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The C-terminal region of Rad52 is essential for Rad52 nuclear and nucleolar localization, and accumulation at DNA damage sites immediately after irradiation

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ABSTRACT

Rad52 plays essential roles in homologous recombination (HR) and repair of DNA double-strand breaks (DSBs) in *Saccharomyces cerevisiae*. However, in vertebrates, knockouts of the Rad52 gene show no hypersensitivity to agents that induce DSBs. Rad52 localizes in the nucleus and forms foci at a late stage following irradiation. Ku70 and Ku80, which play an essential role in nonhomologous DNA-end-joining (NHEJ), are essential for the accumulation of other core NHEJ factors, e.g., XRCC4, and a HR-related factor, e.g., BRCA1. Here, we show that the subcellular localization of EYFP-Rad52(1–418) changes dynamically during the cell cycle. In addition, EYFP-Rad52(1–418) accumulates rapidly at microirradiated sites and colocalizes with the DSB sensor protein Ku80. Moreover, the accumulation of EYFP-Rad52(1–418) at DSB sites is independent of the core NHEJ factors, i.e., Ku80 and XRCC4. Furthermore, we observed that EYFP-Rad52(1–418) localizes in nucleoli in CHO-K1 cells and XRCC4-deficient cells, but not in Ku80-deficient cells. We also found that Rad52 nuclear localization, nucleolar localization, and accumulation at DSB sites are dependent on eight amino acids (411–418) at the end of the C-terminal region of Rad52 (Rad52 CTR). Furthermore, basic amino acids on Rad52 CTR are highly conserved among mammalian, avian, and fish homologues, suggesting that Rad52 CTR is important for the regulation and function of Rad52 in vertebrates. These findings also suggest that the mechanism underlying the regulation of subcellular localization of Rad52 is important for the physiological function of Rad52 not only at a late stage following irradiation, but also at an early stage.

1. Introduction

A DNA double-strand break (DSB) is the most dangerous type of DNA damage and can be induced both exogenously (e.g., by some chemotherapeutic drugs, ionizing radiation, and laser microbeam) and endogenously (e.g., during the process of recombination and replication or as a result of metabolic oxidative stresses) [1,2]. Unrepaired or improperly repaired DSBs can lead to chromosomal truncations and translocations, which can contribute to cancer development in higher eukaryotic organisms. There are two pathways, i.e., nonhomologous DNA-end-joining (NHEJ) and homologous recombination (HR), for DSB repair in mammalian cells [1,2]. The NHEJ repair process, but not HR, is responsible for the repair of a major fraction of DSBs in mammalian cells [1,2]. Recent studies using laser irradiation to induce DNA damage in the nuclei of living cells have shed light on the sequence of recruitment of NHEJ factors to DSB sites [2]. NHEJ repair requires Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV, Artemis, and XLF (also called Cernunnos) [1,2]. The NHEJ repair pathway starts with the binding of Ku70 and Ku80 to a DNA end. Ku70 and Ku80 accumulate immediately at sites of laser-induced DSBs after irradiation, which is essential for the recruitment of NHEJ factors (i.e., DNA-PKcs, XLF and XRCC4) and an HR-related protein BRCA1 at DSB sites [2–6]. These findings suggest that Ku70 and Ku80 provide a platform for the recruitment of other core NHEJ factors and some other DNA repair proteins.

Inactivation of Rad52 results in severe HR and DSB repair deficiencies in *Saccharomyces cerevisiae (S. cerevisiae)*, whereas Rad52 knockouts mice show no hypersensitivity to agents that induce DSBs and HR in these mice is only mildly affected [7]. On the other hand, it has been reported that the overexpression of human Rad52 confers enhanced resistance to γ-rays and induces homologous intrachromosomal recombination in cultured monkey cells [8]. Consistent with its putative function, human Rad52 localizes in the nucleus including nucleoli and forms foci after several hours,

Abbreviations: CTR, C-terminal region; DIC, differential interference contrast; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-strand break; HR, homologous recombination; NLS, nuclear localization signal; NHEJ, nonhomologous DNA-end-joining; S. cerevisiae, Saccharomyces cerevisiae.

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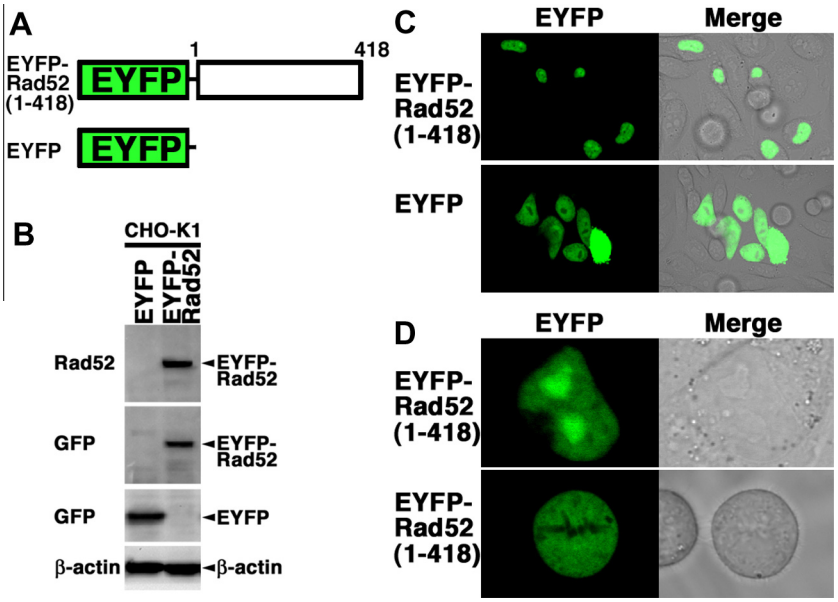

Fig. 1. Subcellular localization of EYFP-Rad52 in living cells. (A) Schematics of EYFP-Rad52 chimeric gene and control gene (EYFP). (B) EYFP-Rad52(1–418) was expressed in CHO-K1 cells, and the expression of EYFP-Rad52(1–418) was examined by Western blotting using the anti-Rad52, anti-GFP or anti-β-actin antibody. (C) Imaging of living EYFP-Rad52(1–418)-transfected cells. Living CHO-K1 cells transiently expressing EYFP-Rad52(1–418) or EYFP were analyzed by confocal laser microscopy. For the same cells, EYFP images are shown alone (left panel) or merge with differential interference contrast (DIC) images (right panel). The images shown are representative of interphase cells (upper panel) and mitotic phase cells (lower panel) (D).

but not immediately, after X-irradiation [9]. However, the mechanisms of localization in the nucleus and at DSB sites have not been clarified in detail. To date, homologues of the Rad52 gene have been found in several eukaryotic organisms. Sequence analysis has revealed that the N-terminal half of the amino acid sequence of the Rad52 protein is highly conserved while the C-terminal half of the sequence is less conserved [8,10]. Human Rad52, which is a 418-amino-acid protein, contains several distinct functional domains, e.g., the N-terminal DNA binding domain (39–80 amino acids), RPA interaction domain (221–280 amino acids), Rad51 interaction domain (290–330 amino acids), and putative nuclear localization signal (NLS) (405–414 amino acids) [8,10–12].

There are some reports about the localization and accumulation of Rad52 at DSB sites [9,13–15]. On the other hand, Ku can interact with two-ended DSBs and block the entry of HR proteins [16]. The HR frequency is high in Ku70−/−, XRCC4−/−, and DNA-PKcs−/− cells, with the increase being particularly striking in Ku70−/− cells. Most recently, it has been reported that Ku binds to DSBs in all cell cycle stages and is likely actively displaced from DSB ends to free the DNA ends for DNA end resection and thus HR to occur [17]. However, it remains unclear whether localization of Rad52 is affected by the deletion or inactivation of any of these core NHEJ factors.

In this study, we examined the subcellular localization of Rad52 and its mutants in the CHO-K1 cell lines and two core NHEJ-factor-deficient cell lines. We also examined whether Rad52 and its mutants accumulate at DNA damaged sites immediately after irradiation.

2. Materials and methods

2.1. Cell lines, cultures, reagents, and transfection

A Chinese hamster ovary cell line CHO-K1 (Riken Cell Bank), a XRCC4-deficient CHO-K1 mutant cell line (XR-1), and a Ku80-deficient CHO-K1 mutant cell line (xrs-6) were cultured as described in previous studies [18–20]. These cells were transiently transfected with pEYFP-Rad52(1–418), pEYFP-Rad52(1–410), pEYFP-Rad52(1–128), pEGFP-Rad52(177–418), or pEYFP-C1 using FuGene6 (Roche Diagnostics K.K., Indianapolis, IN), as described previously. The cells were then cultured for 2 days and monitored under an FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan), as previously described [3].

2.2. Local DNA damage induction using laser and cell imaging

Local DNA damage induction using a laser and cell imaging were performed, as described previously [3,4,18,19]. Briefly, confocal images of living cells or fixed cells expressing EGFP- or EYFP-tagged proteins were obtained using an FV300 confocal scanning laser microscopy system (Olympus). A 30% power scan (for 1 s) from a 405-nm laser was used to induce local DSBs.

2.3. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [3,22]. The fixed cells were first blocked for 10 min with a blocking solution and then incubated for 30 min at room temperature with a rabbit anti-Ku80 polyclonal antibody (AHP317) (Serotec, Oxford, UK). After washing with PBS, antibody binding was detected using appropriate 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 [21,22]. The following antibodies were used: a goat anti-XRCC4 polyclonal antibody (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a rabbit anti-GFP polyclonal antibody (FL) (Santa Cruz Biotechnology), a rabbit anti-Rad52 polyclonal antibody (3425) (Cell Signaling Technology Inc., MA), a rabbit anti-Ku80 polyclonal antibody (AHP317), and a mouse β-actin monoclonal antibody (Sigma, St. Louis, MO).

3. Results

3.1. Expression and subcellular localization of EYFP-Rad52 in living CHO-K1 cells

We examined the expression and subcellular localization of EYFP-Rad52(1–418) in CHO-K1 cells. First, we generated cells transiently expressing EYFP-Rad52(1–418) in CHO-K1 cells. The mammalian expression vector pEYFP-C1 containing human Rad52 (pEYFP-Rad52(1–418)) was transfected into CHO-K1 cells (Fig. 1A). As shown in Fig. 1B, the signal of EYFP-Rad52(1–418) with the expected molecular weight was detected in the transfectants by immunoblotting using the anti-Rad52 antibody and anti-GFP antibody. By confocal laser microscopy, we found that EYFP-Rad52(1–418) was localized in the nuclei of living interphase cells and throughout the cytoplasm, but not in the condensed chromosomes of living mitotic cells in EYFP-Rad52(1–418) transfectants (Fig. 1C and D), indicating that the subcellular localization of EYFP-Rad52(1–418) changes dynamically during the cell cycle. In addition, we found that EYFP-Rad52(1–418) was localized in the nucleoli in a set of interphase cells. On the other hand, in EYFP transfectants, we found that EYFP was distributed throughout the cell but not in the nucleoli (Figs. 1C and 3C), because it has a small molecular mass (27 kDa), which enables it to enter the nucleus by passive diffusion.

3.2. EYFP-Rad52 accumulates rapidly at DSBs induced by laser microirradiation

We examined whether EYFP-Rad52(1–418) accumulates immediately at the irradiated sites. First, we subjected the transfectants to microirradiation using a 405 nm laser. As shown in Fig. 2A, we found that EYFP-Rad52(1–418) accumulated at the irradiated sites in the living cells. Next, we examined whether the microirradiation generated DSBs by detecting the accumulation of Ku80, which is a DSB sensor [2,3,23]. As expected, we observed that Ku80 clearly accumulated at the microirradiated sites (Fig. 2B). Our results also showed that EYFP-Rad52(1–418) colocalized with the DSB sensor protein Ku80. Altogether, these findings suggest that EYFP-Rad52(1–418) is recruited to DSBs. To study the kinetics of EYFP-Rad52(1–418) accumulation after microirradiation, we performed time-lapse imaging of EYFP-Rad52(1–418). As shown in Fig. 2C the intensity of the EYFP signal rapidly increased in EYFP-Rad52 transfectants at the irradiated sites, but not in EYFP transfectants (data not shown). We observed EYFP-Rad52 accumulation in regions with induced DSBs 5 s after irradiation. These findings demonstrate that after irradiation, EYFP–Rad52 immediately accumulated at DSB sites, as shown by our simple live-cell imaging technique.

3.3. The end of the C-terminal region is essential for Rad52 nuclear and nucleolar localization, and recruitment to DSBs in vivo

Sequence analysis has revealed that the N-terminal half of the amino acid sequence of the Rad52 protein is highly conserved whereas the C-terminal half of the sequence is less conserved among Rad52 homologous [8,10] (Fig. 3A). To identify the essential region for the nuclear localization of Rad52, we examined the localization of EYFP-Rad52(1–418) and its mutants (Fig. 3C). The pEYFP-Rad52(1–418) and its mutants were transfected into in CHO-K1 cells. As shown in Fig. 3B, the signal of EYFP-Rad52(1–418) or each mutant with the expected molecular weight was detected in the transfectants by immunoblotting using the anti-GFP antibody. By confocal laser microscopy, we found that two C-terminal deletion mutants, i.e., EYFP-Rad52(1–410) and EYFP-Rad52(1–128), localized mainly in the cytoplasm of CHO-K1 cells, whereas EGFP-Rad52(177–418) as well as EYFP-Rad52(1–418) was localized in the nuclei of living interphase cells (Fig. 3C). We also found that these C-terminal deletion mutants were not localized in the nucleoli. These findings suggest that eight amino acids of the C-terminal region of Rad52 are essential for the nuclear localization of Rad52. We also found that basic amino acids in the C-terminal region of Rad52 are evolutionarily highly conserved among mammalian, avian, and fish species, but not in yeast (Fig. 3D and data not shown), which further suggests the biological importance of the Rad52 C-terminal region in vertebrates. Hereinafter, we refer to the Rad52 C-terminal region (amino acids 411–418) as Rad52 CTR. An N-terminal DNA binding domain of Rad52 is evolutionally conserved among not only mammals, but also other species including yeast [8,10]. To determine which region of Rad52 is required for its recruitment to DSBs in vivo, we examined whether the Rad52 deletion mutant proteins could be recruited to DSBs induced by microirradiation. We observed that all three deletion mutant proteins examined failed to accumulate at DSBs in the nucleus (Fig. 3D). Remarkably, deletion of the eight amino acids of the C-terminal end of Rad52 i.e., Rad52 CTR, completely abolished the recruitment of Rad52 to DSBs (Fig. 3C). On the other hand, mutations of Rad52 at amino acids 405 and 406 (R405A/K406A) in EYFP-Rad52(1–418) did not affect the Rad52 nuclear localization and accumulation of at DSBs (data not shown). Altogether, these findings suggest that the Rad52 CTR is essential, but not sufficient on its own, for the recruitment of Rad52 to DSBs in vivo.

3.4. Core NHEJ factors are not essential for the Rad52 nuclear localization and accumulation at irradiated sites

Next, we examined the localization of EYFP-Rad52(1–418) and its mutants (EYFP-Rad52(1–128)) in core NHEJ factor-deficient cell lines. The pEYFP-Rad52(1–418) or pEYFP-Rad52(1–128) was transfected into the Ku80-deficient cell line xrs-6 or XRCC4-deficient cell line XR-1. As shown in Fig. 4A, a signal of EYFP-Rad52 (1–418) or EYFP-Rad52(1–128) with the expected molecular weight was detected in each transfectant by Western blot analysis using the anti-GFP antibody. As expected, we confirmed that XRCC4 was deficient in XR-1 cells, but not in xrs-6 cells, whereas Ku80 was deficient in xrs-6 cells, but not in XR-1 cells. By confocal laser microscopy, we found that in the two core NHEJ factor-deficient cell lines, EYFP-Rad52(1–418) was localized in the nuclei of living interphase cells (Fig. 4B). Interestingly, EYFP-Rad52(1–418) localized in the nucleoli of a set of XR-1 cells as well as that of CHO-K1 cells, whereas EYFP-Rad52(1–418) did not localize in the nucleoli of xrs-6 cells. On the other hand, EYFP-Rad52(1–128) showed mainly cytoplasmic localization in both XR-1 and xrs-6 cells, as well as in CHO-K1 cells.

We examined whether the accumulation of Rad52 at microirradiated sites is affected by core NHEJ factors. As shown in Fig. 4C and E, EYFP-Rad52(1–418) accumulated at laser-irradiated sites in xrs-6 cells and XR-1 cells. Next, to study the kinetics of EYFP-Rad52(1–418) accumulation after microirradiation, we performed time-lapse imaging of EYFP-Rad52(1–418) in xrs-6 cells. As shown in Fig. 4C, we also observed EYFP-Rad52(1–418) accumulation in regions with induced DSBs 5 s after microirradiation. Next, we examined the accumulation of the Rad52 mutant without the C-terminal half (EYFP-Rad52(1–128)) in xrs-6 cells. As shown in Fig. 4D, the Rad52 mutant failed to accumulate at the irradiated sites in living cells. Similarly, our data showed that EYFP-Rad52(1–410) failed to accumulate at the irradiated sites in both cell lines (data not shown), supporting the importance of Rad52 CTR in responses to damage. These findings demonstrate that Rad52 can localize

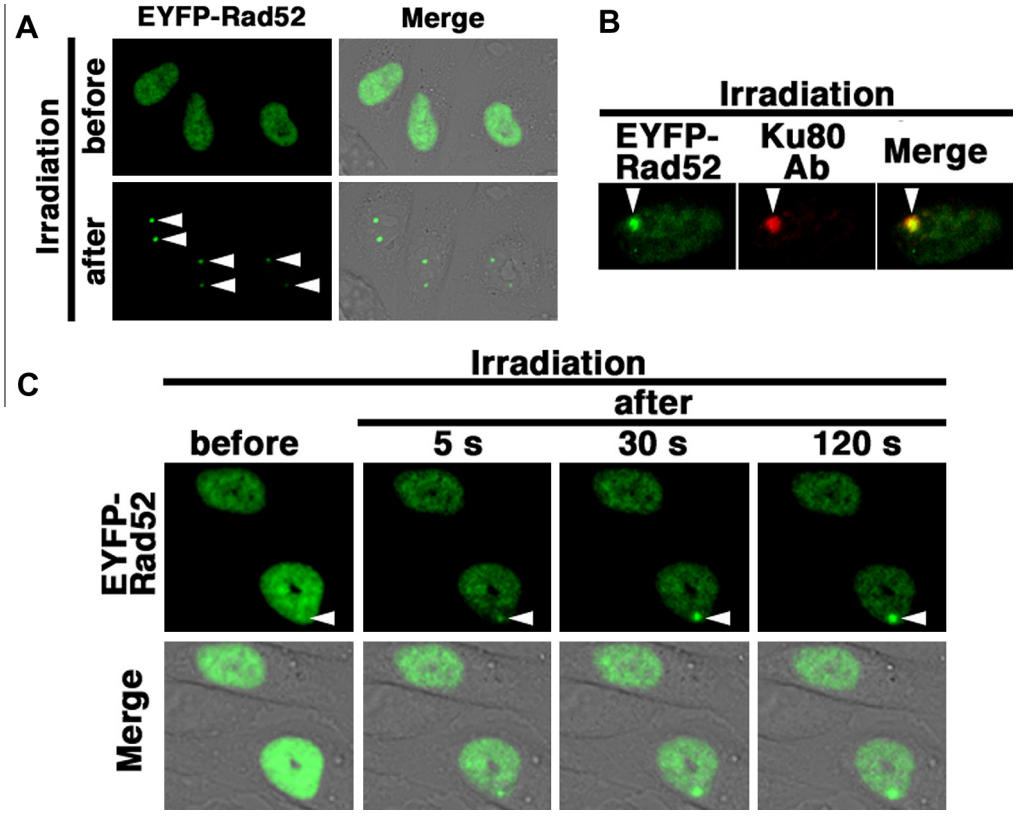

Fig. 2. EYFP-Rad52 accumulated rapidly at DSBs induced by laser microirradiation. (A) Imaging of living EYFP-Rad52(1–418)-transfected CHO-K1 cells before (upper panel) and after (lower panel) microirradiation. (B) Immunostaining of microirradiated EYFP-Rad52(1–418)-transfected cells with anti-Ku80 antibody. At 5 min postirradiation, the cells were fixed and stained with the anti-Ku80 antibody. Left panel, EYFP-Rad52(1–418) image; center panel, Ku80 staining image; right panel, merged image. (C) Time-dependent EYFP-Rad52(1–418) accumulation in living cells (5–120 s) after irradiation. Upper panel, EYFP-Rad52(1–418) image; lower panel, merged image with DIC. Arrowheads indicate the irradiated sites.

in the nuclei and accumulate at the DNA damaged sites in a core-NHEJ-factor-independent manner.

4. Discussion

Rad52 is a multifunctional protein and appears to play a key role in a surprisingly complicated intracellular network of the DNA damage response pathway [13,24]. In this study, by transient expression assay using EYFP-Rad52(1–418), we showed that Rad52 localized within the nucleus and accumulated immediately at the DNA damage sites after irradiation in CHO-K1 cells. On the other hand, EYFP-Rad52(1–418) can localize and accumulate at the DNA damaged sites in a core-NHEJ-factor-independent manner, although EYFP-Rad52(1–418) colocalized with a DSB sensor protein Ku80 at DNA damaged sites. Altogether, these findings suggest that the mechanism underlying the regulation of subcellular localization of Rad52 is important for the physiological function of Rad52 not only at a late cascade of DNA repair following irradiation, but also at an early cascade of repair at DNA damaged sites. Thus, it is important to clarify the mechanisms underlying the spatial and temporal regulation of Rad52 in order to understand the complicated responses to DNA damage.

In *S. cerevisiae*, the Rad52 protein plays a key role in HR [25], wheareas in vertebrates, its role in cells remains unclear. It was reported that Rad52 accumulates at DNA damage sites in the late stage several hours after irradiation [9,13–15], which is consistent with the putative function of Rad52 in the late stages of DSB repair, i.e., HR [24]. In this study, our findings showed that EYFP-Rad52 accumulated immediately at DNA damaged sites after laser irradiation and colocalized with Ku80 at the sites. Studies using Ku80-deficient hamster cells, i.e., xrs-6 or XR-V15B, demonstrate that Ku80 is essential for the accumulation of not only the DNA-PK components, i.e., Ku70 and DNA-PKcs, but also other core NHEJ factors, e.g., XLF and XRCC4 [2,3,6]. Furthermore, recent works indicate that Ku80 plays a critical role in the accumulation of not only core NHEJ factors, but also some of the non-NHEJ factors, e.g., the HR-related factor BRCA1, at DNA damaged sites [5], whereas it has not been clarified whether the accumulation of Rad52 occurs in a Ku80-dependent manner. In this study, we observed that Rad52 accumulated at microirradiated sites with or without a core NHEJ factor, i.e., Ku80 or XRCC4, demonstrating that these core NHEJ factors are not essential for the accumulation of Rad52. The findings in this study might be useful for clarifying mechanisms underlying the spatial and temporal regulation of the DSB repair proteins at DNA damage sites immediately after irradiation. Recently, it has been reported that mammalian RAD52 could respond to DNA DSBs and replication stalling independently of BRCA2, suggesting that Rad52 may provide alternative pathways for Rad51-mediated HR in mammalian cells [13]. Thus, we speculate that the mechanisms underlying the regulation of Rad52 subcellular localization and accumulation at DSBs are important for the physiological function of Rad52.

Until now, homologues of the Rad52 gene have been found in several eukaryotic organisms. Sequence analysis has revealed that the N-terminal half of the amino acid sequence of the Rad52 protein is highly conserved whereas the C-terminal half of the sequence is less conserved [10,11]. Human Rad52, which is a 418-amino-acids protein, contains the N-terminal DNA binding domain (39–80 amino acids) and C-terminal putative NLS (405–414 amino acids) [9,10]. In this study, our findings showed that the eight

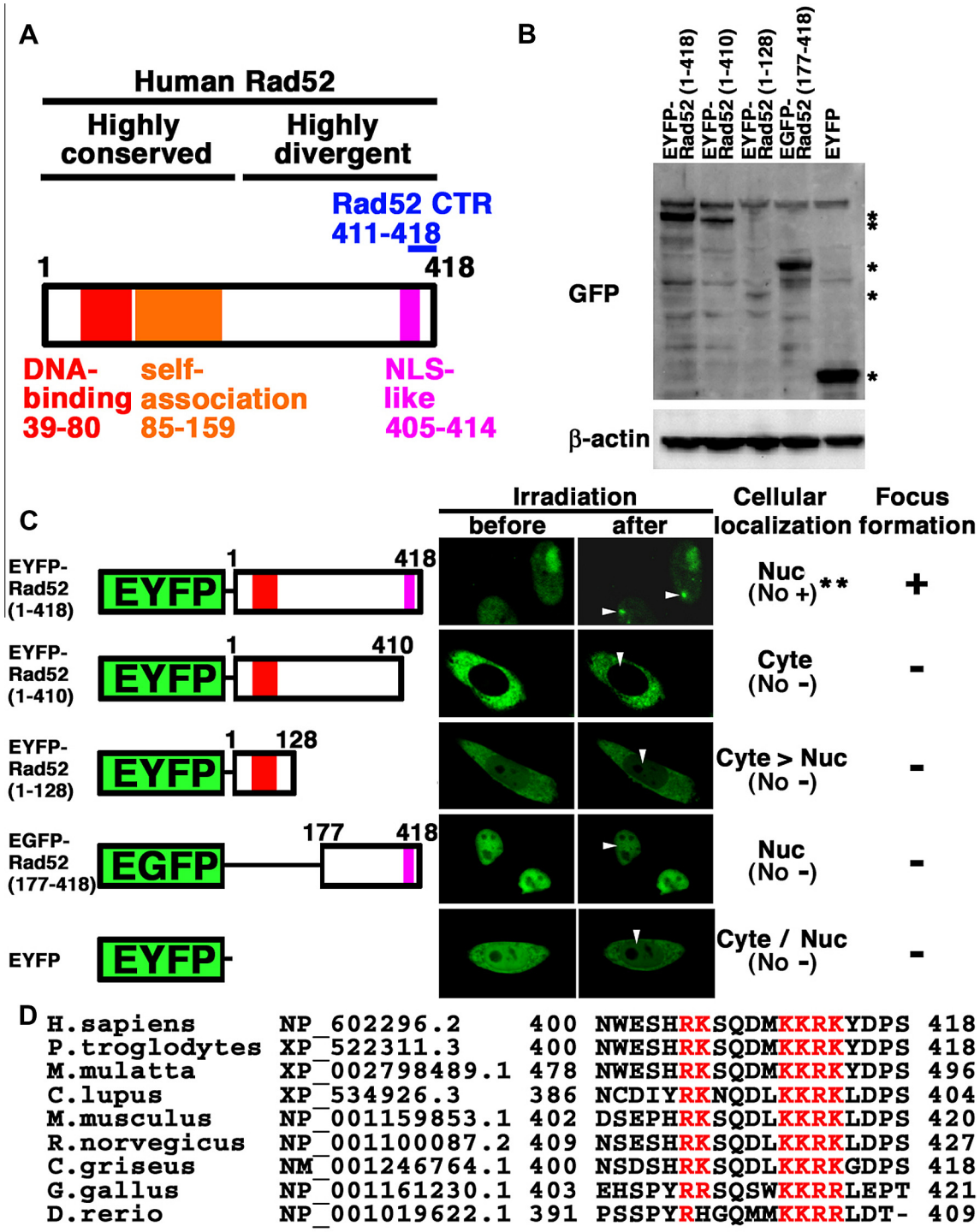

Fig. 3. The end of the C-terminal region is essential for Rad52 nuclear and nucleolar localization, and recruitment to DSBs in vivo. (A) Rad52 consists of a highly conserved N-terminal region and a highly divergent C-terminal region. The regions essential for DNA binding (39–80) and self-association (85–159) are located within the N-terminal half of human Rad52. The C-terminal region of Rad52 (411–418) (Rad52 CTR) contains a portion of putative NLS (405–414). (B) Expression of full-length and deleted Rad52 proteins. Full-length and deleted Rad52 proteins that were fused to the EYFP- or EGFP-tag at their N-terminal ends were transiently expressed in CHO-K1 cells. The expressions were examined by Western blotting using the anti-GFP or anti-β-actin antibody. Asterisks indicate each specific band. (C) Identification of domain of Rad52 essential for nuclear localization, nucleolar localization, and accumulation at DSBs. The localization and accumulation of the chimeric proteins at laser-induced DSBs were examined by live cell imaging. The results are summarized on the right: Cellular localization (Nuc, nucleus; Cyto, cytoplasm), nucleolar localization ([No $_{+}$]**, nucleolar localization; [No $_{-}$], no nucleolar localization), and focus formation (+, accumulated at irradiated sites; –, not accumulated at irradiated sites). Arrowheads indicate the irradiated sites. (D) The alignment of the primary sequence among homologous Rad52 proteins. The basic (red) and nonbasic (black) residues are in different color codes for comparison. The GeneBank accession number of each sequence is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amino acids of the C-terminal region of Rad52, i.e., Rad52 CTR, are essential for the nuclear and nucleolar localization of Rad52. Furthermore, we determined several basic amino acids in the Rad52 CTR that are evolutionarily highly conserved in vertebrates, but not in yeast. We also found that the deletion of the last eight amino acids, i.e., the Rad52 CTR, completely abolished the recruitment of

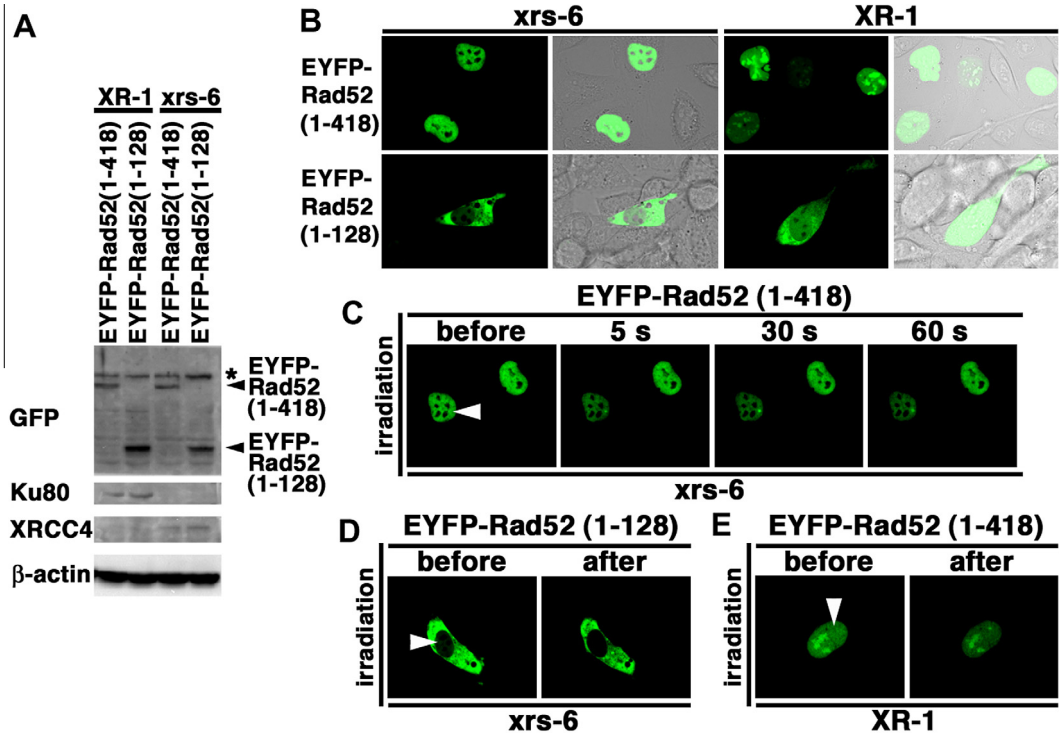

Fig. 4. Core NHEJ factors are not essential for the nuclear localization of Rad52 and accumulation of Rad52 at irradiated sites. (A) Expression of full-length and deleted Rad52 proteins in XR-1 (XRCC4-deficient) and xrs-6 (Ku80-deficient) cells. EYFP-Rad52(1–418) and EYFP-Rad52(1–128) were transiently expressed respectively in XR-1 and xrs-6 cells. The expressions of these proteins were examined by Western blotting using the anti-GFP, anti-Ku80, anti-XRCC4, and anti-β-actin antibodies. An asterisk indicates a nonspecific band. (B) Imaging of living EYFP-Rad52(1–418)- or Rad52(1–128)-transfected cells. Living xrs-6 and XR-1 cells transiently expressing EYFP-Rad52(1–418) (upper panel) or EYFP-Rad52(1–128) (lower panel) were analyzed by confocal laser microscopy. For the same cells, EYFP-Rad52 images are shown alone (left panel) or merged with DIC (right panel). (C) Time-dependent EYFP-Rad52(1–418) accumulation at laser-induced DSBs in living xrs-6 cells after irradiation. (D, E) Accumulation of the chimeric proteins at laser-induced DSBs in xrs-6 (D) and XR-1 (E) cells. Arrowheads indicate the irradiated sites.

Rad52 to DSBs, suggesting that Rad52 might respond to DNA damage stress in a localization-dependent manner. Thus, the Rad52 CTR is essential for both the Rad52 nuclear localization and recruitment to DSBs. Recently, it has been reported that the lysine residues in the putative NLS of human Rad52 are the potential sumoylation sites, but further study is needed to clarify in detail whether the sumoylation plays an important role in the nuclear transport of Rad52 [26]. We suppose that sumoylation and other posttranslational modifications, e.g., acetylation or phosphorylation, modulate the functions of Rad52 CTR. Altogether, the Rad52 CTR might be important for the functional regulation of this protein not only in mammalian cells, but also in other vertebrate cells including fish and avian cells at an early stage following irradiation.

In conclusion, the accumulation of Rad52 at DNA damaged sites is independent of the core NHEJ factors Ku80 and XRCC4. DSBs are repaired by two main pathways: NHEJ and HR. The fate of a damaged cell might be affected by the choice between these pathways. Further study to elucidate the molecular mechanism via the Rad52-dependent DNA damage response pathway at DNA damaged sites will lead to a better understanding of not only the physiological function of Rad52, but also the mechanism underlying the choice of the DSB repair pathway, i.e., NHEJ or HR, in various complicated responses to DNA damage.

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