Post-Translational Regulation of the RSF1 Chromatin Remodeler under DNA Damage

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Chromatin remodeling factors are involved in many cellular processes such as transcription, replication, and DNA damage response by regulating chromatin structure. As one of chromatin remodeling factors, remodeling and spacing factor 1 (RSF1) is recruited at double strand break (DSB) sites and regulates ataxia telangiectasia mutated (ATM) -dependent checkpoint pathway upon DNA damage for the efficient repair. RSF1 is overexpressed in a variety of cancers, but regulation of RSF1 levels remains largely unknown. Here, we showed that protein levels of RSF1 chromatin remodeler are temporally upregulated in response to different DNA damage agents without changing the RSF1 mRNA level. In the absence of SNF2h, a binding partner of RSF1, the RSF1 protein level was significantly diminished. Intriguingly, the level of RSF1-3SA mutant lacking ATM-mediated phosphorylation sites significantly increased, and upregulation of RSF1 levels under DNA damage was not observed in cells overexpressing ATM kinase. Furthermore, failure in the regulation of RSF1 level caused a significant reduction in DNA repair, whereas reconstitution of RSF1, but not of RSF1-3SA mutants, restored DSB repair. Our findings reveal that temporal regulation of RSF1 levels at its post-translational modification by SNF2h and ATM is essential for efficient DNA repair.

Keywords: ATM, DNA double-strand breaks and repair, protein stability, RSF1, SNF2h

INTRODUCTION

In response to DNA damage, DNA damage checkpoint signaling pathways are activated to transduce the signals and to arrest the cell cycle to allow the repair of the broken DNA. Three PI3-kinases are the major transducers, namely ataxia-telangiectasia mutated (ATM) kinase, ATM and Rad3-related (ATR) kinase, and DNA-dependent protein kinase (DNAPK). These kinases phosphorylate their substrates and recruit repair proteins. Two types of DNA repair pathways are well-known; homologous recombination (HR) and non-homologous end joining (NHEJ). HR occurs mostly from the S to G2 phase, whereas NHEJ occurs at the G1 phase. DNA repair and checkpoint pathways are significant in cells as the unrepaired DNA may cause genomic instability, which may lead to the diseases in humans (Ciccia and Elledge, 2010; Jackson and Bartek, 2009).

In order to maintain genomic stability, in human, DNA is wrapping up the histones forming nucleosome, and chromatin remodeling factors exist to regulate the chromatin structure. In general, chromatin remodeling factors regulate the chromatin structure during transcription and differentiation in eukaryotes (Nair and Kumar, 2012). Recently, it has been reported that chromatin remodeling factors play an important role in response to DNA damage for providing access to DNA repair proteins to the site of the broken DNA (Lans et al., 2012; Osley et al., 2007).

There are four conserved families of chromatin remodeling factors in humans; the switch/sucrose non-fermentable (SWI/SNF), the Imitation SWItch (ISWI) ISWI, the chromo-domain-helicase DNA binding protein (CHD), and the INO80 families. Among these families, the ISWI family includes the common ATPase SNF2h, which has different types of binding partners such as RSF1, Williams syndrome transcription factor (WSTF), and ATP-dependent chromatin assembly factor 1 (ACF1). The RSF complex is an ISWI family members, and comprises RSF1 and SNF2h (Aydin et al., 2014; Nair and Kumar, 2012). Previously, published clinical research has reported that RSF1 is overexpressed in various cancers, and that its overexpression is correlated with poor prognosis for overall survival (Maeda et al., 2011; Shih Ie et al., 2005; Tai et al., 2012; Zhang et al., 2017). Furthermore, overexpression of RSF1 activates the DNA damage signaling pathway by activating the ATM-Chk2 kinases (Sheu et al., 2010). Recently, overexpression of RSF1 showed a positive correlation with p53 levels, and this combined expression of RSF1 and p53 was correlated to tumor size and tumor, node, metastasis (TNM) staging in breast cancer patients (Ren et al., 2014). Meanwhile, RSF1 has been reported to facilitate DNA damage response (DDR) signaling and repair. Previously, other groups and we have reported that RSF1 facilitates ATM-dependent checkpoint signaling and double strand break (DSB) repair (Helfricht et al., 2013; Min et al., 2014; Pessina and Lowndes, 2014). Thus, the temporal regulation of RSF1 levels is important for cell homeostasis upon DNA damage to prevent endogenous DNA damage in cells and to facilitate DSB repair in response to DNA damage. In this paper, we reveal that RSF1 level is temporally regulated upon DNA damage by the formation of the RSF complex with SNF2h and its phosphorylation via ATM kinase. In addition, we suggest that the misregulation of RSF1 level causes impaired DSB repair.

MATERIALS AND METHODS

Cell lines and drug treatment
MCF7, 293T, EJ, and U2OS cell lines were cultured in DMEM high glucose (HyClone) supplemented with 10% FBS. The MCF7, U2OS, and EJ cells were treated with DNA damaging drugs, such as phleomycin (50 µg/ml), etoposide (10 nM) and methyl methanesulfonate (MMS; 0.02% and 0.04%). Irradiation was treated with 10 Gray. The cells were harvested at each time points with 1x sample buffer and were subjected to Western blot analysis.

Site-directed mutagenesis
The primers used and procedures were previously described (Min et al., 2014).

Transfection
Cells were harvested after transfecting Flag-ATM and RSF1-GFP using lipofectamine 2000 (Invitrogen) and treating the cells with phleomycin for 2 h or irradiation (10 Gy). For the cycloheximide chase assay, cycloheximide treatment was performed for 36 h after transfection of wild-type and 3SA mutant using lipofectamine 2000 and cells were harvested at the indicated time points. siRNAs of RSF1 and SNF2h were transfected using a electroporator (Neon); the cells were treated with phleomycin after 72 h and harvested at each time point. For MG132 treatment, cells were treated with MG132 at 5 h before harvesting the cells.

Western blot analysis
Gradient acrylamide gels (4-20%) were used and the analysis was performed as the standard procedure using previously described reagents (Min et al., 2014). The primary antibodies used were as follow: RSF1 (Abcam and Abnova), γH2AX (Millipore), SNF2h (Upstate and Abcam), GAPDH, GFP (Santa Cruz), pATM, pATR, phospho-p53(S15), pChk2 (Cell Signaling), Flag (Sigma), and V5 (Invitrogen).

Immunoprecipitation
Cells were prepared as previously described (Min et al., 2014). Briefly, cells were harvested and lysed in NETN buffer and sonicated. The lysate was cleared by centrifugation at 13,000 rpm and the collected supernatant was incubated with the primary antibodies at 4℃ for overnight. Next day, Protein A sepharose (GE) was added and the lysate was incubated for 2 h and washed four times with NETN buffer. For calf intestinal phosphatase (CIP) treatment, CIP (NEB) was incubated with the beads for 30 min at 37℃ after washing the immunoprecipitated beads.

Repair assays
For repair assay, DR-GFP and EJ-GFP cells were seeded in a 12-well plate and transfected with siRNA using lipofectamine RNAiMAX (Invitrogen). Twenty-four hours after siRNA transfection, the cells were transfected with FokI endonuclease and harvested 48 hours after transfection. The harvested cells were washed with PBS, and analyzed by fluorescence-activated cell sorting (FACS).

Statistical analysis
Statistics and graphs were performed using GraphPad Prism (version 5.0). Unpaired student's t test was applied to compare two individual groups, while one-way ANOVA was applied to compare multiple groups. For one-way ANOVA, the post-hoc test (Tukey HSD) was applied to test the significance between multiple groups using SPSS. Asterisks indicates each p-values ($*P < 0.05$; $**P < 0.01$, $***P < 0.001$).

RESULTS

RSF1 stability is regulated at the post-translational level upon DNA damage
Because RSF1 has been reported to contribute in DDR signaling and DSB repair, we examined RSF1 levels in response to DNA damage (Min et al., 2014). The effect of DNA damaging agents such as phleomycin that induces DSB was examined. Interestingly, RSF1 levels increased significantly at the early time point after DNA damage (Fig. 1A). We also examined the effects of etoposide in the U2OS cell line and found that the RSF1 level was upregulated on treatment with etoposide (Fig. 1B). In addition to the drug treatment,
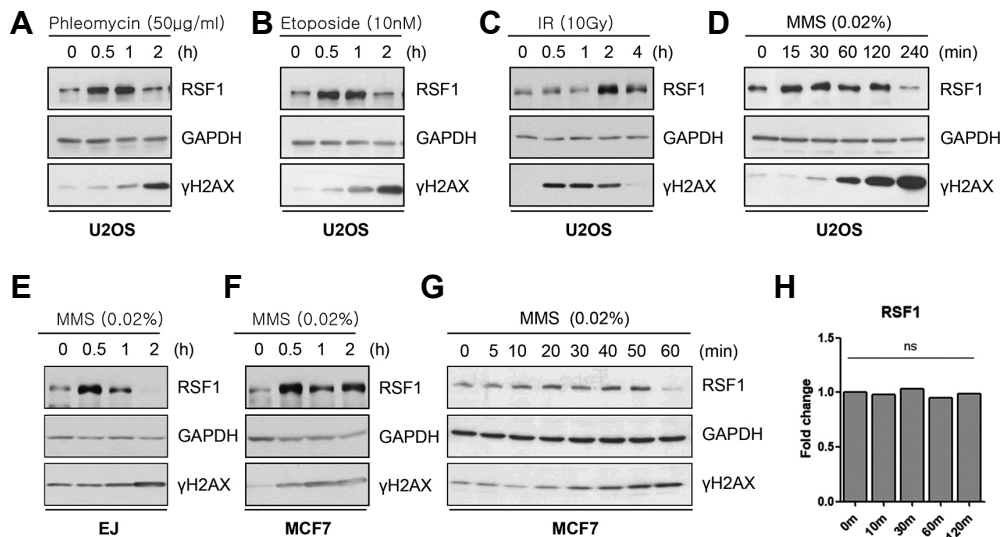
Fig. 1. RSF1 level is upregulated in response to different DNA damaging reagents at post-translational level. (A-D) U2OS cells were directly harvested in the sample buffer after treatment with phleomycin (A), etoposide (B), γ-irradiation (C), and MMS (D), and were analyzed by Western blotting with the indicated antibodies. (E, F) EJ cells (E) and MCF7 cells (F) were treated with MMS, followed by Western blot analysis. (G) MCF7 cells were harvested after treatment with MMS and analyzed by western blotting every ten minutes. (H) Total RNA was isolated from U2OS after treatment with phleomycin, and RNA level of RSF1 was analyzed by real-time PCR and normalized by that of GAPDH.

DNA damage by irradiation also induced stabilization of RSF1 (Fig. 1C). Furthermore, we examined the effects of MMS, which is an alkylating reagent inducing multiple single strand breaks and DSB, and found that RSF1 levels were the same as those observed on treatment with other drugs (Fig. 1D). Because U2OS cell line is derived from osteosarcoma, we also examined the regulation of RSF1 levels in epithelial cell lines. We observed the upregulated RSF1 levels upon DNA damage in EJ and MCF7 cell lines (Figs. 1E and 1F). The data showed that RSF1 level was upregulated immediately after treatment with the four different DNA damage-inducing-drugs. In order to observe the precise regulation of RSF1 stability, we harvested cells every 10 min for 1h, and analysis of the data revealed that the level of RSF1 was temporally regulated in a time-dependent manner (Fig. 1G). These data suggest that the level of RSF1 increased significantly, and the upregulated RSF1 expression was down-regulated at a certain time point depending on the cell line and the damaging sources. These results also indicate that the upregulated RSF1 level requires a fine-tuning mechanism for maintenance of the optimal RSF1 level upon DNA damage.

Next, we measured the expression level of RSF1 mRNA in DDR to examine if the upregulated level was dependent on its transcriptional level. RSF1 mRNA level remained unchanged 2 hr after treatment with phleomycin (Fig. 1H). Thus, this result indicates that RSF1 level is upregulated upon DNA damage through its post-translational regulation.

The binding partner of RSF1, SNF2h, is important for the regulation of its expression upon DNA damage

In general, chromatin remodeling factors exist in a complex, and the subunits comprising the complex stabilize each other (Watanabe et al., 2014). SNF2h is the most well-known
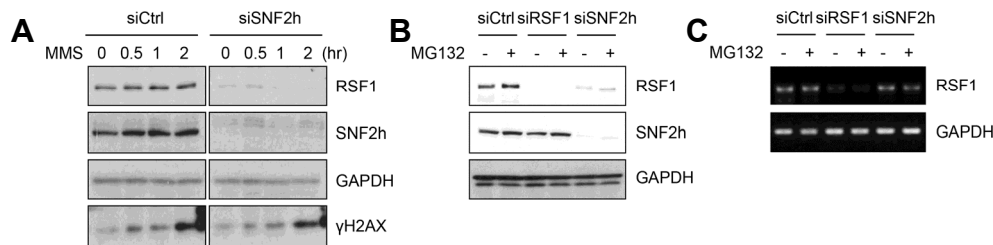

Fig. 2. RSF1 upregulation is dependent on the formation of the RSF complex. (A) U2OS cells were transfected with siCtrl and siSNF2h and treated with MMS (0.02%), followed by Western blot analysis. (B) U2OS cells were treated with siCtrl, siRSF1, and siSNF2h. At 48 h after siRNA transfection, cells were treated with MG132 for 5 h and harvested for Western blot analysis. (C) Total RNA was isolated from U2OS cells transfected with siCtrl, siRSF1, and siSNF2h by treating with MG132 for 5 h.

binding partner of RSF1 and forms the RSF complex with RSF1. We tested if the stability of RSF1 was dependent on SNF2h and found that the absence of its binding partner significantly reduced the level of RSF1 in the presence and absence of DNA damage (Fig. 2A). We next examined if this phenomenon was mediated by ubiquitin-dependent proteolysis; we treated MG132 to block proteasome-dependent degradation. Western blot analysis revealed that the level of RSF1 was slightly, but not fully, recovered after treatment with MG132 in the absence of SNF2h (Fig. 2B). We also checked RSF1 mRNA level in SNF2h-depleted cells and found that the reduced level of RSF1 was dependent on post-translational regulation (Fig. 2C). Thus, we conclude that the formation of RSF complex is required for the protein stability of RSF1 in both absence and presence of DNA damage.

ATM-mediated phosphorylation of RSF1 negatively regulates its level upon DNA damage.

Figure 1 showed that the level of RSF1 was upregulated upon DNA damage, and a fine-tuning mechanism was required for maintenance of the optimal RSF1 level within few hours. Previous reports showed that RSF1 is the direct interacting protein with ATM kinase, which is the major kinase in the DDR signaling pathway, and is the substrate of ATM/ATR kinase (Beli et al., 2012; Matsuoka et al., 2007; Pessina and Lowndes, 2014). In addition to previous studies, RSF1 mass spectrometry by our group revealed that RSF1 harbors several phosphorylation sites and among these sites, three phosphorylation sites are the conserved motif of ATM/ATR substrates. Based on RSF1 mass spectrometry, we performed the phosphatase treatment of immunoprecipitated RSF1 and found that RSF1 was a highly phosphorylated protein without DNA damage (Supplementary Fig. 1A). Furthermore, protein stability is mediated by post-translational modification such as rapid phosphorylation by kinases (Zhao et al., 2017). Thus, we next examined if ATM kinase also influenced the protein stability of RSF1. Next we examined whether RSF1 phosphorylation by ATM regulated RSF1 protein stability upon DNA damage. By generating 3SA mutant (S524A, S1226A, and S1325A), which is unable to be phosphorylated by ATM, we found that 3SA mutant showed high levels of RSF1, compared to WT, even in the equal amount of mRNA level of WT and 3SA mutant (Fig. 3A; Supplementary Fig. 1B). Next, we examined the half-life of 3SA mutant by the cycloheximide chase assay. We treated the cells with cycloheximide to block translation and checked the half-life of the mutants, compared to that of WT. We found that half-life of 3SA mutant was much longer than that of WT (Figs. 3B and 3C). Meanwhile, when we overexpressed ATM kinase, the level of RSF1 did not increase in ATM overexpressed cells (Fig. 3D). These data suggest that RSF1 stability is also regulated by ATM upon DNA damage. Therefore, we suggest that post-translational modification of RSF1 mediated by ATM after DNA damage regulated protein stability of RSF1.
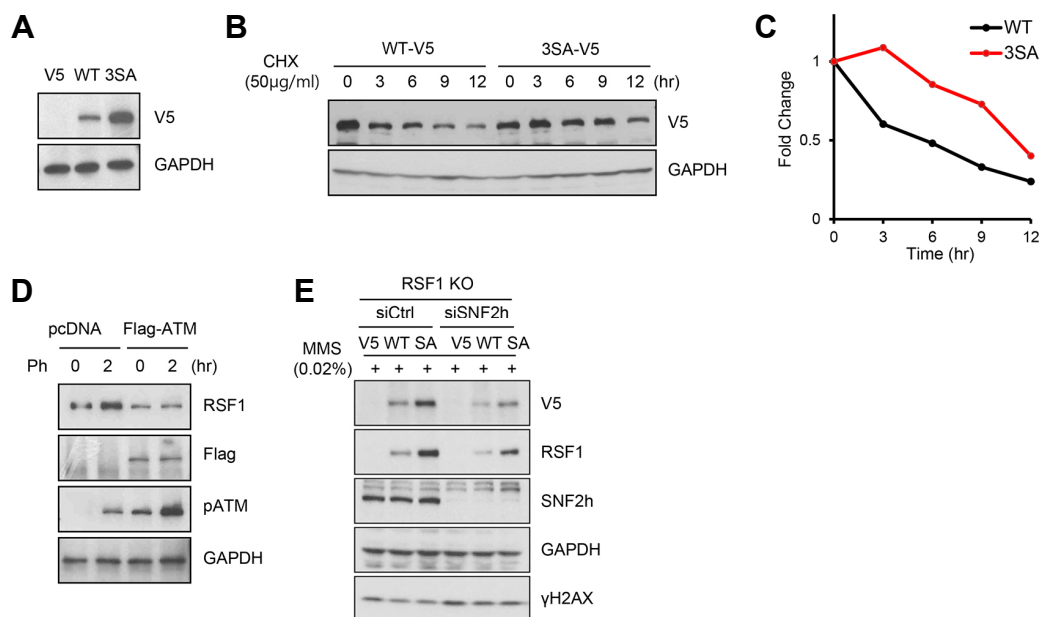

Fig. 3. ATM kinase fine-tunes the upregulated level of RSF1 upon DNA damage. (A) U2OS cells were transfected with V5, RSF1-V5 (WT), and 3SA-V5. At 48 h after transfection, cells were harvested and further analyzed by Western blot. (B) U2OS cells were transfected with RSF1-V5 (WT) and 3SA-V5 and treated with cycloheximide. Cells were harvested at the indicated time points and analyzed by Western blot. (C) Quantitative analysis of the Western blot shown in (B). (D) Flag-ATM was overexpressed in U2OS cell line. At 48 h after transfection, cells were treated with phleomycin for 2 h and analyzed by Western blot with the indicated antibodies. (E) U2OS RSF1 KO cells were transfected with siCtrl and siSNF2h. One day after siRNA transfection, cells were transfected with V5, RSF1 WT-V5, and RSF1 3SA-V5 and treated with MMS at 48 h after transfection, followed by western blot analysis with the indicated antibodies.

Given our observations that the stabilization of RSF1 was accompanied with the presence of SNF2h and post-translational modification by ATM, we tested the combined effect on protein stability of RSF1. Reconstitution of RSF1 3SA mutant and RSF1 WT in the RSF1 KO cell line showed diminished level of RSF1 in the absence of SNF2h upon DNA damage (Fig. 3E). Taken together, we suggest that both the formation of RSF complex and its post-translation modification by ATM are essential for the stabilization of RSF1 in the presence of DNA damage.

Regulation of RSF1 stability is important for DSB repair

To investigate the biological function of temporal regulation of RSF1 stability, we used DR-GFP and EJ-GFP cell lines to measure the efficiency of HR and NHEJ, respectively, after depletion of RSF1 by siRNA transfection. Each cell line produces GFP protein via HR and NHEJ repair system, after DSB is introduced by FokI endonuclease (Gunn et al., 2011). As previously reported, RSF1 depletion reduces the level of HR and NHEJ efficiency (Helfricht et al., 2013; Min et al., 2014; Pessina and Lowndes, 2014). Reintroduction of WT RSF1 slightly recovered HR and NHEJ efficiency, whereas the expression of RSF1 3SA mutant failed to recover HR and NHEJ efficiency upon DNA damage (Figs. 4A and 4B). Thus these results reveal that RSF1 regulation is important for DSB repair.

In addition, to examine the outcome of misregulation of RSF1 stability upon DNA damage, we overexpressed RSF1-GFP in a doxycycline inducible cell line. As previously reported, the overexpression of RSF1 induces an increase in the level of γH2AX, which indicates the activated DDR (Supplementary Fig. 2A) (Sheu et al., 2010). Even after irradiation,

the level of γH2AX was significantly increased in RSF1-overexpressed cells, compared to that in control cells (Supplementary Fig. 2B). To examine the efficiency of DSB repair in RSF1-overexpressed cells, we again counted GFP positive cells in DR-GFP and EJ-GFP cell lines. Overexpression of exogenous RSF1 in DR-GFP and EJ-GFP cells in a dose-dependent manner revealed that RSF1 overexpression also impaired efficient DSB repair, which was similar to the results obtained upon RSF1 depletion (Figs. 4C and 4D; Supplementary Fig. 2C). Thus, these data show that failure in tight regulation of RSF1 levels induced endogenous DNA damage response and prevented efficient DSB repair.

DISCUSSION

Protein homeostasis for maintaining protein levels is usually important for signal transduction, particularly in DDR. p53 is the most well-known tightly regulated protein in response to DNA damage, and its post-transcriptional modification is highly important for its stability (Lakin and Jackson, 1999). Therefore, under urgent stress such as DNA damage, the optimal level of each protein involved in DDR is critical for the maintenance of genomic stability.

In this study, we focused on the protein stability of RSF1, the level of which is significantly important for cell viability in cancer cells. Previous reports have identified a positive correlation between the overexpression of RSF1 by amplification of the *RSF1* gene and the poor prognosis in many patients with cancer (Maeda et al., 2011; Tai et al., 2012; Zhang et al., 2017). In addition to clinical research, molecular studies in many cancer cell lines also showed that RSF1 overexpression induces the endogenous DNA damage by activating the
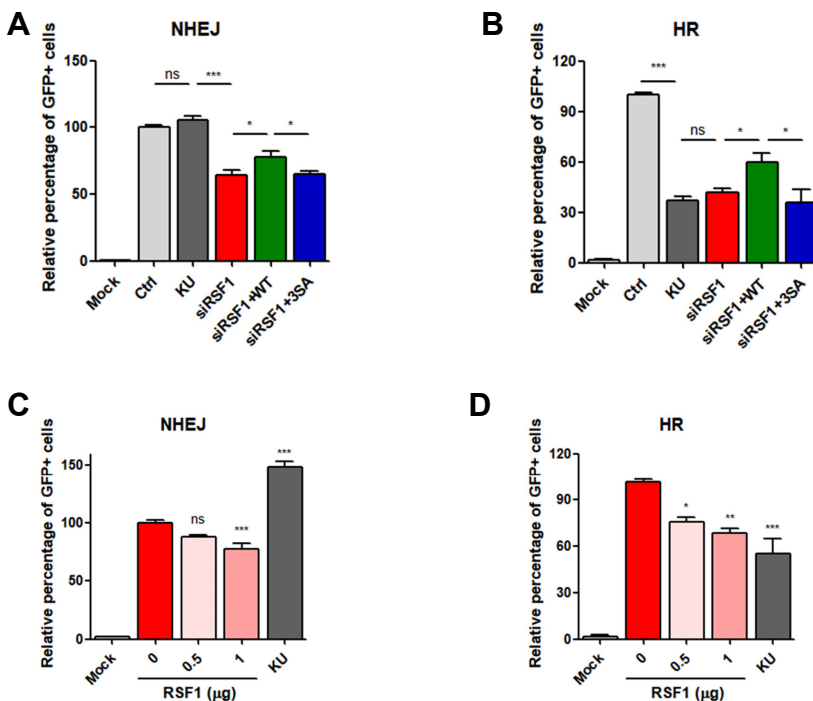

Fig. 4. Regulation of RSF1 level is required for the efficient DSB repair. (A, B) EJ-GFP (A) and DR-GFP (B) cell lines were transfected with siUTR-RSF1, followed by transfection with FokI combined with RSF1-V5 (WT) or 3SA-V5. GFP positive cells were counted by FACS analysis. (C, D) EJ-GFP (C) and DR-GFP (D) cell lines were transfected with RSF1-V5 combined with FokI in a dose dependent manner. GFP positive cells were counted by FACS analysis. *P < 0.05; **P < 0.01; ***P < 0.005, one-way ANOVA with Tukey HSD.

ATM signaling pathway (Sheu et al., 2010). Our data also showed that RSF1 overexpression increased the levels of the endogenous DNA damage signaling pathway and impaired efficient repair upon DNA damage. These data reveal that the optimal RSF1 level is required for cell viability and genome integrity.

Moreover, our data showed that RSF1 level is dynamic upon DNA damage and the upregulation of RSF1 stability is mediated by the formation of RSF complex with SNF2h. RSF1 stability is significantly upregulated in response to DNA damage, followed by downregulation of its stability as γH2AX is induced. Because the maintenance of upregulated RSF1 level may prevent efficient repair, a fine-tuning mechanism of RSF1 level within its optimal level is tightly regulated in DDR. Although the mechanism of upregulated RSF1 needs to be further explored, the presence of SNF2h in RSF complex is critical to RSF1 stability. Importantly, it is known that the level of the subunits of the SWI/SNF complex, a chromatin remodeling factor, are interdependent on each other and the presence of these subunits is required for the recruitment of ATPase, BRM, in SWI/SNF complex at DSB sites (Watanabe et al., 2014). Likewise, in RSF complex, RSF1 is required for SNF2h accumulation at DSB sites (data not shown). However, we cannot exclude the possibility that another subunit in ISWI family is also required for RSF1 stability or SNF2h accumulation at DSB sites.

In addition to SNF2h, ATM kinase is another player in the temporal regulation of RSF1 stability in response to DNA damage. Previous study screening for the ATM/ATR substrates upon DNA damage showed that RSF1 is one of the putative targets of ATM/ATR kinases after irradiation (Beli et al., 2012; Matsuoka et al., 2007). Mass spectrometry revealed that RSF1 has multiple phosphorylation sites. In our previous study, we generated 3SA mutants and showed that 3SA mutant also impaired ATM-dependent DDR signaling pathway (Min et al., 2014). In this study, we suggest another possibility that 3SA mutant upon DNA damage impairs DSB repair because of high expression of RSF1 due to the misregulation of protein stability. As shown in Figs. 4C and 4D, RSF1 overexpression also impaired DSB repair. Thus, tight regulation of RSF1 upon DNA damage is required for efficient repair.

In conclusion, we demonstrated that post-translational regulation of RSF1 is required for the efficient repair in response to DNA damage by the formation of RSF complex with SNF2h and by post-translational modification of RSF1 by ATM kinase. Thus, these results provide insights to explain the mechanism by which RSF1-overexpressed cancer cells respond to DNA damage inducing reagents and how atypical regulation of RSF1 leads to genetic instability in cancer progression.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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