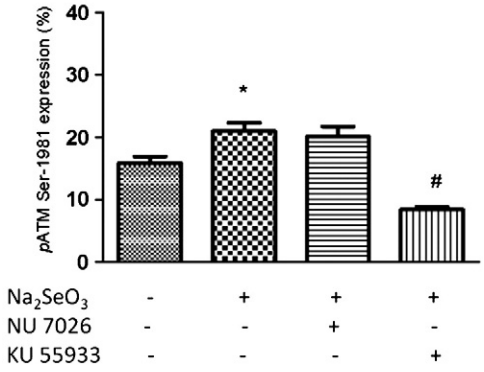

Fig. 3. ATM is upstream of DNA-PK$_{cs}$ in the cellular response to Na_2SeO_3 treatment. MRC-5 cells were sequentially treated with NU 7026 (10 μmol/L, 24 h) or KU 55933 (10 μmol/L, 24 h) then with Na_2SeO_3 (2 μmol/L, 24 h), followed by immunofluorescence analysis of phosphorylation of ATM on Ser-1981. Values are means±S.E.M. ($n=3$; *$P<.05$, compared with cells without treatment; #$P<.05$, compared with the cells treated with Na_2SeO_3 only).

3.3. DNA-PK$_{cs}$ contributes to selenite-induced oxidative stress

Because selenium-induced ATM and DNA-PK$_{cs}$ activation depends on ROS, we assessed cellular ROS status by employing CM-H_2DCFDA. Treatment of MRC-5 cells with Na_2SeO_3 (2–10 μmol/L, 24 h) increased ($P<.05$) levels of ROS in a dose-dependent manner (Fig. 4A). Surprisingly, pretreatment with NU 7026 (10 μmol/L, 24 h) nearly reversed the selenite-induced ROS (2 μmol/L) formation. The validity of the ROS results was verified by results of H_2O_2 treatment, and the selenite-induced ROS was greatly reversed by pretreatment with NAC (10 mmol/L, 24 h). These results implicate that the kinase activity of DNA-PK is necessary for the selenite-induced oxidative stress in MRC-5 cells. To verify this result, we determined ROS status in wild-type and DNA-PK$_{cs}^{-/-}$ MEFs. Na_2SeO_3 treatment (2 μmol/L, 24 h) induced ROS level in wild-type MEFs, but not in DNA-PK$_{cs}^{-/-}$ MEFs, as assessed by immunofluorescent analyses (Fig. 4B) and by flow cytometry (Fig. 4C and D). DNA-PK$_{cs}^{-/-}$ MEFs did not show increased ROS level after H_2O_2 treatment either (10 μmol/L, 24 h, Fig. 4E and F). Altogether, selenite-induced oxidative stress in MRC-5 cells is dependent on DNA-PK$_{cs}$.

3.4. Distinct impact of DNA-PK$_{cs}$ and ATM in the sensitivity of MRC-5 cells to Na_2SeO_3

Because DNA-PK kinase (Fig. 1) and ATM kinase [33] are necessary for the selenium-induced senescence response, we determined whether the kinase activities impact cellular sensitivity of MRC-5 cells to Na_2SeO_3 (2–5 μmol/L, 24 h) coupled with NU 7026 (10 μmol/L) or KU 55933 (10 μmol/L). We found that KU 55933 or NU 7026 sensitized MRC-5 cells to Na_2SeO_3 treatment based on cell counting experiments (Fig. 5A) and to selenite-induced apoptosis (Fig. 5B). Although MRC-5 cells showed increased sensitivity and apoptosis when treated with KU 55933

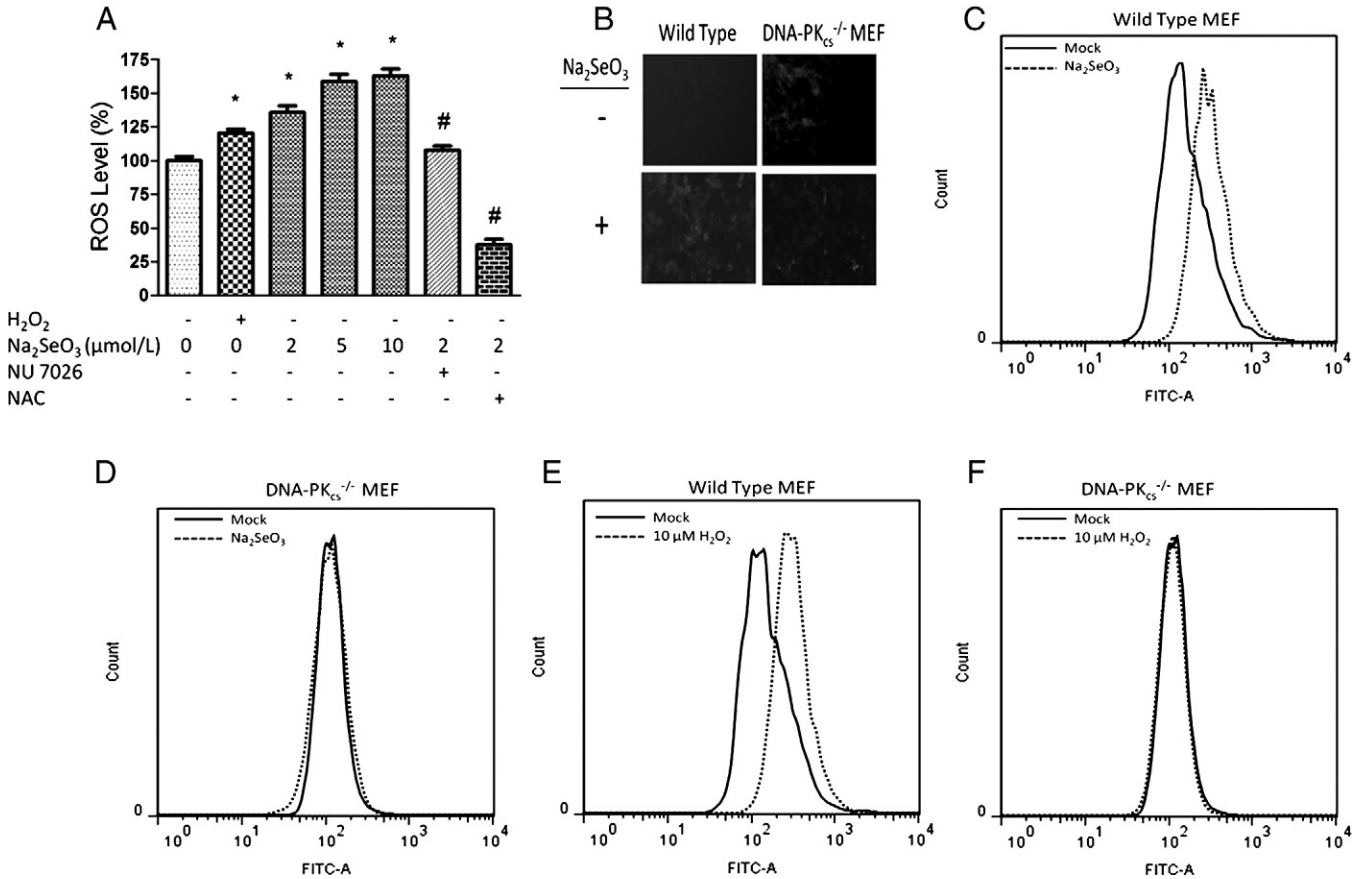

Fig. 4. DNA-PK$_{cs}$ is necessary for the production of ROS in selenite-treated cells. (A) MRC-5 cells were treated with Na_2SeO_3 alone or coupled with NU 7026 (10 μmol/L) or NAC (10 mmol/L) for 24 h, followed by incubation of CM-H_2DCFDA (10 μmol/L) for 45 min at 37°C. H_2O_2 (10 μmol/L, 1 h) was used as the CM-H_2DCFDA positive control. The intensity of the CM-H_2DCFDA signal was determined by fluorescence plate reader and normalized with MRC-5 cells without treatment. Values are means±S.E.M. ($n=3$; *$P<.05$, compared with cells without treatment; #$P<.05$, compared with the cells treated with Na_2SeO_3 at 2 μmol/L). (B–F) Wild-type and DNA-PK$_{cs}^{-/-}$ MEFs were treated with Na_2SeO_3 (2 μmol/L) or H_2O_2 (10 μmol/L) for 24 h, followed by incubation of CM-H_2DCFDA. The CM-H_2DCFDA-positive cells were visualized by using a fluorescence microscope (B) or determined by flow cytometric analyses (C–F).

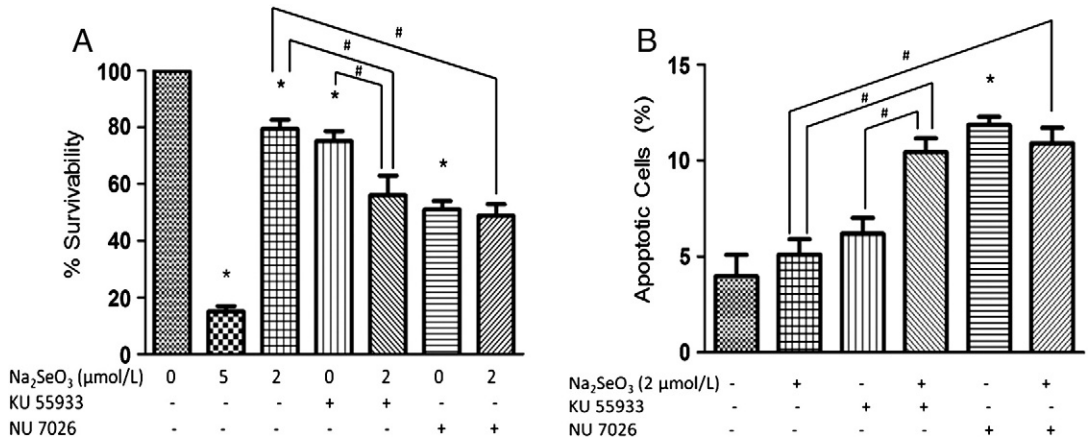

Fig. 5. Effect of KU 55933 and NU 7026 on the sensitivity of MRC-5 cells to Na_2SeO_3 treatment. MRC-5 cells were treated with Na_2SeO_3 alone or coupled with NU 7026 or KU 55933 (10 μmol/L, 48 h), allowed to recover, and counted when the control cells reached 90% confluency (A) or the percentage of apoptotic cells was determined by flow cytometry (B). Values are means±S.E.M. ($n=3$; *$P<.05$, compared with cells without treatment; #$P<.05$).

or NU 7026 alone, Na_2SeO_3 treatment sensitized MRC-5 cells to KU 55933 but not to NU 7026 toxicity. Therefore, Na_2SeO_3 and defective ATM kinase additively promote cell death, while Na_2SeO_3 and defective DNA-PK kinase may function in the same cell death pathway. Taken together, DNA-PK_{cs} and ATM differentially regulate cellular sensitivity to selenite-induced apoptosis.

4. Discussion

In this study, we employed Na_2SeO_3 at levels that are above or at the high end of the nutritional needs and that do not efficiently up-regulate the expression of selenoproteins. Although the majority of selenoproteins exhibit antioxidant activities, the doses of selenium $\leq LD_{50}$ utilized herein instead can induce ROS formation [20]. Consequently, the mild oxidative stress elicits a senescence response that we propose to halt tumorigenesis at an early stage in normal cells. The inorganic forms of selenium, Na_2SeO_3 and Na_2SeO_4, are typically used in animal diets [52,53], and the latter is considered safer than the former. The organic forms of selenium compounds, such as MSeA, have been proven to carry superior anticarcinogenic activities based on animal studies [4,5]. Once inside the cells, it is believed that the various selenium compounds can be metabolized for selenoprotein regulation, ROS formation or excreted, depending on the doses of the selenium compounds.

A proper DNA damage response is critical for cells to remain healthy and prevent malignant changes. Although the DNA-PK complex is well studied in the repair of DNA DSBs by NHEJ, here we have identified an unexpected role of DNA-PK_{cs}, as a positive regulator of the selenium-induced oxidative stress. As depicted in Fig. 6, we propose that selenium compounds, at a supranutritional level, drive ROS formation and the subsequent ATM activation. The ATM-dependent activation of DNA-PK_{cs} maintains and contributes to oxidative stress, which may reinforce the ROS-induced senescence response to selenium exposure that counteracts early-stage tumorigenesis. Selenium-induced senescence may be regulated by p53 and/or $p16^{INK4a}$ that are known to execute or maintain a senescent state. It is also possible that ATM and DNA-PK contribute to selenium-induced senescence through p53 phosphorylation or through modulation of the ATM downstream checkpoint mediators such as Chk2 or SMC1. Consistent with this view, we have recently shown that p53 is indispensable for selenium-induced senescence in MRC-5 cells [54]. Because antioxidant treatment does not reverse selenium-induced senescence as significantly as NU 7026 does, selenium may also use pathways other than the one we describe here, which implicates DNA-PK_{cs} in relaying the selenium-induced ROS formation.

DNA-PK_{cs} Ser-2056 autophosphorylation from ionizing radiation and its localization to laser-induced DNA breaks are independent of ATM; however, phosphorylation of DNA-PK_{cs} on Thr-2647 and other sites in the Thr-2609 cluster is dependent on ATM upon ionizing radiation [55–57]. These DNA-PK_{cs} phosphorylation events, together with the recognition of broken DNA ends and recruitment of DNA-PK_{cs} by the Ku70/Ku80 heterodimer, collectively render a full activation of the NHEJ pathway for the repair of DNA DSBs [31]. Contrary to the response to direct DNA DSBs, induction of *p*DNA-PK_{cs} Ser-2056 by selenium treatment depends on the kinase activity of ATM. Metabolites of selenium compounds do not directly cause DNA DSBs; rather, they promote the formation of ROS [1,6,58]. Oxidative stress is known to induce a nuclear loss of Ku70 and Ku80 in the pancreatic acinar AR42J cells [59], as well as to directly activate the ATM kinase [35,36]. Therefore, upon selenium-induced oxidative stress, the Ku70/Ku80 heterodimer may be uncoupled from DNA-PK_{cs} and transported out from the nucleus. Dissociation from Ku70/Ku80 may allow DNA-PK_{cs} Ser-2056 to become an ATM phosphorylation target. Whatever the mechanism, selenium compounds and DNA DSBs differentially induce *p*DNA-PK_{cs} Ser-2056 phosphorylation in an ATM-dependent or an ATM-independent manner.

The phosphorylation of DNA-PK_{cs} on Thr-2647 upon either ionizing radiation [31] or selenium treatment (Fig. 2B) requires the kinase activity of ATM. We have recently shown that cellular exposure to selenium compounds can induce the formation of the oxidative 8-oxoguanine DNA lesions [42]. If not repaired efficiently, the 8-

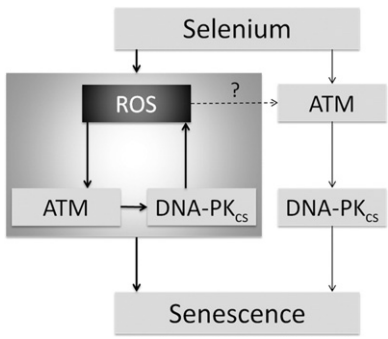

Fig. 6. A model depicting ROS, DNA-PK_{cs} and ATM interplay and how they contribute to selenium-induced senescence in normal diploid fibroblasts.

oxoguanine accumulation can lead to DNA breaks that depend on the mismatch repair proteins MLH1 or MSH2 [42,60]. Interestingly, DNA-PK_{cs} phosphorylation on the Ser-2609 cluster, which includes Thr-2647, is needed for the Artemis endonuclease to process DNA DSBs [61,62]. Furthermore, the DNA-PK-Artemis pathway can be activated by oxidative stress in normal cells [34,61]. Similar to DNA DSBs, selenium-induced phosphorylation of DNA-PK_{cs} on Thr-2647 does not need the kinase activity of DNA-PK. It is thus conceivable that the selenium-induced *p*DNA-PK_{cs} Thr-2647 formation is a downstream event of ATM activation when the unrepaired oxidative DNA damage has manifested into unresolved DNA breaks.

To our knowledge, our research provides the first evidence that DNA-PK_{cs} not only responds to but also contributes to ROS production, particularly in senescent cells. It is known that DNA-PK_{cs} autophosphorylation can facilitate the Artemis endonuclease for the stabilization of the p53 protein in response to oxidative stress [34,62]. Therefore, the role of DNA-PK_{cs} in maintaining ROS levels appears to be a biological necessity to ensure sustained up-regulation of senescence-promoting factors such as p53. Consistent with this notion, we have recently demonstrated that p53 is required for selenium-induced senescence in MRC-5 cells [54]. DNA-PK_{cs} may also feed forward regulation of ROS during the selenium-induced senescence response through oxygen generators. For example, mitochondrial dysfunction occurs under oxidative stress and in senescent cells [63–65], and DNA-PK_{cs} silencing can suppress the expression of the ROS-generating xanthine oxidoreductase [66]. Of note, endogenous oxidative stress and DNA-PK_{cs} phosphorylation exist in MRC-5 cells under our 20% oxygen cell culture condition, as their levels are decreased upon antioxidant treatment (Fig. 4A). To summarize, the full extent to which and how DNA-PK_{cs} promotes ROS formation will require further investigation.

Here we have identified a novel role of DNA-PK_{cs} as a positive regulator of the senescence response as a result of selenium treatment in normal diploid fibroblasts. Upon selenium-induced oxidative stress, DNA-PK_{cs} is downstream of ATM in the DNA damage response pathway. In particular, the DNA-PK kinase activity contributes to oxidative stress, which represents a feed-forward regulation of selenium-induced ROS leading to sustained activation of ATM and DNA-PK and the senescence response. We believe that ROS can act as a signal for precancerous cells to confer and sustain the ATM- and DNA-PK_{cs}-dependent senescence response, an early barrier of tumorigenesis. Cancer cells are immune to this signaling due to their intrinsically high levels of oxidative stress. Although selenium has been touted for its chemopreventive action on cancer cells, our results support a different view, of which selenium can stifle tumorigenesis at the early stages, before a cell turns malignant through the response to and the maintenance of ROS by the ATM-DNA-PK_{cs} pathway.

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Appendix A. Supplementary data

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