Regulation of ionizing radiation-induced Rad52 nuclear foci formation by c-Abl-mediated phosphorylation

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Keywords: c-Abl, Rad52, nuclear foci, ionizing radiation, homologous recombination, tyrosine phosphorylation.

Running title: c-Abl-mediated regulation of Rad52 nuclear foci formation.

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Summary

The RAD52 epistasis group of proteins, including Rad51, Rad52 and Rad54, play an important role in the homologous recombination repair of double strand breaks (DSBs). A well-characterized feature associated with the ability of these proteins to repair DSBs is the inducible nuclear foci formation at the sites of damage. How the process is functionally regulated in response to DNA damage, however, remains elusive. We show here that the c-Abl tyrosine kinase associates with and phosphorylates Rad52 on tyrosine 104. Importantly, the very same site of Rad52 is phosphorylated upon exposure of cells to ionizing radiation (IR). Functional significance of c-Abl-dependent phosphorylation of Rad52 is underscored by our findings that cells expressing the phosphorylation-resistant Rad52 mutant, where the 104-tyrosine was replaced by phenylalanine, exhibit compromised nuclear foci formation in response to IR. Furthermore, IR-induced Rad52 nuclear foci formation is markedly suppressed by the expression of dominant negative c-Abl. Together, our data support a mode of post-translational regulation of Rad52 mediated by the c-Abl tyrosine kinase.

Introduction

DSBs arise on chromosomes when replication forks collapse during DNA replication as well as upon exposure to DNA damaging agents such as IR. To repair DSBs, only one of which is sufficient to cause cell death if left un-repaired, eukaryotes have developed multiple DSBs repair pathways, including non-homologous end joining (NHEJ) and homologous recombination (HR). In yeast Saccharomyces cerevisiae, the genes involved in HR form the RAD52 epistasis
group of genes (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2*) (1). Mutation of these genes is often associated with hypersensitivity to IR and mitotic and meiotic recombination defects (1). Among the *RAD52* group, the *rad51, rad52* and *rad54* mutants exhibit more severe defect in HR repair, suggesting that these genes encode the central components of the recombinational repair machinery (1).

Structural homologues of the *RAD52* group of genes have been cloned from vertebrates (2). Since these proteins share similar biochemical properties with their yeast homologs (3), and since vertebrate cells deficient in the Rad54 (4,5) or Rad51 paralog genes (6,7) are sensitive to IR and show mitotic recombination defect phenotypes, basic molecular machinery of HR is believed to be conserved through evolution. However, mutant phenotypes of *RAD52* epistasis group genes in vertebrate are not always identical to those in yeast. The most prominent examples are Rad51 and Rad52. Rad51 gene knockout mice are embryonic lethal (8,9) and depletion of Rad51 proteins from cells is accompanied by an accumulation of cytologically detectable chromosome aberrations and subsequent cell death, indicating the essential role of Rad51 in the maintenance of chromosomal DNA during normal cell cycle (10). On the contrary, Rad52 gene knockout mice are normal and fertile and Rad52 deficient cells only show slight decrease in gene targeting frequency and don’t exhibit any IR sensitive phenotype (11,12). However, it was recently shown that simultaneous depletion of Rad52 and Xrcc3 proteins renders chicken DT40 cells non-viable and provokes extensive chromosomal breaks (13), demonstrating that
Rad52 plays an essential role in the maintenance of chromosomal DNA in cooperation with Xrcc3, one of five RAD51 paralogs.

HR is important to the maintenance of chromosome integrity. Cells from Ataxia telangiectasia patients, who have mutations in *Ataxia telangiectasia mutated* (ATM) gene, show chromosomal instability, radio-sensitivity, and defective cell cycle checkpoint (14). ATM-deficient cells exhibit altered kinetics of radiation-induced Rad51 and Rad54 focus formation (15,16). Furthermore, genetic analyses have shown that the defect in ATM function is associated with impaired HR-mediated DSBs repair process (15). ATM, c-Abl and Rad51 associate and form protein complex *in vivo* (17,18), and activation of c-Abl kinase in response to IR is in part dependent on ATM function (19,20). c-Abl phosphorylates Rad51 *in vitro and in vivo* (17,18), but the biological significance of this tyrosine phosphorylation is not clear. Here we show that c-Abl phosphorylates Rad52 on Tyr-104 *in vivo*. Inhibition of this tyrosine phosphorylation, either by dominant negative c-Abl expression or substitution of Rad52 Tyr-104 by Phe suppresses IR-induced GFP-Rad52 nuclear foci formation

**Material and Method**

**Cell Culture, transfection and γ-irradiation.** 293T (American Type Culture Collection) and Chinese hamster ovary (CHO) cells (Dr. Hatsumi Nagasawa, Colorado State University) were maintained in Dulbecco’s minimal essential medium (DMEM, Gibco-Invitrogen) containing 10% fetal bovine serum (FBS, Sigma), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Transfections were
performed by the calcium phosphate methods for 293T, and by Lipofectamin 2000 (Gibco-Invitrogen) for CHO cells. To establish GFP-Rad52(WT) and GFP-Rad52(Y104F) expressing CHO cells, 10 µg of plasmids were linearized by digestion with ApaLI (New England Biolabs) overnight at 37 °C, phenol extracted, ethanol precipitated and transfected to semi confluent CHO cells in 100 mm-diameter dishes. Transfected cells were selected in 10% FBS/DMEM containing 0.5 mg/ml geneticin (Gibco-Invitrogen) for 2 weeks. The resistant colonies were isolated and expanded for further analysis. Protein expression was confirmed by Western blot. CHO cells were irradiated at 5.45-5.70 cGy/sec by Cobalt-60 gamma irradiator (model GR-12, US nuclear).

**Plasmids.** Vectors expressing kinase active or inactive c-Abl have been reported previously (21). cDNAs of human Rad51, DFF45 and wild type and mutant Rad52 were subcloned into pcDNA3-Flag and/or pEGFP-C1 vectors. The series of tyrosine to phenylalanine point mutants of human Rad52 were generated by two-step PCR using a set of 30-nucleotide primers carrying the mutated nucleotide.

**Immunoprecipitation and immunoblot analysis.** Cells were transfected in 60-mm-diameter plates with 5 µg of plasmid DNA and harvested at 24 h post-transfection. Cells were lysed in 200 µl of lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol [DTT], 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride [PMSF], 10 µg/ml each of leupeptin and aprotinin) by incubating on ice for 30 min, and the extracts were centrifuged at 13,000 rpm
for 15 min to remove cell debris. Protein concentration was determined using Bio-Rad protein assay (BIO-RAD). After addition of loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 0.005% bromophenol blue, 1% glycerol), the samples were boiled at 95 °C for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoprecipitation, cell lysates were prepared in 0.5% Triton X-100 lysis buffer and incubated with the indicated antibodies for 4 hours and followed by incubation with protein A-protein G beads (Santa Cruz Biotechnology) for an additional 4 hours. For detection of tyrosine phosphorylation of Flag-Rad52, whose molecular size is close to mouse Ig, DTT was replaced by 10 mM N-Ethylmaleimide (NEM, Calbiochem) in lysis buffer and β-mercaptoethanol was eliminated from loading buffer (22). Immune complexes and whole cell lysates were separated by SDS-PAGE. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell) and probed with anti-Flag (M5, Sigma), anti-GFP (Clontech), anti-phosphotyrosine (4G10, Upstate Biotechnology), anti-c-Abl (Ab-2, Oncogene Research Products) and goat polyclonal anti-Rad52 (C-17, Santa Cruz Biotechnology) antibodies followed by incubation with secondary antibodies (goat anti-mouse IgG-HRP or anti-goat IgG-HRP; Santa Cruz Biotechnology). Proteins were visualized with an enhanced chemiluminescence detection system (Perkin Elmer Life Sciences).

**In vitro kinase assay.** Recombinant kinase active c-Abl was prepared from baculovirus-infected insect cells as described previously (23). Immuno-purified Flag-tagged proteins were incubated with the recombinant kinase active c-Abl in
kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 20 µM ATP) and incubated for 30 min at 30 °C. The reaction products were washed 3 times with 500 µl of 0.5% Triton X-100 lysis buffer, resolved on SDS-PAGE and analyzed by Western blot.

**Immunostaining and fluorescent microscopy.** Cells were grown on chamber slides (Nunc, Naperville, IL) and transfected with the indicated vector. To detect transfected proteins, at indicated hours after γ-irradiation, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma) for 30 min at 4 °C. After washing with PBS 3 times, cells were permeabilized with ice-cold 0.2% Triton X-100 for 5 min, blocked with 0.5% bovine serum albumin (BSA) for 30 min, and then incubated with the anti-Flag antibody (1:300 dilution) for 1 h. After washing with PBS 3 times, the slides were incubated with secondary antibody (Texas red X goat anti-mouse IgG, 1:200 dilution, Molecular Probe) and 4′6′ diamidino-2-phenylindole (DAPI; 10 µg/ml) for 1 h. Following 3 times washes with PBS, the slides were mounted with Fluoromount-G (Southern Biotechnology Associates) containing 2.5 mg/ml n-propyl gallate (Sigma) and viewed with a Nikon ECLIPSE E600 fluorescence microscope using a x60 objective. For kinetics analyses, at least 200 cells with green fluorescent signal were counted at each time point and a minimum of five foci per nucleus was scored as a positive cell.

**Results**

**c-Abl associates with Rad52**
Given the findings that Rad52 binds to Rad51 (18,24), with which c-Abl also associates (17,18), we were interested in determining whether there was any interaction between c-Abl and Rad52 using IP-Western analysis. A plasmid encoding Flag-c-Abl was coexpressed with a GFP-Rad52 expressing vector in 293T cells for testing of this possibility. Cell lysates were prepared 24 h post-transfection for immunoprecipitation with an anti-Flag antibody. Anti-GFP immunoblotting analysis of the immunocomplexes revealed an apparent association of Rad52 with the c-Abl protein (Fig. 1A, lane 3). Because of its C-terminal nuclear export sequence (NES), the c-Abl protein actively shuttles between the nucleus and the cytoplasm and consequently distributes to both compartments (25). The Rad52 protein is, however, almost exclusively nuclear localized (26). It was therefore of interest to examine where these two proteins co-localize in the cell. c-Abl C-terminal deletion mutant that lacks the NES and is mainly nuclear-localized (21) was tested for its ability to bind to Rad52. The result indicated that this c-Abl lacking the NES exhibited a markedly increased association with the Rad52 protein when compared with that of wild type c-Abl (Fig. 1A, lane 4), suggesting the interaction between the two proteins is mediated primarily by the nuclear c-Abl. To analyze the interaction between c-Abl and Rad52 further, we performed a reciprocal IP-Western analysis of lysates isolated from cells co-expressing Flag-Rad52 and GFP-c-Abl. Rad51 or an unrelated protein DFF45 was included as positive or negative control, respectively. The readily detectable c-Abl signal in the Rad52 as well as Rad51, but not DFF-45
(Fig. 1B, lanes 4 and 5 versus lane 6), immunocomplexes confirmed specific \textit{in vivo} binding of c-Abl to Rad52.

\textbf{c-Abl phosphorylates Rad52}

The \textit{in vivo} association of c-Abl with Rad52 prompted us to ask whether the Rad52 protein was phosphorylated as a result of binding to the c-Abl tyrosine kinase. To accomplish this, we coexpressed kinase active (KA) or kinase dead (KD) c-Abl (21) with Flag-tagged Rad52 in 293T cells. Flag-Rad51 and Flag-DFF45 were again included as a positive and negative controls, respectively. Tyrosine phosphorylation was assessed by anti-P-Tyr immunoblotting analysis of anti-Flag immunocomplexes. As reported previously (18), coexpression of Rad51 with c-Abl(KA), but not by c-Abl(KD), was associated with Rad51 tyrosine phosphorylation (Figure 2A, lane 3 and 4), whereas c-Abl(KA) did not phosphorylate DFF45 (Figure 2A, lane 5), indicating that this c-Abl-mediated tyrosine phosphorylation is substrate-specific. Under the same experimental conditions, coexpression of c-Abl(KA), but not c-Abl(KD), resulted in Rad52 tyrosine phosphorylation (Figure 2A, lane 1 and 2). To distinguish whether the observed Rad52 tyrosine phosphorylation in the c-Abl(KA) expressing cells was mediated directly by c-Abl or by an intermediate tyrosine kinase, we performed an \textit{in vitro} kinase assay. Recombinant c-Abl protein was purified from insect cells infected with baculovirus containing c-Abl expressing vector and subsequently incubated with purified Flag-tagged substrate proteins. Anti-P-Tyr immunoblot analysis of the reaction products demonstrated tyrosine phosphorylation of full-length Rad52 (data not shown) and Rad52 (1-280)
(Fig.2B, lane 4), but not Rad52 (151-418) (Fig.2B, lane 5), indicating that c-Abl directly phosphorylates Rad52 at its N-terminus. Rad51 was again included as a positive control (Fig. 2B, lane 6). Inspection of the amino acid sequence of Rad52 revealed 8 tyrosine residues within the region spanning amino acid 1-150. We then substituted each of the 8-tyrosine residues with phenylalanine in order to identify the c-Abl phosphorylation site. When coexpressed with the kinase-active c-Abl, only Flag-Rad52(Y104F) exhibited nearly completely diminished reactivity towards anti-P-Tyr, whereas the other mutants remained phosphorylated to the extent comparable to that of wild type Rad52 (Fig. 2C, lane 6 versus lanes 1, 2, 3, 4, 5 and 7). This result clearly demonstrates that, when overexpressed, c-Abl phosphorylates Rad52 in vivo at the Tyr-104 residue.

Consequently, it was important to determine whether this phosphorylation reflects the biological function of endogenous c-Abl. To address this issue and to examine whether IR that activates c-Abl kinase could induce Rad52 tyrosine phosphorylation, we utilized CHO cell lines in which GFP-tagged wild type or Y104F mutant of Rad52 was stably expressed at relatively low levels. At the indicated time after exposure of the cells to ionizing radiation, cell lysates were prepared for anti-P-Tyr immunoprecipitation to enrich tyrosine-phosphorylated proteins, which were then subjected to anti-Rad52 immunoblot. Remarkably, IR indeed induced tyrosine phosphorylation of Rad52, as demonstrated by a distinct Rad52 band detected in the IR-treated cells (Fig. 2D, top panel, lane 6). Significantly, this IR-induced Rad52 phosphorylation was completely absent in the Rad52(Y104F) mutant expressing cells (Fig. 2D, bottom panel, lane 6),
supporting our hypothesis that Rad52 phosphorylation induced by IR is a c-Abl-mediated event.

**Binding of Rad52 to c-Abl is augmented by phosphorylation**

Given the fact that the SH2 domain displays a high affinity towards to the phosphorylated tyrosine residue, we were interested in knowing whether Rad52 tyrosine phosphorylation affected its association with c-Abl, which contains a SH2 domain at its N-terminus. The binding of Rad52 to c-Abl was analyzed by IP-Western from lysates that were prepared from cells coexpressing GFP-Rad52 with either Flag-c-Abl(KA) or Flag-c-Abl(KD). Interestingly, while anti-Flag immunoprecipitation brought down comparable amount of the c-Abl(KA) and c-Abl(KD) proteins (Fig. 3, top panel), anti-GFP immunoblotting detected a markedly stronger Rad52 band in the c-Abl(KA) expressing cells than in the c-Abl(KD) expressing cells (Fig 3, bottom panel, lane 5 versus lane 6), supporting the notion of phosphorylation-enhanced interaction between the two proteins. To further substantiate this observation, the c-Abl phosphorylation-resistant mutant of Rad52, Rad52(Y104F), was tested for its association with c-Abl. In contrast to wild type Rad52, Rad52(Y104F) did not exhibit significant preferential binding toward the kinase active c-Abl (Fig. 3, bottom panel, lane 7 versus lane 8), indicative of importance of c-Abl-mediated phosphorylation to the interaction between Rad52 and kinase active c-Abl.

**The IR-induced GFP-Rad52 nuclear foci formation is regulated by c-Abl**

A well-characterized feature of cellular homologous recombinational repair of DSBs is the formation of nuclear foci that contain the DSBs repair proteins (26-
It has been shown that stably expressed GFP-Rad52 can mimic the endogenous Rad52 protein in forming nuclear foci in response to IR (26,29). Having demonstrated that IR-induced Rad52 phosphorylation was mediated by c-Abl, we were interested in determining whether tyrosine phosphorylation of Rad52 could affect IR-induced nuclear foci formation. To address this issue, we once again utilized stable Rad52 expressing CHO cell lines that we had previously established. Consistent with the published results, exposure of these cells to IR was associated with the induction of the Rad52 nuclear foci formation. While evenly distributed in the nucleus of untreated cells, the green Rad52 proteins became accumulated as distinct nuclear dots in IR-treated cells (Fig. 4A, top row, third panel). The number of cells that contained the Rad52 nuclear foci and the number of Rad52 foci in each cell increased with time and peaked at 6 h post IR (Fig. 4B, open bars). To assess possible involvement of c-Abl in Rad52 foci formation, the GFP-Rad52(Y104F) expressing cells were analyzed for IR-induced foci formation. Significantly, this Rad52 mutant, which is resistant to the c-Abl-mediated phosphorylation, exhibited an impaired ability to form nuclear foci in response to IR treatment (Fig.4A, second row, third panel), as demonstrated by a markedly decrease in the number of Rad52(Y104F)-expressing cells that displayed the nuclear foci compared with wild type Rad52-expressing cells at every time point examined (Fig. 4B, filled bars). To substantiate this observation further, plasmid expressing the kinase dead c-Abl, which functions as a dominant negative mutant, was transfected into the GFP-Rad52 expressing cells (Fig. 4A, third row). Analysis of IR-induced Rad52 nuclear foci revealed a significantly
reduced number of the c-Abl(KD) expressing cells that contained the nuclear foci in comparison to control cells (Fig. 4B, hatched bars), supporting the importance of the kinase activity of c-Abl in the formation of Rad52 nuclear foci induced by IR. Taken together, these results demonstrate that c-Abl functionally regulates Rad52 in response to IR.

Discussion

DNA damage triggers a series of cellular responses that include cell cycle arrest, DNA repair or apoptosis. These events are regulated by coordinated actions of a number of proteins that sense and transmit the damage signal to the transcription, replication, DNA repair, cell cycle and apoptosis machineries. The c-Abl tyrosine kinase is activated in response to DNA damage and has been shown to participate in DNA damage signaling (30). Our findings presented herein reveal a novel activity of c-Abl in the functional regulation of Rad52. The Rad52 protein, which is evenly distributed in the nucleus in unstressed cells, accumulates into foci upon exposure of cells to ionizing radiation (26,29,31). We present here compelling evidence to demonstrate that this IR-induced Rad52 foci formation is regulated by c-Abl via post-translational modification of the Rad52 protein. The Rad52 protein, specifically Tyr-104 residue, is phosphorylated by c-Abl \textit{in vitro} and in IR-treated cells. Functional importance of this Rad52 tyrosine phosphorylation was highlighted by the demonstration that inhibition of c-Abl kinase activity by expression of dominant negative c-Abl was associated with an impediment of Rad52 foci formation induced by IR. An additional support for c-Abl kinase-dependent regulation comes from an independent observation that
the c-Abl phosphorylation-resistant Rad52 mutant exhibited an impaired ability to form nuclear foci in response to ionizing radiation. How phosphorylation augments IR-induced nuclear Rad52 foci formation is currently unclear. The Rad52 proteins form homo-heptameric ring structure in vitro (32). Even though tyrosine 104 is located within the homo-oligomerization domain of Rad52 (33), no apparent effect of phosphorylation on Rad52 homo-complex formation was detected (not shown). Rad52 tyrosine phosphorylation, however, markedly augments its association with the c-Abl protein, which has been shown to interact with ATM. Further studies are necessary to investigate whether this c-Abl-mediated phosphorylation can influence the interaction between Rad52 and other proteins that contribute to the IR-induced nuclear foci formation.

It has been shown that Rad52-mediated HR repair is tightly associated with the S phase during which an identical chromosome, which is needed as a template for the repair, becomes available (34). Interestingly, DNA damage-induced c-Abl activation also occurs during S phase (35), suggesting a temporal correlation between these two events. Additionally, ATM, which is partially responsible for IR-induced c-Abl activation (19,20), contributes to the regulation of HR repair (15). It will be of interest to examine whether the ATM-dependent regulation of HR is mediated through c-Abl. Recently, constitutive association between c-Abl and Brca1 was reported (36). This association was disrupted following exposure to IR in an ATM-dependent manner. c-Abl kinase activity seems to be repressed by this association, since BRCA1-mutated cells exhibit constitutive high c-Abl kinase activity that cannot be further increased upon
exposure to IR (36). Brca1 has also been shown to promote HR-mediated DNA repair (37). The defect in HR repair in BRCA1-mutated cells may be partially attributed to the deregulated c-Abl kinase and tyrosine hyper-phosphorylation of Rad52. Further study will be required to address this possibility.

In summary, our data provide compelling evidence to support a functional role for c-Abl in the post-translational regulation of IR-induced Rad52 nuclear foci formation.

Acknowledgement

This work was supported by a NIH grant (NCI R29 CA76275-01) to Z.Y.

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4020-4032.
Abbreviations: ATM, ataxia telangiectasia mutated; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DAPI, 4′6′-diamidino-2-phenylindole; DSB, double strand break; GFP, green fluorescence protein; HR, homologous recombination; IR, ionizing radiation; IP, Immunoprecipitation; NES, nuclear export sequence; NHEJ, non-homologous end joining

Legends

Figure 1. c-Abl associates with Rad52. A. A vector expressing the Flag-tagged full length (lanes 1 & 3) or C-terminus deletion mutant (1-969; lanes 2 & 4) c-Abl was cotransfected with a plasmid encoding GFP-Rad52 into 293T cells. Cell lysates were prepared at 24 h after transfection and subjected to anti-Flag immunoprecipitation. Whole cell extract (WCE) (lanes 1 & 2) and immunocomplexes (lanes 3 & 4) were subjected to Western analysis using the indicated antibodies. B. A vector expressing GFP-tagged C-terminal deletion mutant c-Abl(1-969) was cotransfected with Flag-Rad52 (lanes 2 & 5), Flag-Rad51 (lanes 1 & 4) and Flag-DFF45 (lanes 3 & 6) into 293T cells. WCE (lanes 1-3) and immunocomplexes (lanes 4-6) were analyzed as described in A.

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Figure 2. c-Abl phosphorylates 104-Tyr of Rad52. A. A vector expressing GFP-tagged c-Abl(KA) (lanes 1, 3 & 5) or c-Abl(KD) (lanes 2, 4 & 6) was cotransfected with a plasmid encoding Flag-Rad52 (lanes 1 & 2), Flag-Rad51 (lanes 3 & 4) or Flag-DFF45 (lanes 5 & 6) into 293T cells and cell lysates were subjected to anti-Flag immunoprecipitation. WCE and immunocomplexes were subjected to Western analysis using the indicated antibodies. B. A vector expressing Flag-Rad52(1-280) (lanes 1, 4 & 7), Flag-Rad52(150-418) (lanes 2, 5 & 8) or Flag-Rad51 (lanes 3, 6 & 9) were transfected into 293T cells and cell lysates were subjected to anti-Flag immunoprecipitation (lanes 1-3). Half of each immunocomplex was further incubated with purified recombinant c-Abl(KA) (lanes 4-6) or c-Abl(KD) (lanes 7-9). All immunocomplexes were subjected to Western analysis using the indicated antibodies. C. A vector expressing Flag-tagged c-Abl(KA) or c-Abl(KD) was cotransfected with a plasmid encoding wild type (lane 1), Y31,36F (lane 2), Y51F (lane 3), Y65F (lane 4), Y81F (lane 5), Y104F (lane 6) and Y120,126F (lane 7) mutant of Flag-Rad52 into 293T cells. Cell lysates isolated at 24 h post-transfection were subjected to Western analysis with the indicated antibodies. D. CHO cells stably expressing GFP-Rad52(WT) (top panel) and GFP-Rad52(Y104F) (bottom panel) were γ-irradiated with 20 Gy (lanes 2, 3, 5 & 6) or left untreated (lanes 1 & 4). Cell lysates were prepared at 0.5 h (lanes 2 & 5) or 3 h (lanes 3 & 6) after the irradiation and subjected to anti-phosphotyrosine antibody (4G10) immunoprecipitation. WCE (lanes 1-3) and the immunocomplexes (lanes 4-6) were subjected to Western analysis using an anti-Rad52 antibody.
Figure 3. Phosphorylation-enhanced binding of Rad52 to c-Abl. A plasmid encoding Flag-c-Abl(KA) (lanes 1, 3, 5 & 7) or Flag-c-Abl(KD) (lanes 2, 4, 6 & 8) was coexpressed with GFP-Rad52(WT) (lanes 1, 2, 5 & 6) or GFP-Rad52(Y104F) (lanes 3, 4, 7 & 8) into 293T cells. Cell lysates were prepared at 24 h post-transfection for anti-Flag immunoprecipitation. WCE (lanes 1-4) and the immunocomplexes (lanes 5-8) were analyzed by immunoblotting with anti-Flag (top panel) or anti-GFP (bottom panel).

Figure 4. c-Abl-dependent regulation of Rad52 nuclear foci formation in response to IR. A. Representative images of Rad52 distribution. CHO cells expressing GFP-Rad52(WT) (top row), GFP-Rad52(Y104F) (second row) and GFP-Rad52(WT) with Flag-c-Abl(KD) (third and bottom rows) were treated with IR (12Gy) or left untreated. The images of GFP-Rad52 (green), c-Abl (red) and nuclei (DAPI, blue) are shown. B. Percentage of CHO cells expressing GFP-Rad52(WT) (open bars), GFP-Rad52(Y104F) (filled bars) and GFP-Rad52(WT) with Flag-c-Abl(KD) (hatched bars) that contained IR-induced GFP-Rad52 nuclear foci at the indicated time after IR with 12Gy. The numbers are mean +/- SD derived from three independent experiments.
### Fig. 1

#### A

|                | WCE | IP: Anti-Flag |
|----------------|-----|---------------|
| Flag-c-Abl FL  | +   | +             |
| Flag-c-Abl(1-969) | +   | +             |
| GFP-Rad52      | +   | +             |

**IB: Anti-Flag**

1. c-Abl

**IB: Anti-GFP**

1. Rad52

#### B

|                | WCE | IP: Anti-Flag |
|----------------|-----|---------------|
| GFP-c-Abl(1-969) | +   | +             |
| Flag-Rad51      | +   | +             |
| Flag-Rad52      | +   | +             |
| Flag-DFF45      | +   | +             |

**IB: Anti-Flag**

1. Ig

2. Rad52

3. DFF45

4. Rad51

**IB: Anti-GFP**

1. c-Abl
Fig. 2

C

| IB: Anti-c-Abl | IB: Anti-Flag | IB: Anti-P-Tyr |
|---------------|--------------|---------------|
| Flag-Rad52(WT) | Flag-Rad52(Y31,36F) | Flag-Rad52(Y51F) |
| Flag-Rad52(Y65F) | Flag-Rad52(Y81F) | Flag-Rad52(Y104F) |
| Flag-Rad52(Y120,126F) |             |               |

1 2 3 4 5 6 7

IB: Anti-c-Abl
IB: Anti-Flag
IB: Anti-P-Tyr

D

| WCE | 0.5 | 3 |
|-----|-----|---|
| GFP-Rad52 (WT) |     |   |
| GFP-Rad52 (Y104F) |   |    |

| IP: Anti-P-Tyr | 0.5 | 3 |
|---------------|-----|---|
| Rad52 |     |   |
| Rad52 |   |    |

IB: Anti-Rad52
**Fig. 3**

|            | WCE |           | IP: Anti-Flag |
|------------|-----|------------|---------------|
| Flag-c-Abl(KA) | +   | +          | +             |
| Flag-c-Abl(KD) | +   | +          | +             |
| GFP-Rad52(WT) | +   | +          | +             |
| GFP-Rad52(Y104F) | +   | +          | +             |

**IB: Anti-Flag**

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |   |

**IB: Anti-GFP**

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |   |
Fig. 4

A

|                  | No IR          | 12 Gy 6 hr     |
|------------------|----------------|----------------|
|                  | GFP            | DAPI           | GFP            | DAPI           |
| GFP-Rad52 (WT)   | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) |
| GFP-Rad52 (Y104F)| ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) |
| GFP-Rad52(WT) + Flag-c-Abl(KD) | ![Image](http://www.jbc.org) | ![Image](http://www.jbc.org) | Anti-Flag        | ![Image](http://www.jbc.org) |

B

![Graph](http://www.jbc.org)
Regulation of ionizing radiation-induced Rad52 nuclear foci formation by c-Abl-mediated phosphorylation
Hiroyuki Kitao and Zhi-Min Yuan

J. Biol. Chem. *published online October 11, 2002*

Access the most updated version of this article at doi: 10.1074/jbc.M208151200

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