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# Oxidative stress preferentially induces a subtype of micronuclei and mediates the genomic instability caused by p53 dysfunction

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## Abstract

Reactive oxygen species (ROS) are known to cause many types of DNA lesions that could be converted into cancer-promoting genetic alterations. Evidence showed that tumor suppressor p53 plays an important role in regulating the generation of cellular ROS, either by reducing oxidative stress under physiological and mildly stressed conditions, or by promoting oxidative stress under highly stressed conditions. In this report we characterized the effect of oxidative stress on the induction of micronuclei, especially the subclass marked by pan-staining of γ-H2AX or MN-γ-H2AX (+). We found that MN-γ-H2AX (+) were more responsive to hydrogen peroxide ($H_2O_2$) than the MN-γ-H2AX (−). In human and mouse cells that are deficient in p53, the frequency of MN-γ-H2AX (+) is significantly elevated, but can be attenuated by antioxidant N-acetylcysteine (NAC). Depletion of p53-regulated antioxidant gene *SESN1* by RNA interference also resulted in an elevation of MN-γ–H2AX (+). Furthermore, we found that in cells that were depleted of p400 by RNAi, and therefore were experiencing increased ROS, the frequency of MN-γ-H2AX (+), but not that of MN-γ-H2AX (−), was significantly induced. We further demonstrated that the induction of MN-γ–H2AX (+) by replication stress can also be attenuated by NAC, and that $H_2O_2$ also leads to increased phosphorylation of Chk1 and Rad17 that mimics replication stress, suggesting that replication stress and oxidative stress are intertwined and may reinforce each other in driving genomic instability. Our findings illustrate the importance of p53-regulated redox level in the maintenance of genomic stability.

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**Keywords**

micronuclei; genomic instability; p53; oxidative stress; replication stress

## 1. Introduction

Reactive oxygen species (ROS), which include $O_2^{\bullet-}$ (superoxide anion radical), $^{\bullet}OH$ (hydroxyl radical) and $H_2O_2$ (hydrogen peroxide), can be generated by endogenous sources, such as mitochondria and NADPH oxidase [1]. ROS also mediates the deleterious effects of ultraviolet (UV) radiation [2, 3], ionizing radiation [4], inflammatory cytokines [5, 6] and arsenic exposure [7]. There are multiple antioxidant defense mechanisms, including enzymatic systems (catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic mechanisms (glutathione, vitamins C and vitamins E) [8, 9]. Maintenance of a homeostatic level of ROS is vital for cell proliferation and various other cellular functions. When ROS overwhelms the antioxidant capacity, damage to cellular macromolecules such as lipids, protein, and DNA ensues [1, 10]. ROS is known to cause at least 100 different types of DNA lesions, including base modifications, singe-strand breaks and double-strand breaks and interstrand crosslinks [10]. Such lesions may also lead to an increased mutant frequency at reporter loci [11–13]. Because oxidative stress is associated with a variety of chronic diseases as well as aging, it remains important to delineate the full spectrum of genomic instability it may cause.

Tumor suppressor p53 plays a critical role in regulating the level of cellular ROS [14, 15]. It upregulates the expression of antioxidant enzymes, such as sestrins and GPX, under physiological conditions. Lack of p53 led to elevated levels of ROS, oxidatively damaged DNA and mutations at the HPRT reporter locus [16]. However, it exhibits prooxidative activities that further increase the levels of stress under high levels of oxidative stress [14, 15]. Mutation within or lack of p53 has long been recognized to lead to increased genomic instability [17–20]. Because p53 may contribute to the maintenance of genomic integrity in multiple pathways, it remains to be determined to what extent each of the p53-regulated pathways contributes.

Micronuclei (MN) in mammalian cells serve as a reliable biomarker of genomic instability and genotoxic exposure [21–23]. Phosphorylation of H2AX, or γ-H2AX, serves as a sensitive marker of DNA damage [24–26]. We observed that a large proportion of MN showed uniform and pan-staining by antibodies against γ-H2AX. We designated them as a MN-γ-H2AX (+), to distinguish them from the MN-γ-H2AX (−) that lack uniform labeling of γ-H2AX in the MN [27]. We had shown that the formation of MN-γ-H2AX (+) probably involves active clustering and disposal of DNA DSBs before the onset of mitosis, and demonstrated that MN-γ-H2AX (+) can be preferentially induced by DNA replication stress.

In this study we studied whether ROS would influence the formation of MN-γ-H2AX (+) and whether p53 function is involved. We found that the frequency of MN-γ-H2AX (+) was significantly increased in cells treated with $H_2O_2$, and in mutant cells with reduced p53 function. We also observed that oxidative stress and replication stress are mutually reinforcing in inducing MN-γ-H2AX (+).

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## 2. Materials and Methods

### 2.1. Cell Culture

Human osteosarcoma cells U2OS were obtained from the American Type Culture Collection (Manassas, VA). HEK-293 cells were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing). The immortalized cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin and 100 mg/ml streptomycin. Primary mouse skin (ear) fibroblasts (MSF) were prepared as described [28]. Briefly, the mouse ears were minced in a well of 12-well plate, treated with collagenase D/dispase at 37°C for 45 min, incubated in 1ml DMEM/10% FBS medium overnight. Cells were obtained by passing the cell suspension through a Cell Strainer (70 mm,Falcon), centrifuged and then incubated in growth medium (DMEM supplemented with 10% FBS, 100 mg/ml penicillin, 100 mg/ml streptomycin and 2.5 mg/ml amphotericin B). All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. N-acetylcysteine (NAC) was purchased from Sigma Chemical Co (St. Louis, MO). Hydrogen peroxide ($H_2O_2$) was from Sangon Biotech. Pifithrin-α was purchased from Beyotime.

### 2.2. Immunofluorescence staining of phospho-H2AX (Ser139)

Antibodies against phospho-H2AX (Ser139) (DAM1661039) were purchased from Millipore (Billerica, MA). TRITC-conjugated Goat anti-mouse secondary antibody was from Jackson Immuno Research Laboratories (West Grove, PA, USA). Cells were grown on coverslips in 6-well plates. After various treatments, cells were washed in PBS, and fixed in 4% paraformaldehyde for 20 min. They were washed three times in PBS for 5 min each and permeabilized for 20 min in 0.2% Triton X-100. The cells were blocked in 10% normal goat serum overnight at 4 ° C. After being washed in PBS, the coverslips were incubated with anti-phospho-H2AX antibody overnight at 4° C. The cells were then washed in PBS, and incubated with TRITC-conjugated secondary antibody for 1 h at room temperature. Cells were washed in PBS four times and counterstained with 4-6-diamidino-2-phenylindole (DAPI). The coverslips were mounted on slides for examination.

### 2.3. RNA Interference

The construction containing p53-shRNA (shp53) and the negative control (shNeg) were purchased from GenePharma (Shanghai, China). For stable transfection, U2OS cells were maintained in culture medium free of antibiotics in a 24-well plate. After overnight incubation, transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated in the medium (containing selection drug) 24 h after transfection. The siRNA duplexes of SESN1 and p400 and negative control siRNA duplexes were purchased from sigma. U2OS cells were transfected with siRNA duplexes (20 μM) using lipofectamine2000 (Invitrogen) for 24 h. Transfected cells were tested by real-time quantitative RT-PCR assay and Western blotting analysis, and then used for further study.

The oligonucleotide sequences were as follows:

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shp53: GACTCCAGTGGTAATCTAC

shNeg: GTTCTCCGAACGTGTCACGT

sip400: CUGAUGAGGAGUUUCAACATT

siSESN1: GUUUGGAUAACAUUACGUUTT

siNeg: UUCUCCGAACGUGUCACGUTT

### 2.4. RNA Isolation and Real-time Quantitative RT-PCR Array

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's introduction. cDNA was obtained by reverse transcription-PCR of 1 μg of total RNA using the AMV reverse transcriptase (Promega). Real-time quantitative RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7500 sequence detection system (Applied Biosystems). Human GAPDH gene was used as control. The real-time PCR was carried out in a final volume of 10 μl. The samples were loaded in quadruples, and the results of each sample were normalized to GAPDH. The primers for SYBR were from primer bank of Harvard Medical School (http://pga.mgh.harvard.edu/primerbank/).

Primer sequences are described as follow:

SESN1

5'- AAGTGGGGAGTGAAGACGC -3' (Forward)

5'- GGCCCATCCATTTGCAGTAGA -3' (Reverse)

p400

5'- GAAGCACAGTAGAGACGGACC -3' (Forward)

5'- CTGGAAAACTACCCCTTGGTG -3' (Reverse)

GAPDH

5'- CAGAACATCATCCCTGCCTCTAC -3' (Forward)

5'- TTGAAGTCAGAGGAGACCACCTG -3' (Reverse)

### 2.5. Western Blotting Analysis

Cells were harvested and lysed with cell lysis buffer for Western and IP (Beyotime) according to the manufacturer's instructions. Protein concentration was determined using the BCA assay. Equal amounts of protein were separated by 12% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA). After blocking with 5% skimmed milk, the membrane was incubated with specific primary antibodies overnight at 4 °C. Antibodies against p53 (sc-126), p-RAD17 (sc-32843) and RAD17 (sc-5613) were purchased from Santa Cruz Biotechnology. SESN1 (ab103121) and p400 (ab5201) were from Abcam (Cambridge, MA). phospho-Chk1 (Ser345) (2348) and Chk1 (2360) were from Cell Signaling Technology Inc. (Beverly, MA). β-actin was from Sigma Chemical Co. (St. Louis, MO). Proteins of interest were detected with appropriate horseradish peroxidase-conjugated

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secondary antibody for 1 h and visualized by ECL Western Blotting Substrate (Thermo Scientific).

### 2.6. Determination of Cellular ROS by Flow Cytometry

To evaluate intracellular ROS levels, a GENMED kit was used. Briefly, cells were washed with PBS, trypsinized and then collected. The cells were then incubated with CM-$H_2$DCFDA in the dark for 20 min at 37 °C. After washing, cells were analyzed by flow cytometry, which was performed using the FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using the FCSExpress V3 program (De Novo Software).

### 2.7. Scoring of MN

The samples were coded and examined with an Olympus DP71 fluorescence microscope. MN and MN-γ-H2AX (+) were scored as previously reported [27]. Briefly, nuclei were scored first for MN using a 100× objective under DAPI filter. MN were then examined for the presence or absence of γ-H2AX signals under Texas Red filter. At least 1000 cells per sample were scored.

### 2.8. Statistical analysis

Statistical calculations were performed using the SPSS 13.0. The statistical significance of the differences in micronuclei data was analyzed using the Chi-square Pearson test. *P* value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Induction of MN-γ-H2AX (+) by $H_2O_2$

Hydrogen peroxide ($H_2O_2$) is frequently used as an inducer of oxidative stress. We treated U2OS cells with increasing concentrations of $H_2O_2$ (50 μM, 100 μM, 150 μM) for different durations (2 h, 6 h, 12 h, 24 h), and examined the frequencies of the MN-γ-H2AX (+) at 48 h after $H_2O_2$ was washed out. We previously showed that the frequency of MN-γ-H2AX (+) usually peaked at 48 h after drug removal [27]. As shown in Figure 1A, at the lowest concentration tested (50 μM), $H_2O_2$ treatment led to a significant increase in the frequency of MN-γ-H2AX (+) in all the treatment durations. Taking $H_2O_2$ treatment for 2 h as an example, the frequency of MN-γ-H2AX (+) was increased to $3.32\times10^{-2}$, from $1.77\times10^{-2}$ in the control ($P<0.05$). At this concentration, $H_2O_2$ −24 h caused the most pronounced increase, a 3.8 fold increase over the control ($P<0.05$). In comparison, the frequency of MN-γ-H2AX (−) did not show a significant increase in any of the increasing treatment durations.

With increasing $H_2O_2$ concentrations, the increase in the frequency of MN-γ-H2AX (+) became more striking (Figure 1B, 1C). However, the higher concentrations of $H_2O_2$ also caused significant increase in the frequency of MN-γ-H2AX (−) (Figure 1B, 1C), albeit to a lesser extent in terms of fold increase over the baseline. These results suggest that MN-γ-H2AX (+) are more responsive to $H_2O_2$ than MN-γ-H2AX (−).

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To confirm that the $H_2O_2$ –induced increase in the frequency of MN was specifically caused by increased oxidative stress, we pretreated cells with antioxidant NAC for 90 min, and then exposed them to $H_2O_2$. Pre-incubation with NAC significantly attenuated the induction of MN-γ-H2AX (+) by $H_2O_2$ in 50 μM-24h, 100 μM-(2 to 24h) and 150 μM-(2 to 24h) groups (Figure 1A, 1B, 1C). In comparison, NAC significantly attenuated the induction of MN-γ-H2AX (−) only in the 100 μM−24h and 150 μM-(4 to 24h) groups (Figure 1B, 1C). These results suggest that induction of MN-γ-H2AX (+) by $H_2O_2$ was mediated by increased oxidative stress.

### 3.2. Elevation of MN-γ-H2AX (+) in p53-deficient cells

We next tested whether p53 deficiency is associated with an elevation in MN-γ-H2AX (+). To this end, we established a p53 knockdown cell line by RNA interference, designated as U2OS-shp53, and a control counterpart as U2OS-shNeg. The downregulation of p53 was confirmed by western blotting (Supplementary Figure S1). As shown in Table 1, 43 of the 1009 U2OS-shp53 cells contained MN-γ-H2AX (+), in comparison to 21 of 1062 U2OS-shNeg cells harboring them ($P$ <0.01), indicating that lack of p53 is associated with increased frequency of MN-γ-H2AX (+).

We also examined the frequency of MN-γ-H2AX (+) in a previously established a U2OS cell line expressing a dominant negative mutant p53 (U2OS-mtp53). As shown in Table 1, U2OS-mtp53 cells also exhibited a higher frequency of MN-γ-H2AX (+) than in the control U2OS-neo cells ($P$ <0.05).

When U2OS cells were subjected to pifithrin-α, a p53 inhibitor, for 48h and then assayed for MN, we also noted a two-fold increase in the frequency of MN-γ-H2AX (+), from $1.29\times10^{-2}$ in the control to $2.67\times10^{-2}$ in the 60 μM treatment group ($P$ <0.05) (Supplemental Table S1). In contrast, the frequency of MN-γ-H2AX (−) remained unchanged.

Similarly, primary mouse skin fibroblasts derived from p53 null mice were observed to have a higher frequency of MN-γ-H2AX (+) than those from p53 wild-type mice (Table 1, $P$ < 0.01). The frequency of MN-γ-H2AX (−) cells showed a less pronounced difference with regard to p53 status ($P$ > 0.05). Together, these results indicate that reduction in or absence of p53 function is preferentially associated with increased production of MN-γ-H2AX (+).

We next tested whether the increased formation of MN-γ-H2AX (+) in U2OS-mtp53 cells is because of ROS. While the frequency of MN-γ-H2AX (+) was significantly higher in U2OS-mtp53 cells than in U2OS-neo cells, the difference became narrow and statistically insignificant when the cells were pre-incubated with NAC (Table 2).

We further tested the effect of NAC on the formation of MN in three pairs of primary mouse skin fibroblasts (MSF) that were p53 wild-type and p53 null, respectively. MSF cells were incubated in the absence or presence of 5mM NAC and then assayed for MN. As shown in Supplementary Figure S2, while the frequency of MN-γ-H2AX (+) was significantly higher in p53-deficient MSF than in the wild-type controls ($P$ < 0.01), elevation of MN-γ-H2AX (+) in p53-deficient MSF was greatly attenuated by NAC. These results strongly support the

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idea that the elevation of MN-γ-H2AX (+) in p53 knockdown or null cells is mediated by increased oxidative stress.

### 3.3. Elevation of MN-γ-H2AX (+) in SESN1 knockdown cells

We next wanted to test whether any of the p53 target genes is responsible for reducing ROS and formation of MN-γ-H2AX (+). Among the genes activated by p53, sestrins (encoded by *SESN1* and *SESN2*, respectively) are known to reduce the spontaneous level of ROS [16, 29]. Thus, we tested the role of *SESN1* by knocking down *SESN1* in HEK-293 cells (Figure 2A). As expected, *SESN1* knockdown led to an increase in the level of ROS (Figure 2B). Examination of MN-γ-H2AX (+) showed that SESN1 depletion led to a significant increase in the frequency of MN-γ–H2AX (+) (Figure 2C), from 1.89% in mock siRNA transfected cell to 2.72% in siSESN1 transfected cells ($P < 0.05$). The frequency of MN-γ-H2AX (−), on the other hand, did not show statistically significant increase. These results suggest that the p53 downstream target SESN1 is one of the factors responsible for reducing spontaneous ROS and MN-γ-H2AX (+).

### 3.4. Depletion of p400 increases the frequency of MN-γ-H2AX (+)

It was recently reported that E1A binding protein p400 is involved in the regulation of ROS homeostasis, and knockdown of p400 led to an increase in intracellular ROS [30]. To test whether downregulation of p400 would cause more MN-γ-H2AX (+), we knocked down p400 in U2OS and HEK-293 cells, respectively, and examined the frequency of MN-γ-H2AX (+). As shown in Figure 3A and 3B, p400 could be efficiently downregulated by RNAi in both cell lines. Consistent with previously reported (30), p400 knockdown led to an elevation in ROS levels in both cell lines (Figure 3C). Moreover, p400 knockdown cells (sip400) exhibited a higher frequency of MN-γ-H2AX (+) when compared to control (siNeg) cells. In each case, the frequency of MN-γ-H2AX (+) was about two fold higher in sip400 than in siNeg cells (Figure 3D), with $P < 0.05$ and $P < 0.01$ for U2OS and HEK-293 cells, respectively. The frequency of MN-γ-H2AX (−), on the other hand, remained unchanged. These results further show that MN-γ-H2AX (+) is more responsive to change in ROS than the MN-γ-H2AX (−).

### 3.5. Reciprocal reinforcement of ROS and replication stress in inducing MN-γ-H2AX (+)

We reported previously that MN-γ-H2AX (+) were preferentially induced by DNA replication stress [27]. If ROS and replication stress can both preferentially induce MN-γ-H2AX (+), then how are the two factors related to each other in the induction of MN-γ-H2AX (+) ? We first tested whether cells experiencing oxidative stress would exhibit signs of replication stress. To this end, we measured the phosphorylation of Chk1 and RAD17, which each reflects replication stress [31–34], in cells treated with $H_2O_2$. We subjected U2OS cells and HEK-293 cells to different doses of $H_2O_2$ for 24 h, and then examined the levels of p-Chk1, Chk1, p-RAD17 and RAD17. As shown in Figure 4A, $H_2O_2$ treatment resulted in a marked dose-dependent increase in the level of p-Chk1 in both cell lines. The level of p-RAD17 was also increased in a dose-dependent manner, albeit to a lesser extent. This result suggests that ROS may mimic replication stress. Our results is consistent with a

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recent report showing that $H_2O_2$ can lead to ATR-dependent activation of Chk1 in *Xenopus* egg extracts [35].

In human endothelial cells, S-phase arrest caused by aphidicolin, an inhibitor of DNA polymerase α and δ, is accompanied by an increase in the level of ROS [36]. Persistent DNA breaks were also shown to be sufficient to elevate the level of ROS [30]. We next tested whether the replication stress-induced formation of MN-γ-H2AX (+) can be attenuated by antioxidant NAC. We previously showed that thymidine could serve as an effective inducer of replication stress and MN-γ-H2AX (+) [27]. Here we pretreated U2OS and MCF-7 cells with NAC (5mM) for 1.5 h before they were treated with thymidine at different concentrations for 24 h. Consistent with our previous report, thymidine caused a significant increase in the frequency of MN-γ-H2AX (+) in both cell lines. Pretreatment with NAC tended to offset the effect of thymidine. It effectively attenuated the induction of MN-γ-H2AX (+) by thymidine at 900 μM (Figure 4B and 4C). Taken together, our results suggest that replication stress and oxidative stress are intertwined and can reinfornce each other in the induction of MN-γ-H2AX (+).

## 4. Discussion

MN-γ–H2AX (+) are distinguished from MN-γ–H2AX (−) by the way they emerge. We previously showed that MN-γ–H2AX (+) are formed in S-phase, are preferentially induced by replication stress and are probably derived from aggregated DNA breaks [27]. A recent study showed that this subtype of MN may occur when their nuclear envelopes collapse and MN without intact envelope are incapable of DNA replication, DNA repair and transcription[37]. In this study, we demonstrated that the MN-γ–H2AX (+) can also be preferentially induced by $H_2O_2$. We showed that both $H_2O_2$ treatment and genetic manipulations that raise the endogenous level of ROS are much more effective for the induction of MN-γ–H2AX (+) than for that of MN-γ–H2AX (−). Application of the antioxidant NAC could significantly attenuate the induction of MN-γ–H2AX (+). Importantly, we showed that p53 function is more important for preventing the formation of MN-γ–H2AX (+) than for that of MN-γ–H2AX (−). Our results provide an important insight into the mechanism by which genomic instability arises when p53 function is lacking or reduced. Besides protecting cells against oxidative stress under physiological and mildly stressed conditions, p53 also functions in other cellular processes, including cell cycle checkpoints, DNA repair and cell death. While failure in any of those processes when p53 is dysfunctional can potentially lead to genomic instability, our results showed that it is the surge in oxidative stress that accounts for the bulk of the elevated MN-γ–H2AX (+) in p53 mutant cells. Considering that human *TP53* germline mutation carriers exhibit a higher level of oxidative metabolism [38], our results may bear an implication in understanding cancer development as well as other clinical aspects associated with p53 dysfunction.

Our results also suggest that oxidative stress mimics replication stress in the induction of MN-γ-H2AX (+). We showed previously that replication stress can preferentially induce MN-γ-H2AX (+) [27]. We here showed that oxidative stress is also involved in the formation of MN-γ-H2AX (+) in cells experiencing replication stress, since antioxidant NAC can effectively neutralize the effect of replication stressor thymidine (Figure 4B and

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4C). It was shown that thymine glycol, the most common oxidative thymine lesion, can stall replicative DNA polymerase [39]. The S-phase arrest caused by resveratrol in human endothelial cells was also shown to be mediated by an increase in the level of ROS [36]. Consistently, we observed that cells treated with $H_2O_2$ exhibited increased phosphorylation of Chk1 and RAD17 (Figure 4A), which mimics replication stress. A recent study showed that $H_2O_2$ can lead to ATR-dependent activation of Chk1 in *Xenopus* egg extracts [35]. It is proposed that APE2 (apurinic/apyrimidinic endonuclease 2), a key component in base excision repair pathway, may generate a stretch of single-stranded DNA that can bind RPA and lead to the activation of ATR-Chk1 pathway. Beacause ATR can phosphorylate H2AX, it is possible that in addition to inducing MN-γ-H2AX (+) indirectly by reinforcing replication stress, oxidative stress may also directly induce MN-γ-H2AX (+). Thus, oxidative stress and replication stress may reinforce each other in exerting their effect as an inducer of MN-γ-H2AX (+) (Figure 4D). Interestingly, DNA replication is restricted to the reductive phase (glycolytic phase) to protect genome integrity in the yeast, and an impediment in metabolic cycle–directed restriction of cell division leads to increased mutation rate [40]. Mammalian cells in S-phase are considerably more sensitive to $H_2O_2$ than in G1 phase [41]. Because replication stress is a major driver of genomic instability [27, 42–44], reducing the level of ROS during replication would understandably reduce replication stress and protect genome integrity. p53 appears to play a critical role in keeping the spontaneous level of ROS in check. In doing so, it alleviates replication stress and maintains genomic integrity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **ROS** | reactive oxygen species |
| **$H_2O_2$** | hydrogen peroxide |
| **NAC** | N-acetylcysteine |
| **MN** | micronuclei |
| **DSBs** | double-strand breaks |
| **MSF** | primary mouse skin fibroblasts |

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## Highlights

A subtype of micronuclei (MN) is preferentially induced by oxidative stress.

Induction of MN subtype by p53 dysfunction is mediated by oxidative stress.

Oxidative and replication stresses reinforce each other in generating MN.

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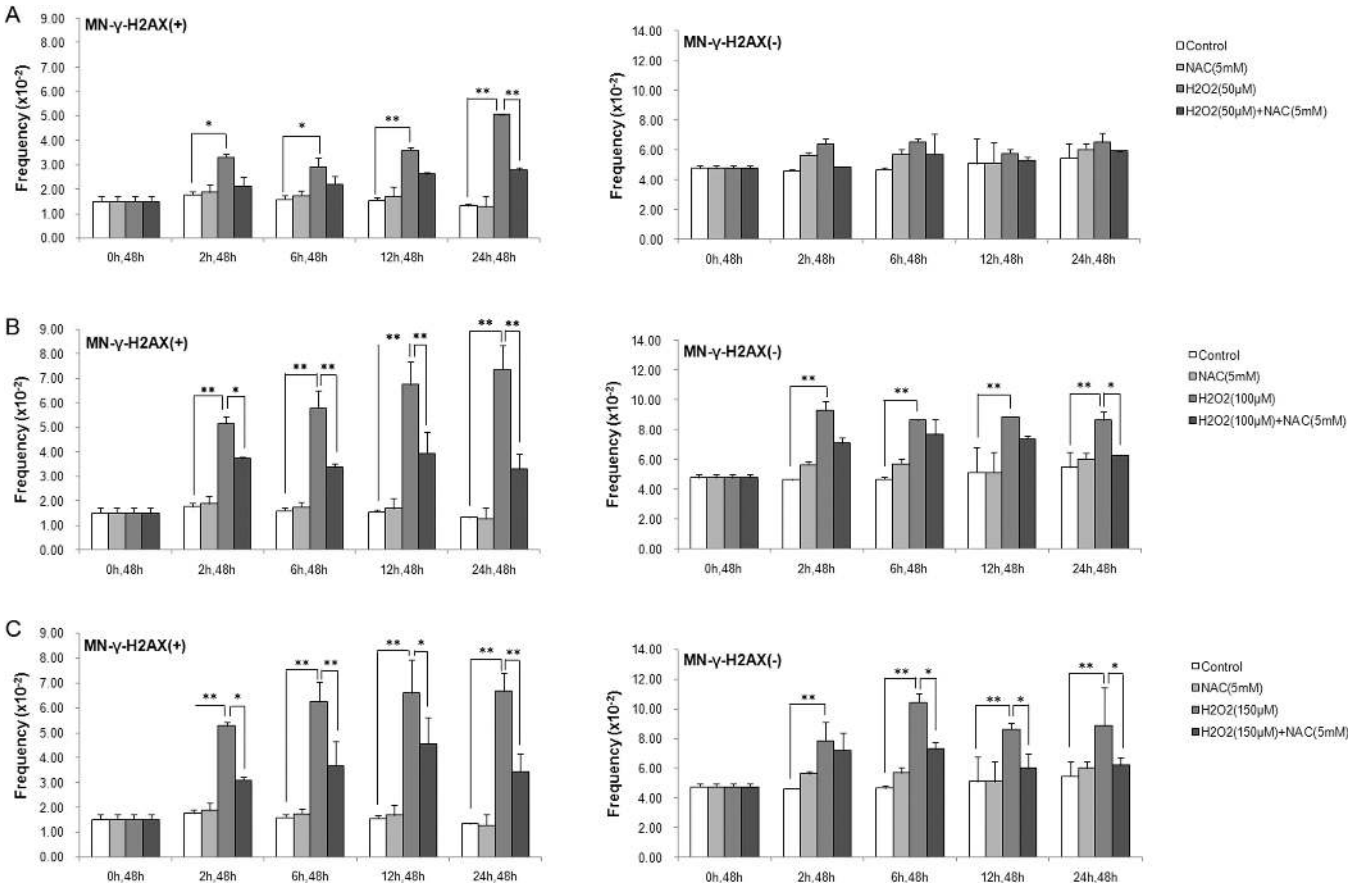


**Fig. 1.**

Induction of MN by $H_2O_2$ and its attenuation by NAC. U2OS cells were pretreated with or without the antioxidant NAC (5mM) for 90 min, and then were treated with 50μM $H_2O_2$ (A), 100μM $H_2O_2$ (B) or 150μM $H_2O_2$ (C) for different durations (2h, 6h, 12h, 24h). Cells were processed for examination of the MN-γ-H2AX (+) and MN-γ-H2AX (−) at 48h after $H_2O_2$ was washed out. At least 1000 cells were examined for each sample. * $P < 0.05$, ** $P < 0.01$.

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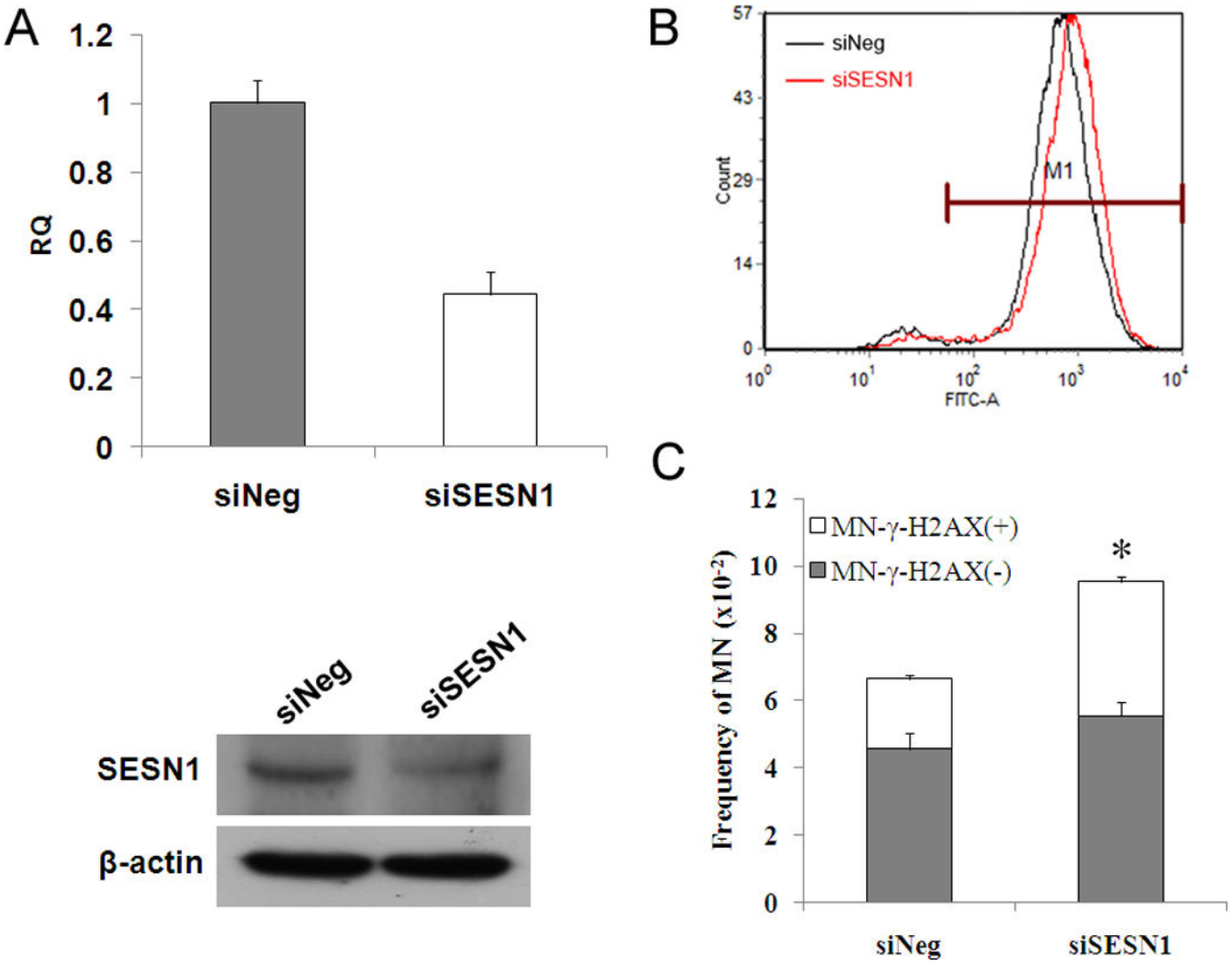


**Fig. 2.**

Elevation of MN-γ-H2AX (+) in SESN1 knockdown HEK-293 cells. A. The expression of *SESN1* mRNA and protein levels in HEK-293 cells. The level of *SESN1* mRNA was quantified by real-time quantitative RT-PCR assay. SESN1 protein level in siSESN1 HEK-293 cells was determined using the antibodies against SESN1. B. Measurement of ROS in SESN1 knockdown cells. HEK-293 cells were transfected using the indicated siRNA. Cells were collected and ROS levels were measured by flow cytometry 24h following transfection. C. Increased MN-γ-H2AX (+) in siSESN1 HEK-293 cells. **P*<0.05, when compared with siNeg HEK-293 cells.

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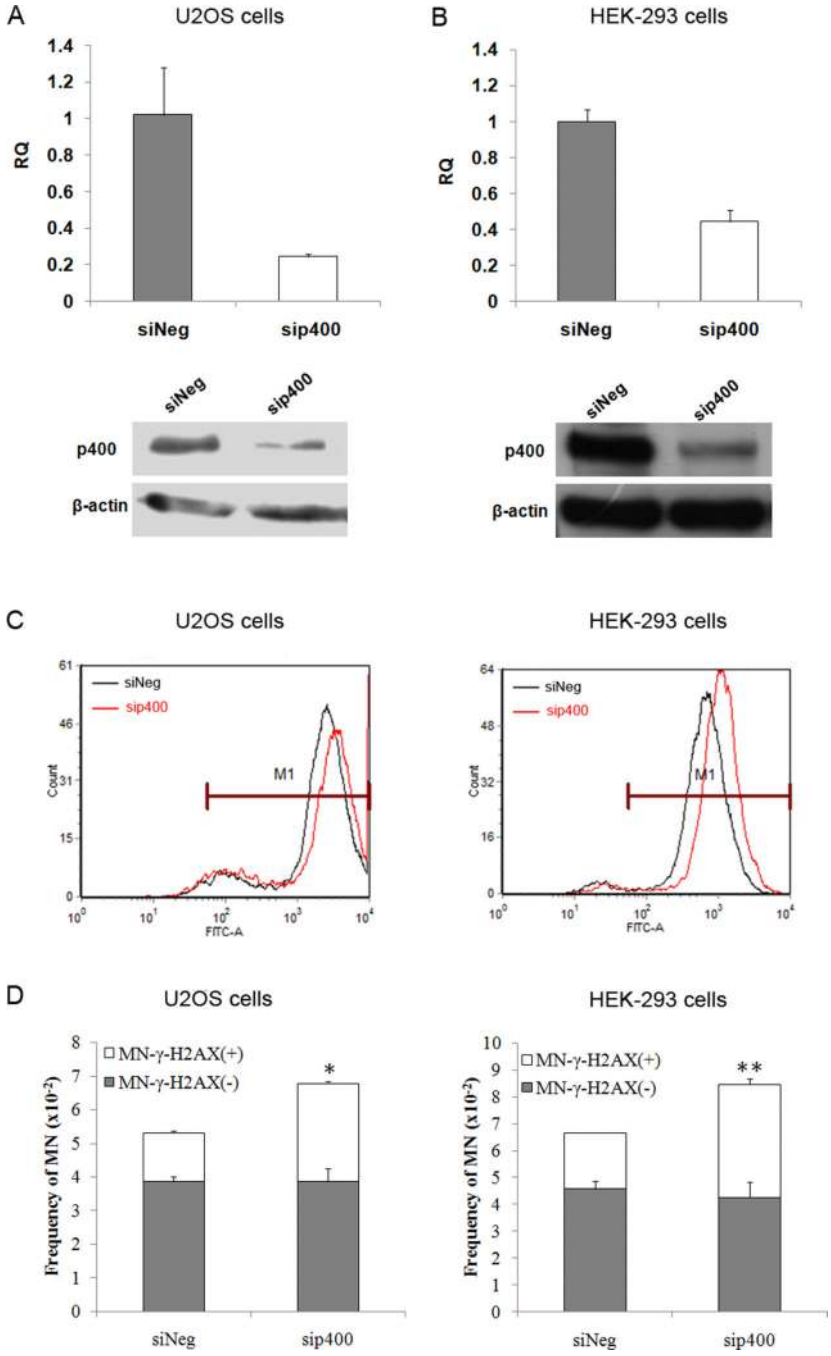


**Fig. 3.**

Elevation of MN-γ-H2AX (+) in p400 knockdown cells. U2OS cells and HEK-293 cells were transfected with siRNA duplexes specific to p400 or negative oligo in serum-free medium for 5h, and then replaced with complete medium for 24 h. The expression of *p400* mRNA and protein levels in U2OS cells (A) and HEK-293 cells (B) were measured by real-time quantitative RT-PCR assay and Western blotting, respectively. C. The levels of ROS in sip400 U2OS cells and sip400 HEK-293 cells were analyzed by flow cytometry. D.

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Increased MN-γ-H2AX (+) formation in sip400 U2OS cells and sip400 HEK-293 cells cells. *$P$<0.05, ** $P$ < 0.01, when compared with each siNeg cells.

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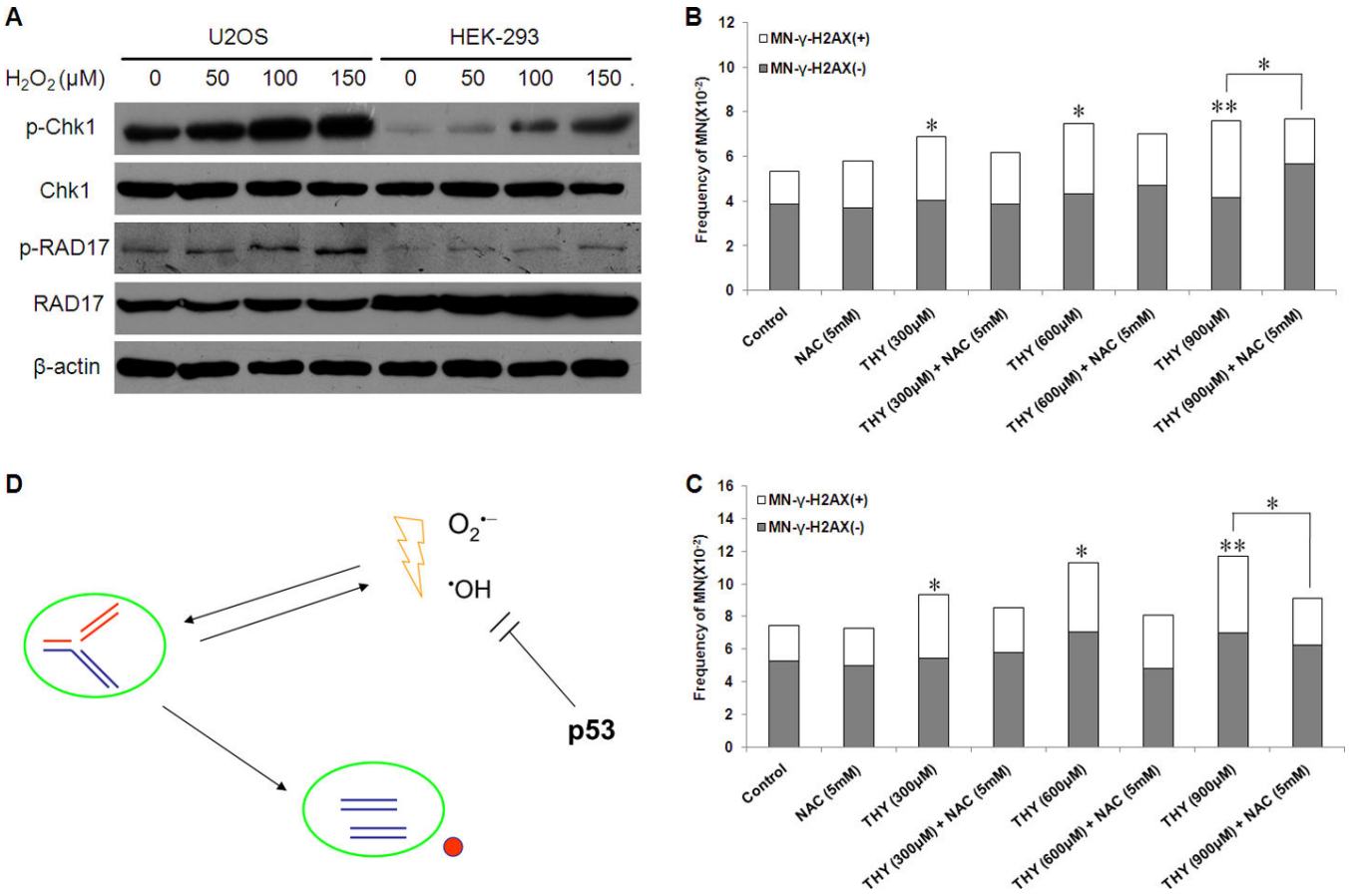


**Fig. 4.**

Mutual reinforcement of oxidative stress and replication stress in inducing micronuclei. A. Evaluation of markers reflecting replication stress. U2OS cells and HEK-293 cells were treated with indicated $H_2O_2$ concentrations for 24h. Cell extracts were analyzed by Western blot using antibodies to detect p-Chk1, Chk1, p-RAD17 and RAD17. β-actin was used as a loading control. Shown is a representative of three independent experiments. B and C. Attenuation of replication stress-induced MN-γ-H2AX (+) by NAC in U2OS cells (B) and MCF-7 cells (C). Cells were pretreated with or without the antioxidant NAC (5mM) for 90 min, and then were co-incubated with different concentrations of thymidine (THY) for 24h. The frequency of MN were examined at 48h after THY was washed out. * $P < 0.05$, # $P < 0.01$. D. A schematic model showing the connection between oxidative stress and replication stress in inducing MN-γ-H2AX (+).

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**Table 1**

Elevation of MN-γ-H2AX (+) in cells with dysfunctional p53

| | MN frequency ($\times 10^{-2}$) | MN-γ-H2AX(+) /MN(%) | MN-γ-H2AX (+) | | MN-γ-H2AX (−) | |
|---|---|---|---|---|---|---|
| | | | Frequency ($\times 10^{-2}$) | Fold change over 0μM | Frequency ($\times 10^{-2}$) | Fold change over 0μM |
| U2OS-shNeg | 5.18 | 38.18 | 1.98 | 1 | 3.2 | 1 |
| U2OS-shp53 | 8.92 | 47.78 | 4.26** | 2.16 | 4.66 | 1.45 |
| U2OS-Neo | 5.99 | 38.33 | 2.3 | 1 | 3.7 | 1 |
| U2OS-mtp53 | 9.91 | 42 | 4.16* | 1.81 | 5.75 | 1.55 |
| MSF-WT | 3.95 | 16.26 | 0.64 | 1 | 3.31 | 1 |
| MSF-p53mt | 8.26 | 26.19 | 2.16** | 3.37 | 6.1 | 1.84 |

*$P < 0.05$,

**$P < 0.01$

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**Table 2**

Attenuation of MN-γ-H2AX (+) by antioxidant NAC in U2OS-mtp53 cells

| | MN frequency ($\times 10^{-2}$) | MN-γ-H2AX(+) /MN(%) | MN-γ-H2AX (+) | | MN-γ-H2AX (−) | |
|---|---|---|---|---|---|---|
| | | | Frequency ($\times 10^{-2}$) | Fold change over 0μM | Frequency ($\times 10^{-2}$) | Fold change over 0μM |
| U2O2-Neo | 6.62 | 37.31 | 2.47 | 1.00 | 4.15 | 1.00 |
| U2OS-mtp53 | 10.28 | 40.45 | 4.16** | 1.68 | 6.12 | 1.48 |
| U2OS-Neo+NAC(5mM) | 7.24 | 30.45 | 2.21 | 1.00 | 5.04 | 1.00 |
| U2OS-mtp53+NAC(5mM) | 8.12 | 34.96 | 2.84 | 1.29 | 5.28 | 1.05 |

*$P < 0.05$,

**$P < 0.01$.