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Identification and Functional Characterizations of Phosphorylation of Serine 97 in RNF168

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Environmental Toxicology

by

Zi Wang

August 2016

Thesis Committee:
Dr. Yinsheng Wang, Chairperson
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The Thesis of Zi Wang is approved:

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ABSTRACT OF THE THESIS

Identification and Functional Characterizations of Phosphorylation of Serine 97 in RNF168

by

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DNA double-strand breaks (DSBs) can be induced endogenously and by exposure to ionizing radiation, and they represent one of the most deleterious forms of DNA damage. To avoid genomic stability arising from DNA DSBs, cells employ DNA damage response (DDR) pathways to detect and promptly repair DNA DSBs. In mammalian cells, the DDR pathway involves a cascade of events of protein recruitments and chromatin modifications near the damaged regions of DNA. An important chromatin modification known to occur during this process is the ubiquitination of histone H2A and H2AX by the E3 ubiquitin ligase RNF168. Herein, we identified, for the first time, the phosphorylation of serine 97 in RNF168, and we found that the level of this phosphorylation was significantly increased in cultured human cells upon exposure to γ rays or a radiomimetic agent, neocarzinostatin. Additionally, the introduction of S97A or S97D mutation to RNF168 substantially reduced the protein's ubiquitination activity in

cells, diminished the efficiency of DNA DSB repair via the non-homologous end-joining pathway, and rendered cells more sensitive toward ionizing radiation. Together, phosphorylation of S97 in RNF168 plays a significant role in the cellular tolerance and repair of DNA DSBs. Thus, our study provided important insights into the post-translational regulation of RNF168 in DNA damage response.

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CHAPTER 1: INTRODUCTION

1.1. Introduction

DNA damage response signaling and repair are crucial for maintaining genomic stability and normal functions of a living cell (Friedberg, 2006). The cell's genetic material is constantly exposed to endogenous and exogenous sources of DNA damaging agents (Lindahl, 1999). DNA double-strand breaks (DSBs), which constitute one of the most deleterious forms of DNA damage, may arise from exposure to ionizing radiation or from the encountering of replication fork with single-strand breaks or other types of DNA (Khanna, 2001). Owing to the cleavage of both strands, DSBs stall DNA replication and transcription, which may give rise to chromosomal rearrangements and losses (Khanna, 2001). Such genetic aberrations may result in the activation of oncogenes and/or inactivation of tumor suppressor genes, which may ultimately lead to cancer development (Khanna, 2001, Harper, 2007). To maintain genomic integrity, cells initiate DNA damage response (DDR) pathways, which elicit a cascade of events of protein recruitments and epigenetic alterations of chromatin, thereby facilitating timely repair of DNA lesions or programmed cell death (Khanna, 2001, Harper, 2007).

Post-translational modifications (PTMs) of proteins, especially phosphorylation and ubiquitination, are an integral component of the DDR process where RING finger nuclear factor 168 (RNF168) plays a crucial role (Mattiroli, 2012, Gatti, 2015). Upon induction of DNA DSBs, Mre11/Rad50/NBS1 complex detects the damage site and recruits phosphatidylinositol 3-like kinases, including ATM and ATR, which subsequently phosphorylate a variant form of histone H2A, i.e. H2AX, in the damage-flanking regions to yield γ-H2AX (Burma, 2001, Ward, 2001). Immediately afterwards, MDC1 is

recruited and becomes phosphorylated at its TQXF motif by ATM (Stucki, 2005). The phosphorylated MDC1 then acts as a scaffold for the recruitment of forkhead-associated domain of E3 ubiquitin ligase RNF8 (Mailand, 2007, Huen, 2007). RNF8 recruits RING-finger E3 ubiquitin ligase RNF168 to the regions flanking the damage site in a ubiquitination-dependent manner, which gives rise to mono-ubiquitination of histone H2A and H2AX at K13/15 (Mattiroli, 2012). After the monoubiquitination of H2A/H2AX near the damage site, RNF8 elongates the ubiquitin chain through the K63 linkage (Mattiroli, 2012). Apart from catalyzing the monoubiquitination of K13/15 in histone H2A and H2AX, RNF168 was found to be essential for the formation of non-canonical K27-linked ubiquitin chain in chromatin that functions in DNA damage response (Gatti, 2015). Depending upon the cell cycle phase and the proteins subsequently recruited, namely 53BP1 or BRCA1, the damage may be repaired via the error-prone non-homologous end-joining (NHEJ) or the error-free homologous recombination (HR) pathway (Khanna, 2001, Harper, 2007, Stewart, Panier and Townsend, 2009).

Aside from DNA damage repair, RNF168 assumes an important role in promoting normal development in higher organisms (Stewart, 2009). Individuals deficient in RNF168 manifest the RIDDLE (radiosensitive, immunodeficiency, dysmorphic features, and learning difficulties) syndrome (Stewart, Panier and Townsend, 2009, Stewart, 2009). The radiosensitive and immune-deficient attributes of this syndrome are thought to stem from compromised recruitment of 53BP1, a protein that modulates NHEJ repair and

V(D)J class-switch recombination during immune system maturation (Ramachandran, 2010, Fradet-Turcotte, 2013).

Chromatin modification machineries are also post-translationally regulated. For instance, RNF20-RNF40 heterodimer is phosphorylated by ATM in response to DNA DSB induction, and the disruption of this phosphorylation could impair the recruitment of downstream HR repair proteins to the damage site (Moyal, 2011). In another scenario, threonine 198 (T198) in RNF8 becomes phosphorylated specifically during mitosis in a CDK1-dependent manner, and this phosphorylation reduces 53BP1 foci formation and compromises the interaction between RNF8 and MDC1, thereby suppressing NHEJ (Orthwein, 2014). This mechanism of NHEJ occlusion during mitosis may constitute a preventive mechanism against telomere fusion in the mitotic phase (Orthwein, 2014).

In the present study, we assessed the post-translational modifications of RNF168 and found that S97 in RNF168 could be phosphorylated. We also observed that this phosphorylation could be stimulated by exposure to a radiomimetic agent or ionizing radiation. Furthermore, we demonstrated that this modification is critical for RNF168's function in NHEJ-mediated repair of DNA DSBs and for conferring cellular resistance towards ionizing radiation.

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CHAPTER 2: RNF168 S97 PHOSPHORYLATION

2.1. Materials and Methods

2.1.1. Plasmids and cell culture

Plasmid for the expression of GFP-tagged full-length RNF168 protein was kindly provided by Dr. Jiri Lukas (Doil, 2009). The coding sequence of human *RNF168* gene was incorporated into the pRK7 plasmid (Addgene), which carried a sequence for expressing 3 tandem repeats of FLAG epitope tag on the C-terminus of RNF168. Plasmids for expressing the S97A and S97D mutants of RNF168 were introduced by site-directed mutagenesis.

HEK293T cells were plated in 6-well plates with Dulbecco's Modified Eagle Medium (DMEM, ATCC) containing 10% FBS and transfected with 1-1.5 μg of plasmid using Lipofectamine 2000 (Invitrogen). After a 48-h incubation at 37°C in 5% CO_2 atmosphere, the cells were washed twice with PBS and processed for subsequent steps. The *H2ax*$^{-/-}$ mouse embryonic stem cells (mESC) were kindly provided by Prof. Jeremy Stark and cultured as previously described (Bassing, 2002).

2.1.2 Immunoprecipitation and on-beads tryptic digestion

The mammalian cells transfected with the RNF168 expression plasmid were lysed with CelLytic M lysis buffer (Sigma) containing 1× protease inhibitor cocktail (Sigma) and phosphatase inhibitors. The lysate containing the C-terminal 3×FLAG-tagged RNF168 was incubated with anti-FLAG M2 agarose beads (Sigma) overnight, washed twice with 1×TBS and subjected to on-beads tryptic digestion. In this respect, the beads were heated at 95°C for 5 min to denature the proteins, and disulfide bonds were subsequently reduced by incubation at 37°C for 45 min in a 50-μL solution containing 1

mM dithiothreitol and 50 mM NH_4HCO_3. The cysteine residues were then alkylated by incubating the beads in a 10-μL solution containing 100 mM iodoacetamide (IAA) and 50 mM NH_4HCO_3 at 55°C for 1 h. The solution was subsequently diluted with 50 mM NH_4HCO_3 solution, to which trypsin (Roche) was added, and the resulting mixture was incubated at 37°C overnight to generate peptide fragments.

2.1.3. Mass spectrometry for the identification of S97 phosphorylation in RNF168

The aforementioned peptides were desalted and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on an LTQ-Orbitrap Velos mass spectrometer (Thermo), which was equipped with a nanoelectrospray ionization source and coupled to an Easy nLC-II system (Thermo). A 75-min linear gradient of 10-90% solvent B (0.1% formic acid in acetonitrile) was employed, and the flow rate was 230 nL/min. The instrument was operated in the data-dependent scan mode, where the 20 most abundant ions found in MS were selected for MS/MS analysis.

The raw MS data were analyzed using the Mascot Daemon (Matrix Science) and Maxquant with a false discovery rate (FDR) of 0.05. The data were searched for phosphorylation of serine, threonine and tyrosine as variable modifications, and carbamidomethylation of cysteine as a fixed modification.

2.1.4. γ ray and NCS treatments

The HEK293T cells were exposed with 10 Gy of γ rays from a ^{137}Cs source (J. L. Shepherd) at a dose rate of 0.61 Gy/min, and subsequently incubated at 37°C in a 5% CO_2 atmosphere for 1 h prior to cell lysis. For NCS treatment, the cells were treated for 1 h in medium containing NCS at a final concentration of 3 μg/mL.

2.1.5. Histone extraction and tryptic digestion

HEK293T cells in six-well plates were co-transfected with RNF168 siRNA, N-terminal 3×FLAG-tagged wild-type ubiquitin plasmid (kindly provided by Dr. Sybille Krauss) and various siRNA-resistant RNF168 plasmids. After a 48-h incubation at 37°C in 5% CO_2 atmosphere, the cells were washed twice with 1× PBS and the histone proteins were isolated using a previously published acid extraction procedure (Mattiroli, 2012). The extracted histones were neutralized with a buffer containing 0.4 M Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM $MgCl_2$, along with phosphatase and protease inhibitors, and then incubated with anti-FLAG beads. The proteins were then subjected to on-beads digestion and mass spectrometry analysis in the multiple-reaction monitoring (MRM) mode.

2.1.6. LC-MS/MS in the MRM mode for monitoring the levels of phosphorylation on serine 97 in RNF168 and K27-linked ubiquitin

The immunoprecipitated FLAG-RNF168 and acid-extracted chromatin samples were reduced, alkylated and digested with trypsin following the aforementioned procedures, and the resulting peptides were analyzed by LC-MS/MS in the MRM mode on a TSQ Vantage triple quadrupole mass spectrometer (Thermo). A 85-min linear gradient of 10-90% acetonitrile in 0.1% formic acid was employed for the LC separation and the flow rate was 230 nL/min. MRM transitions for the formation of at least three abundant fragment ions were set up for the analysis of the peptide samples.

For the targeted quantification of serine 97 phosphorylation, the MRM transitions were *m/z* 980.4 → *m/z* 777.4, 892.4 and 899.4, which monitored the formation of y_6, y_7

and [$b_9 - H_3PO_4$] ions from the $[M + 2H]^{2+}$ ion of the phosphorylated peptide (Figure 1). The peak intensity found in the selected-ion chromatogram (SIC) was then divided by that found in the corresponding SIC for monitoring the *m/z* 699.4 → *m/z* 586.4, 677.4, 814.5, 943.5, 1171.6 transitions (i.e. the formation of y_9^{2+}, y_5, y_6, y_7, and y_9 ions from the $[M+2H]^{2+}$ ion of LIDLEHLLFER, a peptide derived from RNF168 that is known not to carry any PTM, Figure 1). The above ratios found for the cells treated with γ rays and NCS were then normalized against that observed for control cells to determine the relative phosphorylation level of serine 97.

Similar LC-MRM analysis was employed for measuring the level of K27-linked ubiquitin in chromatin proteins. In this vein, we monitored the formations of y_3, [$b_7 - H_2O$] and y_{11} ions from the $[M + 2H]^{2+}$ ion of the tryptic peptide harboring the ubiquitin-derived diglycine remnant on K27, i.e. TITLEVEPSDTIENVK(GG)AK, through the MRM transitions of *m/z* 1051.0 → *m/z* 460.3, 768.4 and 1315.7. The corresponding K27-containing tryptic peptide without modification on K27, namely TITLEVEPSDTIENVK, was employed as the reference for relative quantification, where we monitored the formations of y_9, y_{10}, y_{11}, and y_{12} ions from the $[M + 2H]^{2+}$ ion via the MRM transitions of *m/z* 894.5 → *m/z* 1002.5, 1131.5, 1230.6 and 1359.7 (Figure 2).

2.1.7. DNA damage repair assay

EJ5-GFP and DR-GFP U2OS cells were maintained in DMEM without sodium pyruvate (Gibco 11965-092) and with 10% FBS at 37°C in 5% CO_2 atmosphere. The U2OS cells were plated on 6-well plates and transfected with 100 pmol of RNF168-targeting siRNA using Lipofectamine 2000 for 24 h. The sequence of siRNF168 was 5′-

GGCGAAGAGCGAUGGAAGATT-3′ (Poulsen, 2012). After removal of siRNA, the cells were transfected simultaneously with 100 pmol of siRNA, 1.5 µg of I-SceI plasmid and various siRNA-resistant RNF168 plasmids and incubated for 48 h. The cells were washed twice with 1× PBS and stored in a sorting buffer (1×PBS, 1 mM EDTA, 25 mM HEPES, 1% FBS, pH 7.0) for flow cytometry analysis (BD FACS Aria).

2.1.8. Western blot

Western blot was employed for monitoring the siRNA knockdown efficiency of RNF168 gene and for assessing the relative expression of different forms of RNF168 in mammalian cells. A total of 10 µg of whole cell lysate was used. The antibody that specifically recognizes human RNF168 (R&D Systems) was used at a dilution suggested by the vendor. FLAG epitope tag antibody (Cell Signaling technology) was used at a 1:30,000 dilution.

Human β-actin antibody (Abcam) was used at a 1:10,000 dilution. Horseradish peroxidase-conjugated secondary goat anti-mouse antibody (Santa Cruz Biotechnology), donkey anti-goat antibody (Santa Cruz Biotechnology), and goat anti-rabbit antibody (Abcam) were used at a 1:10,000 dilution.

2.1.9. H2AX ubiquitination assay

To monitor ubiquitination, $H2ax^{-/-}$ mouse embryonic stem cells (mESC) were transfected with vectors for RNF168 and/or various constructs of H2AX using 1-2 µg of each plasmid and 5 µL of Lipofectamine 2000 in a 2.5-mL total volume for 3 h. After the transfection, the cells were incubated at 37°C for 48 h and treated with 10 Gy of γ rays. After a 2-h recovery, the cells were lysed using a buffer containing 0.25 M sucrose, 10

mM $MgCl_2$, 50 mM Tris (pH 7.4), 0.5% Triton-X, 0.5 mM PMSF, 10 mM *N*-ethylmaleimide, and a protease inhibitor cocktail. The histones were again isolated from the resulting pellet using an acid extraction procedure (Mattiroli, 2012). H2AX was detected using anti-H2AX antibody (abcam), and developed with the HRP-conjugated secondary antibody (Sigma) and ECL reagent (Amersham Biosciences), as described previously (Munoz, 2014).

2.1.10. Clonogenic survival assay

The impact of RNF168 S97 phosphorylation on cell survival was assessed using U2OS cells. Briefly, the cells were co-transfected with RNF168 siRNA and various siRNA-resistant RNF168 plasmids, and replated onto six-well plates. After attachment, the cells were exposed to a series of doses of γ rays (0-10 Gy) and subsequently incubated at 37°C in the presence of 5% CO_2 for 10 days. The colonies under each treatment conditions were counted after fixation and staining with a solution containing 0.5% crystal violet and 6% glutaraldehyde.

2.2. Results

2.2.1. S97 in RNF168 is phosphorylated in cultured human cells and this phosphorylation could be stimulated by DNA DSB-inducing agents

While the role of RNF168-mediated ubiquitination in DNA damage response has been well established, it remains not well understood whether its role in DNA damage response is post-translationally regulated. Thus, we set out to assess systematically the PTMs of RNF168. To this end, we isolated ectopically expressed FLAG-tagged RNF168 from HEK293T cells, digested the protein with trypsin, and subjected the resulting

peptides to LC-MS/MS analysis. The LC-MS/MS data led to the identification of a novel site of phosphorylation in RNF168, i.e. S97 (Figure 1a).

We next asked whether this phosphorylation could be stimulated by exposure to DNA damaging agents. Thus, we estimated the levels of this phosphorylation by using LC-MS/MS in the multiple-reaction monitoring mode, where we normalized the signal intensity for the phosphorylated peptide to that of an unmodified tryptic peptide of RNF168, i.e., LIDLEHLLFER (amino acid residues 429-439, details are described in Experimental Procedures, Figure 1b&c). The latter peptide is not known to undergo any type of post-translational modification. Our results showed that exposure of cells to neocarzinostatin (NCS) or γ rays led to significant elevations in the levels of S97 phosphorylation (Figure 1d).

2.2.2. Disruption of S97 phosphorylation compromises RNF168's ubiquitination activity

Because the major function of RNF168 in DNA damage response and repair resides in its E3 ubiquitin ligase activity, we examined the impact of S97 phosphorylation on RNF168's ubiquitination activity in cells using two independent assays. First, we assessed the role of this phosphorylation in the ubiquitination of K13/15 in histone H2AX. Considering that K118 and K119 are the major ubiquitination sites of histone H2AX (Mattiroli, 2012, Munoz, 2014), we examined the RNF168-mediated ubiquitination of K13/15 in H2AX by monitoring the ubiquitination of the ectopically expressed K118/119Q mutant of H2AX in *H2ax*$^{-/-}$ mouse embryonic stem cells (mESC). Consistent with the previous observations (Mattiroli, 2012, Munoz, 2014), we found that the ectopically expressed wild-type and K13/15Q mutant of histone H2AX, but not the

corresponding K118/119Q or K13/15/118/119Q mutant, were monoubiquitinated in *H2ax*$^{-/-}$ mESC upon exposure to 10 Gy of γ rays. Ectopic expression of wild-type RNF168, but not its phospho-mimetic (S97D) or phospho-deficient (S97A) mutant, could result in the monoubiquitination of K118/119Q mutant of H2AX (Figure 2a). In this vein, wild-type RNF168 and its S97A or S97D mutant are expressed at similar levels in *H2ax*$^{-/-}$ mESC, as revealed by Western blot analysis (Figure 2b).

A recent study showed that RNF168 stimulates the formation of K27-linked ubiquitin chain, a non-canonical ubiquitin topology, in chromatin proteins and the K27-linked ubiquitin constitutes a major ubiquitin-based modification that marks chromatin upon DNA damage (Gatti, 2015). Hence, we also investigated the impact of S97 phosphorylation on RNF168-mediated formation of K27-linked ubiquitin chain in histone proteins. In agreement with previous findings (Gatti, 2015), we observed a significantly elevated K27-linked ubiquitination in histone proteins extracted from HEK293T cells with ectopic expression of FLAG-tagged ubiquitin and wild-type RNF168. This increase in K27-linked ubiquitination was, however, not found in cells with ectopic expression of FLAG-tagged ubiquitin and the S97D or S97A mutant of RNF168 (Figure 2c). Along this line, our Western blot analysis showed that the expression levels of the wild-type RNF168 and its S97A or S97D mutant were again very similar. Taken together, the above findings demonstrated that S97 phosphorylation plays a crucial role in the ubiquitination activity of RNF168.

2.2.3. The role of S97 phosphorylation in NHEJ repair

We next assessed the role of S97 phosphorylation on the efficiency of DNA DSB repair. Toward this end, we monitored the efficiencies of DNA DSB repair via the NHEJ and HR pathways by employing U2OS cells with a chromosomally integrated EJ5-GFP and DR-GFP reporter genes, respectively (Gunn, 2012). In EJ5-GFP U2OS cells, a puromycin resistance cassette flanked by two I-SceI restriction sites prevents the expression of the downstream GFP gene. Excisions of the cassette by I-SceI and the subsequent rejoining via the NHEJ pathway restore GFP expression, which could be monitored by flow cytometry analysis (Gunn, 2012). On the other hand, the DR-GFP U2OS cells harbor two complementary mutant GFP reporter genes in direct repeats, which can result in GFP expression after HR-mediated repair of I-SceI-induced DNA DSB in the upstream GFP construct (Gunn, 2012).

Prior to I-SceI expression, we knocked down endogenous RNF168 in U2OS cells using siRNA and complemented the cells with siRNA-resistant plasmid for expressing the wild-type RNF168, or its corresponding S97A or S97D mutant. We subsequently assessed the repair efficiency by flow cytometry analysis. Our results obtained from the use of EJ5-GFP U2OS cells showed that the efficiency of NHEJ-mediated repair of DNA DSB was reduced by siRNA-mediated knockdown of RNF168, and this drop could be restored by reconstituting the cells with wild-type RNF168, but not the S97A or S97D mutant. (Figure 3a). In agreement with the role of RNF168 in suppressing HR (Munoz, 2014, Munoz, 2012), we found that genetic depletion of RNF168 led to a substantial increase in HR efficiency (Figure 3b). This elevation in HR could be markedly

diminished by reconstituting the cells with wild-type RNF168 to a similar extent as its S97A or S97D mutant (Figure 3b).

2.2.4. The impact of S97 phosphorylation on the cellular sensitivity toward ionizing radiation

Having established the importance of S97 phosphorylation of RNF168 in its ubiquitination activity and in the cellular repair of DNA DSB via the NHEJ pathway, we next examined how this phosphorylation affects the cellular sensitivity toward ionizing radiation. In line with the previous findings (Doil, 2009), we found that depletion of endogenous RNF168 with siRNA rendered U2OS cells more sensitive toward ionizing radiation (Figure 4). As expected, the elevated sensitivity toward ionizing radiation could be rescued by complementing these cells with an siRNA-resistant construct for expressing wild-type RNF168. Reconstitution with the corresponding S97A or S97D mutant of RNF168, however, failed to restore the defect introduced by the depletion of endogenous RNF168. Thus, dynamic S97 phosphorylation confers cellular resistance toward ionizing radiation.

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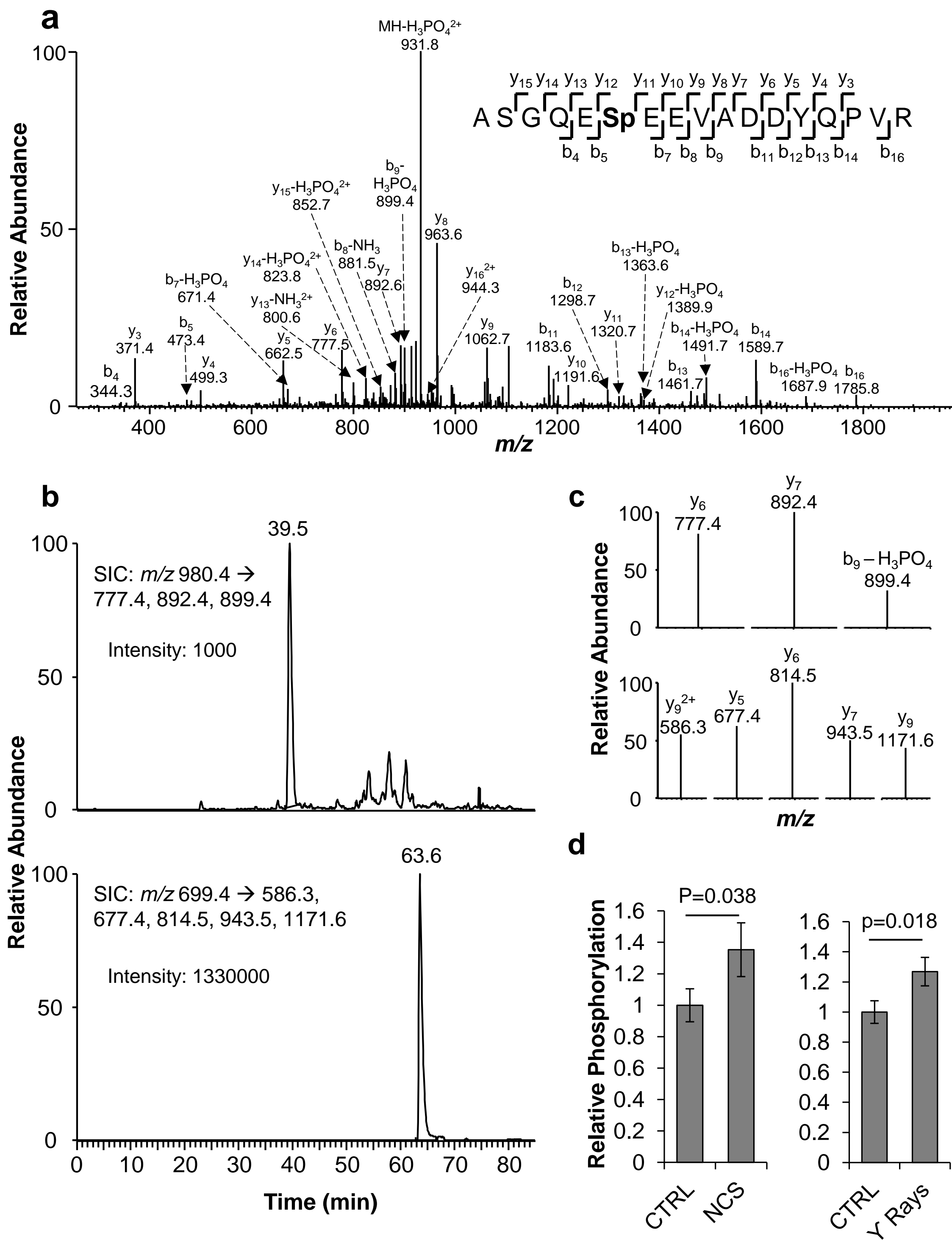
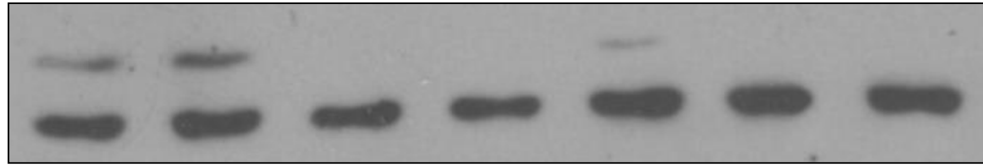
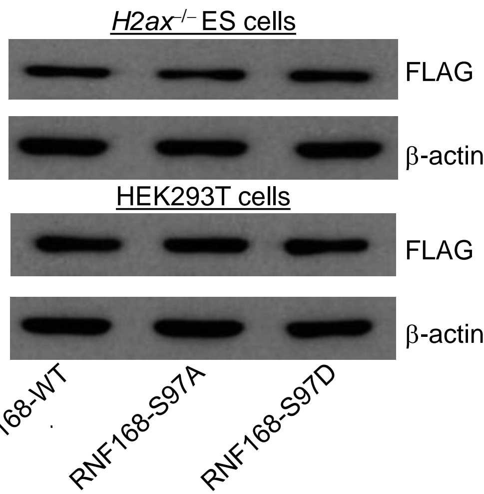

a
b
c
d
Relative Abundance
m/z
MH-H3PO4 2+ 931.8
A S G Q E Sp E E V A D D Y Q P V R
SIC: m/z 980.4 → 777.4, 892.4, 899.4
Intensity: 1000
SIC: m/z 699.4 → 586.3, 677.4, 814.5, 943.5, 1171.6
Intensity: 1330000
39.5
63.6
Time (min)
Relative Phosphorylation
P=0.038
p=0.018
CTRL
NCS
Y Rays

FIGURE 1. Phosphorylation of serine 97 in RNF168. (a) The product-ion spectrum (MS/MS) of the $[M+2H]^{2+}$ ion of the RNF168 peptide containing amino acid residues 92-108 with S97 being phosphorylated. Shown in the inset is a scheme summarizing the fragment ions observed for the phosphopeptide. **(b-c)** LC-MRM for monitoring the relative level of S97 phosphorylation. Shown are the selected-ion chromatograms for monitoring the indicated transitions for phosphorylated S97-containing peptide as shown in (a) and a reference peptide derived from RNF168 (i.e. LIDLEHLLFER), as well as the MS/MS observed in MRM analysis. **(d)** The level of S97 phosphorylation could be stimulated in cultured HEK293T cells upon exposure to γ rays or neocarzinostatin, a radiomimetic agent. The phosphorylation level was determined based on ratios of peak intensities found in the selected-ion chromatograms for the two peptides, and the levels were normalized to that found for mock-treated cells. Details are provided in Experimental procedures. The data represent the means and standard deviations of results obtained from three separate transfection and LC-MS/MS measurements. The p values were calculated by using unpaired, two-tailed student's *t*-test.

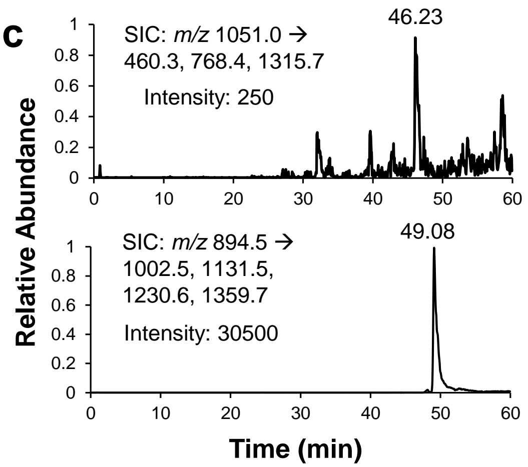
a



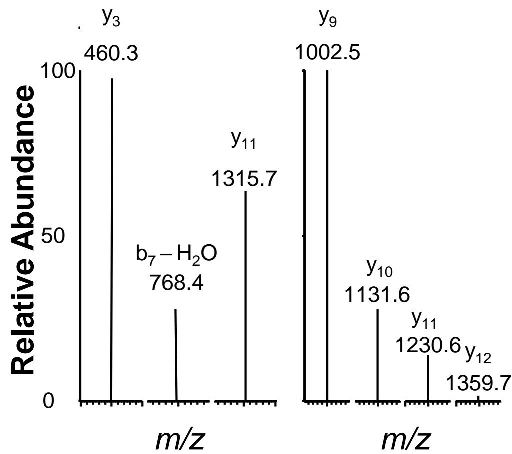
b



c



d



e

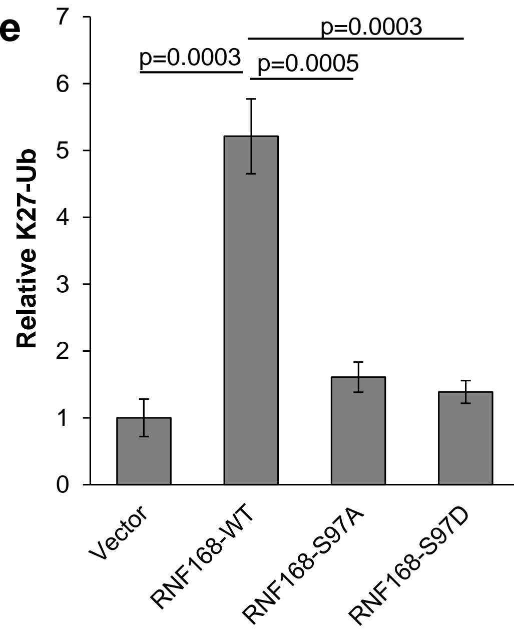


FIGURE 2. The role of S97 phosphorylation in RNF168's ubiquitination activity. (a) S97 phosphorylation is important in ionizing radiation-induced ubiquitination of K13/15 in histone H2AX. Overexpression of S97-mutant RNF168 suppresses the efficient ubiquitination of histone H2AX K118/119Q in *H2ax*$^{-/-}$ mouse embryonic stem cells. The cells were pretreated with 10 Gy of γ rays and a 2-hr recovery in 37C prior to lysis. **(b)** Western blot results showing the similar levels of expression of FLAG-RNF168 as well as its S97A and S97D mutant in *H2ax*$^{-/-}$ mouse embryonic stem cells and HEK293T cells. **(c-d)** LC-MRM analysis for monitoring the level of K27-linked ubiquitin in chromatin proteins isolated from HEK293T cells with ectopic expression of FLAG-tagged RNF168, RNF168-S97D or RNF168-S97A. Shown are the selected-ion chromatograms (c) and MS/MS for monitoring the indicated transitions for the diglycine-modified K27-containing tryptic peptide from ubiquitin and the unmodified K27-containing tryptic peptide. **(e)** S97 phosphorylation plays an instrumental role in non-canonical K27-linked ubiquitin chain in chromatin. The relative levels of K27-linked ubiquitination were measured by MRM analysis of diglycine-modified K27-containing ubiquitin peptide in histone mixture isolated from 293T cells with ectopic expression of wild-type RNF168, its S97D or S97A mutant. The data represent the mean ± S.D. of results obtained from three independent experiments. The p-values were calculated using unpaired, two-tailed student's *t*-test.

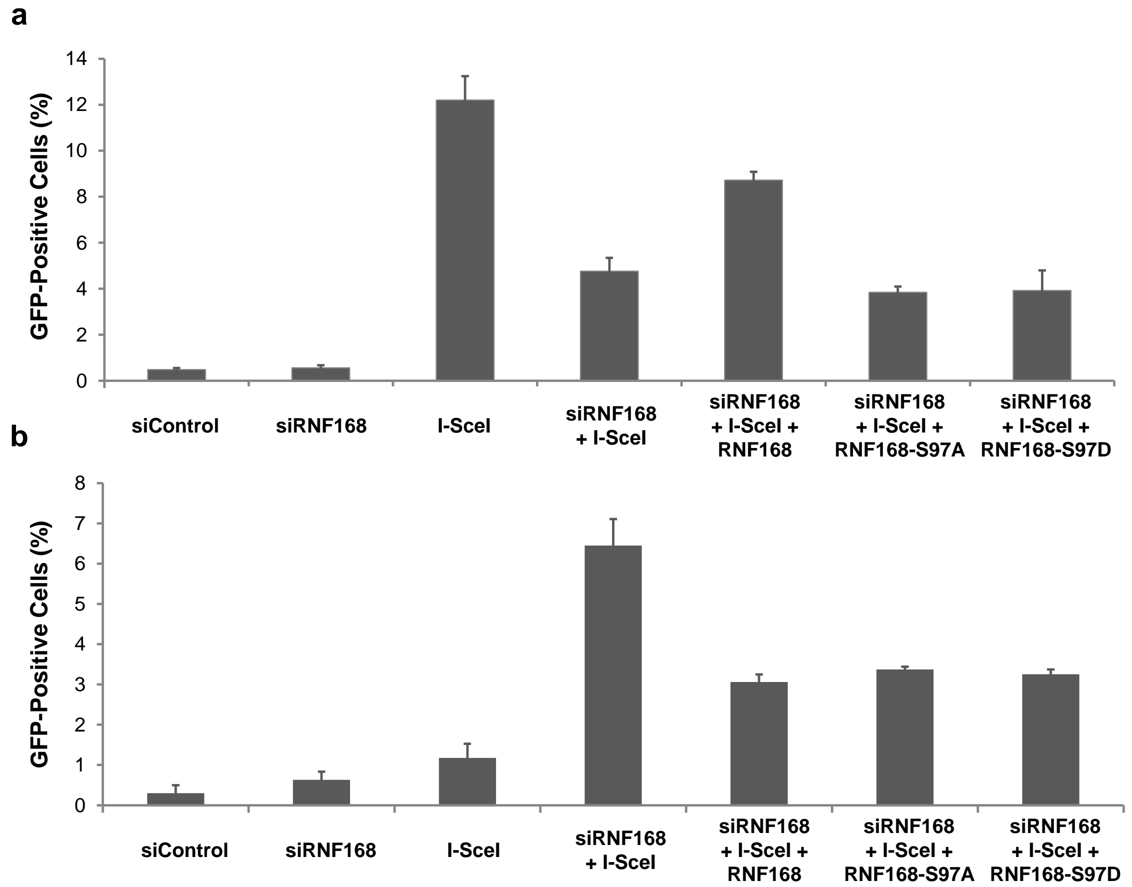
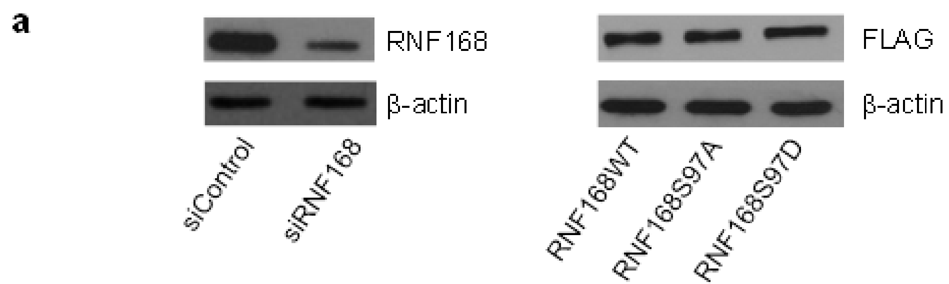

a
14
12
10
8
6
4
2
0
GFP-Positive Cells (%)
siControl
siRNF168
I-SceI
siRNF168
+ I-SceI
siRNF168
+ I-SceI +
RNF168
siRNF168
+ I-SceI +
RNF168-S97A
siRNF168
+ I-SceI +
RNF168-S97D
b
8
7
6
5
4
3
2
1
0
GFP-Positive Cells (%)
siControl
siRNF168
I-SceI
siRNF168
+ I-SceI
siRNF168
+ I-SceI +
RNF168
siRNF168
+ I-SceI +
RNF168-S97A
siRNF168
+ I-SceI +
RNF168-S97D

FIGURE 3. Defective S97 phosphorylation compromises cellular repair of DNA DSB via the NHEJ pathway, but does not impair HR-mediated repair. **(a)** The efficiencies of NHEJ repair in EJ5-GFP U2OS cells were reduced upon depletion of endogenous RNF168 by siRNA, and this decrease could be partially restored by complementing cells with siRNA-resistant plasmid for the expression of wild-type RNF168, but not the S97A or S97D mutant. The efficiency in NHEJ repair represents the percentage of GFP-positive cells as scored by flow cytometry analysis. **(b)** The efficiencies of HR repair in DR-GFP U2OS cells where the endogenous RNF168 was depleted by siRNA, and were subsequently complemented with siRNA-resistant plasmid for the expression of wild-type, S97A, or S97D mutant of RNF168. The efficiency in HR repair represents the percentage of GFP-positive cells as scored by flow cytometry analysis. The data represent the mean ± S.D. of results obtained from three independent experiments.

a



b

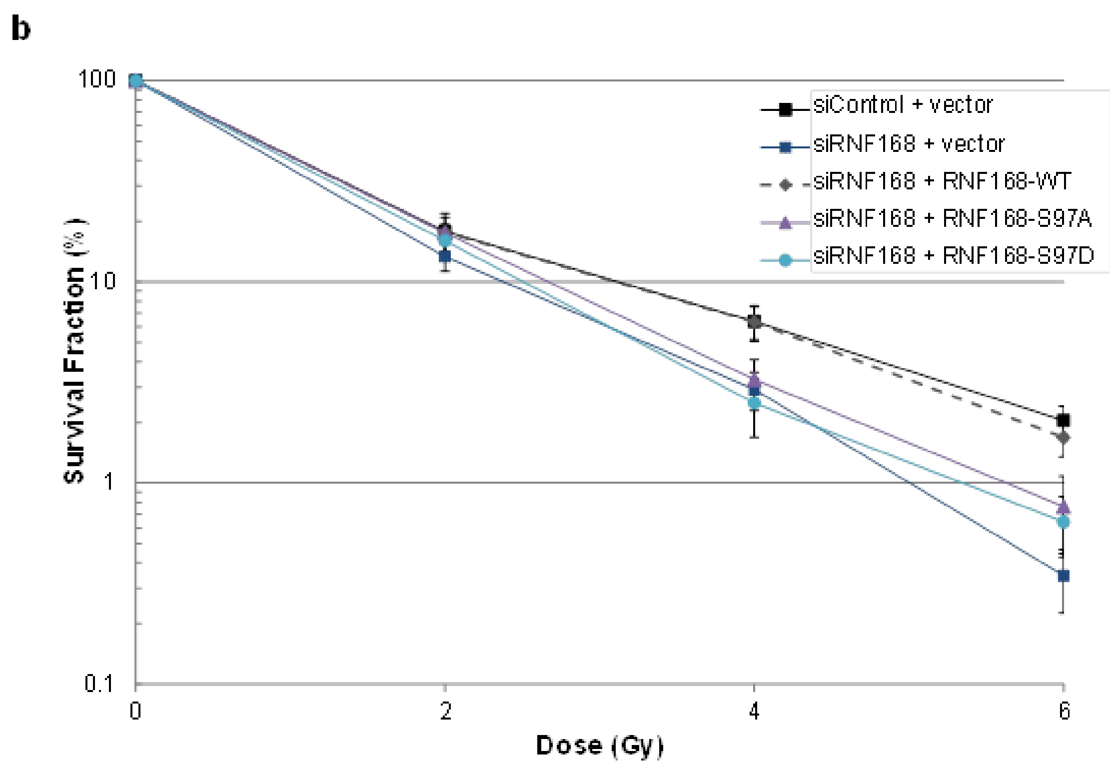


FIGURE 4. S97 phosphorylation confers cellular resistance toward ionizing radiation. **(a)** Western blot results showing that the siRNA gave rise to efficient knockdown of endogenous RNF168 in U2OS cells, and that the wild-type, S97A, and S97D-RNF168 were expressed at similar levels in U2OS cells. **(b)** Clonogenic survival assay results illustrating that the siRNA-mediated knock-down of RNF168 led to diminished survival of U2OS cells following exposure to different doses of γ rays, and this reduced survival could be restored by reconstituting cells with siRNA-resistant plasmid for the expression of wild-type RNF168, but the corresponding S97A or S97D mutant.

CHAPTER 3: CONCLUSIONS

3.1. Conclusions

Although many proteins and events necessary for DNA damage response signaling and repair in mammalian cells have been studied, the precise mechanisms involved are complex and not well elucidated. The results from the present study demonstrated the significant role of S97 phosphorylation in the function of RNF168 in DNA damage signaling. In response to DNA DSB formation, RNF168 ubiquitinates histone H2A/H2AX (Burma, 2001, Ward, 2001) and it plays a major role in establishing K27-linked ubiquitin chain (Gatti, 2015), which facilitates the recruitment of the ubiquitin-binding proteins that function in DNA DSB repair. The lack of proper RNF168-induced ubiquitination after DNA damage may hamper the preparation of a chromatin environment that is conducive for DNA repair.

The results from the present study unveiled an important role of phosphorylation of S97 in RNF168 in DNA damage-induced ubiquitination, NHEJ-mediated repair of DNA DSB, and cellular resistance toward ionizing radiation. On the grounds that the NHEJ repair efficiencies were attenuated with both phospho-deficient and phospho-mimetic mutants of RNF168, our results are congruent with the notion that transient phosphorylation of S97 may be crucial for RNF168's ubiquitination activity and its function in DNA damage response. The decrease in NHEJ repair introduced by the S97A or S97D mutation may be attributed to the diminished recruitment of proteins involved in NHEJ, such as 53BP1, to DNA damage site. The failure to observe any defect in HR repair emanating from the lack of this transient S97 phosphorylation is not surprising, especially in the context that RNF168 is known to inhibit homology-directed repair and

single-strand annealing, two variations of HR repair pathway (Munoz, 2014, Munoz, 2012). Along this line, cells depleted of RNF168 can still undergo HR through formation of ionizing radiation-induced foci of RAD51 without the presence of ionizing radiation-induced BRCA1 foci (Munoz, 2012). To further investigate the role of S97 phosphorylation on DSB repair, the use of glutamic acid (S97E) that contains an additional methylene group on its side chain may provide useful insights. Taken together, S97 in RNF168 may be a unique residue that is necessary component for proper maintenance of genomic integrity.

3.2. Reference

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