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Author(s)	Yamamori, Tohru; Meike, Shunsuke; Nagane, Masaki; Yasui, Hironobu; Inanami, Osamu
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**ER stress suppresses DNA double-strand break repair and sensitizes tumor cells to ionizing radiation by stimulating proteasomal degradation of Rad51**

Tohru Yamamori, Shunsuke Meike, Masaki Nagane, Hironobu Yasui, Osamu Inanami\*

Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan.

\*Corresponding author: Prof. Osamu Inanami

Address: Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan.

E-mail: [inanami@vetmed.hokudai.ac.jp](mailto:inanami@vetmed.hokudai.ac.jp)

Tel: +81-11-706-5235

Fax: +81-11-706-7373

## **Abstract**

In this study, we provide evidence that endoplasmic reticulum (ER) stress suppresses DNA double-strand break (DSB) repair and increases radiosensitivity of tumor cells by altering Rad51 levels. We show that the ER stress inducer tunicamycin stimulates selective degradation of Rad51 via the 26S proteasome, impairing DSB repair and enhancing radiosensitivity in human lung cancer A549 cells. We also found that glucose deprivation, which is a physiological inducer of ER stress, triggered similar events. These findings suggest that ER stress caused by the intratumoral environment influences tumor radiosensitivity, and that it has potential as a novel target to improve cancer radiotherapy.

Key words: ER stress; Rad51; DNA double-strand break repair; radiosensitivity; unfolded protein response

Abbreviations: DSB, double-strand break; ER, endoplasmic reticulum; ERAD, ER-associated degradation; IR, ionizing radiation; HR, homologous recombination; NHEJ, non-homologous end joining; RT-PCR, reverse transcription-PCR; UPR, unfolded protein response

## 1. Introduction

A number of cellular stresses lead to accumulation of unfolded and/or misfolded proteins in the ER lumen and cause so-called ER stress [1-3]. ER stress activates various intracellular signaling pathways, known as the unfolded protein response (UPR) [1-3]. When cells undergo ER stress, the UPR triggers three main responses: the inhibition of general protein translation, the induction of genes such as ER chaperones to increase the protein-folding capacity of the ER, and up-regulation of aberrant protein degradation in the ER. The last process is called ER-associated degradation (ERAD). The ERAD system eliminates aberrant proteins via degradation in the cytosol [4]. Aberrant ER proteins are retrotranslocated across the ER membrane into the cytosol, where ubiquitin-conjugating enzymes target them for proteasomal degradation [5]. Fundamentally, UPR is a cyto-protective response. However, excessive or prolonged UPR results in cell death. The UPR pathways are activated in a variety of tumor types, and are essential for tumor cells to survive the unfavorable environment of solid tumors, which is typically characterized by hypoxia, low pH, and nutrient deprivation [3,6,7]. In addition, recent evidence suggests that the UPR is an important mechanism required for cancer cells to maintain malignancy and therapy resistance [3,7].

In cancer radiotherapy, DNA damage caused by ionizing radiation (IR) and inherent DNA repair capacity of tumor cells are important factors that determine therapeutic outcome. Therefore, interfering with the DNA repair machinery of tumor cells is one of the major strategies to treat cancer [8]. Among the various types of DNA damage induced by IR, DSB is regarded as the most lethal to the cell [8,9]. In mammalian cells, DSBs are repaired by one of two distinct processes, known as non-homologous end joining (NHEJ) or homologous recombination (HR) [9]. Rad51, a recA homolog that binds the single-strand DNA generated by Mre11-Rad50-NBS1 complex, is essential for recombinational repair. Cells lacking functional Rad51 are unable to form subnuclear foci, characteristic of HR, and are significantly more sensitive to IR [10,11]. Furthermore, elevated Rad51 expression in tumor tissues is associated with an unfavorable prognosis in lung cancer [12]. These observations indicate that Rad51 is a promising target to improve the therapeutic efficacy of radiotherapy. This concept is partly supported by our recent study demonstrating that a novel anticancer drug, TAS106, radiosensitized tumor cells by down-regulating Rad51 and Brca2 expression [13].

Recent evidence suggests that ER stress imposed on tumor cells due to the surrounding environment may affect the outcome of various anticancer strategies. For example, it has been

reported that ER stress influences chemosensitivity of tumor cells to anticancer drugs such as cisplatin and doxorubicin [14,15]. In addition, Contessa *et al.* have shown that the classic ER stress inducer tunicamycin causes radiosensitization in tumor cells, but not in normal cells [16]. However, it remains unclear how ER stress influences DSB repair capacity and radiosensitivity of tumor cells. Therefore, we sought to decipher the effect of ER stress using different types of ER stress-inducing treatments.

## **2. Materials and methods**

### **2.1. Reagents**

Regular and glucose-free RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA, USA). Tunicamycin was obtained from Enzo Life Sciences (Farmingdale, NY, USA), and cisplatin was obtained from Wako Pure Chemical Industries (Osaka, Japan). MG132 was purchased from Cayman Chemical (Ann Arbor, MI, USA). ALLN was obtained from Merck (Darmstadt, Germany). Protein A Sepharose was obtained from GE healthcare (Buckinghamshire, UK). The following antibodies were used for western blotting and immunostaining: anti-NBS1, anti-Mre11 (Abcam, Cambridge, MA, USA), anti-phospho-eIF2 $\alpha$ , anti- $\gamma$ -H2AX (Cell Signaling Technology, Beverly, MA, USA), anti-Rad51, anti-DNA-PKcs, anti-Ku70, anti-actin, anti-ubiquitin, HRP-conjugated

secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Alexa Fluor<sup>®</sup> 488 anti-rabbit IgG (Invitrogen). The chemiluminescence detection kit, Western Lightning<sup>®</sup> Plus-ECL, was purchased from Perkin-Elmer (Boston, MA, USA).

## 2.2. Cell culture and X-irradiation

The human lung carcinoma cell line A549 was grown in RPMI 1640 containing 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. X-irradiation was performed with a Shimadzu PANTAK HF-350 X-ray generator (Shimadzu, Kyoto, Japan) at 200 kVp, 20 mA with a 1.0-mm aluminum filter.

## 2.3. SDS-PAGE and western blotting

Cells were collected and lysed in lysis buffer (20 mM HEPES-NaOH [pH 7.4], 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin). After centrifugation at 20,000g for 15 min at 4°C, supernatants were collected. Three-fold concentrated Laemmli's sample buffer (0.1875 M Tris-HCl [pH 6.8], 15% β-mercaptoethanol, 6% SDS, 30% glycerol and 0.006% bromophenol blue) was added to the supernatants, and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Advantec Toyo, Tokyo, Japan). The membrane was probed with

specific antibodies diluted with TBST (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl and 0.1% Tween-20) containing 5% nonfat skim milk, overnight at 4°C. After probing with HRP-conjugated secondary antibodies, bound antibodies were detected with Western Lightning® Plus-ECL (Perkin-Elmer).

#### 2.4. Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using a Reverse Transcription System (Promega, Madison, WI, USA) and resulting cDNA amplified with GoTaq™ DNA Polymerase (Promega). The specific primer sequences for PCR were as follows: for Rad51, 5'-TTTGGAGAATTCCGAACTGG-3' and 5'-AGGAAGACAGGGAGAGTCG-3'; for actin 5'-GACCCAGATCATGTTTGAGACC-3' and 5'-GGTGAGGATCTTCATGAGGTAG-3'. The PCR profile was as follows: initial denaturation at 95°C for 2 min, followed by 31 cycles (actin) or 40 cycles (Rad51) at 95°C for 1 min, annealing at 63°C (actin) or 64°C (Rad51) for 1 min and extension at 72°C for 1 min. The final extension was performed by incubation at 72°C for 5 min. PCR products were subjected to agarose gel electrophoresis and visualized using ethidium bromide (Sigma, St. Louis, MO, USA).

## 2.5. Immunofluorescence staining

At the times indicated following X-irradiation, cells attached on glass coverslips were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. After permeabilization with 0.5% Triton X-100/PBS for 5 min at 4°C, cells were treated with PBS containing 6% goat serum for 30 min at room temperature. Subsequently, they were incubated with the anti- $\gamma$ -H2AX antibody (1:500) in 3% goat serum overnight at 4°C. They were then incubated in the dark with the Alexa Fluor® 488-conjugated anti-rabbit secondary antibody at a 1:500 dilution for 1.5 h. After incubation, they were counterstained with 300 nM 4',6-diamidino-2-phenylindole (Invitrogen) for 5 min at room temperature. Coverslips were mounted with Prolong Gold antifade reagent (Invitrogen). Fluorescent microscopic analysis was performed using an Olympus BX61 microscope with a reflected light fluorescence and foci were counted manually.

## 2.6. Clonogenic survival assay

Cells were seeded on 6-cm dishes and treated with 5  $\mu$ M tunicamycin for 12 h, followed by X-irradiation. Where necessary, cells were treated with cisplatin for 3 h after tunicamycin treatment. After incubation for 10 days, they were fixed with methanol and stained with Giemsa solution (Sigma).

Colonies containing more than 50 cells were scored as surviving cells. Surviving fractions were corrected using the plating efficiency of the non-irradiated control. The survival curves of X-irradiated cells were obtained by fitting to a linear-quadratic model using data analysis software Origin Pro 7 (OriginLab Co. Northampton, MA, USA).

## 2.7 Rad51 ubiquitination

A549 cells were treated with DMSO or 5  $\mu$ M tunicamycin in the presence or absence of 10  $\mu$ M MG132 for 12 h and lysed in IP lysis buffer (50 mM Tris-HCl [pH 7.5], 1%(v/v) Triton X-100, 5%(v/v) glycerol, 5 mM EDTA, 150 mM NaCl, 1 $\times$  protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), 10 mM iodoacetamide and 50  $\mu$ M MG132). After centrifugation at 20,000g for 15 min at 4°C, whole cell extracts (WCE) were collected. WCE were rotated with Protein A Sepharose for 30 min at 4°C and precleared WCE were collected. Two micrograms of Rad51 antibody was added to the samples and they were rotated overnight at 4°C. After 30  $\mu$ l of 50% slurry Protein A Sepharose was added, the samples were rotated for 1 h at 4°C. After beads were washed 7 times, they were mixed with 3x Laemmli's sample buffer and subjected to SDS-PAGE, followed by immunoblotting using the specified primary antibody and the appropriate secondary antibody.

### 3. Results

#### 3.1. ER stress suppresses Rad51 levels

To determine whether ER stress affects DSB repair, we examined the effect of tunicamycin, a classic ER stress inducer, on the expression levels of DSB repair-related proteins in human lung cancer A549 cells. Since ER stress is known to activate PERK, which leads to the phosphorylation of eIF2 $\alpha$  and suppression of protein translation [17], we used phospho-eIF2 $\alpha$  as a marker of ER stress. As shown in Fig. 1A, treatment with tunicamycin for 12 h increased phospho-eIF2 $\alpha$  in a concentration-dependent manner, indicating that tunicamycin caused ER stress in A549 cells, as expected. We then examined the expression levels of various DSB repair proteins after tunicamycin treatment and found that it led to a decrease in Rad51 levels, a key HR pathway protein, in a concentration-dependent manner. Meanwhile, tunicamycin did not alter expression levels of other HR-related proteins (Mre11 and NBS1) or NHEJ proteins (DNA-PKcs and Ku70). Furthermore, we observed that 5  $\mu$ M tunicamycin induced a time-dependent increase of eIF2 $\alpha$  phosphorylation and the simultaneous reduction of Rad51 (Fig. 1B). To examine whether tunicamycin-induced down-regulation of Rad51 was due to a decrease in Rad51 mRNA, semi-quantitative RT-PCR analysis was performed. As shown in Fig. 1C, tunicamycin treatment did not

change Rad51 mRNA levels. In addition, we also observed that another ER stress inducer, thapsigargin, suppressed Rad51 protein levels without affecting expression levels of other DSB repair-related proteins (Fig. S1). These results indicate that ER stress down-regulates Rad51 protein levels without affecting its mRNA levels.

### 3.2. ER stress triggers proteasomal degradation of Rad51

We assessed the involvement of intracellular protein degradation pathways to investigate the mechanism of tunicamycin-induced Rad51 down-regulation. To this end, we tested the effect of proteinase inhibitors on tunicamycin-induced Rad51 down-regulation. Whereas 10  $\mu$ M MG132, a 26S proteasome inhibitor, did not affect basal Rad51 levels in A549 cells, it clearly inhibited tunicamycin-induced Rad51 down-regulation (Fig. 2A). In contrast, 20  $\mu$ M ALLN, a calpain inhibitor, had no effect on Rad51 levels with and without tunicamycin treatment. In addition, we also found that tunicamycin stimulated Rad51 ubiquitination as shown in Fig. 2B. These results suggest that tunicamycin leads to Rad51 down-regulation through the ubiquitin-proteasome system.

### 3.3. ER stress attenuates DSB repair and sensitizes cells to genotoxic stress

Because RNAi-mediated Rad51 down-regulation sensitizes tumor cells to IR [10], we next analyzed whether tunicamycin treatment influenced cellular radiosensitivity using a clonogenic survival assay. The survival curves of cells exposed to X-rays is shown in Fig. 3A. Cells pretreated with tunicamycin displayed an increase in radiosensitivity. The 10% lethal dose ( $D_{10}$ ) of the surviving fraction was reduced from 6.85 Gy in the control cells to 5.45 Gy in the tunicamycin-treated cells (dose enhancement ratio = 1.26). We further tested the effect of tunicamycin on chemosensitivity of A549 cells to cisplatin and found that it was also enhanced by tunicamycin treatment (Fig. 3B).

To elucidate the mechanism of tunicamycin-induced radiosensitization, we evaluated the effect of tunicamycin on the DSB repair capacity in irradiated cells by  $\gamma$ -H2AX foci formation assay. Because histone H2AX is phosphorylated at serine 139 ( $\gamma$ -H2AX) immediately after DSB induction and forms nuclear foci [18], the number of  $\gamma$ -H2AX foci per cell serves as an indicator of DSBs. A549 cells were exposed to X-rays with or without tunicamycin, and the formation of  $\gamma$ -H2AX foci was analyzed. As shown in Fig. 3C, the number of  $\gamma$ -H2AX foci spiked 30 min after irradiation, and then decreased in a time-dependent manner in both cell types. No significant effect by tunicamycin was observed in the initial peak of the radiation-induced  $\gamma$ -H2AX

foci formation. However, the number of  $\gamma$ -H2AX foci decreased much more slowly in tunicamycin-treated cells than in control cells, suggesting the inhibition of DSB repair by tunicamycin. Together, these data suggest that tunicamycin sensitizes cells to genotoxic stress by suppressing DSB repair via Rad51 down-regulation.

#### 3.4. Glucose deprivation suppresses Rad51 levels and DSB repair

In an intratumoral environment, tumor cells are often exposed to nutrient starvation, which leads to ER stress [3,7]. Therefore, to examine whether nutrient stress induces the ER stress-associated down-regulation of Rad51 and DSB repair, we tested the effect of glucose deprivation in A549 cells. As expected, glucose deprivation stimulated eIF2 $\alpha$  phosphorylation, indicating a starvation-induced ER stress (Fig. 4A). Under this condition, we observed a time-dependent decrease of Rad51 levels. In addition, treatment with glucose-containing media did not alter the levels of either phospho-eIF2 $\alpha$  or Rad51. Whereas Rad51 mRNA levels were maintained after glucose deprivation (Fig. 4B), MG132 abrogated the starvation-induced reduction of Rad51 protein levels (Fig. 4C). Furthermore, when we evaluated the effect of glucose deprivation on radiation-induced DSB formation, the cells under nutrient stress had more  $\gamma$ -H2AX foci after 1 Gy irradiation than

those under normal conditions (Fig. 4D). These results suggest that nutrient stress by glucose deprivation induces ER stress-associated down-regulation of Rad51 and the reduction of DSB repair.

#### **4. Discussion**

The UPR in response to ER stress is activated in various tumors [3,7]. Accumulating evidence suggests that the ER stress-induced UPR is an important mechanism by which tumor cells determine their malignancy and resulting therapy resistance [7]. Nevertheless, few studies have investigated how ER stress affects radiotherapy outcomes. In the present study, we examined the radio-/chemo-sensitivity of tumor cells that underwent ER stress and found that ER stress sensitized them toward genotoxic stress (Fig. 3). Increased radiosensitivity by the chemical ER stress inducer, tunicamycin, observed in this study is consistent with the data reported by Contessa *et al.* [16]. We found that ER stress resulted in the proteasomal degradation of Rad51, one of the HR proteins, and the consequential reduction of DSB repair (Figs. 1-3). We consider this as the central mechanism of ER stress-induced radiosensitization because cells lacking functional Rad51 are defective in HR repair and more susceptible to IR [10,11]. However, it remains elusive how Rad51 is degraded by the

ubiquitin-proteasome system. Interestingly, gemcitabine treatment increases Rad51 ubiquitination and degradation via the 26S proteasome by celecoxib (a cyclooxygenase-2 inhibitor) or gefitinib (an epidermal growth factor receptor kinase inhibitor) [19,20]. These findings imply that various treatments, not limited to ER stress inducers, might be able to stimulate the proteasomal degradation of Rad51. An alternative possibility is that these drugs might also cause ER stress, thereby leading to Rad51 degradation. Further study will be required to elucidate how ER stress causes polyubiquitination and proteasomal degradation of Rad51.

We revealed in this study that, similar to tunicamycin, glucose deprivation - a physiological condition to induce ER stress - also resulted in the proteasomal degradation of Rad51 and the suppression of DSB repair (Fig. 4). As far as we know, this provides the first evidence that nutrient stress leads to inhibition of the HR pathway. The effect of glucose withdrawal on the DNA repair machinery has not been studied extensively. One exception is the report by Li *et al.* [21], who demonstrated that glucose deprivation induces expression of Ku70, one of the NHEJ proteins, and enhances radioresistance, which is at odds with our data. Here, we found no effect of tunicamycin on the expression level of Ku70. In addition, their results showed that glucose deprivation did not induce the ER stress maker GRP78

in HT29 cells and DU145 cells. Together, these data suggest that enhanced Ku70 expression following glucose deprivation is unrelated to the glucose deprivation-induced ER stress we observed here, providing a potential explanation for the discrepancy between the two studies.

Although we report here what we believe are the first data suggesting that ER stress impairs DSB repair, hypoxia - another physiological condition that induces ER stress - also has been documented to decrease it [6,22,23]. Chronic hypoxia leads to down-regulation of various HR proteins including Brcal/2 and Rad51 [6,22,23]. It is noteworthy that chronic hypoxia reduces Rad51 expression at the protein as well as mRNA levels, accompanied by HR inhibition and increased radiosensitivity [6,22]. Whereas the authors have suggested that Rad51 down-regulation is due to transcriptional suppression, it might be attributable, at least in part, to the enhanced degradation of HR proteins by hypoxia-induced ER stress and ERAD. Regarding the biological significance of compromised DNA repair by chronic hypoxia, Bristow *et al.* have suggested the possibility that it drives tumor cells to become more malignant by acquiring a mutator phenotype that consists of an increased mutation rate and increased chromosomal instability [24]. In fact, several groups have already reported increased mutation rates, using mutation reporter constructs, in cells exposed *in vitro* or *in*

*vivo* to hypoxic conditions [25-27], supporting this idea. In contrast, the fact that defective DNA repair in hypoxic cells influences their radiosensitivity implies that targeting hypoxic tumor cells with compromised DNA repair capacity could be a potential strategy to improve therapeutic effectiveness [24]. Considering that ER stress affected cellular radiosensitivity in a similar way as chronic hypoxia did (*i.e.* Rad51 down-regulation and decreased DSB repair), tumor cells under ER stress might be potential candidates to ameliorate the efficacy of radiotherapy.

In conclusion, the present study demonstrates ER stress reduces DSB repair and enhances radiosensitivity of tumor cells via down-regulation of Rad51 levels. The proteasomal degradation of Rad51 triggered by ER stress is highly likely to be responsible for the suppression of DSB repair after IR. These findings indicate that ER stress imposed by the intratumoral environment affects tumor radiosensitivity and has potential as a novel target to improve cancer radiotherapy.

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## Figure legends

### **Fig. 1. Effect of tunicamycin on the expression levels of DSB repair-related proteins**

(A) Cells were treated with tunicamycin (TM) at the indicated concentrations for 12 h. (B) Cells were treated with vehicle or tunicamycin (5  $\mu$ M) for the indicated times. After incubation, cell extracts were analyzed by western blotting. Actin was used as a loading control. (top) Representative blots. (bottom) The intensities of Rad51 bands were normalized to those of actin bands. Data are expressed as means  $\pm$  SE of three experiments. \* $p$  < 0.05; \*\* $p$  < 0.01 vs 0 h (Student's  $t$ -test). (C) Cells were treated with tunicamycin (5  $\mu$ M) for 6 or 12 h. After incubation, total RNA was isolated and mRNA levels were analyzed by RT-PCR. Actin was used as an internal control.

### **Fig. 2. Effect of proteinase inhibitors on tunicamycin-induced Rad51 down-regulation**

(A) Cells were treated with vehicle or tunicamycin (TM) (5  $\mu$ M) in the presence or absence of the proteasome inhibitor MG132 (10  $\mu$ M), or the calpain inhibitor ALLN (20  $\mu$ M), for 12 h as indicated. After incubation, cell extracts were analyzed by western blotting. Actin was used as a loading control. (B) Immunoblot showing incorporation of ubiquitin (Ub) moieties into Rad51. Cells were treated with vehicle or tunicamycin (TM)

(5  $\mu\text{M}$ ) in the presence or absence of MG132 (10  $\mu\text{M}$ ) for 12 h, followed by immunoprecipitation and western blotting. IP: immunoprecipitates; WCE: whole cell extracts.

**Fig. 3. Effect of tunicamycin on cellular sensitivity to genotoxic stress and DSB repair**

(A) Cells were pretreated with or without tunicamycin (5  $\mu\text{M}$ ) for 12 h, followed by X-irradiation. The cellular sensitivity to IR was assessed by clonogenic survival assay. Data are expressed as mean  $\pm$  SE of three experiments. \* $p$  < 0.05; \*\* $p$  < 0.01 vs -tunicamycin (Student's  $t$ -test). (B) The cellular sensitivity to cisplatin assessed as described above. Data are expressed as mean  $\pm$  SE of three experiments. \*\* $p$  < 0.01 vs -tunicamycin (Student's  $t$ -test). (C) Cells were pretreated with or without tunicamycin (5  $\mu\text{M}$ ) for 12 h, followed by X-irradiation (1 Gy). After irradiation, cells were collected at the times indicated to evaluate the nuclear  $\gamma$ -H2AX foci formation. The number of foci in at least 30 cells was scored and the averages were plotted in the graph. Data are expressed as mean  $\pm$  SE of three experiments. \* $p$  < 0.05; \*\* $p$  < 0.01 vs -tunicamycin (Student's  $t$ -test).

**Fig. 4. Effect of glucose deprivation on Rad51 levels and DSB repair**

(A) Cells were cultured with or without glucose for 12 h. After incubation, cell extracts were analyzed by western blotting. Actin was used as a loading control. (B) Cells were cultured without glucose for 6 or 12 h. After incubation, total RNA was isolated and the mRNA levels were analyzed by RT-PCR. Actin was used as an internal control. (C) Cells were cultured with or without glucose in the presence or absence of the proteasome inhibitor MG132 (10  $\mu$ M) for 12 h, as indicated. After incubation, cell extracts were analyzed by western blotting. Actin was used as a loading control. (D) Cells were cultured with or without glucose for 12 h, followed by X-irradiation (1 Gy). After irradiation, cells were collected at the times indicated to evaluate the nuclear  $\gamma$ -H2AX foci formation. The number of foci in at least 30 cells was scored and the averages were plotted. Data are expressed as mean  $\pm$  SE of three experiments. \* $p$  < 0.05 vs glucose(+) (Student's  $t$ -test).

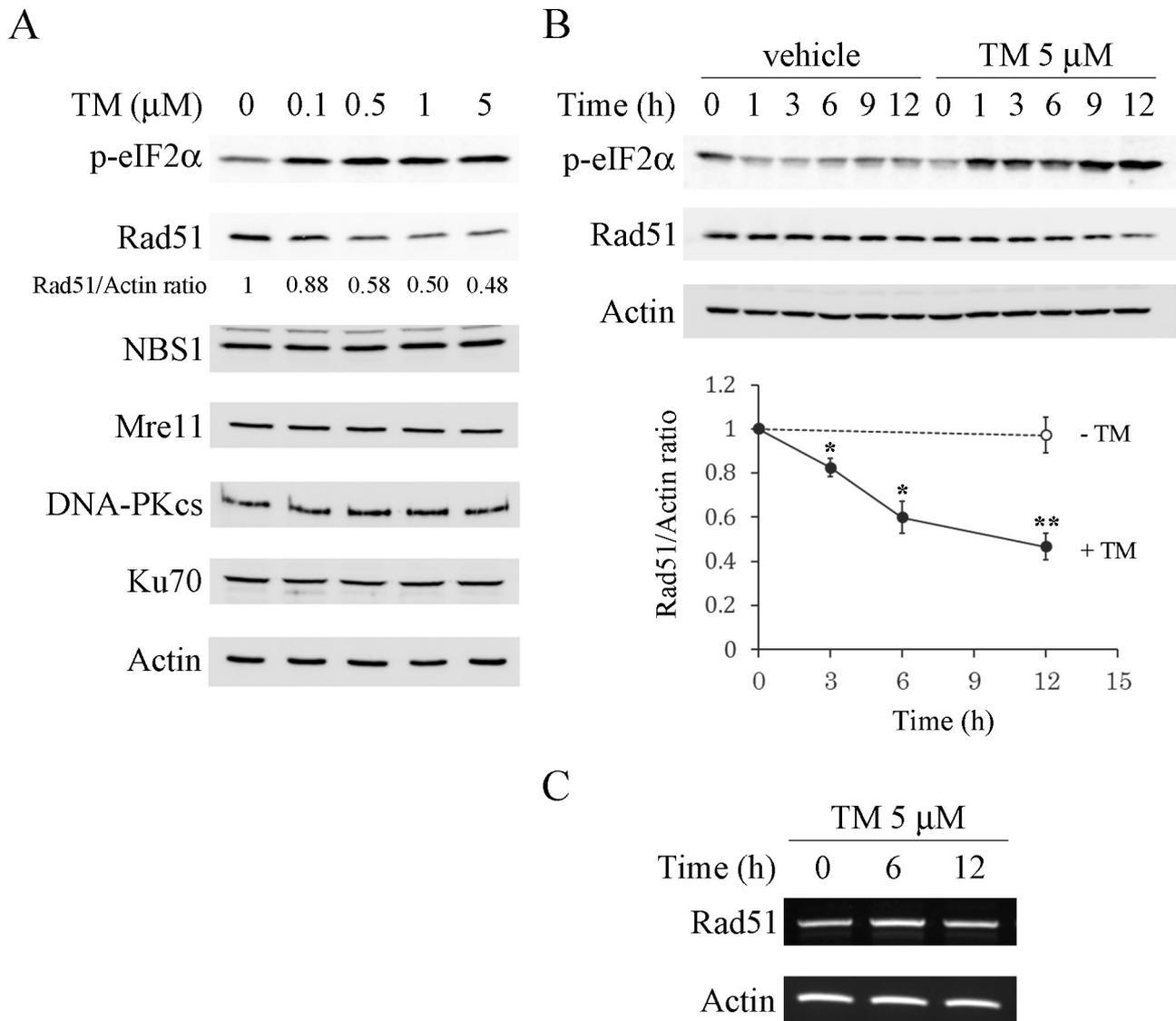
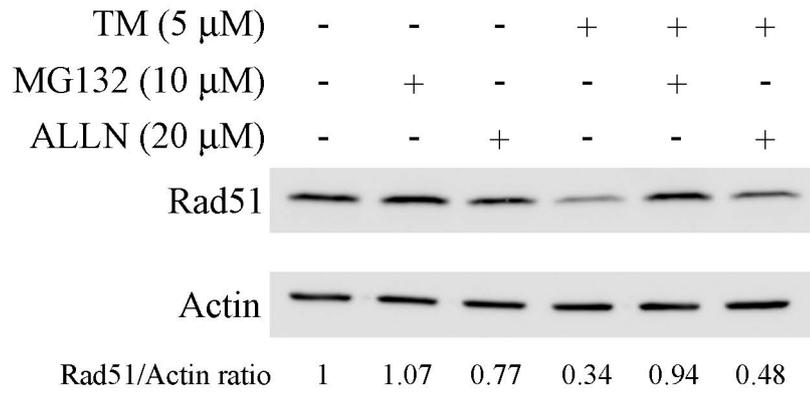


Fig. 1 Yamamori *et al*

A



B

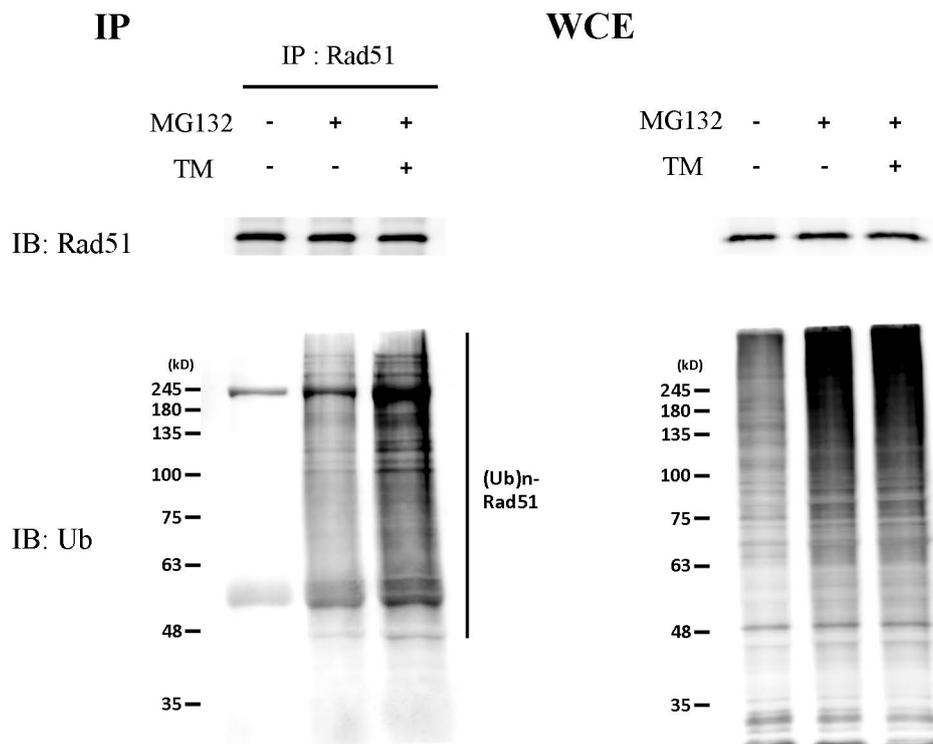


Fig. 2 Yamamori *et al*

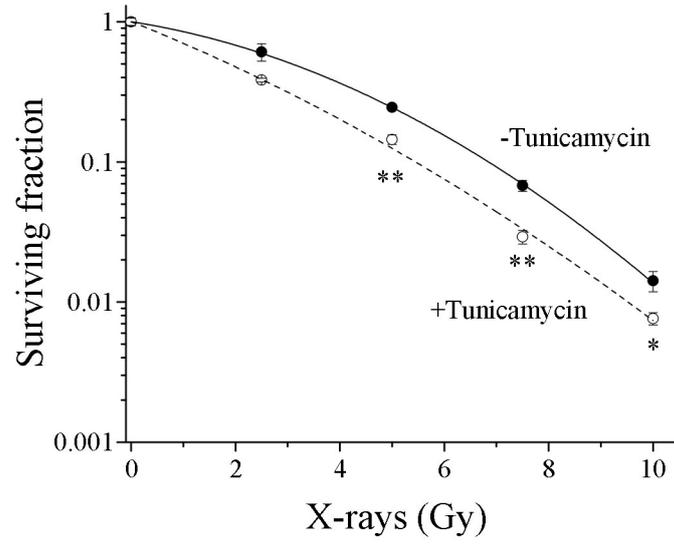
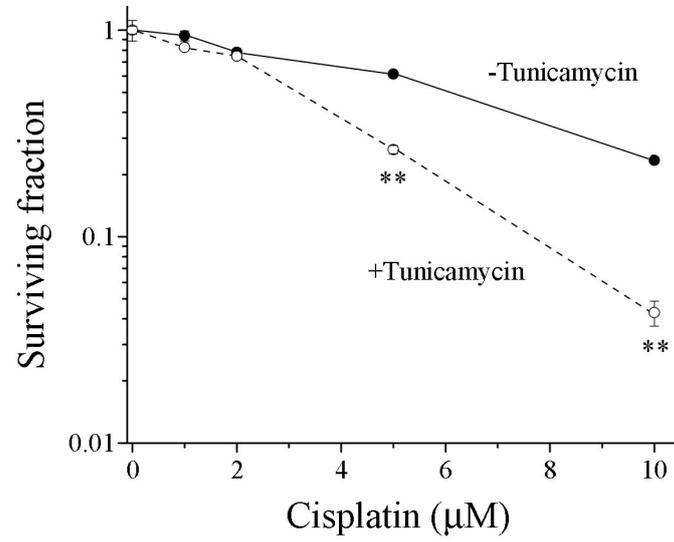
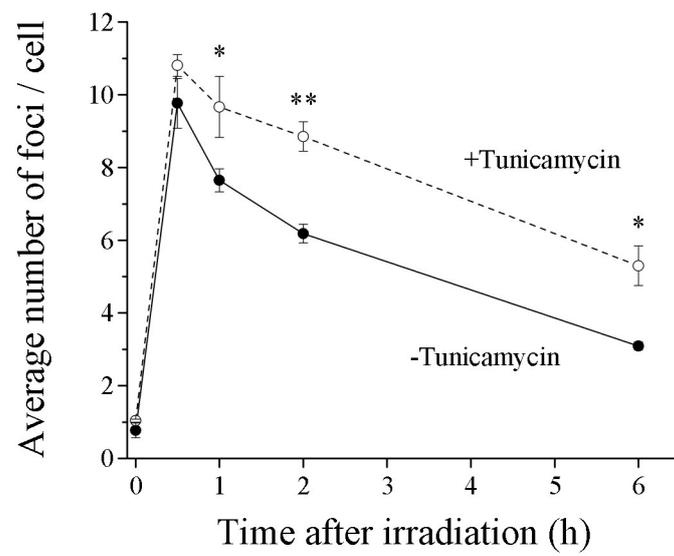
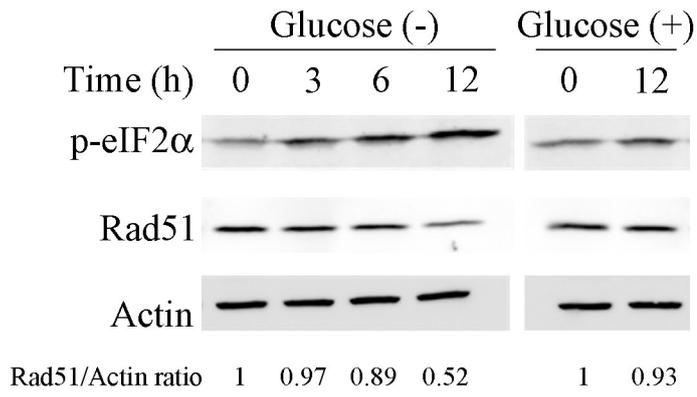
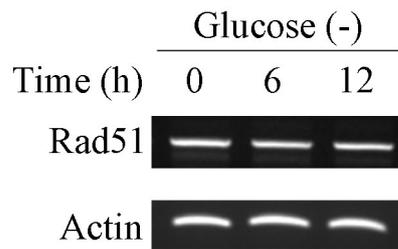
**A****B****C**

Fig. 3 Yamamori *et al*

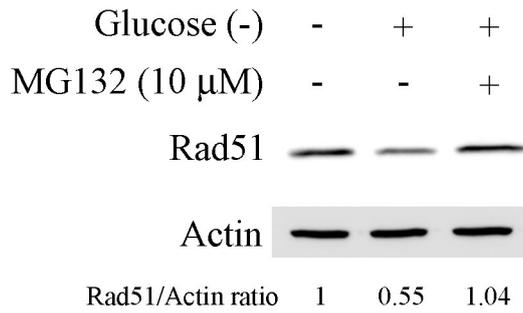
A



B



C



D

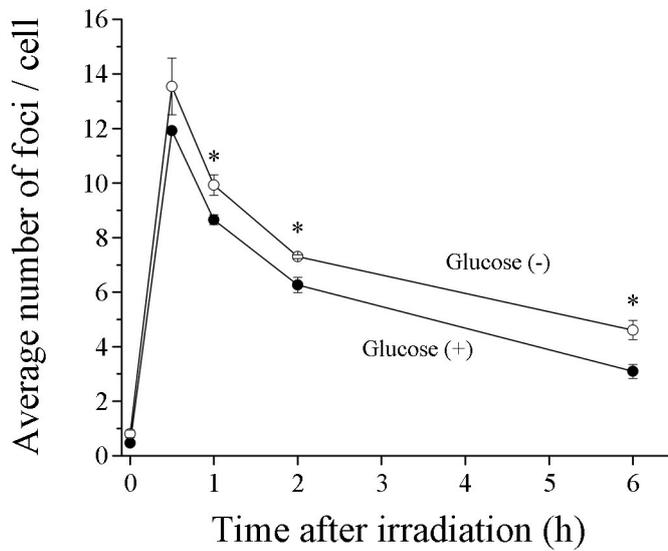


Fig. 4 Yamamori *et al*