## Original Article

# p21 is Responsible for Ionizing Radiation-induced Bypass of Mitosis*

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**Abstract**

**Objective** To explore the role of p21 in ionizing radiation-induced changes in protein levels during the G2/M transition and long-term G2 arrest.

**Methods** Protein expression levels were assessed by western blot in the human uveal melanoma 92-1 cells after treatment with ionizing radiation. Depletion of p21 was carried out by employing the siRNA technique. Cell cycle distribution was determined by flow cytometry combined with histone H3 phosphorylation at Ser28, an M-phase marker. Senescence was assessed by senescence-associated-β-galactosidase (SA-β-gal) staining combined with Ki67 staining, a cell proliferation marker.

**Results** Accompanying increased p21, the protein levels of G2/M transition genes declined significantly in 92-1 cells irradiated with 5 Gy of X-rays. Furthermore, these irradiated cells were blocked at the G2 phase followed by cellular senescence. Depletion of p21 rescued radiation-induced G2 arrest as demonstrated by the upregulation of G2/M transition kinases, as well as the high expression of histone H3 phosphorylated at Ser28. Knockdown of p21 resulted in entry into mitosis of irradiated 92-1 cells. However, cells with serious DNA damage failed to undergo cytokinesis, leading to the accumulation of multinucleated cells.

**Conclusion** Our results indicated that p21 was responsible for the downregulation of G2/M transition regulatory proteins and the bypass of mitosis induced by irradiation. Downregulation of p21 by siRNA resulted in G2-arrested cells entering into mitosis with serious DNA damage. This is the first report on elucidating the role of p21 in the bypass of mitosis.

**Key words:** G2/M transition; DNA damage; Ionizing radiation; G2 arrest

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## INTRODUCTION

Environmental factors, such as toxic chemicals, oxidative stress, and ionizing radiation, could induce DNA damage in organisms.

Once DNA damage is generated, proliferating cells appear to enter a sustained arrest in the G1 or G2 phase of the cell cycle, which allows the cells to repair the damage. If the damage is repaired, cells reenter the cell cycle and become normal. If the damage is not repaired, cellular senescence will be initiated by activation of the persistent DNA damage response (DDR) pathway[1]. Normally, senescent cells

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contain diploid DNA, a typical characteristic of the G1 phase in somatic or *in vitro* cultured cells[2-3]. However, DNA damage-induced senescent cells contained a sizeable subpopulation with tetraploid DNA content[4-6]. These observations imply that the arrested cells with tetraploid DNA enter senescence at the G2 phase. This is inconsistent with the notion that all intermediate phases are unstable, except for G0 and the early G1 phase[7-9]. Our previous work[10] and other studies[4,11] suggested that these G2-arrested cells with DNA damage bypassed mitosis and directly slipped into G1 phase, then underwent senescence.

In this progression, proteins essential for G2/M transition, such as cyclin B1[10,12-14], cyclin-dependent kinase 1 (CDK1)[10], aurora kinase A (Aurora A)[10], and polo-like kinase1 (PLK1)[10] declined dramatically. cyclin B1/CDK1 is a master mitotic regulator[15]. In cycling cells, cyclin B1 accumulated during G2 is degraded at the metaphase to anaphase transition, and is predominantly localized in the cytoplasm until the beginning of mitosis[4,16]. Rapid nuclear translocation of cyclin B1/CDK1 is a key event for nuclear envelope breakdown. Aurora A and PLK1 are two other key mitotic kinases[15]. Aurora A is required for mitotic entry. The initial activation of Aurora A in late G2 phase is essential for recruitment of the cyclin B1/CDK1 complex to centrosomes, where it becomes activated and commits cells to mitosis[17-19]. PLK1 plays an essential role in both the onset of the G2/M transition and cytokinesis[15]. The overexpression of PLK1 is strongly correlated with a wide spectrum of human cancers and poor prognosis[20]. In normal cell cycle progression, these kinases are accumulated at G2 phase and degraded at anaphase by the anaphase-promoting complex/cyclostome (APC/C) to promote the exit of cells from mitosis[21]. The unscheduled degradation of these kinases is believed to result in long-term G2 arrest. However, the types of mechanisms that drive the unscheduled degradation of these mitotic kinases remain unknown.

Interestingly, accompanying the degradation of mitotic kinases, tumor protein p53 (TP53/p53) and cyclin-dependent kinase inhibitor 1A (CDKN1A/p21) were stably accumulated after ionizing radiation (IR)[10]. p21 is a multifunctional protein and a key player in regulating different cellular processes. The transcription of p21 is regulated by p53-dependent and -independent pathways[22]. It is clear that p53 and p21 are required to maintain G2 arrest in human cells[23-24]. Earlier and recent studies strongly suggested that p21 upregulation mediated cyclin B1 degradation by driving nuclear translocation of cyclin B1 in response to DNA damage[4,14,16]. However, it is still unclear whether p21 is responsible for the degradation of these mitotic kinases during G2 arrest after irradiation. In this study, the relationship between the upregulation of p21 and the changes in the protein levels of these mitotic kinases was investigated after exposure of human uveal melanoma 92-1 cells to ionizing radiation, the most common treatment method in cancer therapy.

## MATERIALS AND METHODS

### *Cell Culture and Irradiation*

Human uveal melanoma 92-1 cells[25] and normal fibroblast GM-08398 cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, NY, USA), 100 μg/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere with 5% $CO_2$. Cells were seeded in 12-well plates ($1\times10^5$ cells per well), 35-mm culture dishes ($2\times10^5$ cells per dish) or 60-mm culture dishes ($5\times10^5$ cells per dish), incubated for 48 h to 70% confluence, and irradiated at room temperature with X-rays generated by a Faxitron RX650 (Faxitron RX650, Faxitron Bioptics, Lincolnshire, IL, USA) at a dose rate of 1 Gy/min. Iron ion (LET 400 KeV/μm) irradiation was performed at the HIRFL (Heavy Ion Research Facility of Lanzhou, Institute of Modern Physics, Lanzhou, China). The dose rate ranged from 0.2 to 0.3 Gy/min.

### *Cell Cycle Assay*

After various periods of post-treatment incubation, cells were harvested and fixed with 70% pre-chilled ethanol for over 24 h at -20 °C. The fixed cells were washed twice with PBS, treated with 100 μg/mL RNase A, and stained with 50 μg/mL propidium iodide mixed buffer (BD Biosciences, San Jose, CA) for 30 min at 37 °C. Cell cycle distribution was analyzed with FlowJo. DNA content was measured with CellQuest (Benton Dickinson).

### *Immunofluorescence*

For immunostaining, cells were seeded on sterile coverslips at a density of $4\times10^5$ cells per dish in 60-mm culture dishes, cultured for 24 h, then irradiated and incubated (37 °C, 5% $CO_2$) for the indicated time. The irradiated cells were fixed with

4% paraformaldehyde for 10 min at room temperature and in pre-cooling methanol for 20 min at -20 °C, then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Nonspecific binding sites were blocked with 5% nonfat dried milk in PBS for 2 h at room temperature before probing with primary antibodies. Anti-Ki67 rabbit polyclonal (ab833, Abcam, Cambridge, MA, UK), and anti-53BP1 rabbit polyclonal (ab36823, Abcam, Cambridge, MA, UK) antibodies were used. Secondary antibodies (anti-rabbit, Santa Cruz, TX, USA) conjugated with AlexaFluor 594 were incubated for 1 h. Images were obtained using a Zeiss LSM 700 Meta confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

### *SA-β-Gal Staining*

Human uveal melanoma 92-1 cells ($1\times10^5$) were plated in 35-mm cell culture dishes and incubated for 48 h before exposure. At each indicated time point after irradiation, cells were stained with the Senescence-associated β-Galactosidase Staining Kit (C0602, Beyotime, Haimen, China) following the standard protocol suggested by the manufacturer. Senescent cells were identified under a light microscope.

### *Western Blotting*

Cells in 35-mm culture dishes were lysed in RIPA buffer (P0013C, Beyotime, Haimen, China) on ice and collected by gentle scraping with cell scraper. Samples were centrifuged at 10,000 ×g for 15 min at 4 °C, and total protein concentrations were determined from supernatants using the BCA protein assay kit (Pierce, Rockford, IL, USA). Thereafter, samples were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Beijing, China). Membranes were blocked for 2 h in blocking buffer (5% nonfat dry milk in PBS) and incubated with primary antibodies for 2 h. Next, membranes were washed three times with PBS containing 0.1% Tween-20, incubated with secondary antibody for 2 h at room temperature, and then washed with PBS containing 0.1% Tween-20. Protein bands were visualized using the enhanced chemiluminescence system (Amersham-Buchler, Braunschweig, Germany) and exposed to X-ray medical film (Kodak, Tokyo, Japan). GAPDH and β-actin were used as the loading controls. The antibodies employed in this study were anti-p53 (ab2433, Abcam, Cambridge, MA, UK), anti-p21 (sc-397, Santa Cruz, TX, USA), anti-β-actin (sc-130065, Santa Cruz, TX, USA), anti-PLK1 (36-298, Abcam, Cambridge, MA, UK), anti-Aurora A (35C1, Abcam, Cambridge, MA, UK), anti-cyclin B1 (GNS1, Santa Cruz, TX, USA), anti-CDK1 (ab32384, Abcam, Cambridge, MA, UK), and anti-GAPDH (sc-25778, Santa Cruz, TX, USA).

### *siRNAs and Transfections*

siRNA targeting p21 and the negative control siRNA sequence (siRNA-Neg) were purchased from Fulen Gen (Guangzhou, China) and were transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol in a final concentration of 50 nmol/L. All siRNA transfections were performed 24 h before the start of the experiment.

### *Statistical Analysis*

The differences between means were tested for significance using one-way ANOVA in the SPSS statistical package (version 17.0; SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was considered to indicate a statistically significant result ($^*$) and a value of $P<0.01$ was considered to indicate an extremely significant result ($^{**}$). All experiments were repeated at least three times and the values shown on the graphs represent the means±SEM.

## RESULTS

### *Ionizing Radiation Induces a Decline of Mitotic Kinases and Long-term G2 Arrest Followed by Senescence*

Previous studies have shown that upon exposure to high dose of IR, the cell cycle of exponentially growing 92-1 cells was suspended[12] and long-term G2 arrest was established, accompanying the decline in proteins essential for the G2/M transition, then these cells skipped the M phase, directly slipped into G1 phase, and underwent senescence[10]. In the current study, we confirmed this phenomenon in 92-1 cells after treatment with 5 Gy of X-ray irradiation. As shown in Figure 1A, untreated cells showed typical cell cycle progression and normal cell cycle distribution. Cells exposed to X-rays also resumed cell cycle progression, but failed to undergo cell division, leading to tetraploid cell accumulation. Notably, tetraploid cells might be cells that are arrested at the G2 or M phase. To confirm whether these tetraploid cells were arrested at the G2 or M phase, we used an antibody specific for histone H3 phosphorylated at

Ser28, a marker of the M phase, to assess whether these tetraploid cells had entered into M phase. However, immunoblot analysis did not detect histone H3 phosphorylation at Ser28 in the irradiated cells (Figure 1B), revealing that tetraploid 92-1 cells induced by X-rays were blocked at the G2 phase, not at entry into mitosis.

Cyclin B1 is essential for the G2/M transition. It has been confirmed that cyclin B1 accumulates during G2, is degraded at the metaphase to anaphase transition, and is predominantly localized in the cytoplasm until the beginning of mitosis[4,26]. However, in cells that failed to recover from G2 arrest, we observed a rapid decrease in cyclin B1 during the first 24 h after IR[4] (Figure 1B). Similarly, after IR, we also detected a remarkable decrease in CDK1, Plk1, and Aurora A[17-18], collectively known as mitotic kinases and essential for the G2/M transition[27] (Figure 1C). These results implied that the IR-induced decrease in G2/M transition regulatory proteins contributed to long-term G2 arrest.

Five days later, we determined the survival of these irradiated cells by staining for SA-β-gal (Figure 1D, 1E), a widely used marker of cellular senescence[28]. The irradiated 92-1 cells showed a marked increase in the amount of SA-β-gal-positive cells 5 days after IR (Figure 1D, E).

Taken together, these results confirmed that the majority of G2-arrested cells failed to enter into mitosis but entered senescence in the G2-arrested state in response to ionizing radiation (Figures 1D, E).

### *p21 Accumulates in Irradiated 92-1 Cells*

p21 is the principle mediator of cell cycle arrest in response to DNA damage. It primarily mediates G1 cell cycle arrest by inactivating G1-associated cyclin A- and cyclin E-containing cyclin/CDK complexes. However, some other reports also suggested a role for p21 in mediating G2 cell cycle arrest[23]. Here, we first exposed 92-1 cells to 0.5 Gy, 5 Gy of X-rays or 5 Gy of iron ion beams, and protein level analysis by western blot showed that p21 increased sharply after irradiation (Figure 2A, 2B). Notably, we observed that the kinetics and magnitude of p21 upregulation after DNA damage were dose and time dependent. Low-dose (0.5 Gy) X-ray treatment only induced a slight increase in p21 in 92-1 cells, while high-dose

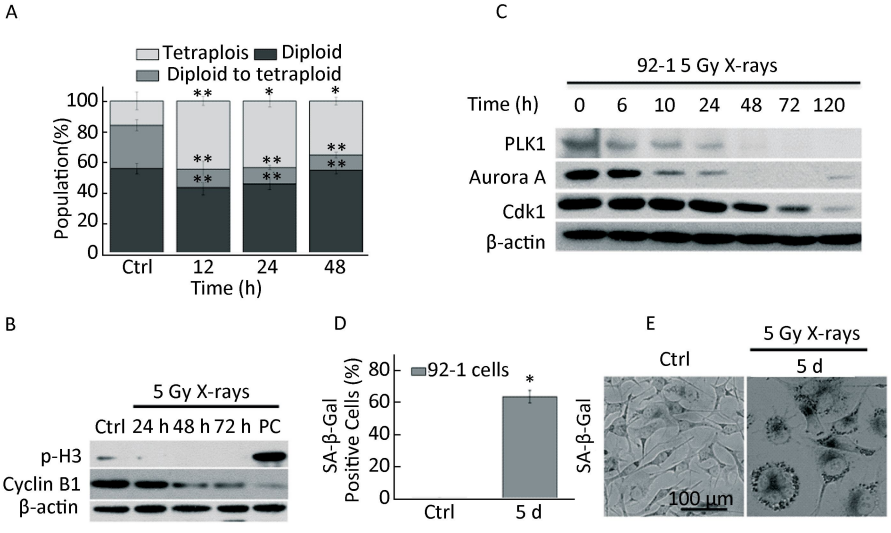


**Figure 1.** Ionizing radiation induced long-term G2 arrested cells underwent senescence. (A) The percentage of cells in each cell cycle phase is shown after 5 Gy X-rays. (B) The protein levels of cyclin B1 protein and H3 phosphorylated at Ser28 are shown after irradiation; PC: as a positive control for p-H3 protein detection. (C) Western blotting of the protein levels essential for G2/M transition in cells irradiated with 5 Gy of X-rays. (D) Quantification of irradiated cells that arrest for 5 days and become SA-β-gal positive. Means±SEM; *n*=3. (E) Representative micrographs of control and 5 Gy irradiated cells at 5 days after IR stained for SA-β-gal. Ctrl: non-irradiated samples. β-actin was used as the loading control. Scale bar: 100 μm. For the quantification analysis, 500 cells were analyzed for SA-β-gal experiments. $^{*}P<0.05$, $^{**}P<0.01$.

X-ray (5 Gy) treatment caused a dramatic increase in p21 24 h after irradiation (Figure 2A). However, the protein levels of p21 displayed a declining trend from 24 h to 5 d after IR in high-dose X-ray irradiated 92-1 cells (Figure 2A). In contrast, p21 in cells irradiated with 5 Gy of iron ions was maintained at high levels for up to 5 d after treatment (Figure 2B).

Many studies have confirmed that p21 is a master modulator of cellular senescence[29-30]. In agreement with this notion, we observed that the population of SA-β-gal-positive cells showed a correlation with the protein levels of p21. As shown in Figure 2C, high-dose iron ion irradiation, which maintained high levels of p21 in 92-1 cells, resulted in more than 90% of irradiated cells being stained by SA-β-gal 5 d after IR. In contrast, when low levels of p21 were induced by 0.5 Gy of X-rays, only 19% of cells were stained by SA-β-gal. This result implied that the accumulation of p21 was necessary to maintain G2 arrest and trigger the onset of senescence.

p21 expression is regulated in large part by p53, which acts as a transcription factor. Some studies suggested that p53 was not required for the initial arrest of cells in the G2 phase, but was essential for the long-term maintenance of the arrest[23-24]. In addition, p53 was required for DNA damage-induced senescence[4]. In this study, we also detected the protein level of p53 in irradiated 92-1 cells. As expected, p53 was upregulated quickly and maintained at high levels after IR (Figure 2B). However, we did not

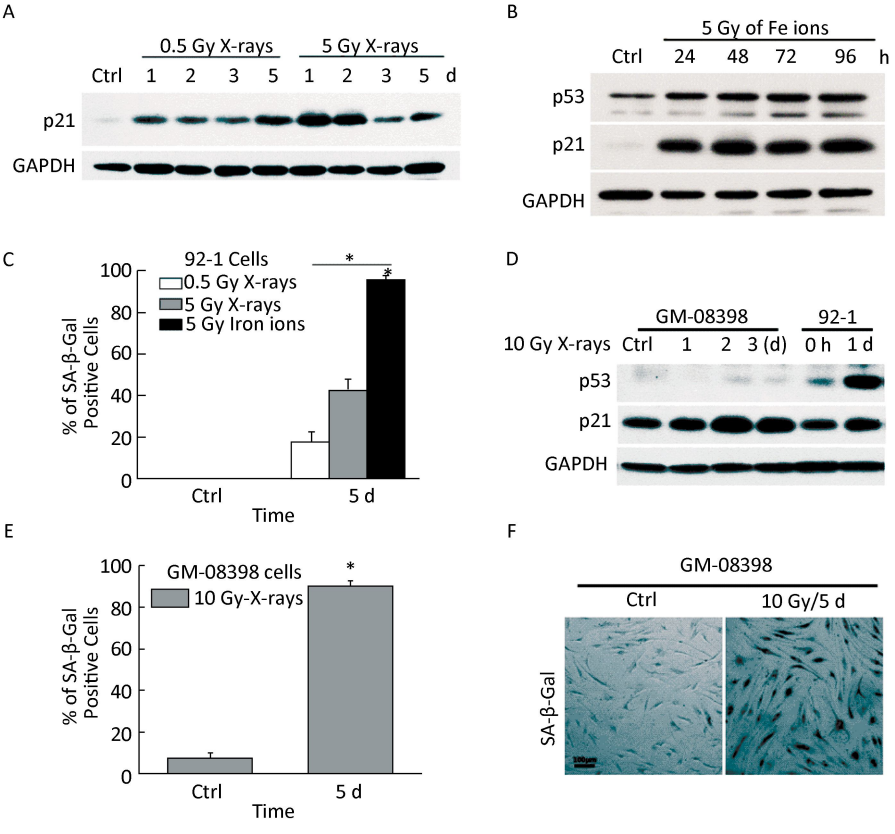


**Figure 2.** p21 accumulation induced by irradiation. Western blotting of p21 and p53 expression in cells irradiated with 0.5 Gy or 5 Gy of X-rays (A) or 5 Gy of iron ions (B) at the indicated time points post irradiation. (C) Quantification of irradiated cells that arrested for 5 d and became SA-β-gal-positive in 92-1 cells. Means±SEM; *n*=3. (D) Western blotting of p53 and p21 expression in GM-08398 cells irradiated with 10 Gy of X-rays; the panels of 92-1 cells are shown as a positive control for p53. (E) Quantification of irradiated cells that arrested for 5 d and become SA-β-gal-positive in GM-08398 cells. Mean±SEM; *n*=3. (F) Representative micrographs of control and irradiated cells (treated with 10 Gy of X-rays) 5 d after IR stained for SA-β-gal. Ctrl: non-irradiated samples. Scale bar: 100 μm. For the quantification analysis, 500 cells were analyzed in SA-β-gal experiments.

observe an increase in p53 expression in irradiated GM-08398 cells (Figure 2D), a cell line in which long-term G2 arrest also occurred followed by a senescent state 5 d after IR (Figure 2E, 2F). Interestingly, p21 expression was dramatically upregulated after 10 Gy of X-ray irradiation (Figure 2D). In addition, the p21 protein was maintained at a relatively high level in untreated GM-09398 cells (Figure 2D). This might be because some p53-independent p21 synthesis occurred after the irradiation of GM-08398 cells. These results were consistent with the fact that p53 was a major, but not the sole, transcriptional regulator of p21 in mammalian cells. Taken together, the accumulation of p21 was essential for IR-induced G2 arrest and senescent entry, though we cannot confirm whether p53 contributed to G2 arrest and senescence.

### *Depletion of p21 Induces G2/M Transition by Rescuing IR-induced Decline of G2/M Regulatory Proteins*

To further reveal the connection between the upregulation of p21 in response to DNA damage and the maintenance of G2 arrest in irradiated 92-1 cells, we used siRNA to knock down the expression of p21 (Figure 3A), and assessed cell cycle progression in irradiated 92-1 cells. As seen in Figure 3C and 3D, knockdown of p21 in irradiated 92-1 cells significantly impaired their ability to block cells at the G2 phase, thus leading to entry into mitosis with DNA damage. However, these cells never completed cytokinesis[31], and they were eventually flattened and were found to appear abnormal in shape and multinuclear (or to have abnormal nuclei) (Figure 3C, 3D, 3E, 3G). To confirm whether these cells entered into mitosis, we used immunoblot analysis to detect the M-phase protein marker histone H3, phosphorylated at Ser28, in p21 depleted 92-1 cells. After treatment with high dose of X-rays, cells transfected with negative control siRNA lacked the expression of histone H3 phosphorylated at Ser28 (in agreement with Figure 1B). However, the protein level of histone H3 phosphorylated at Ser28 was dramatically upregulated at 48 h after IR in p21 depleted cells (Figure 3B). Ki67 is a cell proliferation marker and Ki67 staining can be used to detect proliferation potential in all phases except the rest phase of cells. We also detected Ki67-positive cells in these irradiated cells. The results showed that high-dose X-ray treatment efficiently abolished cell proliferation 3 d post-irradiation. In contrast, depletion of p21 could partly rescue cells from radiation-induced cell proliferation abolishment (Figure 3E, 3F), which was also proven by the high percentage of Ki67 staining that colocalized with abnormal nuclei (Figure 3E, 3H). Together, these results suggested that depletion of p21 led to the G2/M transition in cells treated with IR.

The progression of cells from G2 phase to mitosis is a tightly regulated cellular process that requires activation of the mitotic kinases, such as CDK1[15], PLK1[15,20], and Aurora A[18], which determines the onset of mitosis in all eukaryotic cells[27]. Once DNA was damaged, the elimination of these G2/M transition kinases helped to maintain cells in G2 phase. This provided an opportunity for damage repair and to stop the proliferation of damaged cells. Here we found that G2 arrest was abrogated if p21 was depleted before irradiation. To determine whether these mitotic kinases were responsible for mitotic entry in p21-depleted cells, we detected the protein levels of these mitotic kinases in p21-depleted cells treated with 5 Gy of X-rays. As shown in Figure 4A and 4B, the protein levels of CDK1, cyclin B1, Aurora A, and PLK1 were decreased dramatically in cells transfected with negative control siRNA sequence (siRNA-Neg) before treatment with 5 Gy of X-rays. Strikingly, a decrease in all of these kinases and cyclin B1 induced by X-rays was rescued by depletion of p21 before irradiation. This result suggested that p21 was a master regulator of G2 arrest in response to DNA damage, which also displayed a tightly negative connection between p21 and mitotic kinase activation.

### *M-phase Cells with Serious DNA Damage Fail to Undergo Cytokinesis*

Although depletion of p21 led to G2/M transition in irradiated 92-1 cells, the M-phase cells failed to undergo cytokinesis with serious DNA damage (Figure 5A), confirmed by the accumulation of multinuclear cells (Figure 3E, G). It was not yet clear whether cells with serious DNA damage failed to undergo cytokinesis because of a cytokinesis checkpoint or because of a simple mechanical problem. However, the cells that entered mitosis with DNA damage lost the ability to divide into two daughter cells and became large and flattened, displaying a senescent-like phenotype. To investigate the fate of these M-phase arrested cells, we stained the p21-depleted cells for SA-β-gal 5 d post-irradiation. Notably, depletion of p21 did not reverse the senescence entry of 92-1 cells in response to 5 Gy of X-rays, though it abolished G2 arrest

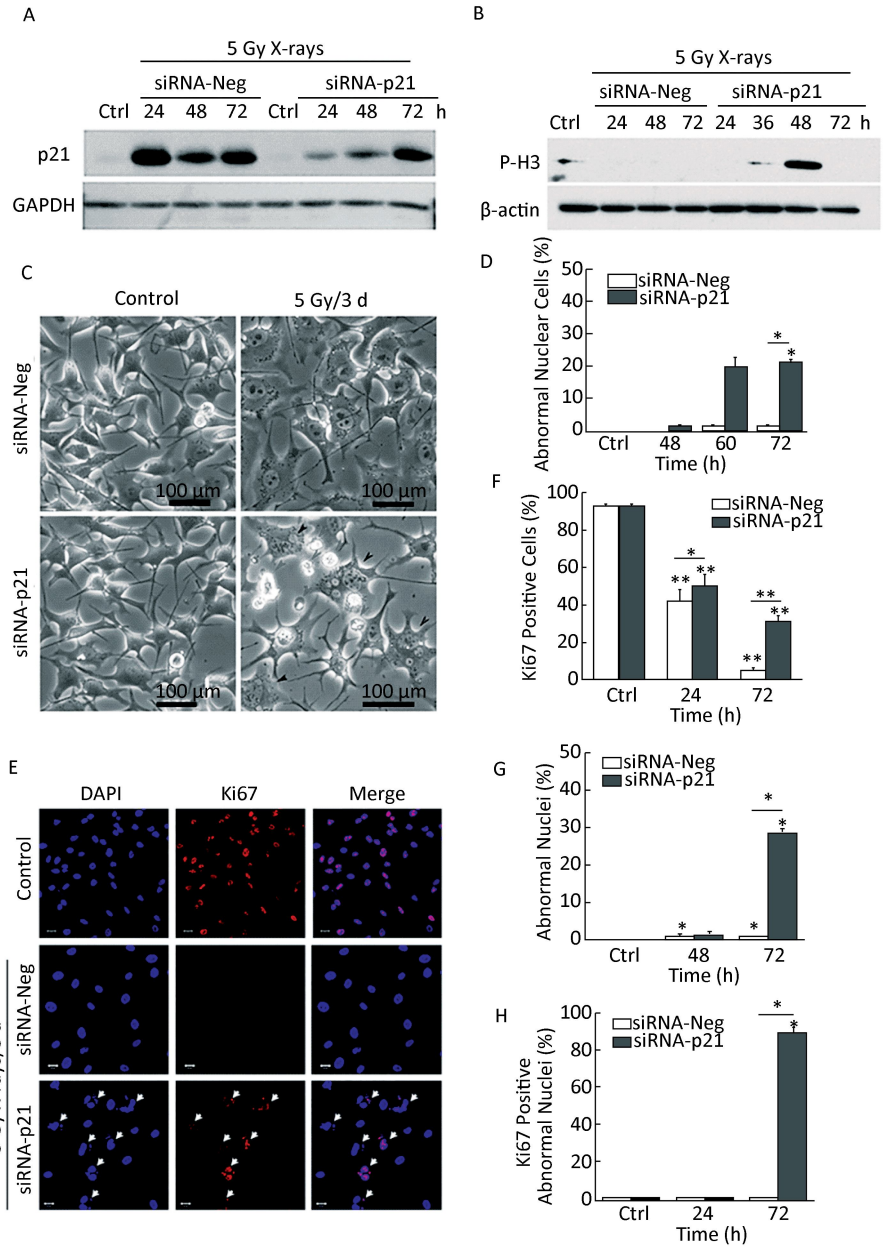


**Figure 3.** Cells lacking p21 were unable to sustain G2 cell cycle arrest in response to DNA damage. (A) Western blotting shows the relative expression levels of the indicated proteins in 92-1 cells used in (C)-(H)**.** (B) Western blotting shows the relative expression levels of phosphorylated H3 Ser28 in control cells and in p21 depleted 92-1 cells after irradiation with 5 Gy X-rays. (C, D) Control cells and cells depleted of p21 were irradiated with 5 Gy of X-rays and subsequently imaged for 3 days. (C) Representative micrographs and (D) quantification of abnormal nuclear cells in 5 Gy X-ray-irradiated cells are shown. Scar bar: 100 μm. (E) Representative micrographs of Ki67-positive cells and abnormal nuclei (white arrows) in control cells or p21-depleted cells 3 d after irradiation with 5 Gy of X-rays. Scar bar: 10 μm. (F-H) Same as before, control cells and cells depleted of p21 were irradiated with 5 Gy of X-rays. Quantification of Ki67-positive cells (F), abnormal nuclei (G), or Ki67-positive abnormal nuclei (H) is shown. For the quantification analysis, 500 cells per time point were analyzed.

induced by DNA damage (Figure 5B, C). This result implied that M-phase cells with serious DNA damage failed to undergo cytokinesis and then entered senescence with abnormal nuclei.

## DISCUSSION

Substantial evidence demonstrates that long-term G2 arrest followed by bypassing of mitosis is a critical mechanism for G2 phase cells in response to DNA damage in some cell lines[10-11]. Here, our data reveal that serious DNA damage induces long-term G2 cell cycle arrest in 92-1 cells. From the onset of cell cycle arrest, the protein levels of G2/M transition genes, such as cyclin B1, CDK1 (CDC2), Aurora A, and PLK1, started to decline quickly. Until now, it was not clear whether changes in the levels of G2/M regulatory proteins were the early triggers of G2 arrest. However, the down regulation of G2/M transition kinases was essential for maintenance of

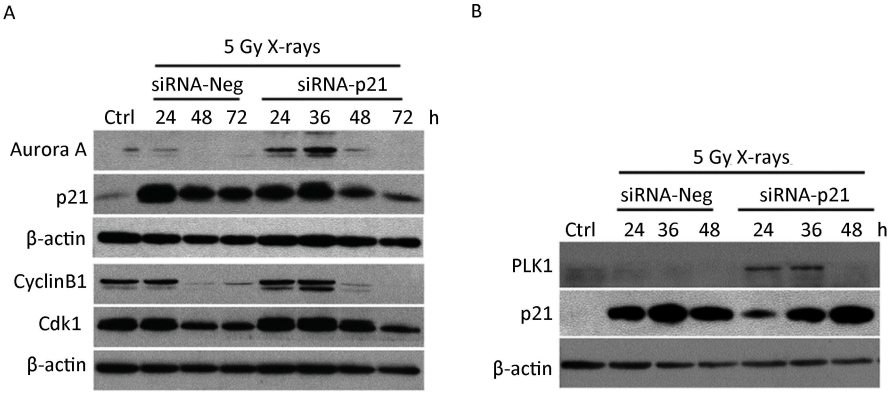


**Figure 4.** Deletion of p21 in irradiated 92-1 cells rescued the decline in IR-induced mitotic kinases. Western blotting of p21, CDK1, Aurora A, cyclin B1 (A), and PLK1 (B) protein levels at the indicated time points in control siRNA and p21 siRNA-transfected 92-1 cells after treatment with 5 Gy of X-rays. To knock down p21, 92-1 cells were transfected with a sequence of siRNA targeting p21 or a nonspecific siRNA sequence.

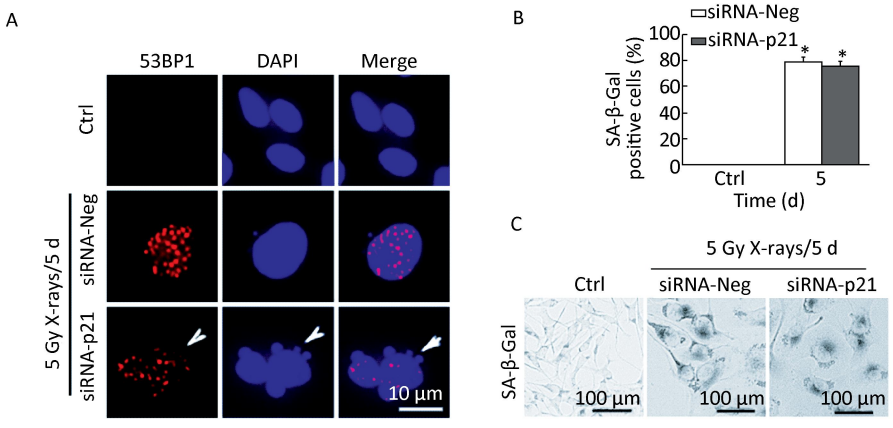


**Figure 5.** M-phase cells with serious DNA damage failed to undergo cytokinesis and entered senescence with abnormal nuclei. (A) Representative micrographs showing recruitment and retention of 53BP1 (indicates DNA double-strand break sites) at the sites of damaged DNA induced by 5 Gy X-rays in 92-1 cells. Scale bar: 10 μm. (B) Quantification of irradiated cells that arrest for 5 days and become SA-β-gal-positive in control cells and cells depleted of p21. Means±SEM; *n*=3. For the quantifications analysis, 500 cells were analyzed per time point. (C) Representative micrographs of control and 5 Gy irradiated cells 5 d after irradiation stained for SA-β-gal. Ctrl: non-irradiated samples. Scale bar: 100 μm.

long-term G2 arrest[4,14]. In addition, some studies demonstrated that p21 was essential for maintaining the G2 checkpoint[4,6,23]. In this study, we confirmed that p21 was required for long-term G2 arrest in 92-1 cells. Depletion of p21 by siRNA resulted in the arrested cells partly getting through the G2 block, and entering into the M phase with DNA damage (Figure 3). However, most of these M-phase cells failed to undergo cytokinesis, which led to the accumulation of cells with abnormal nuclei and serious DNA damage (Figure 3B, C, D, and F).

These results helped us to confirm that p21 is indeed essential for the establishment of G2 arrest and maintenance in response to DNA damage. We also enquired whether p21 was involved in maintaining G2 arrest by causing downregulation of G2/M regulatory proteins. Indeed, western blotting showed that depletion of p21 in irradiated 92-1 cells rescued G2/M transition kinases from radiation-induced downregulation (Figure 4). Combined with these results, we recommended that p21 sustained long-term G2 arrest by inducing a decline in key mitotic kinases in response to DNA damage, though the exact mechanism of this process is still unknown. One cogent model suggested that high levels of p21 expression helped cyclin B1 to enter into nuclear regions and was followed by cyclin B1 degradation[4]. Overexpression of p21 in U2OS cells has been observed to result in nuclear retention of cyclin B1 in G2 phase[32]. These studies recommended that p21 is a major regulator for unscheduled degradation of cyclin B1. But how p21 mediates CDK1, Aurora A, and PLK1 downregulation in response to DNA damage is still an interesting issue that requires further study.

After long-term G2 arrest, the irradiated 92-1 cells entered into senescence. We also wondered whether downregulation of p21 in irradiated 92-1 cells reversed the fate of these cells. However, most of the multinuclear cells with DNA damage entered a senescent state 5 d after IR (Figure 5), though a high percentage of abnormal nuclei still maintained proliferation competence (indicated by Ki67-positive staining) 72 h after IR (Figure 3D, 3E, 3G). Notably, in most of the multinuclear cells, the number of nuclei was fewer than three in a single cell. This implied that the cells blocked at the M phase did not enter into next cell cycle, though they broke through the G2 block caused by the downregulation of p21.

Although we have established the necessity for p21 in mitotic kinase downregulation in response to DNA damage, it remained unclear whether p21 played a direct or indirect role in the mechanism of mitotic kinase degradation. Some studies suggested that p21 might play a direct role in cyclin B1 degradation[14]. However, more evidence was still needed. Other studies suggested that the disassembly of nuclear pore complexes (NPCs) was a key event during nuclear disintegration in organisms undergoing mitosis. During this progress, nucleoporins were hyperphosphorylated. Direct evidence confirmed that CDK1, Aurora A, and PLK1 activities were essential for nucleoporin phosphorylation[11,18,23,26-27]. Thus, combined with this notion, our data imply that p21 upregulation suppresses nuclear disintegration during the G2/M transition, and this suppression leads to G2 arrested cells bypassing mitosis. Finally, although p21 mutations are relatively rare in cancers, p53 mutations are very common and cells with mutant p53 are impaired in their ability to upregulate p21 in response to DNA damage. Our work highlights the implications of intact p21 functions in the treatment of cancers with DNA-damaging agents and provides insight into the multiple roles of p21 as a cell cycle regulator.

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## COMPETING INTERESTS

The authors declare no competing financial interests.

## AUTHOR CONTRIBUTIONS

ZXR, WJF, and LSW designed this study. ZXR, LYA, SF, and LH performed the experiments and analyzed the data. ZXR and WJF wrote the manuscript.

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