Abstract. Ribosomes are important cellular components that maintain cellular homeostasis through overall protein synthesis. The nucleolus is a prominent subnuclear structure that contains ribosomal DNA (rDNA) encoding ribosomal RNA (rRNA), an essential component of ribosomes. Despite the significant role of the rDNA-rRNA-ribosome axis in cellular homeostasis, the stability of rDNA in the context of the DNA damage response has not been fully investigated. In the present study, the number and morphological changes of nucleolin, a marker of the nucleolus, were examined following ionizing radiation (IR) in order to investigate the impact of DNA damage on nucleolar stability. An increase in the number of nucleoli per cell was found in HCT116 and U2OS cells following IR. Interestingly, the IR-dependent increase in nucleolar fragmentation was enhanced by p53 deficiency. In addition, the morphological analysis revealed several distinct types of nucleolar fragmentation following IR. The pattern of nucleolar morphology differed between HCT116 and U2OS cells, and the p53 deficiency altered the pattern of nucleolar morphology. Finally, a significant decrease in rRNA synthesis was observed in HCT116 p53−/− cells following IR, suggesting that severe nucleolar fragmentation downregulates rRNA transcription. The findings of the present study suggest that p53 plays a key role in protecting the transcriptional activity of rDNA in response to DNA damage.

Introduction

The nucleolus is an intra-nuclear organelle that contains ribosomal DNA (rDNA) that encodes ribosomal RNA (rRNA), which is an essential component of ribosomes. The rRNA and a variety of ribosomal proteins form ribosomes, which serve as the site of biological protein synthesis. Previous studies have demonstrated that ribosome biogenesis is associated with multiple cellular activities. For example, limiting ribosome biogenesis extends the lifespan, whereas increased ribosome biogenesis has been reported as a hallmark of premature aging (1). In the context of cancer, alterations in ribosome biogenesis have been suggested to be involved in tumorigenesis by downregulating tumor suppressors (2,3). In addition, rDNA rearrangements have been observed in over half of adult solid tumors (4). This observation suggests that rDNA restructuring is one of the most common chromosomal alterations in adult solid tumors and may be involved in tumorigenesis or malignancy. Conversely, for cancer treatment, an inhibitor targeting RNA polymerase I, which is required for rDNA transcription, induces apoptosis in cancer cells by activating the ATM/ATR pathway (5).

Ionizing radiation (IR) generates multiple types of DNA damage, such as DNA double-strand breaks (DSBs), single-strand breaks (SSBs), and base damage (6). Among the various types of DNA damage, DSBs are considered to be a critical factor causing severe genome instability, such as deletion, duplication, inversion and translocation (7). Linear energy transfer (LET) is a parameter used to describe the pattern of energy deposition. X-rays or γ-rays are known as low LET IR, whereas heavy charged particle IR is high LET radiation. High LET particle irradiation causes complex DNA lesions, defined...
DNA damage was recently identified, observed as clustered foci of p53. The sequence of the p53 siRNA oligonucleotide knockdown was confirmed by immunoblotting for the detection of p53. The efficiency of the siRNA transfection was performed using HiPerFect (Qiagen GmbH), as previously described (18). The efficiency of the transfection was performed using a Faxitron RX-650 at 100 kVp and 40 mA with a dose rate of 1.14 Gy/min (Faxitron Biopsics). Carbon ion irradiation was performed at Gunma University Heavy Ion Medical Center using a spread-out Bragg-peak (SOBP) beam (290 MeV/nucleon; mean LET at the center of a 1-cm SOBP, 50 keV/μm).

siRNA knockdown and p53 overexpression. In U2OS cells, p53 siRNA transfection was performed using HiPerFect (Qiagen GmbH), as previously described (18). The efficiency of the knockdown was confirmed by immunoblotting for the detection of p53. The sequence of the p53 siRNA oligonucleotide used was 5′-GUGCCAGCUGUGGGUUGAUUdTdT-3′. To examine the effect of p53 overexpression on nucleolar stability after IR, Flag-p53 WT was transfected in HCT116 cells using the Neon® Transfection System (Thermo Fisher Scientific, Inc.). Transfection efficiency was confirmed by immunoblotting using p53 antibodies (1:500 anti-p53 antibody, sc-6243, Santa Cruz Biotechnology, Inc.) and flag (1:1,000 anti-flag antibody, F3165, Sigma-Aldrich; Merck KGaA). Cells were incubated in fresh MEM for 24 h and then irradiated and processed for immunofluorescence analysis.

Immunofluorescence staining. Cells were seeded on Fisherbrand microscope cover glasses (12-545-80 12CIR-1, Thermo Fisher Scientific, Inc.) 48 h prior to irradiation. At the indicated time points after irradiation, cells were fixed with 3% paraformaldehyde-2% sucrose for 10 min. After washing with phosphate-buffered saline (PBS), permeabilization was carried out by treatment with 0.2% TritonX-100-PBS (Sigma-Aldrich; Merck KGaA) for 2 min and 30 sec. Cells were then washed twice with PBS and incubated with the primary antibody in 2% bovine serum albumin (BSA)-PBS (1:1,000 anti-nucleolin antibody, ab22758, Abcam) at 37°C for 30 min. The cells were washed twice with PBS before being incubated with the appropriate secondary antibody conjugated to Alexa-Fluor-555 (1:500 in 2% BSA-PBS) at 37°C for 30 min. The cells were again washed twice with PBS and mounted onto slides using ProLong Gold antifade mountant (Thermo Fisher Scientific, Inc.).

Morphological evaluation of nucleoli. Immunofluorescence slides were examined with a Nikon microscope (ECLIPSE Ni-U with DS-Q2i camera and NIS-Elements D) using a 60x magnification lens. From a representative area of each sample, at least 100 consecutive cells were evaluated by direct visualization. First, each cell was examined simultaneously for nuclear staining and nucleolar morphology, and then categorized into one of the seven nucleolar types. The number of nucleoli per cell was noted for all cells, excluding type 7 cells (cells undergoing apoptosis, mitosis, or mitotic catastrophe). Nucleolin signal within micronuclei (types 3 and 6) were also counted individually, and the total number was a combination of the signal within the main nuclei and micronuclei.

Quantification of 47S rRNA expression levels using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Cells were seeded and incubated for 24 h prior to irradiation. At 72 h of irradiation, total RNA was extracted from the cells using NucleoSpin RNA (Macherey-Nagel GmbH). PrimeScript RT Reagent Kit (Perfect Real Time; Takara) was used to reverse-transcribe cDNA from total RNA, according to the manufacturer’s instructions, and qPCR was performed using StepOnePlus (Thermo Fisher Scientific, Inc.). Reactions (20 μl each) were prepared in duplicate in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each reaction contained 0.5 μM of each primer, 0.2 μM probe, 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), and cDNA as a template. The expression levels were normalized to GAPDH and were calculated using the 2−ΔΔCq method (19). The qPCR settings were as follows: Initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. The primers and probes used for the qPCR are listed below:
47S rRNA forward: 5’-GAA CGG TGG TGT GTC GTT C-3’
47S rRNA reverse: 5’-GCG TCT CGT CTC GTC TCA CT-3’
47S rRNA probe: 5’-CGG TGT GGG GTT CGA GGC GGT TT-3’
GAPDH forward: 5’-CTC CTC TGA CTT CAA CAG CGA-3’
GAPDH reverse: 5’-CCA AAT TCG TTG TCA TAC CAG GA-3’
GAPDH probe: 5’-ATG CCA GCC CCA GCG TCA AAG GT-3’

Statistical analysis. Box plots were constructed using SigmaPlot 12.3 (Systat Software, Inc.) from the combined data of three independent experiments. The statistical significance was analyzed using the Student’s two-tailed t-test or Mann-Whitney U test. For datasets where multiple comparisons were performed using single group, to avoid the high rate of familywise error, Bonferroni’s correction was applied. The level of significance was determined using the α-value obtained by Bonferroni’s correction and is specified in each individual figure legend.

Results

X-rays cause nucleolar fragmentation in human cancer cell lines. To investigate the nucleolar stability in response to IR in cancer cells, the number of nucleoli per cell and its morphological changes were examined following X-ray irradiation (n.b., nucleolin is localized within the nucleolus; therefore, it is used as a marker of nucleolus). In HCT116 p53<sup>+/+</sup> and U2OS cell lines, there was an increase in the number of nucleoli per cell over time after 2 Gy of X-ray irradiation (Fig. 1A and B). Next, the morphological patterns were categorized to investigate the morphological changes after IR (Fig. 1C). Interestingly, a marked increase in type 3 HCT116 p53<sup>+/+</sup> cells was observed following IR (Fig. 1D). Type 3 represents a cell containing micronuclei with a nucleolin signal. The peak of type 3 among HCT116 p53<sup>+/+</sup> cells was observed at 24 h following IR. By contrast, U2OS cells exhibited an increase in types 2 and 3 following X-ray irradiation (Fig. 1E). The majority of the nucleolar aberrations in U2OS cells were type 2 (cell containing micronuclei without nucleolin signal). These data suggest that nucleolar instability is caused in cancer cells following IR, and the type of morphological changes is cell type-dependent (Fig. 1F-H, supplementary information Fig. S1A). Furthermore, the levels of nucleolar instability were examined after different doses of X-ray irradiation. Importantly, the incidence of nucleolar fragmentation and type 3 morphology increased with the dose of X-ray irradiation until it peaked at 2-4 Gy at all the evaluated time points (Fig. 2A and B). Next, the number of nucleoli in normal human fibroblasts was examined following IR to investigate the nucleolar stability in normal cells. In contrast to the observation of the increase in...
The number of nucleoli in cancer cell lines, X-ray irradiation did not increase the number of nucleoli in normal human fibroblasts, even at markedly higher radiation doses (Fig. 3).

**p53 deficiency augments nucleolar fragmentation following exposure to X-rays.** Furthermore, to address whether the genetic status involving genome stability affects nucleolar fragmentation after IR, the number of nucleoli per cell and any morphological changes were examined in cancer cells with p53 deficiency. Then, we compared the nucleolin signal in HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> cells to investigate the impact of the p53 gene status on nucleolar stability. Importantly, a statistically significant (P=0.003) increase in nucleolar number was observed in HCT116 p53<sup>-/-</sup> cells at 72 h following exposure to X-rays (Fig. 4A). Furthermore, a dose-dependent increase in the nucleolar number of HCT116 p53<sup>-/-</sup> cells up to 8 Gy was found at 72 h (Fig. 4B). To investigate the effect of p53 on U2OS cells, siRNA knockdown targeting p53 was performed in U2OS cells. Similar to the results in HCT116 cells, there was a significant increase in the number of nucleoli in p53-depleted U2OS cells (Fig. 4C), with this increase being evident at later time points of 48-72 h after X-ray irradiation.

Next, morphological changes following the categorization shown in Fig. 1C in p53-defective cells were analyzed following IR. A morphological categorization analysis revealed the appearance of type 4 morphology (multiple nucleolar fragmentations without micronuclei) in HCT116 p53<sup>-/-</sup> cells at 72 h (Fig. 4D, supplementary information Figs. S1B and S2A-E). In addition, dose-dependent analysis confirmed more prominent type 4, type 6 and type 7 characteristics in HCT116 p53<sup>-/-</sup> cells (Fig. 4E). Alternatively, the morphological analysis in p53-depleted U2OS cells revealed a change in the nucleolar structure from type 2 to types 3 and 7 (Fig. 4F, supplementary information Figs. S1B and S2F-J). These results suggest that p53 is an important gene for suppressing nucleolar fragmentation following X-rays.
By contrast, when examining nucleolar fragmentation levels in HCT116 \(+/-\) cells exogenously overexpressing p53 (Fig. 5A), it was observed that, compared with mock cells, the HCT116 \(+/-\) cells overexpressing p53 exhibited a marginal increase in nucleolar fragmentation at 72 h after irradiation with 2 Gy of X-rays (Fig. 5B and C).

Carbon ions induce moderately greater nucleolar fragmentation compared with X-rays. High LET carbon ion irradiation is one of the most promising next-generation radiotherapies (20). Following high LET carbon ion irradiation, critical genome instability causes greater cell-killing effect due to the formation of complex DNA lesions and clustered DSBs, leading to large deletions or chromosomal translocations (21,22). To evaluate the biological effects of high LET carbon ion irradiation on nucleolar stability, the number of nucleoli per cell and the morphology were examined after 2 Gy of X-rays or carbon ion irradiation. Similar results were obtained by two independent experiments. (F) Percentage of nucleolar types in the entire set of U2OS cells. Cells after transfection with control siRNA (left panel) or p53 siRNA (right panel) were harvested at 24, 48 and 72 h after irradiation with 2 Gy of X-rays. The comparison of individual nucleolar types is shown in the supplementary information (Fig. S2F-J). The error bars represent the standard deviation of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. NS, not significant, for panels A and C. IR, ionizing radiation.

By contrast, when examining nucleolar fragmentation levels in HCT116 \(p53^{+/+}\) cells exogenously overexpressing p53 (Fig. 5A), it was observed that, compared with mock cells, the HCT116 \(p53^{+/+}\) cells overexpressing p53 exhibited a marginal increase in nucleolar fragmentation at 72 h after irradiation with 2 Gy of X-rays (Fig. 5B and C).
Transcriptional activity of rDNA is reduced under conditions of p53 deficiency following IR. To address the question whether nucleolar fragmentation after IR affects transcriptional activity of rDNA, qPCR was performed to measure the level of 47S rRNA, an immediate transcript of rDNA, which ultimately yields mature rRNA after RNA processing. Importantly, consistent with the results of nucleolar fragmentation, a significant decrease in rRNA synthesis was observed in HCT116 p53<sup>-/-</sup> cells 72 h after irradiation with 4-16 Gy of X-rays; however, X-ray irradiation did not change rRNA synthesis in HCT116 p53<sup>+/+</sup> cells after 2, 4 or 8 Gy (Fig. 8A and B). Instead, an increase was observed in HCT116 p53<sup>+/+</sup> cells after 16 Gy.

Discussion

In the present study, it was observed that IR causes nucleolar fragmentation, suggesting that DNA damage disrupts the nucleolar structure. Furthermore, it was observed that cancer cells lacking p53 or subjected to p53 siRNA display increased nucleolar fragmentation following IR. Compared with X-ray irradiation, carbon ion irradiation, generating complex DNA lesions and clustered DSBs, caused only slightly greater nucleolar fragmentation. In addition, the analysis of 47S rRNA using qPCR revealed a significant reduction of ribosomal RNA expression in p53-defective cells following IR. These results suggest that IR causes nucleolar instability, and p53 plays a key role in its prevention.

A chromosomal rearrangement, such as a large deletion, duplication, inversion or translocation, is a characteristic manifestation of dynamic genome instability following IR. The nucleolar fragmentation detected in the present study likely occurs during chromosomal rearrangements. In human cells, ~150-200 copies of rDNA encoding rRNA are located on five distinct chromosomes. Each chromosome contains a repetitive rDNA sequence. As rRNA forms secondary structures, such as hairpins, stem-loops, or cruciform structures (23), such loop structures are also expected to be formed in rDNA when DNA becomes single-stranded (e.g., during DNA replication or transcription). In the present study, however, it may be inferred that DSBs at rDNA regions are unlikely to be induced by IR, since the percentage of rDNA in the total genome is <1%. For example, 2 Gy of X-rays induce ~60 DSBs per cell in G1 phase cells and ~120 DSBs in G2 phase cells (24). Therefore, only 1 or <1 DSB per cell are induced at the rDNA region after 2-8 Gy of X-rays. Since such isolated DSBs are unlikely to be the cause of nucleolar fragmentation, it may be inferred that additional steps are required to induce nucleolar fragmentation following
IR. One possibility is that DNA replication-associated DSBs in S phase cells may be the cause of nucleolar fragmentation. IR causes multiple types of DNA damage, such as DSBs, base damage, SSBs and cross-link damage. The amount of base damage and SSBs is estimated to be ~1,000-3,000 per cell per Gy (25). The number of direct DSBs after 1 Gy X-ray is ~30 per G1 cell (24). However, if a DNA replication fork encounters unrepaired base damage or SSBs, the damage is converted into DSBs, namely replication-associated DSBs (i.e., several thousand DSBs may be formed at replication sites) (26). The generation of DNA replication-associated DSBs may be augmented under p53 deficiency. Since p53 plays an important role in the G1/S checkpoint arrest following IR (27), the lack of a p53-dependent G1 arrest leads to the enhancement of the replication-associated DSBs. This model is supported by the findings of the present study, showing that nucleolar fragmentation following IR is augmented in p53-defective cells.

At the beginning of this study, it was expected that carbon ion irradiation may cause substantially greater nucleolar fragmentation compared with X-rays. (A and B) Distribution of the number of nucleoli per cell at 24, 48 and 72 h after irradiation with 2 Gy of X-rays or 2 Gy of carbon ions in (A) HCT116 p53+/+ and (B) U2OS cells. X: X-ray, C: carbon ion. (C and D) Percentage of nucleolar types in the total set of evaluated HCT116 p53+/+ cells. Cells were harvested at 24, 48 and 72 h after irradiation with (C) 2 Gy of X-rays or (D) 2 Gy of carbon ions. The comparison of individual nucleolar types is shown in the supplementary information (Fig. S3A-E). (E and F) Percentage of nucleolar types in the total set of evaluated U2OS cells. Cells were harvested at 24, 48 and 72 h after irradiation with (E) 2 Gy of X-rays or (F) 2 Gy of carbon ions. The comparison of individual nucleolar types is shown in the supplementary information (Fig. S3F-H). The statistical significance in (A and B) was examined by comparing X-ray- and carbon ion-irradiated samples at the respective time points. The error bars represent the standard deviation of three independent experiments. **P<0.01, ***P<0.001. NS, not significant; IR, ionizing radiation.

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At the beginning of this study, it was expected that carbon ion irradiation may cause substantially greater nucleolar fragmentation, as it is associated with a higher frequency of chromosomal rearrangements compared with that observed after X-rays; however, the present analysis uncovered only a subtle enhancement of nucleolar fragmentation following carbon ion exposure. Replication-associated DSBs from base damage and SSBs may be the primary source of nucleolar fragmentation, as carbon ion-specific DSBs may not be implicated in the enhancement of nucleolar fragmentation. Consistent with this model, it may be inferred that p53 deficiency, which causes failure of G1/S checkpoint arrest (i.e., increase in S phase entry), led to nucleolar fragmentation. Additionally, G2/M checkpoint and mis-segregation during mitosis may be a major factor inducing nucleolar fragmentation. On morphological analysis, an increase in the formation of micronuclei with nucleolin signals was observed following IR, particularly in HCT116 cells and p53-depleted U2OS cells. As micronuclei are formed following mis-segregation in mitosis (28,29) the sensitivity of the G2/M checkpoint arrest is associated with nucleolar fragmentation. Although the role of p53 in G2/M checkpoint arrest following IR may be less significant, the lack of p53 may promote the formation of micronuclei with nucleolin signals through mis-segregation in mitosis following inefficient G2/M checkpoint arrest, particularly in U2OS cells. Additionally, it was observed that the primary type of fragmented nucleoli following IR in HCT116 cells was type 3, whereas in U2OS cells it was type 2. Although the precise mechanism underlying such a distinct type of formation is unclear, different responses between HCT116 and U2OS cells against the G1/S and G2/M checkpoint arrest may be involved. In contrast to the results observed in cancer cell lines, the normal human fibroblasts (1BR hTERT) did not exhibit an increase in the number of nucleolar fragments. Since normal fibroblasts display an intact G1/S and G2/M checkpoint arrest, nucleolar fragmentation and formation of micronuclei may not be efficiently induced via DNA replication and mitosis, respectively. In addition, following persistent checkpoint arrest, fibroblast cells tend to undergo cellular senescence rather than apoptosis (30); these distinct characteristics may explain the different levels
of nucleolar fragmentation following IR. By contrast, cancer cells, which normally exhibit the predominant modes of cell death after irradiation, preferentially undergo apoptosis and mitotic catastrophe under p53 intact and deficient conditions, respectively (12). Thus, it appears that the checkpoint machinery involving G1/S and G2/M checkpoint arrest and apoptosis under the regulation of p53 plays a critical role in preventing nucleolar fragmentation following IR (Fig. 9).

In addition to nucleolar fragmentation, rRNA production was found to be reduced in p53-defective cells following IR. Since rRNA is an essential component of the ribosome, which is required for all protein synthesis, the reduction of

![Figure 7](image-url) Carbon ion irradiation augments nucleolar fragmentation in p53-deficient cells. (A and B) The number of nucleoli per cell in (A) HCT116 p53wt cells was examined at 24, 48 and 72 h after 2 Gy of X-rays or 2 Gy of carbon ions. (B) Similarly, the number of nucleoli per cell in U2OS p53 siRNA cells was examined. X, X-ray; C, carbon ion. (C and D) Percentage of nucleolar types in the total set of evaluated HCT116 p53wt cells at 24, 48 and 72 h after irradiation with (C) 2 Gy of X-rays or (D) 2 Gy of carbon ions. The comparison of individual nucleolar types is shown in the supplementary information (Fig. S4A-F). (E and F) Percentage of nucleolar types in the total set of evaluated U2OS p53 siRNA cells. Cells were harvested at 24, 48 and 72 h after irradiation with (E) 2 Gy of X-rays or (F) 2 Gy of carbon ions. The comparison of the individual nucleolar types is shown in the supplementary information (Fig. S4G-K). The statistical significance in A and B was examined by a comparison of X-ray- and carbon ion-irradiated samples at the respective time points. The error bars represent the standard deviation of three independent experiments. *P<0.01, **P<0.001. NS, not significant; IR, ionizing radiation.

![Figure 8](image-url) Transcriptional activity of rDNA is reduced under conditions of p53 deficiency following IR. (A and B) Levels of 47S precursor rRNA, quantified by qPCR analysis 72 h after irradiation of HCT116 (A) p53wt (B) or p53−/− cells with 2, 4, 8, or 16 Gy of X-rays. The error bars represent the standard deviation of three independent experiments. The statistical significance was examined between irradiated cells at the indicated dose and non-irradiated cells, using Bonferroni’s correction. *P<0.0125, **P<0.0025, ***P<0.00025. NS, not significant; IR, ionizing radiation; qPCR, quantitative polymerase chain reaction.
ribosomal biogenesis would impair cellular activity and viability. In contrast to p53-defective cells, rRNA production in p53-proficient cells was not reduced, but rather enhanced, following high-dose IR. As described above, a proportion of cells undergo mitosis due to a checkpoint release following a prolonged G2/M checkpoint arrest, which results in micronuclei formation. Following mitosis, cells with micronuclei may undergo apoptosis in the next G1 phase if the p53-dependent apoptosis pathway is intact. Thus, in p53-proficient cells, normal levels of rDNA transcription may be sustained in the remaining cells with intact nucleoli, as cells with fragmented nucleoli are eliminated by apoptosis. On the other hand, in p53-deficient cancer cells, the remaining cells exhibit inefficient rDNA transcriptional activity due to the nucleolar fragmentation, as cells displaying fragmented nucleoli with micronuclei cannot efficiently undergo apoptosis.

Consistently with the present findings, evidence supporting the involvement of p53 in nucleolar biogenesis has also been provided by previous studies (31,32). The downregulation of ribosome biogenesis leads to the release of ribosomal proteins, including Arf, from the nucleolus to the nucleoplasm, which promotes the binding between Murine Double Minute 2 (MDM2) and released ribosomal proteins (31). Thus, the prevention of MDM2-dependent p53 degradation results in p53 activation. In addition, nucleolin and ribosomal protein L26 (RPL26) bind to the 5' untranslated region of p53 mRNA after DNA damage, thereby controlling the translation of p53 (32). In conclusion, the present study demonstrated that p53 plays an important role in maintaining the transcriptional activity of rDNA in response to DNA damage. The downregulation of the rDNA-rRNA axis (i.e., the pathway of ribosomal biogenesis) under conditions of p53 deficiency may be involved in cancer development via the impairment of cellular homeostasis, particularly after IR or genotoxic stress. These pathways play a significant role in the maintenance of ribosomal biogenesis as one of the mechanisms of cancer prevention, as the downregulation of the rDNA-rRNA axis may be caused by the two key functions of p53, including G1/S and G2/M checkpoint arrest and apoptosis.

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Availability of materials and data

All the datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AS designed the experiments and wrote the paper with SK. The experiments including immunofluorescence, immunoblotting and qPCR were performed by SK, YH and AS. Acquired data were analyzed and interpreted by SK, WG, YH, TO, HS, MY, RK, TY and AS. The manuscript was reviewed by TO, HS, KDH, MY and TY. Administrative, technical, or material support was provided by TO, SL and TN. The study was supervised by AS.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

References

Figure S1. Heat map summary showing the effects of IR and p53 on nucleolar type. (A-D) Heat maps summarizing the statistical significance of results obtained comparing individual nucleolar types in HCT116 and U2OS cells. Comparison (A) between 2 Gy of X-rays and the control, (B) between p53-proficient and -deficient status after irradiation with 2 Gy of X-rays, (C) between X-rays and carbon ions in p53-proficient cells and (D) between X-rays and carbon ions in p53-deficient cells. IR, ionizing radiation; NA, not available.
Figure S2. Distribution of individual nucleolar types in p53-proficient and -deficient cells following X-rays. (A-J) The percentage of individual nucleolar types at 24, 48 and 72 h after irradiation with 2 Gy of X-rays was compared between p53<sup>+/+</sup> and p53<sup>−/−</sup> HCT116 cells (A-E). Similarly, the percentage of individual nucleolar types in control or p53-depleted U2OS cells is shown (F-J). The error bars represent the standard deviation of three independent experiments. *P<0.05, **P<0.01. NS, not significant; IR, ionizing radiation.
Figure S3. Distribution of individual nucleolar types following X-rays and carbon ions in p53-proficient cells. (A-H) Percentage of individual nucleolar types at 24, 48 and 72 h after irradiation with 2 Gy of X-rays or 2 Gy of carbon ions in (A-E) HCT116 p53<sup>+/+</sup> and (F-H) U2OS cells. Error bars represent the standard deviation of three independent experiments. *P<0.05, **P<0.01. NS, not significant; IR, ionizing radiation.
Figure S4. Distribution of individual nucleolar types following X-rays and carbon ions in p53-deficient cells. (A-K) The percentage of individual nucleolar types in the total set of evaluated (A-F) HCT116 p53−/− and (G-K) p53-depleted U2OS cells after 2 Gy of X-rays or 2 Gy of carbon ions was examined. The error bars represent the standard deviation of three independent experiments. *P<0.05. NS, not significant; IR, ionizing radiation.
Figure S5. Carbon ion irradiation does not increase the number of nucleoli in normal human fibroblasts. Distribution of the number of nucleoli per cell in 1BR hTERT cells at 72 h following irradiation with 2 or 10 Gy of carbon ions. The statistical significance was examined by comparison with non-irradiated cells, using Bonferroni's correction. *P<0.025. NS, not significant; IR, ionizing radiation.