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USP49 is a novel deubiquitylating enzyme for γ H2AX in DNA double-strand break repair

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ABSTRACT

DNA double-strand break (DSB) that is one of the most serious DNA lesions is mainly repaired by two mutually exclusive pathways, homologous recombination and non-homologous end-joining. Proper choice of DSB repair pathway, in which recruitment of 53BP1 to chromatin around DSB sites plays a pivotal role, is crucial for maintaining genome integrity. Ubiquitylations of histone H2A and H2AX on Lys15 are prerequisite for 53BP1 loading onto chromatin. Although ubiquitylation mechanism of H2A and H2AX had been extensively studied, mechanism regulating deubiquitylation of γH2AX that is a phosphorylated form of H2AX remains elusive. Here, we identified USP49 as a novel deubiquitylating enzyme targeting DSB-induced γH2AX ubiquitylation. Over-expressed USP49 suppressed ubiquitylation of γH2AX in an enzymatic activity-dependent manner. Catalytic dead mutant of USP49 interacted and colocalized with γH2AX. Consequently, over-expression of USP49 inhibited the DSB-induced foci formation of 53BP1 and resulted in higher cell sensitivity to DSB-inducing drug treatment. Furthermore, endogenous USP49 protein was degraded via the proteasome upon DSB induction, indicating the importance of modulating USP49 protein level for γH2AX deubiquitylation. Consistent with our cell-based data, kidney renal clear cell carcinoma patients with higher expression of USP49 showed poor survival rate in comparison to the patients with unaltered USP49 expression. In conclusion, these data suggest that fine tuning of protein level of USP49 and USP49-mediated deubiquitylation of γH2AX are important for genome integrity.

1. Introduction

Genomic DNA is constantly exposed to various DNA-damaging sources. Among DNA damages, DNA double-strand breaks (DSBs) are known as one of the most serious DNA lesions. DSBs can be generated not only by external sources including ionizing radiation (IR) and anti-tumor drugs but also by internal factors such as reactive oxygen species (Mehta and Haber, 2014). If the repair mechanism of DSBs does not function normally, it can cause cell death and genomic instability (Jackson and Bartek, 2009). To avoid such risks, rapid and proper DSB repair is essential for living organisms. In human cells, non-homologous end-joining (NHEJ) and homologous recombination (HR) function as the major repair mechanisms of DSBs (Ciccia and Elledge, 2010). NHEJ directly ligates two ends of cleaved DNA to each other. Even though generally NHEJ is a dominant pathway in human cells, it is prone to cause insertion or deletion (Lieber, 2010). On the other hand, HR is thought to be an error-free pathway, since HR copies the DNA sequence from the undamaged sister chromatid (Jasin and Rothstein, 2013). One of the major differences between these two pathways is how they are regulated by the cell cycle. NHEJ works regardless of the cell cycle except M phase, while HR functions only in S and G2 phases, where sister chromatids are available.

It is well known that these DSB repair machineries are strictly regulated by protein post-translational modifications such as phosphorylation and ubiquitylation. Upon induction of DSB, ataxia telangiectasia mutated (ATM) phosphorylates histone H2AX on Ser139, which

Abbreviations: DSB, DNA double-strand break; NHEJ, non-homologous end-joining; HR, homologous recombination; DUB, deubiquitylating enzyme; 53BP1, tumor protein p53 binding protein 1; Znf-UBP, zinc-finger ubiquitin-specific protease; DDR, DNA damage response; ATM, ataxia telangiectasia mutated; RIF1, Rap1-interacting factor 1; PTIP, Pax transactivation domain-interacting protein.

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is named as γH2AX (Rogakou et al., 1998). Subsequently, γH2AX and also H2A are ubiquitylated on Lys13 and/or Lys15 by the E3 ligase RNF168 that is recruited to DSB sites in RNF8, another E3 ligase, dependent manner (Doil et al., 2009; Gatti et al., 2012; Mattiroli, 2012; Pinato, 2009; Stewart et al., 2009). Ubiquitylation on Lys15 of H2As in a nucleosome is directly recognized by the tumor protein p53 binding protein 1 (53BP1), resulting in the accumulation of 53BP1 on chromatin around the DSB sites (Fradet-Turcotte et al., 2013; Salguero et al., 2019; Wilson et al., 2016). Furthermore, 53BP1 phosphorylated by ATM recruits Rap1-interacting factor 1 (RIF1) and Pax transactivation domain-interacting protein (PTIP) (Callen et al., 2013; Feng et al., 2013; Chapman, 2013; Escribano-Díaz, 2013; Di Virgilio et al., 2013; Zimmermann et al., 2013). Together with these proteins, 53BP1 promotes DSB repair via NHEJ by suppressing the DNA end-resection that is essential nucleolytic degradation for initiating HR. Therefore, recruitment of 53BP1 to DSB sites that is modulated by ubiquitylation of γH2AX and H2A plays a key role in deciding by which pathway DSBs will be repaired.

Ubiquitin is a protein consisting of 76 amino acids (a. a.) and covalently binds to target proteins as a monomer or poly-ubiquitin chain via its Carboxyl-terminus Gly. Ubiquitylation of proteins is carried out by sequential reactions involving three classes of enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3 ligase) (Kerscher et al., 2006). Ubiquitin bound to E1 is transferred to E2 and then ligated to substrates via E3 ligase. Importantly, ubiquitylation is a reversible reaction, in which ubiquitin is removed by deubiquitylating enzymes (DUBs) (Reyes-Turcu et al., 2009; Komander et al., 2009). While ubiquitylation plays important roles in DSB repair, it is now obvious that deubiquitylating reactions should be fine-tuned as well during DSB repair (Nishi, 2017). Multiple DUBs have been reported to be involved in deubiquitylation of γH2AX and H2A in the context of DSB response. Namely, DUB3, BRCC36, USP3, USP11, USP16, USP22, USP26, USP37, USP44 and USP51 are known DUBs that are directly or indirectly involved in the deubiquitylation of γH2AX and H2A, (Shanbhag et al. 2010, Sharma et al., 2014; Nicassio, 2007; Wang, 2017; Wang, 2016; Mosbech et al., 2013; Yu, 2016; Zhang et al., 2014; Delgado-Díaz et al., 2017; Typas, 2016; Shao, 2009). Although some screenings had been carried out for DUBs targeting H2As, it remains uncertain whether any other DUBs can regulate γH2AX ubiquitylation. Of these aforementioned DUBs, USP3, USP16, USP22, USP44 and USP51 contain a Zinc-finger ubiquitin binding domain (ZnF-UBP) at the Amino-terminus (N-terminus), indicating that the ZnF-UBP domain may be a common feature of DUBs deubiquitylating γH2AX or H2A. In our previous screen for DUBs involved in DSB responses, USP49 that also contains a ZnF-UBP domain in its N-terminus was identified as a strong candidate (Nishi et al., 2014), while its potential role in deubiquitylating γH2AX was not investigated. Since post-translational modifications of H2AX play pivotal roles in DSB responses, in this study, we investigated the potential role of USP49 in controlling γH2AX ubiquitylation and modulating cellular response to DSBs.

2. Materials and Methods

2.1. Cell lines and cell culture

All cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere. All cells were cultured with Dulbecco's modified Eagle's medium (DMEM, Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS, SIGMA-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Nacalai tesque), 100 μg/ml streptomycin (Nacalai tesque) and 584 μg/ml l-glutamine. A U2OS cell line stably expressing mCherry-tagged 53BP1 (mCherry-53BP1) was established by culturing cells with identical media but containing 500 μg/ml Geneticin (Thermo Fisher Scientific, MA, USA) for two weeks after transfection of pmCherry-C1 plasmid (Takara Bio, Shiga, Japan) coding 53BP1.

2.2. Transfection of plasmids and siRNAs

HEK293T cells were transfected with the plasmid or siRNA (2 nM at final concentration) with Polyethylenimine "Max" (PEI-MAX, MW 40,000, Polyscience, PA, USA) or HiperFect (Qiagen, Dusseldorf, Germany), respectively as described below. The plasmid was diluted in OPTI-MEM media (Thermo Fisher Scientific) and then incubated for 5 min at room temperature. The plasmid containing media was mixed with OPTI-MEM supplemented with PEI-MAX and further incubated for 20 min at room temperature. Finally, the mixture was added to cells cultured with DMEM supplemented only with 10% FBS. For transfecting cells in 35, 60 or 100 mm dish, 2, 4 or 8 μg of plasmid was used, respectively. Cells used for Direct-repeat GFP (DR-GFP) assay were transfected with the appropriate plasmids by Mirus TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's instructions (also see homologous recombination assay section). The siRNA targeting Luciferase (5'-AACGUACGCGGAAUACUUCGA-3', Eurofins Genomics, Tokyo, Japan) or USP49 (Thermo Fisher Scientific, s24638) was mixed with HiperFect in OPTI-MEM media. After incubation for 15 min at room temperature, the mixture was added to cells cultured with DMEM supplemented with 10% FBS and antibiotics.

2.3. Cell extract preparation and immunoblotting analysis

Except for sample preparation for immunoprecipitation, cell extracts were prepared with CSK buffer [10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, 1 mM ethylene glycol bis (2-aminoethyl ether)-N, N, N', N'-tetraacetic acid, 0.1% Triton X-100 and 300 mM sucrose] containing 300 mM NaCl, 1 × Protease Inhibitor cocktail ethylenediamine tetraacetic acid (EDTA)-free (PI, Roche, Basel, Switzerland), 10 mM NaF (Nacalai tesque), 20 mM N-ethylmaleimide (NEM, Nacalai tesque), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF, SIGMA-Aldrich). The cells were washed twice with ice-cold phosphate buffered saline (PBS) and incubated with an appropriate volume of CSK buffer containing 300 mM NaCl, 1 × PI, 10 mM NaF, 20 mM NEM, and 0.25 mM PMSF for 1 h on ice with occasional mixing. Soluble fractions were collected by centrifugation at 20,000 × g for 10 min at 4 °C. Residual chromatin fractions (pellet fractions) were washed twice, resuspended with the identical buffer, and then solubilized by sonication (UD-100, 40% output, 30 s, TOMY, Tokyo, Japan). Where indicated, cells were incubated with 500 μM phleomycin (InvivoGen, CA, USA) for 1 h. For immunoprecipitation, cells were washed twice and collected with an appropriate volume of ice-cold PBS, followed by centrifugation at 10,000 × g, for 1 min. Cells were solubilized by sonication with CSK buffer containing 300 mM NaCl, 1 × PI, 10 mM NaF, 20 mM NEM, and 0.25 mM PMSF. Insoluble fractions were removed by centrifugation at 20,000 × g for 10 min at 4 °C. The protein concentrations of cell extracts were determined with Coomassie Protein Assay Reagent (Thermo Fisher Scientific, MA, USA) with bovine serum albumin as a standard (Takara Bio). The antibodies used for immunoblotting in this research are anti-GFP antibody (Roche, 11814460001, 1:1,000 dilution), anti-γH2AX antibody (Millipore, 05–636, MA, USA, 1:1,000 dilution), anti-USP49 antibody (Proteintech, 18066–1-AP, 1:2,000 dilution), anti-USP49 antibody (Abcam, ab127574, Cambridge, UK, 1:1,000 dilution) and anti-α-tubulin antibody (SIGMA-Aldrich, T9026, 1:5,000 dilution). All immunoblotting experiments were carried out at least twice in the laboratory.

2.4. Immunoprecipitation

Soluble fractions of cell extracts were subjected to immunoprecipitation with an anti-GFP antibody coupled with magnetic beads (GFP-Trap_MA, ChromoTek, Planegg-Martinsried, Germany) by rotating overnight at 4 °C. The beads were washed six times with the buffer used for cell extract preparation and bound proteins were eluted by boiling at 95 °C for 10 min with 1 × Laemmli buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulphate, 10% glycerol, 0.02% bromophenol blue

and 6.25% β-mercaptoethanol].

2.5. Immunofluorescence staining

To investigate the localization of GFP-fused USP49 (GFP-USP49) without exogenous DNA damage, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized by incubating with 0.2% Triton X-100 in PBS for 5 min at room temperature. Hereafter, the samples were washed twice with 0.1% Tween 20 in PBS after each procedure. Nuclei were stained with 1 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) solution for 10 min. To survey DSB-induced foci formation of mCherry-53BP1, cells irradiated with 2 Gy of IR (Faxitron RX-650, AZ, USA) were incubated for 15 min or cells were treated with 40 μg/ml phleomycin for 2 h. When colocalization of GFP-USP49 and γH2AX was examined, cells were pre-extracted prior to fixation with pre-extraction buffer [10 mM Pipes (pH 6.8), 3 mM $MgCl_2$, 3 mM EDTA, 0.5% Triton X-100, 300 mM sucrose and 50 mM NaCl] for 5 min on ice. Following fixation and permeabilization, cells were incubated with blocking buffer (5% FBS, 0.1% Triton X-100 in PBS) for 30 min. Subsequently, cells were incubated with the primary antibody for 1 h and then with the secondary antibody for 30 min diluted in blocking buffer. Following nuclei staining with DAPI as described above, all samples were sealed with VECTASHIELD (VECTOR LABORATORIES, Burlingame, CA, USA). Images were taken with a confocal microscope (TCS SP5, Leica, Wetzlar, Germany or FV3000, Olympus, Tokyo, Japan). The primary antibody used for immunofluorescence staining in this research was anti-γH2AX antibody (Millipore, 05–636, 1:200 dilution).

2.6. Cell survival assay

HEK293T cells were seeded in a 96 well plate and, on the following day, treated with various doses of phleomycin for 48 h. Cell viability was analyzed using Cell Count Reagent SF (Nacalai tesque) according to the manufacturer's instructions.

2.7. Homologous recombination assay

The efficiency of homologous recombination was assessed with DR-GFP assay as previously described (Matsui et al., 2020). Briefly, cells were transfected with expression plasmids of FLAG-USP49 (WT or CA), I-SceI and mCherry. As a negative control, cells were transfected with an empty plasmid instead of I-SceI coding plasmid. Forty-eight hour after transfection, cells were analyzed with cell sorter (SH800S, Sony, Tokyo, Japan). HR efficiency was determined as population of GFP positive cells in mCherry positive cells.

3. Results

3.1. DNA damage-induced ubiquitylation of γH2AX was suppressed with the over- expressed USP49

H2AX is one of the H2A variants and makes up about only 10% of total H2A family in mammalian cells, however, it plays important roles in DNA damage response (DDR)(Salguero et al., 2019; Lowndes and Toh, 2005). To reveal hitherto unidentified DUBs targeting ubiquitylation of γH2AX, we compared domain structures of the DUBs that are previously reported to be involved in deubiquitylation of γH2AX and H2A either directly or indirectly (Fig. 1A and Supplementary Table ST1). As shown in Fig. 1A, USP3, USP16, USP22, USP44 and USP51 carry a ZnF-UBP domain at the N-terminus, suggesting that ZnF-UBP may play roles in the deubiquitylation of γH2AX. In human genome about a hundred DUBs are encoded, and seven of them contains ZnF-UBP domain in addition to aforementioned five DUBs. Phylogenic analysis of human DUBs revealed that among these DUBs, USP49 shows relatively high amino acid sequence similarity to USP3, USP22, USP44 and USP51 (Nishi et al., 2014). Furthermore, we previously identified USP49 as a potential DDR factor in a screen, although how USP49 contributes to DDR was not investigated (Nishi et al., 2014). Based on these knowledges we examined whether USP49 influences on γH2AX deubiquitylation. For this purpose, HEK293T cells transiently over-expressing either GFP or GFP-tagged USP49 [wild-type: GFP-USP49 (WT)] were treated with phleomycin, which is a radio-mimetic drug. Phleomycin treatment induced ubiquitylation of γH2AX that was suppressed by the transient over-expression of GFP-USP49 (WT) in comparison to GFP expression (Fig. 1B). Contrary, γH2AX ubiquitylation was not suppressed by the transient over-expression of a catalytically inactive mutant GFP-USP49 [GFP-USP49 (CA)] in which Cys262 was replaced with Ala (Fig. 1B) (Zhang, 2013). Immunoblotting analysis with anti-USP49 antibody indicated that expression level of exogenous GFP-USP49 (WT) or GFP-USP49 (CA) was 9.2-fold or 15.7-fold higher than that of endogenous USP49, respectively (Supplementary Fig. S1), suggesting that GFP-USP49 dominantly functioned in these transfected cells. Furthermore, quantitative analysis of γH2AX ubiquitylation, in which the expression level of GFP-USP49 (WT and CA) was considered, showed that their effects on γH2AX ubiquitylation were significant (Fig. 1C). These results suggested that over-expression of USP49 suppressed phleomycin-induced γH2AX ubiquitylation in a DUB activity dependent manner. Subsequently, the importance of ZnF-UBP domain of USP49 in deubiquitylating γH2AX was examined by transfecting cells with a plasmid coding truncated mutant of USP49 lacking the N-terminal domain that contains ZnF-UBP domain (ΔN: 253–688 a. a.). Transiently over-expressed GFP-USP49 (ΔN) suppressed ubiquitylation of γH2AX after phleomycin treatment in a similar manner with GFP-USP49 (WT) (Fig. 1D). To investigate whether C-terminus region of USP49 that is not a part of catalytic domain is required for suppressing γH2AX ubiquitylation, similar experiment was performed with a USP49 mutant lacking the C-terminus region (ΔC: 1–657 a. a.). As shown in Fig. 1D., GFP-USP49 (ΔC) was still able to suppress ubiquitylation of γH2AX. These results suggest that USP49 negatively regulates ubiquitylation of γH2AX not in a ZnF-UBP domain but a DUB activity dependent manner.

3.2. USP49 colocalized and interacted with γH2AX

To further understand the involvement of USP49 in DDR, we examined subcellular localization of USP49. Both wild-type and catalytically inactive mutant of USP49 localized to nucleus without DNA damage (Fig. 2A), suggesting that USP49 may exert its function mainly in nucleus. Subsequently, localization of USP49 to DSB sites were examined. Since USP49 was suggested to bind histone H2B (Zhang, 2013), to specifically detect USP49 localized to DSB sites immunofluorescent staining was performed with extraction prior to fixation, which is a generally used method for investigating localization of DDR proteins. GFP-USP49 (CA) colocalized with γH2AX after phleomycin treatment, suggesting that USP49 could function at sites of DSB (Fig. 2B). Although recruitment of GFP-USP49 (WT) to DSB sites was not detectable, this may be because of transient interaction between USP49 and a potential substrate such as ubiquitylated-γH2AX. Consistent with this idea, it was previously reported that localization of USP44 to DSB sites was enhanced when its catalytically dead mutant was used (Mosbech et al., 2013). In addition, interactions between DUBs (USP3 and USP11) and histone substrates were more easily detected with enzymatically dead DUBs (Yu, 2016). The colocalization of USP49 (CA) and γH2AX prompted us to investigate the protein–protein interaction between USP49 (CA) and γH2AX (Fig. 2C). GFP-USP49 (CA) immunoprecipitated with an anti-GFP antibody co-precipitated γH2AX (Fig. 2C), suggesting interaction between these proteins. Altogether, these results suggest that USP49 could deubiquitylate γH2AX at sites of DSBs.

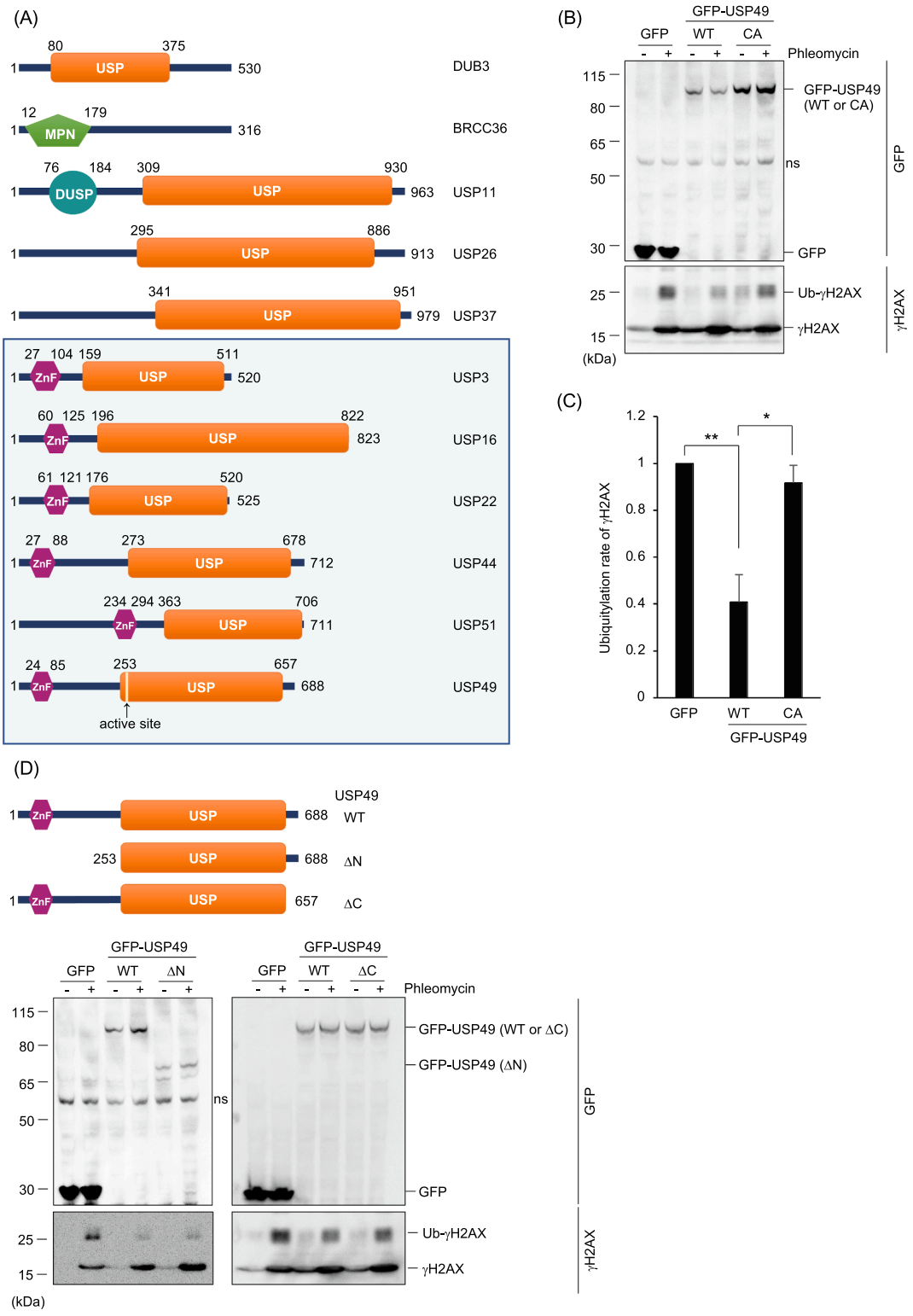

Fig. 1. DNA damage-induced ubiquitylation of γH2AX was suppressed by the over-expressed USP49 (A) Schematic representation of human DUBs targeting γH2AX and H2A. Domain structure of USP49 is also depicted. The numbers represent amino acid residues. USP: ubiquitin-specific protease, MPN: JAB1/MPN/MOV34 metalloenzyme catalytic domain, DUSP: domain present in ubiquitin-specific proteases, ZnF: zinc-finger ubiquitin-specific protease (ZnF-UBP). DUBs containing ZnF-UBP domain are indicated with blue box. (B) HEK293T cells transfected either with expression plasmid of GFP or GFP-USP49 (WT or CA) were treated with phleomycin or mock treated. Cell extracts were analyzed by immunoblotting with the indicated antibodies. Ub-γH2AX: ubiquitylated γH2AX. The non-specific band detected with anti-GFP antibody was indicated as ns. (C) Quantification of the data presented in (B) for the residual ubiquitylated γH2AX (mean ± SEM, n = 3, *$p < 0.05$, **$p < 0.01$) . (D) Schematic representation of human USP49 and its truncated mutants (upper). HEK293T cells transfected with the expression plasmid coding the indicated gene were treated with phleomycin or mock treated. Cell extracts were analyzed by immunoblotting with the indicated antibodies (lower). Ub-γH2AX: ubiquitylated γH2AX. The non-specific band detected with anti-GFP antibody was indicated as ns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

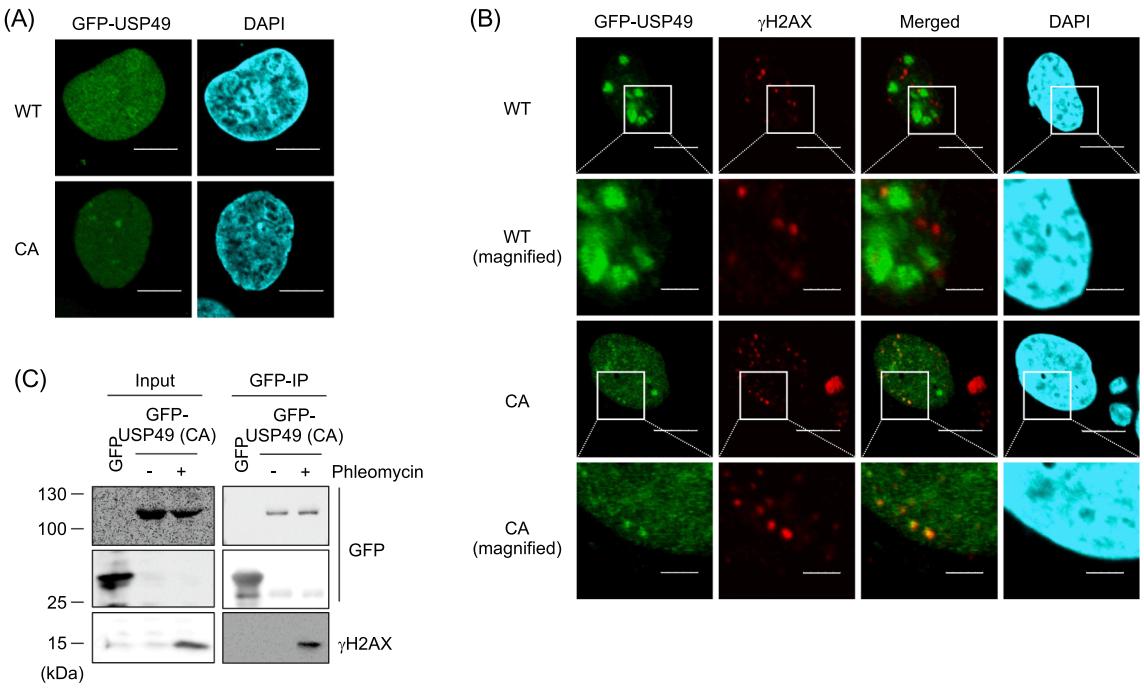

Fig. 2. USP49 colocalized and interacted with γH2AX (A) Subcellular localization of GFP-USP49 (WT or CA) in U2OS cells. Scale bar: 10 μm. (B) Subcellular localization of GFP-USP49 (WT or CA) and γH2AX in phleomycin treated U2OS cells. Scale bar: 10 μm. The area indicated with the white square in the figure are magnified and shown directly below each panel. Scale bar: 3 μm. (C) HEK293T cells were transfected with the plasmids encoding either GFP or GFP-USP49 (CA). Following phleomycin treatment, cell extracts were subjected to immunoprecipitation with an anti-GFP antibody followed by immunoblotting analysis with the indicated antibodies.

3.3. Over-expression of USP49 inhibited DSB-induced 53BP1 foci formation and sensitized cells to a radio-mimetic drug

It has been reported that ubiquitylation of γH2AX on Lys15, which is induced by DSBs, is directly recognized by 53BP1 and recruits 53BP1 to sites of DSBs (Fradet-Turcotte et al., 2013; Salguero et al., 2019). In addition, we found that USP49 negatively regulates DSB-induced γH2AX ubiquitylation (Fig. 1B). Thus, we studied the effect of USP49 over-expression on DSB-induced foci formation of 53BP1 with a cell line stably expressing mCherry-53BP1. As previously reported, mCherry-53BP1 distributed uniformly in nucleus except nucleoli. Even without DNA damage, a few bright spots of mCherry-53BP1 were detected that are thought to be OPT domain (Harrigan, 2011). This distribution pattern of mCherry-53BP1 was not altered by the over-expression of GFP-USP49 (WT and CA) (Fig. 3A). When cells were treated with phleomycin, mCherry-53BP1 formed distinct foci in GFP expressing cells as expected. In line with the effect on γH2AX ubiquitylation, phleomycin-induced 53BP1 foci formation was inhibited in GFP-USP49 (WT) expressing cells (Fig. 3A). On the other hand, foci formation of 53BP1 in cells expressing GFP-USP49 (CA) was similar to the one with GFP expression (Fig. 3A). Importantly, similar results were obtained with IR-irradiated cells (Supplementary Fig. S2), suggesting that excess amount of USP49 antagonized DSB-induced 53BP1 foci formation by suppressing γH2AX ubiquitylation. Subsequently, we investigated whether USP49 over-expression affects HR efficiency with DR-GFP assay, because 53BP1 promotes NHEJ and inhibits HR (Daley and Sung, 2014). Over-expression of USP49 (WT) significantly increased the efficiency of HR compared to the control (Fig. 3B). To our surprise, over-expression of USP49 (CA), which affected neither γH2AX ubiquitylation nor 53BP1 foci formation, strongly suppressed HR efficiency (Fig. 3B). The transfection efficiency of plasmids, which was confirmed by the expression of mCherry from the co-transfected plasmid, was similar between experiments (data not shown), therefore, HR suppression with USP49 (CA) was not likely due to impaired expression of I-SceI restriction enzyme. Furthermore, since over-expression of USP49 affected the choice of DSB repair pathway, its effect on cellular sensitivity to DSB-inducing reagent was also investigated. As shown in Fig. 3C, GFP-USP49 (WT) over-expression sensitized cells to phleomycin compared to GFP expression, suggesting that excessive USP49 (WT) could compromise DSB repair pathway choice and result in higher sensitivity to phleomycin. Although over-expression of USP49 led to increased HR repair (Fig. 3B), total DSB repair activity may not be enough to maintain cell survival. Interestingly, USP49 (CA) over-expression also reduced viability following phleomycin treatment, while it did not affect γH2AX ubiquitylation and not hinder 53BP1 foci formation. It suggested that other than regulating γH2AX-53BP1 axis, USP49 may have additional roles in DSB responses. These results suggest that regulating the protein level and catalytic activity of USP49 is important for DSB repair pathway choice and cell viability.

3.4. Knockdown of USP49 did not affect γH2AX ubiquitylation, HR efficiency and cell survival

Although we had revealed involvement of USP49 in DSB repair by over-expressing USP49, the consequences of depleting endogenous USP49 were also assessed. Firstly, HEK293T cells in which endogenous USP49 was knocked down by a siRNA were treated with phleomycin, and ubiquitylation of γH2AX was examined. While expression of USP49 was effectively suppressed by siRNA transfection, no significant difference in ubiquitylation of γH2AX was observed compared to control (Fig. 4A and 4B). As in the over-expression experiments, the HR efficiency and cell viability of USP49 knockdowned cells were also examined. The results showed that depletion of USP49 significantly altered neither HR efficiency nor cell survival (Fig. 4C and 4D). These data may suggest that unregulated over-expression of USP49 is problematic for cells rather than loss of USP49. No or quite weak phenotype caused by USP49 depletion was maybe because other DUBs, such as USP3, USP11, DUB3 and BRCC36, are able to complement the function of UPS49.

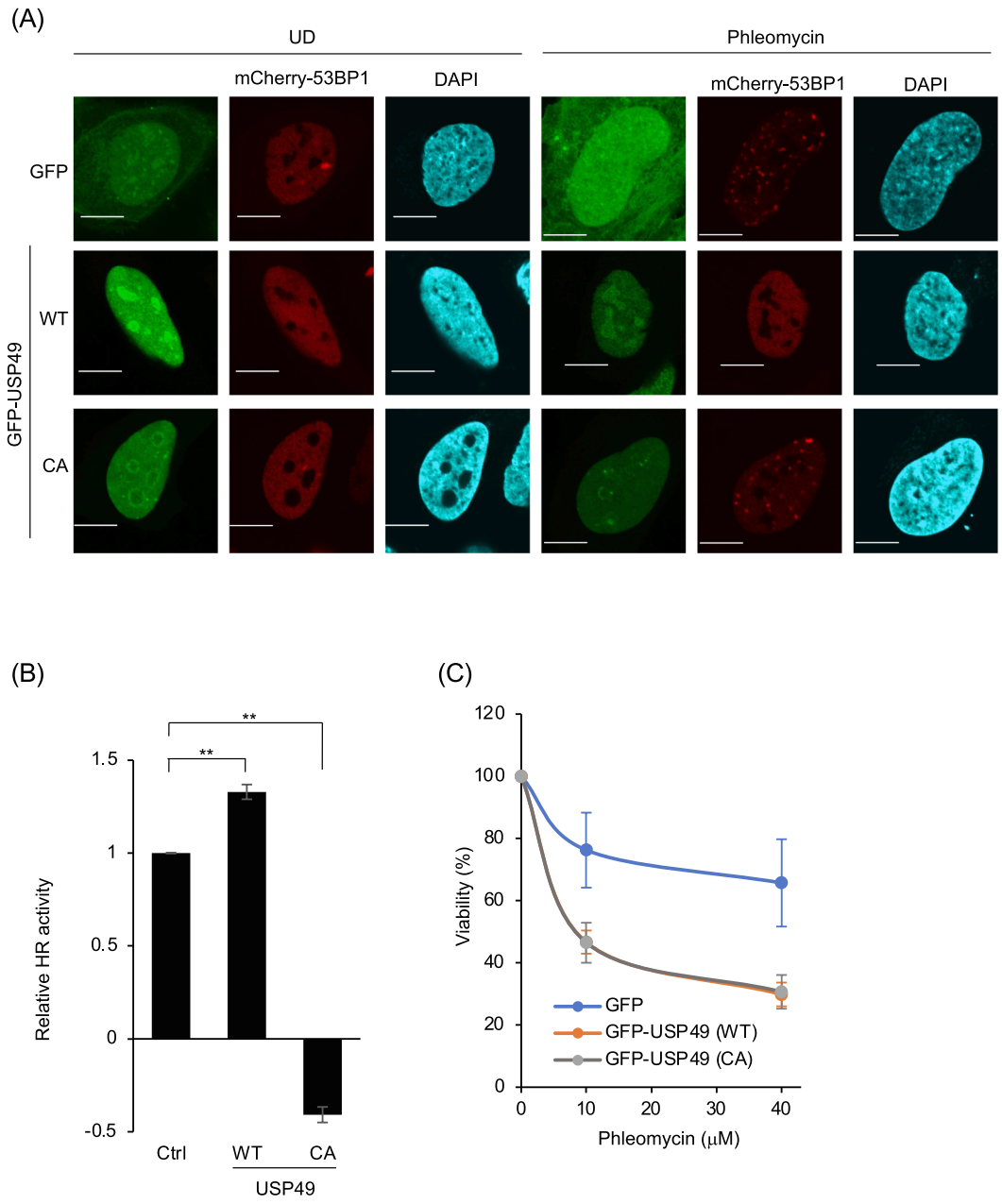

Fig. 3. Over-expression of USP49 inhibited 53BP1 foci formation induced with phleomycin treatment and sensitized cells to phleomycin (A) U2OS cells stably expressing mCherry-53BP1 were transfected with the plasmid coding either GFP or GFP-USP49 (WT or CA). Cells were then treated with phleomycin or mock treated (undamaged: UD). Scale bar: 10 μm. (B) DR-GFP assay was carried out with exogenous expression of FLAG-tagged USP49 (WT or CA) or FLAG-tag (Ctrl). GFP-positive cell populations were normalized to FLAG-tag expression sample, which was set to 1 (mean ± SEM, n = 3, **p < 0.01). (C) HEK293T cells transfected with the expression plasmid coding GFP or GFP-USP49 (WT or CA) were treated with phleomycin for 48 h. Cell viability was assessed as described in Materials and Methods section (mean ± SEM, n = 3).

3.5. Expression of endogenous USP49 was controlled in a DSB-dependent manner

Since over-expression of USP49 inhibited γH2AX ubiquitylation after DNA damage and increased cellular sensitivity to phleomycin, we examined whether the expression of endogenous USP49 protein is controlled upon induction of DSB. Endogenous USP49 was reduced when cells were treated with phleomycin (Fig. 5A), which was further confirmed by performing the identical experiment with cycloheximide (CHX), a protein synthesis inhibitor (Fig. 5A). This DSB-induced reduction of USP49 was inhibited, if cells were treated with phleomycin in the presence of MG132, a proteasome inhibitor (Fig. 5A). These data suggest that endogenous USP49 is degraded by a proteasome after DSB induction. These findings indicated that protein level of USP49 should be regulated properly, and prompted us to investigate the relationship between expression level of USP49 and clinical outcomes. Since we mainly used transformed kidney cells in this study, expression of USP49 in kidney cancer patients was investigated using the Cancer Genome Atlas (TCGA) data set (Cerami, 2012; Gao et al., 2013). Among 488 patients of kidney renal clear cell carcinoma, 24 patients were found to express USP49 at higher than two Z-score (high USP49-expression group). We then examined disease specific survival of these patients, indicating that high USP49-expression group showed significantly poor survival compared to another group (unaltered USP49-expression group) ($p = 0.048$) (Fig. 5B). Higher expression of USP49 was also found in some other cancers. In uterine corpus endometrial carcinoma,

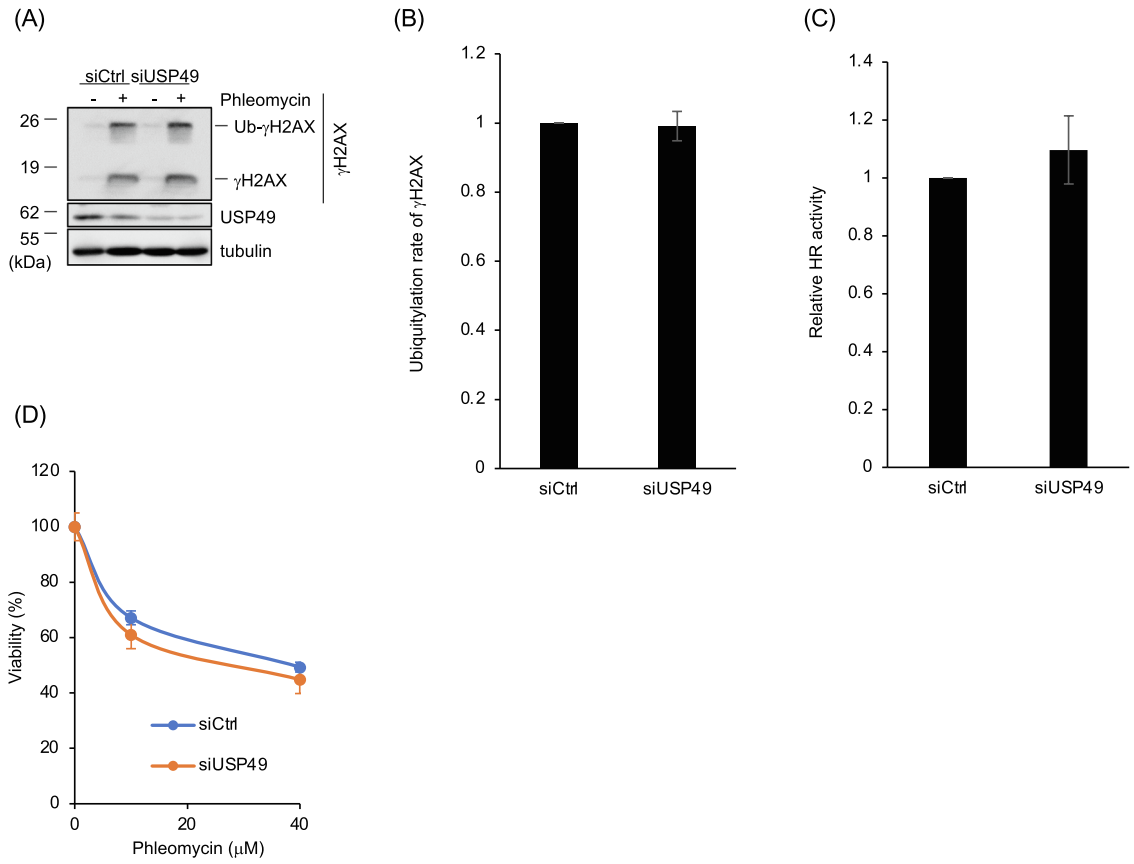

Fig. 4. Knockdown of USP49 did not affect γH2AX ubiquitylation, HR efficiency and cell survival (A) HEK293T cells were transfected with either control siRNA (siCtrl) or siRNA targeting USP49 (siUSP49). These cells treated with phleomycin or mock treated were subjected to immunoblotting analysis with the indicated antibodies. Ub-γH2AX: ubiquitylated γH2AX. (B) Quantification of the data presented in (A) for the ratio of ubiquitylated γH2AX (mean ± SEM, n = 3) . (C) DR-GFP assay was carried out with cells transfected with the indicated siRNAs (mean ± SEM, n = 3). (D) HEK293T cells transfected with the indicated siRNAs were treated with phleomycin for 48 h. Cell viability was assessed as described in Materials and Methods section (mean ± SEM, n = 3).

for example, significantly shorter survival was seen with the patients expressing higher level of USP49 protein (data not shown), suggesting that increased expression of USP49 could be corelated with poor survival and prognosis.

4. Discussion

In this study, we identified USP49 as a novel DUB targeting ubiquitylation of γH2AX that was induced by DSBs based on a series of evidence. 1) Over-expression of USP49 suppressed DSB-induced ubiquitylation of γH2AX in its catalytic activity dependent manner. 2) USP49 interacted and colocalized with γH2AX. 3) Subsequently, excess USP49 inhibited DSB-induced 53BP1 foci formation, increased HR efficiency and conferred higher radio-mimetic drug sensitivity to cells. 4) Importantly, kidney renal clear cell carcinoma patients with higher expression of USP49 showed poor disease specific survival. These findings suggest that USP49 is a key factor in regulating DSB responses and for clinical outcome. Although knockdown of USP49 did not significantly affect these phenotypes, we interpreted this as other DUBs, such as USP3, USP11, DUB3 and BRCC36, can function in the absence of USP49.

The appropriate choice of DSB repair pathway in which recruitment of 53BP1 to sites of DSB is critical is important for maintaining genome integrity (Scully et al., 2019). Namely, inhibition or deletion of 53BP1 resulted in increased HR (Chen, 2020; Xie, 2007; Canny, 2018), which is consistent with our results showing that over-expression of USP49 suppressed 53BP1 loading to DSB sites and increased HR (Fig. 3A and 3B). Direct binding of 53BP1 to Lys15 ubiquitylated H2A, together with recognition of dimethylated H4Lys20, is essential for 53BP1 loading to DSB sites, (Botuyan et al., 2006; Sanders et al., 2004). Although H2AX was not essential for 53BP1 loading to DSB sites, but it enhanced 53BP1 loading to DSB sites (Salguero et al., 2019). Thus far, there are variety of DUBs targeting ubiquitylated H2A. However, only a few DUBs (USP3, USP11, DUB3 and BRCC36) had been suggested as DUBs targeting ubiquitylated γH2AX with some discrepancies, (Ting, 2019). Here we showed that endogenous USP49 protein was degraded by proteasome upon induction of DSBs (Fig. 5A). This could suggest that USP49 suppresses 53BP1 binding to chromatin by deubiquitylating γH2AX when cells were not challenged. But once DSBs were generated, 53BP1 loading on chromatin and NHEJ could be promoted by proteasomal degradation of USP49. Since it was suggested that such a timely (de)ubiquitylation is essential for repairing DSBs with appropriate pathway (Nakada, 2016), USP49 may be part of this process. In view of domain analysis, ZnF-UBP domain of USP49 was not required for suppressing γH2AX ubiquitylation (Fig. 1D). On the other hand, USP49 was also known to deubiquitylate H2BLys120, which promoted both HR and NHEJ, (Moyal 2011, Nakamura, 2011; So et al., 2019), suggesting that ZnF-UBP domain of USP49 might function in deubiquitylating H2B. In line with this idea, while catalytically inactive USP49 did not affect γH2AX-53BP1 axis, this mutant still sensitized cells to phleomycin, suggesting that USP49 could have multiple targets in DSB repair. This should be addressed in the future study.

It is worth mentioning that USP49 had been reported to be involved in tumorigenesis by deubiquitylating other substrates. USP49 deubiquitylated and stabilized FKBP51 and, as a result, inhibited the growth of pancreatic cancer cells (Luo, 2017). In addition, p53 is stabilized by

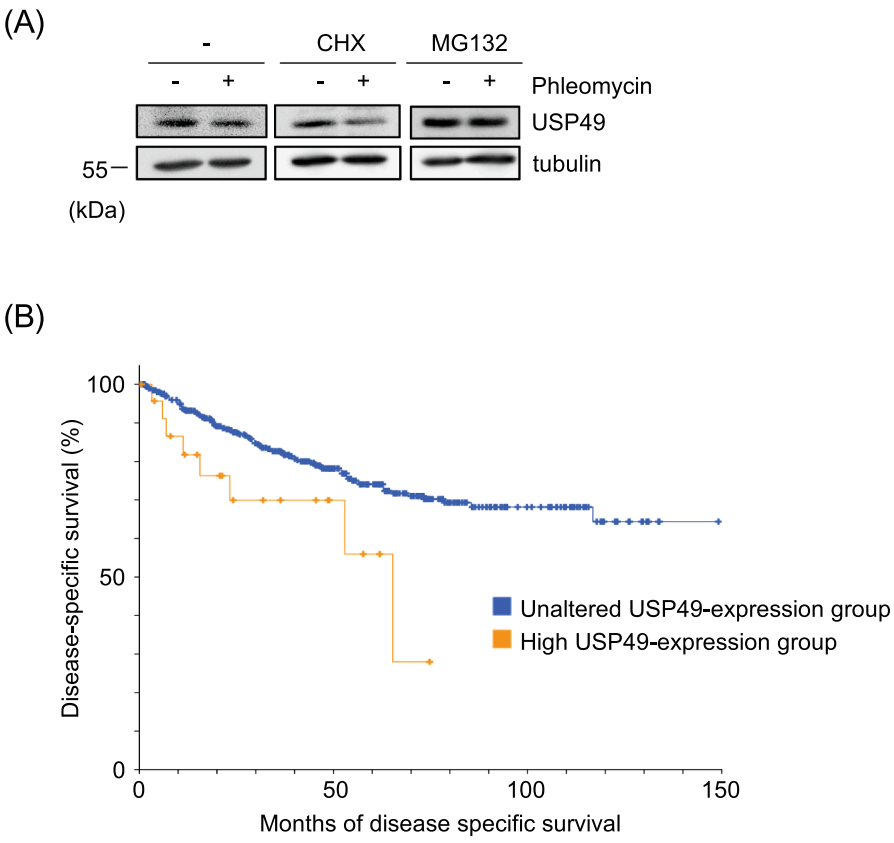

Fig. 5. Protein level of endogenous USP49 was controlled in a DSB-dependent manner (A) Prior to phleomycin treatment, HEK293T cells were incubated with 100 μg/ml CHX for 2 h or 10 μM MG132 for 1 h. As a control, cells were also mock treated (-). Cell extracts were analyzed by immunoblotting with the indicated antibodies. (B) Disease-specific survival rate (%) of the patients with high USP49-expression (orange) or the patients with unaltered USP49-expression (blue) was plotted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

USP49 (Tu, 2018). More importantly, consistent with our notion, HCT116 cells expressing exogenous FLAG-tagged USP49 were more sensitive to etoposide (Tu, 2018). While higher USP49 expression in kidney renal clear cell carcinoma patients was corelated with poor survival, in gastric cancer and lung cancer, recurrence free survival was significantly better with patients expressing higher level of USP49. These results suggested that consequence of altered USP49 expression may vary depending on tissues, which requires further investigation.

CRediT authorship contribution statement

Misaki Matsui: Conceptualization, Investigation, Writing – original draft. **Shoki Kajita:** Investigation. **Yuina Tsuchiya:** Investigation. **Wakana Torii:** Investigation. **Shiori Tamekuni:** Investigation. **Ryotaro Nishi:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146599.

References

Mehta, A., Haber, J.E., 2014. Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harb. Perspect. Biol. 6 (9).
Jackson, S.P., Bartek, J., 2009. The DNA-damage response in human biology and disease. Nature 461 (7267), 1071–1078.
Ciccia, A., Elledge, S.J., 2010. The DNA damage response: making it safe to play with knives. Mol. Cell 40, 179–204.
Lieber, M.R., 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu. Rev. Biochem. 79 (1), 181–211.
Jasin, M., Rothstein, R., 2013. Repair of strand breaks by homologous recombination. Cold Spring Harb. Perspect. Biol. 5, a012740.
Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., Bonner, W.M., 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. 273, 5858–5868.
Doil, C., Mailand, N., Bekker-Jensen, S., Menard, P., Larsen, D.H., Pepperkok, R., Ellenberg, J., Panier, S., Durocher, D., Bartek, J., Lukas, J., Lukas, C., 2009. RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell 136 (3), 435–446.
Gatti, M., Pinato, S., Maspero, E., Soffientini, P., Polo, S., Penengo, L., 2012. A novel ubiquitin mark at the N-terminal tail of histone H2As targeted by RNF168 ubiquitin ligase. Cell Cycle 11 (13), 2538–2544.
Mattiroli, F., et al., 2012. RNF168 ubiquitinates K13–15 on H2A/H2AX to drive DNA damage signaling. Cell 150, 1182–1195.
Pinato, S., et al., 2009. RNF168, a new RING finger, MIU-containing protein that modifies chromatin by ubiquitination of histones H2A and H2AX. BMC Mol. Biol. 10, 55.
Stewart, G.S., Panier, S., Townsend, K., Al-Hakim, A.K., Kolas, N.K., Miller, E.S., Nakada, S., Ylanko, J., Olivarius, S., Mendez, M., Oldreive, C., Wildenhain, J., Tagliaferro, A., Pelletier, L., Taubenheim, N., Durandy, A., Byrd, P.J., Stankovic, T., Taylor, A.M.R., Durocher, D., 2009. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. Cell 136 (3), 420–434.
Fradet-Turcotte, A., Canny, M.D., Escribano-Díaz, C., Orthwein, A., Leung, C.C.Y., Huang, H., Landry, M.-C., Kitevski-LeBlanc, J., Noordermeer, S.M., Sicheri, F., Durocher, D., 2013. 53BP1 is a reader of the DNA-damage-induced H2A Lys 15 ubiquitin mark. Nature 499 (7456), 50–54.
Salguero, I., Belotserkovskaya, R., Coates, J., Sczaniecka-Clift, M., Demir, M., Jhujh, S., Wilson, M.D., Jackson, S.P., 2019. MDC1 PST-repeat region promotes histone H2AX-independent chromatin association and DNA damage tolerance. Nat Commun 10 (1). https://doi.org/10.1038/s41467-019-12929-5.

Wilson, M.D., Benlekbir, S., Fradet-Turcotte, A., Sherker, A., Julien, J.-P., McEwan, A., Noordermeer, S.M., Sicheri, F., Rubinstein, J.L., Durocher, D., 2016. The structural basis of modified nucleosome recognition by 53BP1. Nature 536 (7614), 100–103.
Callen, E. et al. 53BP1 mediates productive and mutagenic DNA repair through distinct phosphoprotein interactions. *Cell* **153,** 1266-1280 (2013).
Feng, L., Fong, K.W., Wang, J., Wang, W., Chen, J., 2013. RIF1 counteracts BRCA1-mediated end resection during DNA repair. J. Biol. Chem. 288, 11135–11143.
Chapman, J.R., et al., 2013. RIF1 is essential for 53BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection. Mol. Cell 49, 858–871.
Escribano-Díaz, C., et al., 2013. A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice. Mol. Cell 49, 872–883.
Di Virgilio, M., Callen, E., Yamane, A., Zhang, W., Jankovic, M., Gitlin, A.D., Feldhahn, N., Resch, W., Oliveira, T.Y., Chait, B.T., Nussenzweig, A., Casellas, R., Robbiani, D.F., Nussenzweig, M.C., 2013. Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching. Science 339 (6120), 711–715.
Zimmermann, M., Lottersberger, F., Buonomo, S.B., Sfeir, A., de Lange, T., 2013. 53BP1 regulates DSB repair using Rif1 to control 5' end resection. Science 339 (6120), 700–704.
Kerscher, O., Felberbaum, R., Hochstrasser, M., 2006. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell Dev. Biol. 22, 159–180.
Reyes-Turcu, F.E., Ventii, K.H., Wilkinson, K.D., 2009. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. 78, 363–397.
Komander, D., Clague, M.J., Urbé, S., 2009. Breaking the chains: structure and function of the deubiquitinases. Nat. Rev. Mol. Cell Biol. 10, 550–563.
Nishi, R., 2017. Balancing act: To be, or not to be ubiquitylated. Mutat. Res. 803–805, 43–50.
Shanbhag, N.M., Rafalska-Metcalf, I.U., Balane-Bolivar, C., Janicki, S.M., Greenberg, R. A., 2010. ATM-dependent chromatin changes silence transcription in cis to DNA double-strand breaks. Cell 141 (6), 970–981.
Sharma, N., Zhu, Q., Wani, G., He, J., Wang, Q.-E., Wani, A.A., 2014. USP3 counteracts RNF168 via deubiquitinating H2A and γH2AX at lysine 13 and 15. Cell Cycle 13 (1), 106–114.
Nicassio, F., et al., 2007. Human USP3 is a chromatin modifier required for S phase progression and genome stability. Curr. Biol. 17, 1972–1977.
Wang, A., et al., 2017. USP22 Induces Cisplatin Resistance in Lung Adenocarcinoma by Regulating γH2AX-Mediated DNA Damage Repair and Ku70/Bax-Mediated Apoptosis. Front. Pharmacol. 8, 274.
Wang, Z., et al., 2016. USP51 deubiquitylates H2AK13,15ub and regulates DNA damage response. Genes Dev. 30, 946–959.
Mosbech, A., Lukas, C., Bekker-Jensen, S., Mailand, N., 2013. The deubiquitylating enzyme USP44 counteracts the DNA double-strand break response mediated by the RNF8 and RNF168 ubiquitin ligases. J. Biol. Chem. 288, 16579–16587.
Yu, M., et al., 2016. USP11 Is a Negative Regulator to γH2AX Ubiquitylation by RNF8/RNF168. J. Biol. Chem. 291, 959–967.
Zhang, Z., Yang, H., Wang, H., 2014. The histone H2A deubiquitinase USP16 interacts with HERC2 and fine-tunes cellular response to DNA damage. J. Biol. Chem. 289, 32883–32894.
Delgado-Díaz, M.R., Martín, Y., Berg, A., Freire, R., Smits, V.A.J., 2017. Dub3 controls DNA damage signalling by direct deubiquitination of H2AX. Mol. Oncol. 11, 1112.
Typas, D., et al., 2016. The de-ubiquitylating enzymes USP26 and USP37 regulate homologous recombination by counteracting RAP80. Nucleic Acids Res. 44, 2976.
Shao, G., et al., 2009. The Rap80-BRCC36 de-ubiquitinating enzyme complex antagonizes RNF8-Ubc13-dependent ubiquitination events at DNA double strand breaks. Proc. Natl. Acad. Sci. U. S. A. 106, 3166–3171.
Nishi, R., Wijnhoven, P., le Sage, C., Tjeertes, J., Galanty, Y., Forment, J.V., Clague, M.J., Urbé, S., Jackson, S.P., 2014. Systematic characterization of deubiquitylating enzymes for roles in maintaining genome integrity. Nat. Cell Biol. 16 (10), 1016–1026.
Matsui, M., Sakasai, R., Abe, M., Kimura, Y., Kajita, S., Torii, W., Katsuki, Y., Ishiai, M., Iwabuchi, K., Takata, M., Nishi, R., 2020. USP42 enhances homologous recombination repair by promoting R-loop resolution with a DNA-RNA helicase DHX9. Oncogenesis 9 (6). https://doi.org/10.1038/s41389-020-00244-4.
Lowndes, N.F., Toh, G.-L., 2005. DNA repair: the importance of phosphorylating histone H2AX. Curr. Biol. 15 (3), R99–R102.
Zhang, Z., et al., 2013. USP49 deubiquitinates histone H2B and regulates cotranscriptional pre-mRNA splicing. Genes Dev. 27, 1581–1595.
Harrigan, J.A., et al., 2011. Replication stress induces 53BP1-containing OPT domains in G1 cells. J. Cell Biol. 193, 97–108.
Daley, J.M., Sung, P., 2014. 53BP1, BRCA1, and the choice between recombination and end joining at DNA double-strand breaks. Mol. Cell. Biol. 34, 1380–1388.
Cerami, E., et al., 2012. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2, 401–404.
Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C., Schultz, N., 2013. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6 (269). https://doi.org/10.1126/scisignal.2004088.
Scully, R., Panday, A., Elango, R., Willis, N.A., 2019. DNA double-strand break repair-pathway choice in somatic mammalian cells. Nat. Rev. Mol. Cell Biol. 20, 698–714.
Chen, J., et al., 2020. 53BP1 loss rescues embryonic lethality but not genomic instability of BRCA1 total knockout mice. Cell Death Differ. 27, 2552–2567.
Xie, A., et al., 2007. Distinct roles of chromatin-associated proteins MDC1 and 53BP1 in mammalian double-strand break repair. Mol. Cell 28, 1045–1057.
Canny, M.D., et al., 2018. Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency. Nat. Biotechnol. 36, 95–102.
Botuyan, M.V., Lee, J., Ward, I.M., Kim, J.-E., Thompson, J.R., Chen, J., Mer, G., 2006. Structural basis for the methylation state-specific recognition of histone H4–K20 by 53BP1 and Crb2 in DNA repair. Cell 127 (7), 1361–1373.
Sanders, S.L., Portoso, M., Mata, J., Bähler, J., Allshire, R.C., Kouzarides, T., 2004. Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. Cell 119 (5), 603–614.
Ting, X., et al., 2019. USP11 acts as a histone deubiquitinase functioning in chromatin reorganization during DNA repair. Nucleic Acids Res. 47, 9721–9740.
Nakada, S., 2016. Opposing roles of RNF8/RNF168 and deubiquitinating enzymes in ubiquitination-dependent DNA double-strand break response signaling and DNA-repair pathway choice. J Radiat Res 57 (S1), i33–i40.
Moyal, L., et al., 2011. Requirement of ATM-dependent monoubiquitylation of histone H2B for timely repair of DNA double-strand breaks. Mol. Cell 41, 529–542.
Nakamura, K., et al., 2011. Regulation of homologous recombination by RNF20-dependent H2B ubiquitination. Mol. Cell 41, 515–528.
So, C.C., Ramachandran, S., Martin, A., 2019. E3 Ubiquitin Ligases RNF20 and RNF40 Are Required for Double-Stranded Break (DSB) Repair: Evidence for Monoubiquitination of Histone H2B Lysine 120 as a Novel Axis of DSB Signaling and Repair. Mol. Cell. Biol. 39 (8) https://doi.org/10.1128/MCB.00488-18.
Luo, K., et al., 2017. USP49 negatively regulates tumorigenesis and chemoresistance through FKBP51-AKT signaling. EMBO J. 36, 1434–1446.
Tu, R., et al., 2018. USP49 participates in the DNA damage response by forming a positive feedback loop with p53. Cell Death Dis. 9, 553.