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Accumulation of p21 proteins at DNA damage sites independent of p53 and core NHEJ factors following irradiation

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ABSTRACT

The cyclin-dependent kinase (CDK) inhibitor p21 plays key roles in p53-dependent DNA-damage responses, i.e., cell cycle checkpoints, senescence, or apoptosis. p21 might also play a role in DNA repair. p21 foci arise at heavy-ion-irradiated DNA-double-strand break (DSB) sites, which are mainly repaired by nonhomologous DNA-end-joining (NHEJ). However, no mechanisms of p21 accumulation at double-strand break (DSB) sites have been clarified in detail. Recent works indicate that Ku70 and Ku80 are essential for the accumulation of other NHEJ core factors, e.g., DNA-PKcs, XRCC4 and XLF, and other DNA damage response factors, e.g., BRCA1. Here, we show that p21 foci arise at laser-irradiated sites in cells from various tissues from various species. The accumulation of EGFP-p21 was detected in not only normal cells, but also transformed or cancer cells. Our results also showed that EGFP-p21 accumulated rapidly at irradiated sites, and colocalized with the DSB marker γ-H2AX and with the DSB sensor protein Ku80. On the other hand, the accumulation occurred in Ku70-, Ku80-, or DNA-PKcs-deficient cell lines and in human papillomavirus 18-positive cells, whereas the p21 mutant without the PCNA-binding region (EGFP-p21(1–146)) failed to accumulate at the irradiated sites. These findings suggest that the accumulation of p21, but not functional p53 and the NHEJ core factors, is dependent on PCNA. These findings also suggest that the accumulation activity of p21 at DNA damaged sites is conserved among human and animal cells, and p21 is a useful tool as a detection marker of DNA damaged sites.

1. Introduction

A DNA double-strand break (DSB) is the most deleterious type of DNA damage and is induced by some chemotherapeutic drugs, ionizing radiation, ultraviolet radiation, laser microbeam, and heavy-ion irradiation [1–4]. Unrepaired or improperly repaired DSBs can lead to chromosomal truncations and translocations, which can contribute to cancer development in higher eukaryotic organisms. Two major pathways exist in mammalian cells for the repair of DSBs: nonhomologous DNA-end-joining (NHEJ) repair and homologous recombination (HR) [1,2]. The golden standard marker of DSBs is γ-H2AX foci, which are formed by the rapid phosphorylation of H2AX on serine 139 at DNA DSBs [5,6]. The NHEJ repair process, but not HR, is responsible for repairing a major fraction of DSBs in mammalian cells [1,2].

NHEJ repair requires Ku70, Ku80, a DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV, Artemis, and XLF (also called Cernunnos) [1,2]. Recent studies using laser irradiation to induce DNA damage in the nuclei of living cells have shed light on the order of recruitment of NHEJ factors to DSB sites [2]. The NHEJ repair pathway starts with the binding of Ku70 and Ku80 to a DNA end. Ku70 and Ku80 accumulates at laser-induced DSB sites immediately following irradiation, and these are essential for the accumulation of DNA-PKcs, XLF, and XRCC4 at DSB sites. These findings suggest that Ku70 and Ku80 provide a platform for the recruitment of other NHEJ core factors.

p21/CDKN1A (hereafter referred to as p21), a member of the cyclin-dependent kinase (CDK) inhibitor family, plays multiple roles not only as a cell-cycle regulator in response to DNA damage, but also as a regulator of transcription, senescence, apoptosis, and DNA repair [7,8]. Although the activity of p21 in cell cycle regulation is usually associated with CDK inhibition, p21 also interacts directly with the proliferating cell nuclear antigen (PCNA), thereby inhibiting DNA replication [7,8]. There are some reports about the localization and accumulation of p21 at DSB sites [3,9,10]. In addition, p21 interacts with the NHEJ core factor Ku70 after irradiation [10]. However, it is not clarified whether NHEJ core factors provide a platform for the recruitment of p21.

Abbreviations: CDK, cyclin-dependent kinase; DIC, differential interference contrast; DNA-PK, DNA-dependent protein kinase; DSB, DNA double-strand break; HPV, human papillomaviruses; HR, homologous recombination; MLE, murine lung epithelial cell line; NHEJ, nonhomologous DNA-end-joining; PCNA, proliferating cell nuclear antigen.

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In this study, we examined the localization and accumulation of p21 in seven laser-irradiated cell lines including NHEJ core factor-deficient- or functional p53 deficient-cell lines. We also examined whether a p21 mutant unable to bind to PCNA accumulates at DNA damaged sites after irradiation.

2. Materials and methods

2.1. Cell lines, cultures, reagents and transfection

A Ku70-deficient murine lung epithelial cell line (Ku70−/− MLE), a human papillomavirus (HPV) 18-positive human cervical carcinoma cell line (HeLa (Riken Cell Bank, Tsukuba, Japan)), a normal human diploid lung fibroblast cell line (TIG-1 (HSRRB, Osaka, Japan)), a Chinese hamster ovary cell line (CHO-K1 (Riken Cell Bank)), and a Ku80-deficient CHO-K1 mutant cell line (xrs-6) were cultured as described in previous studies [12–15]. A DNA-PKcs-deficient human glioblastoma cell line (MO59J) and a mouse embryonic fibroblast cell line (NIH3T3 (Riken Cell Bank)) were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. MO59J was purchased from the American Type Culture Collection (Rockville, MD). Transient transfections of pEGFP-p21, pEGFP-p21(1–146), or pEGFP-C2 were performed in cells using FuGene6 (Roche Diagnostics K.K., Indianapolis, IN) as described previously, and the cells were cultured for 2 days and then monitored under an FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) as previously described [13].

2.2. Local DNA damage induction using laser and cell imaging

Local DNA damage induction using laser and cell imaging were performed as described previously [13]. Briefly, confocal images of living cells or fixed cells expressing EGFP-tagged proteins were obtained using an FV-300 confocal scanning laser microscopy system (Olympus, Tokyo, Japan), as described previously [13]. A 90% power scan (for 1 s) from a 405-nm laser was used to induce local DSBs. Each experiment was performed on at least 10 cells. The signal intensity of accumulated EGFP at the microirradiated sites was converted into a numerical value using the confocal scanning laser microscopy system's software (Olympus).

2.3. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [12,13]. The fixed cells were first blocked for 10 min using a blocking solution and then incubated for 30 min at room temperature with a mouse anti-PCNA monoclonal antibody (PC10) (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-Ku80 polyclonal antibody (AHP317) (Serotec, Oxford, UK) or a mouse anti-γ-H2AX monoclonal antibody (JBW301) (Upstate Biotechnology Inc., Charlottesville, VA). After washing with PBS, antibody binding was detected using Alexa fluor 568-conjugated secondary antibodies (Molecular Probes, OR, USA).

2.4. Immunoblotting

The extraction of total lysates and Western blot analysis were performed as described previously [12,14,16]. The following antibodies were used: a rabbit anti-GFP polyclonal antibody (FL) (Santa Cruz Biotechnology), a mouse anti-p21 monoclonal antibody (HJ21) (Invitrogen, Carlsbad, CA), or a mouse β-actin monoclonal antibody (Sigma, St. Louis, MO).

3. Results

γ-H2AX is a sensitive indicator of DSBs induced by drugs, e.g., bleomycin and etoposide, as well as by X-rays [6,17]. We examined the expression levels of p21 and γ-H2AX in the normal human fibroblast cell line TIG-1 and the immortalized mouse fibroblast cell line NIH3T3, treated the drugs by Western blot analysis using the anti-p21 antibody and anti-γ-H2AX antibody. As shown in sFig.1(A–D), the expression levels of γ-H2AX and p21 increased in response to bleomycin or etoposide in a dose-dependent manner. These results indicate that the expression of p21 is induced by the two DSB inducers in normal human and mouse fibroblasts. It has been reported that in a p21 knock down cell line stably expressing p21 shRNA, DNA damage-induced H2AX foci remains elevated after etoposide have been removed [18]. To confirm this, we examined γ-H2AX focus formation after etoposide treatment and monitored the reversibility post-etoposide washout in p21-deficient cells, HCT116 (p21−/−). As shown in sFig. 1E, 1 h treatment with etoposide induced prominent γ-H2AX foci in both HCT116 cells and HCT116 (p21−/−) cells. In HCT116 cells, γ-H2AX staining was decreased, and by 6 h after etoposide removal, the γ-H2AX staining was almost undetectable. In contrast, HCT116 (p21−/−) cells displayed a sustained γ-H2AX staining up to 6 h after etoposide removal. In addition, HCT116 (p21−/−) cells, but not HCT116 cells, displayed a sustained γ-H2AX staining up to 6 h after bleomycin removal (data not shown). These results strongly support the idea that there is interplay between p21 and DSB repair.

Most recently, it has been reported that EGFP-p21 accumulates and colocalizes with γ-H2AX at irradiated sites in charged-particle-irradiated HeLa cells, indicating that p21 accumulates at DSB sites [3]. Previously, we showed that 405-nm laser microirradiation without presensitization treatments induces DSBs at microirradiated sites [13]. We examined whether exogenous EGFP-p21 is expressed and accumulates at microirradiated sites in human and mouse fibroblasts (Fig. 1). As shown in Fig. 1B, a signal of EGFP-p21 with the expected molecular weight was detected in the transfectants by immunoblotting using the anti-p21 antibody and anti-EGFP antibody. To examine whether EGFP-p21 localizes and accumulates at the microirradiated sites, we exposed the EGFP-p21-transfected cells by microirradiation using a 405-nm laser. As shown in sFig. 2, EGFP-p21 localized within the nucleus of both cell lines. In addition, we found that EGFP-p21 localized and accumulated at the irradiated sites in living cells after irradiation (Fig. 1C). Ku70 and Ku80 starts NHEJ repair. Next, we examined whether the laser treatment generates DSBs and activates the NHEJ repair pathway by staining with an antibody that recognizes γ-

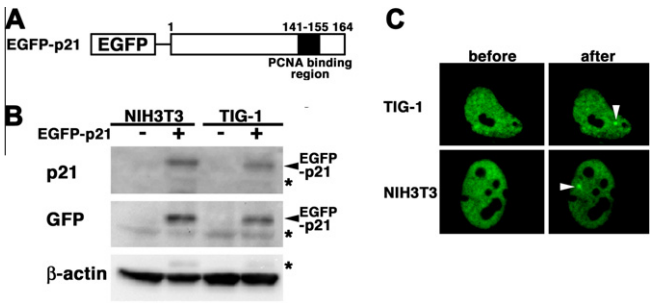


Fig. 1. EGFP-p21 accumulated at 405-nm-laser-irradiated sites. (A) Schematics of EGFP-p21. (B) EGFP-p21 was expressed in TIG-1 or NIH3T3 cells, and the expression of EGFP-p21 was examined by Western blotting using the anti-p21, anti-GFP or anti-β-actin antibody. Asterisk, nonspecific band. (C) Imaging of living EGFP-p21-transfected cells before (left panel) and after (right panel) microirradiation. Arrowheads indicate sites of irradiation.

H2AX or Ku80. As shown in Fig. 2A, EGFP-p21 clearly colocalized with γ-H2AX and Ku80 at microirradiated sites in NIH3T3 cells, suggesting that p21 accumulates at 405-nm-laser-induced DSB sites, where the NHEJ repair pathway is activated. Next, we performed time-lapse imaging of EGFP-p21-transfected NIH3T3 cells and EGFP-transfected NIH3T3 cells (Fig. 2B). We observed EGFP-p21 accumulation at the irradiated sites 5 s after irradiation. The intensity of the EGFP signal rapidly increased in EGFP-p21-transfected cells, but not in EGFP-transfected cells. These observations demonstrate that after irradiation, EGFP-p21 immediately accumulates at laser-induced DSBs in living cells.

Next, we examined whether p21 accumulates at irradiated sites in not only normal and transformed fibroblasts, but also epithelial cells and cancer cells. As shown in Fig. 3A and B, p21 accumulation was also detected in HPV-positive p53-dysfunctional human cervical carcinoma cells (HeLa cells) and hamster ovary epithelial cells (CHO-K1 cells). We examined whether the accumulation of p21 at laser-irradiated sites is affected by NHEJ core factors. As shown in Fig. 3B, EGFP-p21 showed normal accumulation in the human glioblastoma cell line MO59J (DNA-PKcs-deficient) and the murine lung epithelial cell line Ku70−/− MLE (Ku70-deficient). EGFP-p21 also accumulated at laser-irradiated sites in xrs-6 cells (Ku80-deficient) derived from CHO-K1 cells (Fig. 3B). These findings demonstrate that the accumulation of p21 occurred in Ku70-, Ku80-, or DNA-PKcs-deficient cell lines and in HPV-positive p53-dysfunctional cells (Fig. 3C). Altogether, p21 can localize and accumulate at the DNA damaged sites in an NHEJ core factor-independent manner.

Previously, a pool of p21 is rapidly recruited to and colocalizes with PCNA at UV-induced DNA damaged sites [10]. We examined whether EGFP-p21 colocalized with PCNA at laser-irradiated sites in NIH3T3 cells. As shown in Fig. 3D, EGFP-p21 colocalized with PCNA at laser-irradiated sites. It was shown that the PCNA-binding region of p21 is the region of amino acids 141–155, and deletion of amino acids 149–154 had no ability to bind to PCNA [19]. Next, we examined the localization and accumulation of the p21 mutant without the PCNA-binding ability (EGFP-p21(1–146)) in NIH3T3 and TIG-1 cells. As shown in Fig. 3E, the mutant was found in not only the nucleus, but also the cytoplasm. The p21 mutant without the PCNA-binding ability (EGFP-p21(1–146)) failed to accumulate at the irradiated sites in living cells (Fig. 3E), indicating the importance of the C-terminal region including the PCNA-binding region of p21 for the damage response.

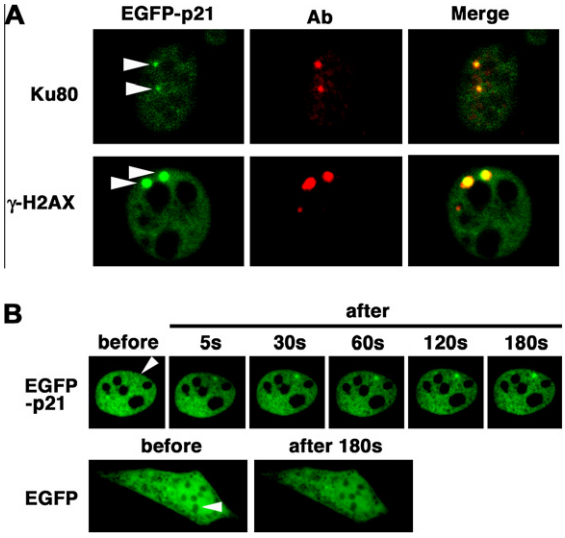


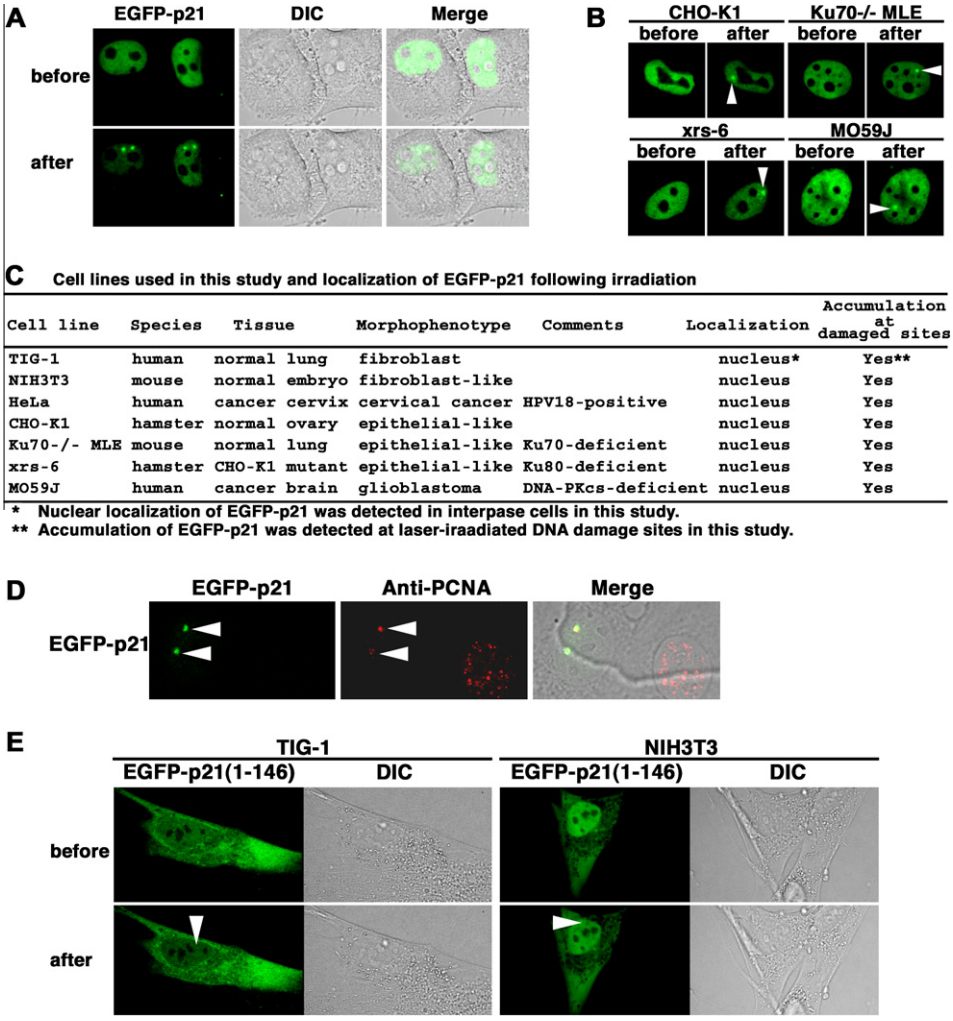
Fig. 2. EGFP-p21 accumulated rapidly at DSBs induced by laser microirradiation. (A) Immunostaining of microirradiated EGFP-p21-transfected NIH3T3 cells with anti-Ku80 (upper panel) or anti-γ-H2AX (lower panel) antibody. At 5 min postirradiation, the cells were fixed and stained with the anti-Ku80 or anti-γ-H2AX antibody. Left panel, EGFP-p21; center panel, Ku80 (upper panel) or γ-H2AX (lower panel) image; right panel, merged image. (B) Time-dependent EGFP-p21 accumulation in transfected NIH3T3 living cells (5–180 s) after irradiation. Upper panel, EGFP-p21; lower panel, EGFP alone. Arrowheads indicate the irradiated sites.

4. Discussion

p21 is a multifunctional protein and appears to play a central role in a surprisingly complicated intracellular network of the DNA damage response pathway [7,8]. In this study, we showed that two inducers, bleomycin and etoposide, which induce DSBs with different efficiencies and by different mechanisms, induced the expression of p21 in the human normal diploid fibroblast cell line TIG-1 and the mouse fibroblast cell line NIH3T3. Furthermore, by transient expression assay using EGFP-tagged p21, we found that p21 localized within the nucleus and accumulated at laser-irradiated sites in seven cell lines including the normal- and tumor-derived cell lines. These findings demonstrated the accumulation of p21 at the laser-irradiated sites in all tested human, hamster, and mouse cell lines derived from various tissues. Altogether, these findings suggest that in addition to the p21 induction mechanism in the DNA damage response pathway, the localization and accumulation mechanisms of p21 at DNA damaged sites play important roles in DNA damage response in mammalian cells, although further studies are required to clarify these mechanisms at the molecular level.

In this study, we confirmed that the 405-nm laser induced DSBs, and we showed that EGFP-p21 colocalized with γ-H2AX foci at laser-irradiated sites. In addition, p21 localized at laser-irradiated sites via its C-terminal region. Most recently, it has been reported that EGFP-p21 colocalizes with γ-H2AX at heavy-ion-irradiated sites in HeLa cells expressing EGFP-p21 [3]. The rapid recruitment of p21 was previously observed after heavy-ion-induced DNA damage in human fibroblasts, confirming the role of p21 in early processing of DSBs [9]. Perucca et al. (2008) reported that the p21 response seems to be independent of the type of lesion, but related to PCNA-dependent repair pathways [10]. They observed the rapid relocation of p21 under irradiation conditions (337-nm laser) producing DSBs, and also observed the rapid relocation of p21 under irradiation conditions (405-nm laser or UV-C) producing CPDs, a typical NER substrate [10]. On the other hand, most recently, it has clearly been shown that UV-C induces DSBs in human fibroblasts [4]. Further studies to clarify accumulation mechanisms and the role of p21 at DNA damaged site are required under each irradiation condition.

The accumulation of p21 at heavy-ion-induced DNA damaged sites is not dependent on p53 and occurs independent of focus formation of the Mre11/Rad50/NBS1 (MRN) complex, which plays a key role in the ATM-dependent signaling pathway at DSB sites [9]. Recent works indicate that Ku80 is essential for the accumulation of NHEJ core factors, e.g., Ku70, XRCC4, and XLF and other factors, e.g., BRCA1, at DNA damaged sites [2,13,20], although it has not been clarified whether the accumulation of p21 occurs in a Ku80-dependent manner. In this study, our data suggested that the accumulation of p21 is dependent on PCNA, but not functional p53 and the NHEJ core factors, i.e., Ku70, Ku80, and DNA-PKcs. On the other hand, it has been shown that p21 interacts with the NHEJ core factor Ku70 after irradiation [11], and the p21-binding partner PCNA also interacts with Ku70 and Ku80 in DSB-damage-dependent manner [21,22]. Ku70 and Ku80 were shown to interact physically with PCNA, which can be significantly enhanced by γ-irradiation [21]. In addition, it was reported that treatment of HeLa cells with γ-rays induces the colocalization of the Ku complex



C Cell lines used in this study and localization of EGFP-p21 following irradiation

Cell line	Species	Tissue	Morphophenotype	Comments	Localization	Accumulation at damaged sites
TIG-1	human	normal lung	fibroblast		nucleus*	Yes**
NIH3T3	mouse	normal embryo	fibroblast-like		nucleus	Yes
HeLa	human	cancer cervix	cervical cancer	HPV18-positive	nucleus	Yes
CHO-K1	hamster	normal ovary	epithelial-like		nucleus	Yes
Ku70-/- MLE	mouse	normal lung	epithelial-like	Ku70-deficient	nucleus	Yes
xrs-6	hamster	CHO-K1 mutant	epithelial-like	Ku80-deficient	nucleus	Yes
MO59J	human	cancer brain	glioblastoma	DNA-PKcs-deficient	nucleus	Yes

* Nuclear localization of EGFP-p21 was detected in interpase cells in this study.
** Accumulation of EGFP-p21 was detected at laser-iraadiated DNA damage sites in this study.

Fig. 3. Accumulation of p21 at irradiated sites is dependent on PCNA, but not p53 and NHEJ core factors. (A) EGFP-p21 accumulated at irradiated sites in the HPV-positive p53-dysfunctional human cervical carcinoma cell line (HeLa). Imaging of living EGFP-p21-transfected cells before (upper panel) and after (lower panel) microirradiation. DIC, differential interference contrast (DIC) image. Merge, EGFP-p21 image and DIC image. (B) EGFP-p21 accumulated at irradiated sites in the hamster ovary epithelial cell line (CHO-K1), Ku70-deficient murine lung epithelial cell line (Ku70−/− MLE), Ku80-deficient CHO-K1 mutant cell line (xrs-6), DNA-PKcs-deficient human glioblastoma cell line (MO59J), and XRCC4-deficient CHO-K1 mutant cell line (XR-1). (C) Characterization of cell lines used in this study and localization of EGFP-p21 following irradiation. (D) Immunostaining of microirradiated EGFP-p21-transfected NIH3T3 cells with anti-PCNA antibody. At 5 min postirradiation, the cells were fixed and stained with the anti-PCNA antibody. Left panel, EGFP-p21 image; center panel, PCNA image; right panel, merged image. (E) A deletion mutant of p21 that is unable to bind to PCNA (EGFP-p21(1–146)) cannot accumulate at irradiated sites in TIG-1 and NIH3T3 cells. DIC, DIC image. Arrowheads indicate the irradiated sites.

(Ku70/Ku80) with PCNA on chromosomes, and the interaction between Ku70/Ku80 and PCNA occurs at DNA ends [22]. In this study, our data showed that EGFP-p21 colocalized with Ku80 at DNA damaged sites, although the accumulation of p21 is not dependent on Ku80. Now, we are interested in determining whether or not p21 and/or PCNA associates with NHEJ core factors and plays a novel role in the modulating DNA damage responses after its accumulation at DNA damaged sites.

Detection of DNA damage level in cells is useful in not only basic research e.g., research on aging, anti-cancer drug development, apoptosis, DNA repair, radiation biology, and environmental biology, but also in the clinical settings for humans and animals. There are also potential clinical applications of DNA damage detection in tissues of cancer patients and animals during radiotherapy and chemotherapy. Immunohistochemistry using antibodies against γ-H2AX is commonly utilized as a sensitive method determining DNA damaged sites in fixed cells, whereas it is difficult to apply it in living cells [5,6,23–25]. Data from us and others show that it is easy to detect the accumulation of p21 at DNA damaged sites not only in fixed cells by immunocytochemistry using an anti-p21 antibody, but also in living cells using live-cell imaging techniques with GFP-tagged p21 [3,9,10]. Thus, p21 might be a useful tool as a detection marker of DNA damaged sites in both living and fixed cells from humans and animals.

In conclusion, the accumulation activity of p21 at DNA damaged sites is conserved among human and animal cells, and the accumulation of p21 is independent of the NHEJ core factors Ku70, Ku80, and DNA-PKcs. Most recently, Cazzalini et al. (2010) demonstrated that DNA damage except DSB can trigger p21 accumulation at damaged sites which can, through PCNA interaction modulate BER and PARP activity [26]. On the other hand, it was shown that PARP-1 and Ku70/Ku80 compete for repair of DSBs by distinct NHEJ pathways. [27]. In addition, it was suggested that PARP-1 might interact with the Ku70/DNA LigIV complex in order to block its activity at the DNA lesion [28]. Further study to elucidate the molecular mechanism via the p21-dependent DNA damage response pathway at DNA damaged sites will lead to a better understanding of not only the physiological function of p21, but also the mechanism underlying the regulation of various complicated DNA damage responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.032.

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