



A Novel Reciprocal Crosstalk between RNF168 and PARP1 to Regulate DNA Repair Processes

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Emerging evidence has suggested that cellular crosstalk between RNF168 and poly(ADP-ribose) polymerase 1 (PARP1) contributes to the precise control of the DNA damage response (DDR). However, the direct and reciprocal functional link between them remains unclear. In this report, we identified that RNF168 ubiquitinates PARP1 via direct interaction and accelerates PARP1 degradation in the presence of poly(ADP-ribose) (PAR) chains, metabolites of activated PARP1. Through mass spectrometric analysis, we revealed that RNF168 ubiquitinated multiple lysine residues on PARP1 via K48-linked ubiquitin chain formation. Consistent with this, micro-irradiation-induced PARP1 accumulation at damaged chromatin was significantly increased by knockdown of endogenous RNF168. In addition, it was confirmed that abnormal changes of HR and NHEJ due to knockdown of RNF168 were restored by overexpression of WT RNF168 but not by reintroduction of mutants lacking E3 ligase activity or PAR binding ability. The comet assay also revealed that both PAR-binding and ubiquitin-conjugation activities are indispensable for the RNF168-mediated DNA repair process. Taken together, our results suggest that RNF168 acts as a counterpart of PARP1 in DDR and regulates the HR/NHEJ repair processes through the ubiquitination of PARP1.

Keywords: DNA repair, PARP1, PARylation, RNF168, ubiquitination

INTRODUCTION

DNA damage response (DDR) is a specialized cellular defense system to repair damaged chromatin arising from various intrinsic or extrinsic risk factors, including oxidative stress, ionizing radiation, DNA replication errors, and DNA damage-inducing reagents. DNA double-strand breaks (DSBs) are the most toxic form of genomic DNA damage. The defects of the DNA repair process have been linked to various human diseases, including cancers or neurodegenerative disorders (Kulkarni and Wilson, 2008; Lord and Ashwort, 2012; Rass et al., 2007). During DDR, DSBs are promptly recognized by master regulatory proteins, namely, ataxia telangiectasia mutated (ATM) or poly-ADP ribose polymerase 1 (PARP1). At damaged chromatin, ATM is activated and recruited by the MRN complex (Mre11-NBS1-Rad50) to DNA breaks (Ciccia and Elledge, 2010; Uziel et al., 2003; You et al., 2005). Many DDR-related proteins, such as histone H2AX, were shown to be phosphorylated by ATM to amplify the repair signal (Polo and Jackson, 2011). In parallel with this, PARP1 also rapidly recognizes DNA insults and immediately activates itself to induce poly(ADP-ribose) (PAR) synthesis, thereby triggering the PARylation of substrates or the generation of multi-branched free PAR, which are non-covalently attached to substrates.

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PARYlation is an important post-translational modification, which regulates diverse intracellular signals, such as the DDR process, transcriptional regulation, RNA interference, cell division, cell survival, and death (Gibson and Kraus, 2012; Luo and Kraus). In particular, the PAR chain binds to and modifies many DDR-associated proteins for efficient DNA repair (Polo and Jackson, 2011; Wei and Yu, 2016). Consequently, ATM and PARP1 can recruit a number of DDR-associated factors, such as ubiquitin E3 ligases (E3s), 53BP1, MDC1, and BRCA1, onto chromatin surrounding DSB sites for the precise control of DDR. Among them, E3s catalyze the ubiquitination of substrates and are engaged in the assembly of repair enzymes (Schwertman et al., 2016).

Recently, it has been reported that two RING-type E3s, RNF8 and RNF168, play important roles in the cellular response to DDR by modifying H2A and its variant H2AX. The ubiquitinated H2A/H2AX during DDR is the most crucial signal for the recruitment of downstream effector proteins in DNA lesions. Therefore, RNF168-dependent ubiquitination is a vital element in the DNA repair pathway (Gatti et al., 2015; Mattioli et al., 2012). In contrast, RNF168-mediated signaling amplification can be turned off by TRIP12/UBR5, which are E3s that lead to the degradation of RNF168 protein for the fine-tuning of DDR. TRIP12/UBR5 determine the size of the RNF168 nuclear pool, suppressing the excessive spread of ubiquitination to undamaged chromosomes in the vicinity of DNA lesions (Gudjonsson et al., 2012). Interestingly, TRIP12 contains a WWE domain, which is a well-known PAR-binding motif (Gibson and Kraus, 2012). There is another route of DDR, wherein the RNF168-associated DDR cascade is regulated by SMARCA5/SNF2H in a PARP1-dependent manner, promoting RNF168 accumulation at DSBs for efficient ubiquitin conjugation (Smeenk et al., 2013). It has also been revealed that RNF168 is PARYlated by PARP1 at DNA lesions (Smeenk et al., 2013). Taken together, these findings suggest that RNF168 is controlled by PARP1 at damaged chromatin.

Although RNF168-coupled DDR is ultimately connected to ATM and RNF8 activation, it is not clear how they crosstalk with the PAR-signaling pathway at DNA lesions. Here, we show that RNF168 has PAR-binding ability and that this ability is required for the robust ubiquitination of PARP1 to accelerate its degradation via the proteasome, thereby orchestrating DNA repair.

MATERIALS AND METHODS

Cell lines and siRNA

HeLa and HEK293FT cell lines were purchased from ATCC, and these lines were maintained in DMEM or MEM supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO), respectively. U2OS-based DR-GFP and EJ5-GFP cells were kindly provided by Dr. Jeremy Stark; they were cultured with DMEM containing 10% FBS and 1 µg/ml puromycin (Sigma-Aldrich). siRNA was transfected into cells using Lipofectamine RNAiMAX (Invitrogen), with the following sequences: sc_{ctrl} sense sequence, CCUCGUGCCGUCCAUCAGGUAG; siRNF168 sense sequence, GTTTAGTCGGCTTACTAA.

Plasmids and antibodies

To generate point mutants of RNF168, mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene). Truncation mutants of RNF168 were constructed by a classical PCR method. All mutation regions were confirmed by DNA sequencing analysis. pDEST53 or pDEST15 vector was used for subcloning of RNF168 wild type (WT) and its mutants to generate GFP or GST fusion proteins, respectively. Antibodies used for immunoblot analysis and immunocytochemistry were as follows: anti-RNF168 from R&D Systems; anti-histone H4 and anti-β-actin from Abcam; anti-ubiquitin from Dako; anti-GST from GE Healthcare; anti-HA from Thermo Fisher; anti-PARP1 from BD Biosciences, and anti-PAR from Trevigen.

Laser micro-irradiation and immunocytochemistry

HeLa cells were plated onto glass-bottomed culture dishes (SPL Lifescience) and transfected with target plasmids. Cells were presensitized with 10 µM 5-bromo-2'-deoxyuridine (BrdU, Sigma) for 30 h and then subjected to laser micro-irradiation using a confocal microscopy (Nikon A1) in the setting of 405 nm wave length during 3 s (32 lines/sec) at 37°C chamber supplying 5% CO₂. After laser treatment, cells were fixed with 4% PFA for 10 min at room temperature and washed three times with PBS. Subsequently, cells were permeabilized with 0.25% Triton X-100 in PBS for 15 min at room temperature and blocking with 1% BSA in PBS for 30 min. Primary antibody was incubated for 18 hr at 4°C. Cells were then washed and next incubated with secondary antibody for 60 min at room temperature. After washed, nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min and each well was mounted onto 1.2 mm glass slides using Vectashield mounting medium (Vector Labs).

Homologous recombination (HR) and non-homologous end joining (NHEJ) analysis

HR or NHEJ efficiency was measured by U2OS-DR-GFP (HR) or U2OS-EJ5-GFP (NHEJ) reporter cell lines, respectively. U2OS-DR-GFP and U2OS-EJ5-GFP cells were transfected with RNF168-targeting siRNA. The following day, I-SceI and siRNA-resistant V5-RNF168 constructs were delivered to each reporter cell, and 72 h later, they were assayed for GFP-positive by the flow cytometry.

Neutral comet assay

To monitor extent of DNA damage from individual cells, cells were transfected with 40 µM of siRNAs alone or in combination with siRNA-resistant plasmid. After 48 h, each cell was treated with 40 µg/ml Zeocin for 2 h and then washed three times with PBS. Next, these cells were further incubated for 2 h in the medium without Zeocin. The neutral comet assay was performed in accordance with the manufacturer's protocol, and the tail moments were measured using OpenComet V1.3 software.

Purification of recombinant protein from *Escherichia coli*

To purify GST-fusion proteins from *E. coli*, pENTRY vector of RNF168 were subcloned into pDEST15 vector using the

Gateway LR cloning system (Invitrogen). Generated pDEST15-RNF168 vectors were transformed into the *E. coli* BL21-Gold (DE3) strain (Agilent). All GST fusion proteins were purified by sonication method. Glutathione-Sepharose4B resin (GE Healthcare Life Science) binding proteins were eluted with reduced glutathione containing elution buffer (50 mM HEPES, pH 7.5, 40 mM reduced glutathione, 100 mM NaCl, 30% glycerol, and 0.03% Triton X-100).

PAR overlay assay

For the PAR overlay assay, each NC membrane was blocked with 5% skim milk (BD Bioscience) in PBS-T (0.05% Tween 20). After blocking, the membrane was incubated for 2 h at room temperature with PAR polymer and PAR-binding proteins were detected by anti-PAR antibody. Immunoblots were visualized in X-ray films (AGFA) by an ECL method (Thermo Scientific).

In vitro ubiquitination assay

To measure the ubiquitination activity of PARP1 by RNF168, 50 nM E1, 50 nM UbcH5c, E3 (WT or mutants of GST-RNF168), and 1 unit of PARP1 were incubated with 200 mM ubiquitin at 37°C in reaction buffer containing 50 mM Tris-Cl (pH 7.5), 2.5 mM MgCl₂, 2 mM DTT, and 2 mM ATP. ubiquitinated proteins were detected by immunoblot with anti-ubiquitin antibody. All proteins were separately visualized by Coomassie Brilliant Blue (Bio-Rad). Recombinant E1, UbcH5c and ubiquitin were purchased from Boston Biochem.

Chromatin fractionation

Cells were harvested and lysed in NETN buffer (50 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, and 5 mM EDTA) with protease and phosphatase inhibitors. The lysate was sonicated and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was measured by Bradford assay and the equal amount of protein lysate was separated SDS-PAGE.

Clonogenic survival assay

Clonogenic viability was examined using a colony forming assay. Cells were transfected with the siRNA and siRNA-resistant DNA, 48 h later, cells were harvested and seeded using the appropriate number on a 6-cm dish. The following day, cells were treated with Zeocin (0-50 µg/ml) for 2 h and then washed with PBS. Next, cells were further incubated in the medium without Zeocin for 14 days. Resulting colonies were fixed with methanol and stained with 0.5% Crystal violet (Sigma). Colonies were counted and normalized to plating efficiencies.

Mass spectrometry

To analysis of ubiquitin linkages for ubiquitinated PARP1, ubiquitinated PARP1 sample by RNF168 was subjected to SDS-PAGE. The gels were stained with Coomassie Brilliant Blue (Bio-Rad). Mass spectrometric analysis was performed by the Biological Mass Spectrometry Facility.

Statistical analysis

Graphs were created, and statistics were calculated using

Prism software (GraphPad). One-way analysis of variance (ANOVA) was used followed by Tukey-Kramer's *post hoc* test. Data represents means ± s.d. or s.e.m. *P* < 0.05 was considered statistically significant.

RESULTS

RNF168 is a PAR-binding ubiquitin E3 ligase

It has been reported that the PARylation of RNF168 is crucial for the sequential recruitment of chromatin-remodeling factors to DNA break sites (Smeenk et al., 2013). This finding suggests that the covalent conjugation of PAR to RNF168 is an essential step for the downstream signaling cascade of DDR. However, the mechanism underlying this remains unclear. To investigate this issue, a PAR overlay assay was performed with GST-fused RNF168 and PAR (Fig. 1A). Intriguingly, we observed that GST-RNF168 and histone H4, known as PAR-binding proteins, strongly bind to PAR in a non-covalent manner, whereas while GST protein failed to bind to PAR. To identify the region of RNF168 that associated with PAR, we generated two deletion mutants of RNF168: the N-terminal region of RNF168 (N; amino acids 1-196), which contains a zinc finger, the first MIU1, UIM, and the first LR motif; and the C-terminal region of RNF168 (C; amino acids 197-572), which includes the second MIU2 and the second LR motif (Fig. 1B). RNF168 and its mutant proteins were purified from *E. coli* and then applied to a PAR overlay assay under either non-denaturing or denaturing conditions (Figs. 1C and 1D). As expected, we observed that the C-terminal domain of RNF168 strongly interacted with PAR in both experimental conditions. To characterize the PAR-binding motif on the C-terminal of RNF168, potential PAR-binding sites were screened by a comparative sequence analysis with known classical PAR-binding sequences (Fig. 1E). Based on the sequence similarity, we generated various point mutants against the WT of RNF168 and tested their PAR-binding ability using a PAR overlay assay (Fig. 1F). Finally, we identified that lysine residues in positions 213 and 492 are necessary for the PAR-binding ability of RNF168 because the mutations of lysine 213/492 to alanine (K213A/K492A, referred to hereafter as KK/AA) led to a failure of PAR to bind with RNF168. These findings suggest that RNF168 strongly binds to PAR via its K213 and K492 residues in a non-covalent manner.

RNF168 targets PARP1 for ubiquitination-mediated degradation

Because RNF168 interacts with PAR, we surmised that RNF168 has the ability to bind to PAR-modified proteins, such as PAR-bound and/or PARylated proteins. PARP1 is a major synthesizer of PAR and is well known to be the major PARylated protein under conditions of DNA insult. Thus, to investigate whether RNF168 ubiquitinates PARP, we performed an *in vitro* ubiquitination assay. Remarkably, we observed that PARP1 is robustly ubiquitinated by RNF168 but not by E3 ligase-dead mutant (ED), which lacks the ubiquitin-conjugating activity of RNF168 (Figs. 2A and 2B). We also found that the PAR-binding ability of RNF168 does not affect PARP1's ubiquitination in the setting of the *in vitro*

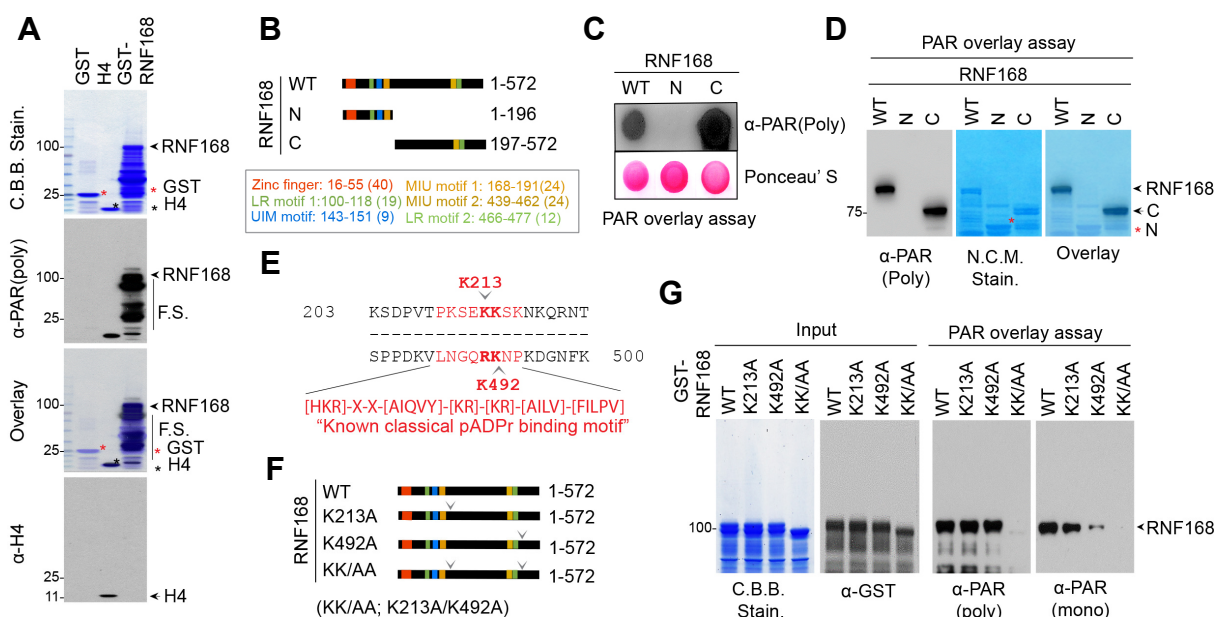


Fig. 1. RNF168 is a PAR-binding ubiquitin E3 ligase. (A) Recombinant GST-RNF168 was loaded onto SDS-PAGE and then transferred to the NC membrane, followed by PAR overlay assay. PAR-binding activity of RNF168 was analyzed by immunoblotting with anti-PAR antibody. GST or H4 was used negative or positive control, respectively. Black and red star indicate GST and H4 protein, respectively. (B) Construction of RNF168 deletion mutants. (C, D) PAR overlay assay was performed with WT, N terminal (N)-, and C terminal (C)-mutants of RNF168 in setting of non-denaturing (C) or denaturing condition (D). (E) Potential PAR-binding sites of RNF168 were analyzed in comparison with the classical PAR-binding motif as indicated. Gray arrowheads indicate postulated PAR-binding residues of RNF168 (F) To validate PAR binding activity of RNF168, its point mutants were generated by a site-direct mutagenesis as indicated. Gray arrow indicates the mutated sites of RNF168 (G) PAR overlay assay was performed along with WT and point mutants of RNF168 as indicated. F.S represents fragment spectrum of GST-RNF168.

ubiquitination assay (Fig. 2B). However, we could not rule out the possibility that the *in vitro* conditions differ from those *in vivo* (intracellularly). To address this point, we confirmed PARP1's ubiquitination by RNF168 in HeLa cells. To this end, we first tested the knockdown efficacy of siRNAs targeting endogenous RNF168 and found that endogenous RNF168 is significantly diminished by siRNA overexpression, whereas siRNA-resistant RNF168 was still expressed well in a setting of siRNA overexpression (Fig. 2C). Following this, we examined whether the knockdown and/or overexpression of RNF168 affects PARP1 stability. The PARP1 level was slightly decreased by RNF168 WT and not ED and KK/AA mutants in total cell lysates (Fig. 2D). To further confirm this result, we monitored the protein level of PARP1 from the chromatin fraction under the same experimental conditions. As expected, we observed that the PARP1 level drastically decreased by RNF168 overexpression (Fig. 2E), suggesting that PARP1 is a genuine target of RNF168 in the PAR-coupled DDR path. To determine the molecular mechanism underlying RNF168-mediated PARP1 degradation, chromatin-bound PARP1, which was derived from damaged chromatin by treatment with Zeocin, was subjected to an *in vitro* ubiquitination assay along with recombinant WT or ED or KK/AA mutants of RNF168. The results showed that RNF168 WT strongly induces PARP1 ubiquitination, but the ED and KK/AA mutants did not. This suggests that the interplay

between PAR and RNF168 is critical for the ubiquitination of PARP1 at damaged chromatin (Fig. 2F). Next, we examined whether ubiquitinated PARP1 is degraded by the ubiquitin-proteasome system (UPS). Cells expressing RNF168 WT were treated with MG132, a proteasome inhibitor, and the level of PARP1 protein was monitored. As expected, we observed that the PARP1 downregulated by RNF168 was perfectly restored with MG132, suggesting that UPS is a major pathway controlling the PARP1 level in cells (Fig. 2G). The mass spectrometric analysis revealed that RNF168 utilizes K11, K48, and K63 ubiquitin linkages for PARP1 ubiquitination (Fig. 2H). Taken together, these results indicated that PAR-associated RNF168 not only induces the ubiquitination of PARP1 at damaged chromatin but also accelerates UPS-mediated PARP1 degradation through robust ubiquitination in a PAR-binding-dependent fashion.

RNF168 regulates PARP1 levels at DNA damage sites

To reveal the correlation between the PAR-binding ability of RNF168 and PARP1 at DNA damage sites, we monitored the recruitment of RNF168 to DNA lesions using a live micro-irradiation (mIR) system in the presence of the PARP1 inhibitor (PJ34) or ATM inhibitor (KU55933). Interestingly, we observed that RNF168 was recruited to laser strips in an ATM-dependent manner (Figs. 3A and 3B), and the KK/AA mutant of RNF168 was recruited to DNA breaks in a manner

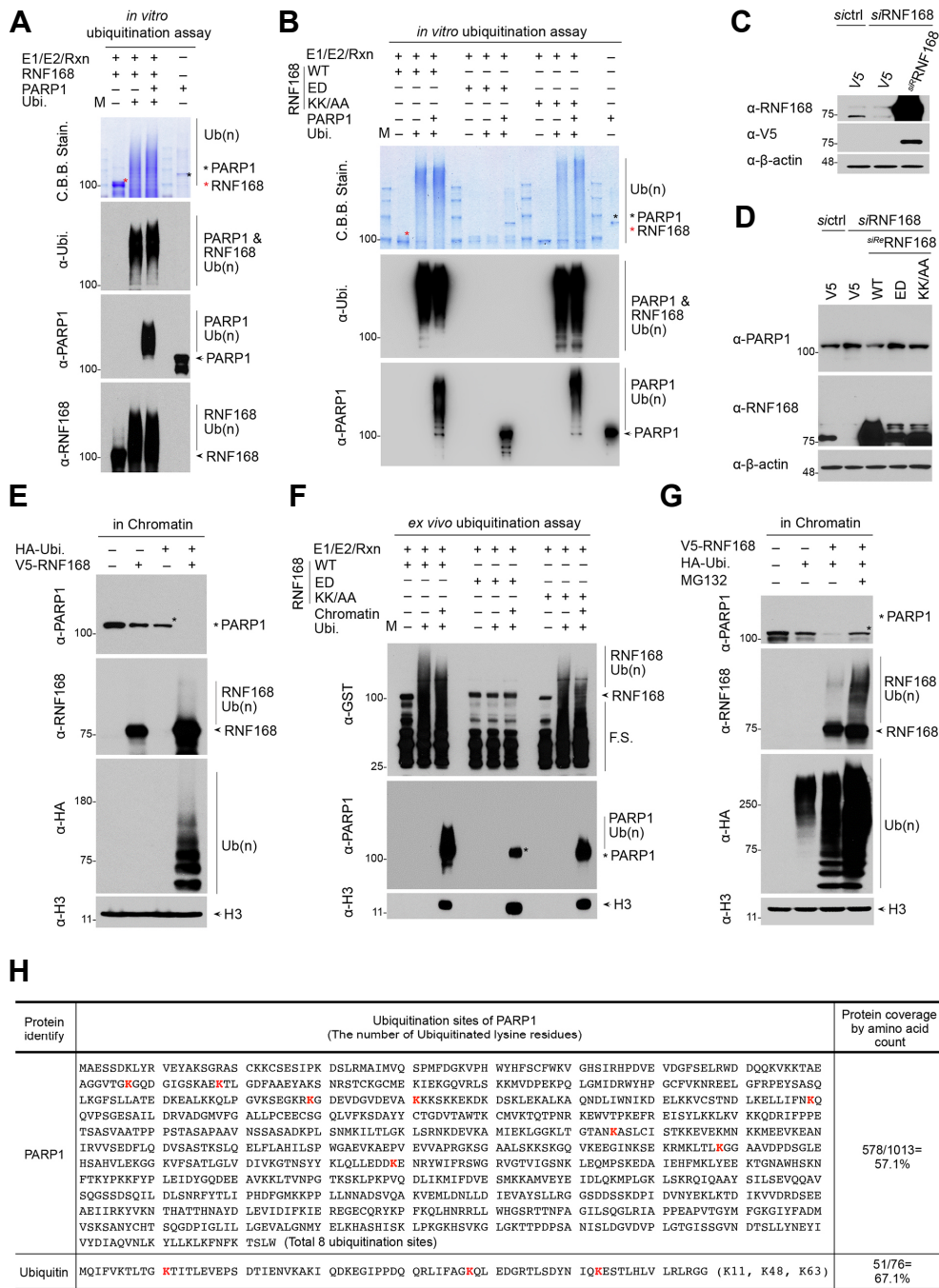


Fig. 2. RNF168 ubiquitinates PARP1 for its proteasomal degradation in the context of chromatin. (A) Recombinant PARP1 and RNF168 were subjected into *in vitro* ubiquitination assay as indicated. Samples were analyzed by immunoblotting with indicated antibodies. (B) Recombinant PARP1 was subjected into *in vitro* ubiquitination assay along with RNF168 WT or its point mutants as indicated. Black and red star indicate PARP1 and RNF168, respectively. (C) Validation of siRNAs and siRNA resistant (siRe) mutants of RNF168. (D) The intracellular level of PARP1 was monitored by transfection with RNF168 WT and its mutants as indicated. (E) The change of PARP1 level in the chromatin fraction was analyzed by overexpression with HA-ubiquitin and RNF168 as indicated. Black star indicates endogenous PARP1. (F) Chromatin fraction was subjected into *in vitro* ubiquitination assay as indicated. RNF168 mediated PARP1's ubiquitination was monitored by immunoblotting with anti-GST and PARP1 antibodies. Black star indicates intact PARP1 in the chromatin fraction. F.S. represents fragment spectrum of GST-RNF168 (G) RNF168 induces PAR dependent degradation of PARP1 by ubiquitination in a chromatin context-dependent manner. PARP1's protein level regulated by RNF168 was monitored in absence or presence of MG132 as indicated. (H) RNF168 mediated PARP1's ubiquitination sites were analyzed by a mass-spectrometry analysis. Bold type K letters as marked red color indicate ubiquitinated lysine residues on PARP1 or ubiquitin. Ub(n), denotes polyubiquitinated chains of target proteins; RNF168 Ub(n) or PARP1 Ub(n) represents ubiquitinated RNF168 or PARP1, respectively.

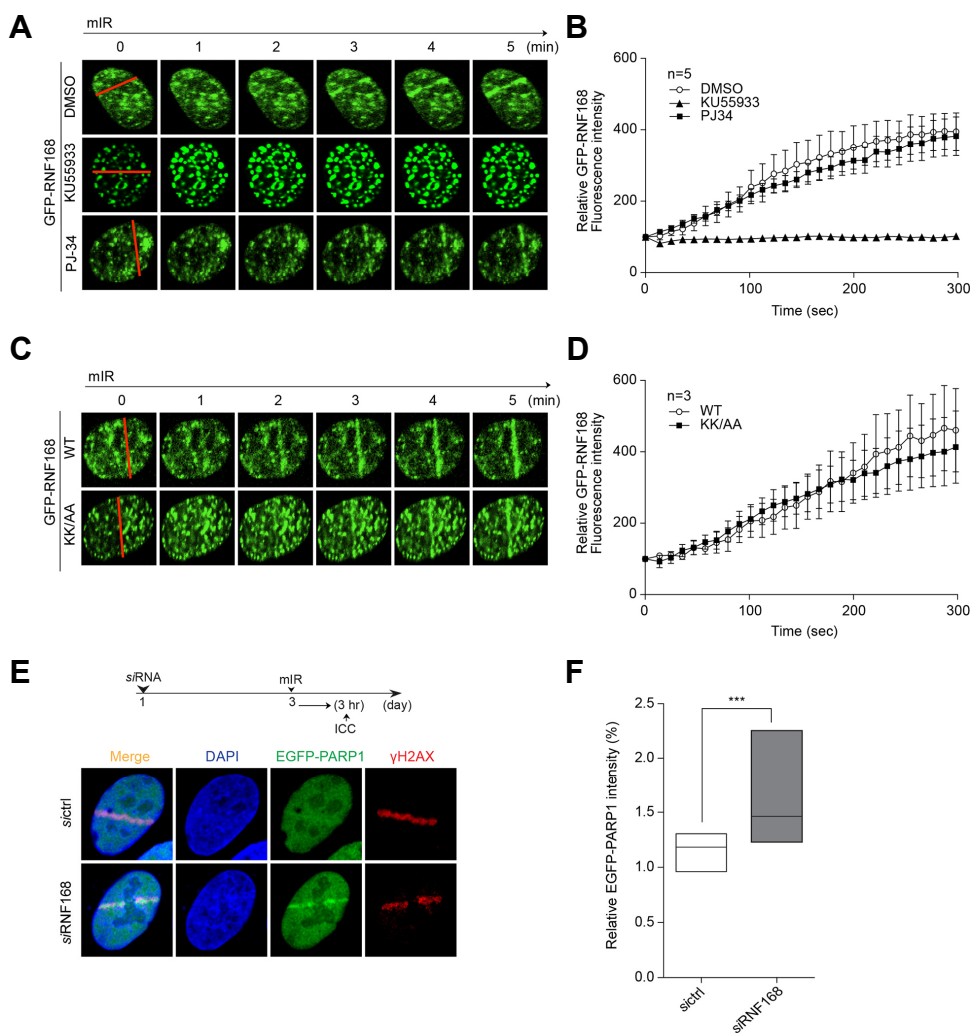


Fig. 3. RNF168 regulates the protein level of PARP1 at the damaged chromatin. (A, B) RNF168 was recruited to DNA lesions in ATM-dependent manner. HeLa cells expressing GFP-RNF168 were subjected into a laser micro-irradiation in the presence of PARP1 or ATM inhibitor as indicated. The GFP intensity representing accumulated PARP1 or ATM at the damaged chromatin was measured by Nikon NIS elements program. Data represent mean \pm s.e.m., from five cells. (C, D) PAR-binding ability of RNF168 did not affect its translocation to DNA lesions. (E, F) The protein level of PARP1 in damaged chromatin was controlled by RNF168. EGFP-PARP1 and indicated siRNAs (*siCtrl* or *siRNF168*) were transfected into HeLa cells and then subjected into a laser micro-irradiation. After 3 h, cells were fixed and stained with indicated antibodies. Data represent mean \pm s.d., from 30 cells; * $P < 0.05$. Straight-red lines indicate the laser-induced damaged areas.

similar to that of the WT (Figs. 3C and 3D). These results indicated that PAR does not affect RNF168 translocation to sites of DNA damage. Thus far, we revealed that RNF168 strongly interacts with PAR and accelerates the degradation of PARP1 via its PAR-binding ability. Next, we further analyzed the steady-state level of PARP1 at damaged chromatin induced by mIR and observed that the level of PARP1 dramatically increased at the laser strips in a setting of RNF168 knockdown, in comparison with that in cells expressing control siRNA (Figs. 3E and 3F). Taken together, these results indicated that RNF168 moves to DNA lesions in a PAR-independent manner but controls PARP1's stability at damaged chromatin in a PAR-dependent fashion.

Regulation of PARP1 by RNF168 is critical for DNA damage repair and cell viability

To investigate whether PARP1's degradation by RNF168 is necessary for the DNA damage repair process, we assessed the DNA repair efficiency by HR/NHEJ repair analysis and a comet assay. HR and NHEJ are major, well-known mechanisms for DSB repair. Using verified RNF168-targeted siRNA and siRNA-resistant constructs, we measured the repair efficiency achieved by RNF168 and revealed that both E3 ligase and the PAR-binding activity of RNF168 are critical for HR and NHEJ repair efficiency (Figs. 4A and 4B). To validate these observations, we performed a comet assay after inducing DNA damage by applying treatment with Zeocin. RNF168

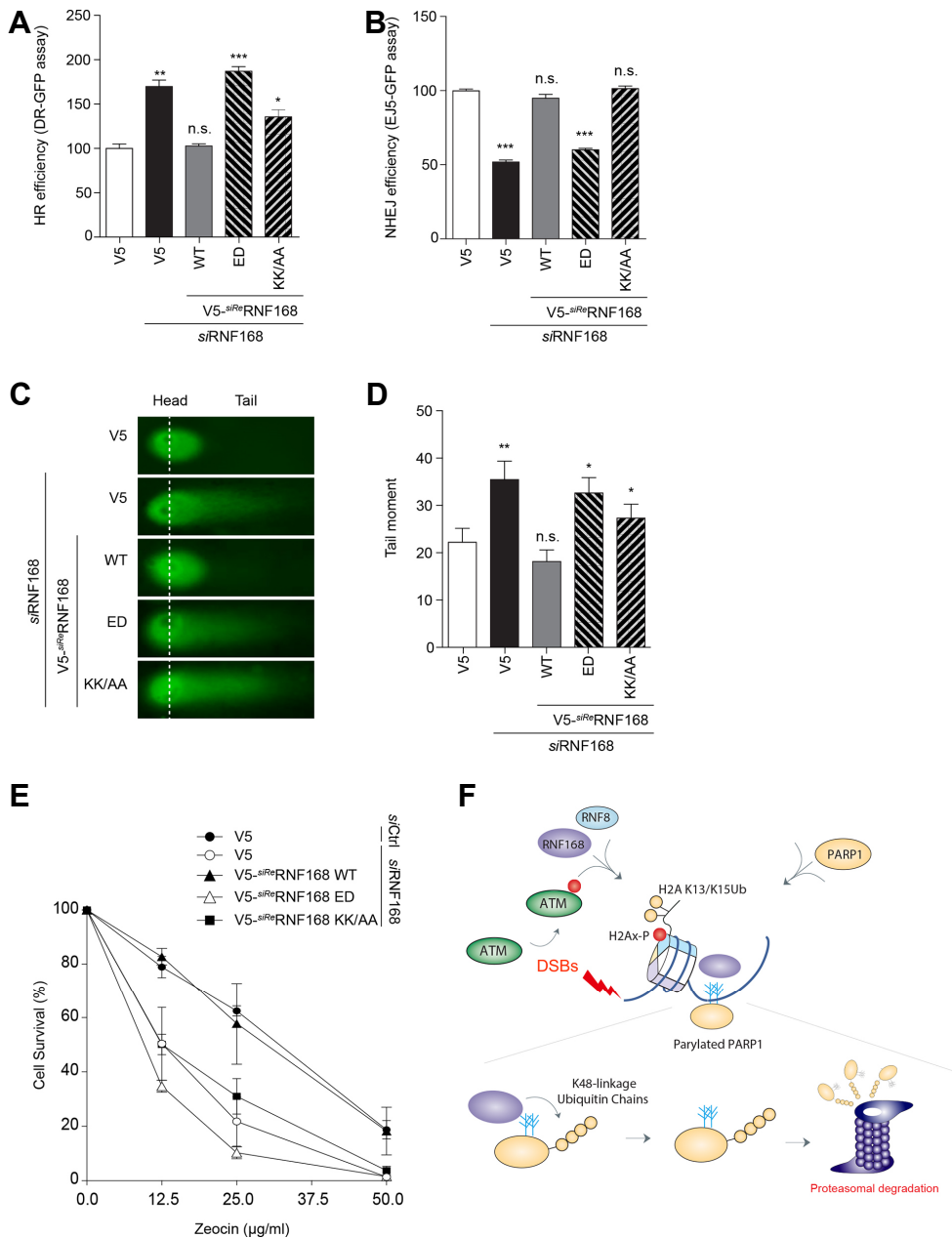


Fig. 4. RNF168 mediated PARP1 degradation in the chromatin context is a key step to forward DNA repair process. (A, B) RNF168-targeted siRNA and siRNA-resistant constructs were transfected into HR- or NHEJ- reporter cell lines with I-SceI as indicated. After 72 h, GFP-positive cells were counted by FACS analysis. (C, D) The genomic stability regulated by RNF168 mediated PARP1's degradation was monitored by neutral comet assay in the same experimental condition as outlined above. The intensity of head, and length of tail were analyzed by Open-Comet V1.3 software. Data was achieved from at least 100 cells per sample. (E) Clonogenic analysis. RNF168 WT, ED or KK/AA mutant was transfected into U2OS cells depleted endogenous RNF168 and then colony forming assay was performed as indicated. (F) Summary figure of all observed results. Data represent mean \pm s.d., from three independent experiments; * $P < 0.05$. n.s., not significant.

knockdown remarkably increased the comet tail length compared with that in control siRNA-transfected cells, and this increase in the comet tail was completely abolished by siRNA-resistant RNF168 WT. In contrast, ED or KK/AA mutants of RNF168 failed to recover the comet tail length in RNF168 knockdown cells (Figs. 4C and 4D). Furthermore,

Zeocin-inducing clonogenic survival assay revealed that the KK/AA mutant failed to rescue RNF168 knockdown-mediated cell death (Fig. 4E). These findings indicate that the ability of RNF168 to induce PARP1 degradation is essential for an efficient DNA repair process to maintain genomic integrity under physiological conditions.

DISCUSSION

Recently, it has been reported that the level of PAR gradually decreases after DNA repair for the fine-tuning of DDR. However, when PAR is highly accumulated in cells, it leads to cell death, termed parthanatos (Andrabi et al., 2011; David et al., 2009; Lee et al., 2013). Intriguingly, it has also been reported that the PAR level is sustained in ATM-deficient cells (GM09607, GM03189), but not in control cells (Haince et al., 2007). In addition, fibroblasts derived from an ataxia telangiectasia (A-T) patient harboring ATM mutations showed increased PARP1 activity (58-96%) compared with the level in normal individuals, so these cells finally undergo cell death. It has been suggested that PARP1's hyperactivation triggers parthanatos in ATM-deficient cells. Indeed, the proportion of surviving A-T fibroblasts was dramatically increased by treatment with the PARP inhibitor (Marecki and McCord, 2002). These results suggest that PARP1 is negatively correlated with ATM. However, it remains unclear how ATM cross-talks with PARP1 in DDR.

Here, we identified a new PAR-binding E3, RNF168, which is known to be a major downstream effector of ATM in DDR. Intriguingly, we found that RNF168 has a classical PAR-binding motif in its C-terminal region (K219 and K492A), and its PAR-binding ability is critical for PARP1's degradation via ubiquitination at damaged chromatin (Fig. 4F). Our findings strongly suggest that the ATM pathway is a counterpart for PARP1-linked DDR because ATM-coupled RNF168 accelerates the degradation of PARP1 in a PAR-dependent fashion. However, this has raised two major questions. The first question is how cells maintain the balance of RNF168 and PARP1 proteins in the DDR. In the present study, we found that RNF168 induces the ubiquitination of PARP1 in the absence of PAR, although it led to the robust ubiquitination of PARP1 in the absence of PAR *in vitro*. In cells, however, RNF168 only ubiquitinates PARP1 in a PAR-dependent fashion, suggesting that the RNF168-mediated ubiquitination of PARP1 occurs only at sites where PAR has accumulated, thereby precisely controlling the level of PAR-associated PARP1 but not intact PARP1. One possible explanation for this differential dependency of *in vitro* and *in vivo* RNF168-mediated PARP1 ubiquitination on PAR-binding ability of RNF168 is that *in vitro* ubiquitination setting, i.e. at high enzyme (RNF168) and substrate (PARP1) concentration, can promote ubiquitination reaction without PAR-dependent binding to PARP1 of RNF168. The other reason for the *in vitro* PAR-independency can be that RNF168 may interact with PARP1 with very low affinity in absence of PAR, thereby allows RNF168 mediated PARP1's ubiquitination *in vitro*. However, *in vivo* condition, where the number of RNF168, PARP, or both available can be limited, the RNF168's PAR-binding acts as modular element to recognize and bind to PARylated or PAR binding PARP1. The last question is why RNF168 should crosstalk with both ATM and PARP1. Upon DNA damage condition, ATM-MDC1-RNF8 pathway contributes to RNF168 recruitment in an ubiquitination-dependent fashion. Its ubiquitin interaction motif serves as a binding module to ubiquitinated histones at the damaged chromatin. Thus, RNF168 recruitment at sites of damaged

DNA depends on its ubiquitin interaction but not PAR-binding activity. However, when RNF168 ubiquitinates the substrates, it recognizes, binds, and ubiquitinates the PARylated or PAR binding substrates. In this study we showed that PARP1 is one of the PARylated or PAR binding substrates, and RNF168 mediated PARP1's ubiquitination at the DNA lesions could promote PARP1 degradation. These results allow us to interpret that the PAR moiety may be able to confer specificity for substrate selection of RNF168 in DNA lesions, thereby coordinating DDR spatially or temporally. Therefore, degradation of PARP1 by RNF168 during DDR induces a change in the amount of PARP1 in a PAR dependent manner, which may affect not only DNA repair process but also cell survival or death. Further, from a clinical perspective, these findings also imply that an inhibitor that blocks PARP1's activation may be useful for the treatment of ATM and/or RNF168-associated diseases.

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