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Loss of *Smu1* function de-represses DNA replication and over-activates ATR-dependent replication checkpoint

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ABSTRACT

Smu1 is an evolutionarily conserved gene that encodes a member of the WD40-repeat protein family. Disruption of *Smu1* function leads to multiple cellular defects including chromosomal instability, aberrant DNA replication and alternative RNA splicing events. In this paper, we show that *Smu1* is a chromatin-bound protein that functions as a negative regulator of DNA replication. Knockdown of *Smu1* gene expression promotes excessive incorporation of dNTP analogue, implicating the acceleration of DNA synthesis. *Smu1*-silenced cells show an excessive activation of replication checkpoint in response to ultraviolate (UV) or hydroxyurea treatment, indicating that abnormal stimulation of DNA replication leads to instability of genomic structure. Hence, we propose that *Smu1* participates in the protection of genomic integrity by negatively regulating the process of DNA synthesis.

1. Introduction

DNA damages induced by endogenous or exogenous genotoxic agents threaten the maintenance of genome stability [1]. To preserve genome and chromosomal stability, co-ordinations between DNA metabolism and the genome surveillance system including DNA replication, repair, and cell cycle checkpoint are required. DNA damages that remained undetected or left unrepaired can lead to genome instability, mutation, cell death and human diseases such as cancer [2]. DNA replication is mediated by sophisticated multi-protein complexes and regulated by the cell cycle checkpoint. Under physiological conditions, DNA replication is driven by PCNA and DNA polymerase to processively incorporate dNTP into complementary strands of parental DNA [3]. DNA replication is highly sensitive to DNA lesions on the template DNA strand; replication fork would stall when the checkpoint is activated upon detection of DNA damage [4,5]. Therefore, the damage-sensitive checkpoint signals form a negative regulatory mechanism of replication forks [6]. On the other level, the velocity of DNA synthesis in terms of dNTP incorporation is also finely tuned by other factors. For example, disruption of the Cul4-Ddb1-CSN ubiquitin ligase in fission yeast impedes DNA synthesis by inhibiting the ribonucleotide reductase [7,8]. However, there is no previous report, at least to our knowledge, describing a protein or mechanism negatively regulating the efficiency of dNTP incorporation.

The WD40-repeat protein *Smu1* is encoded by an evolutionarily conserved gene found in most eukaryotic organisms. Genetic and cell biology studies have revealed that *Smu1* can regulate the alternative splicing of unc-52/perlecan pre-mRNA in *Caenorhabditis elegans*, suggesting a possible function of *Smu1* in RNA splicing [9–11]. *Smu1* is also involved in the genome stability maintenance of higher organisms. A temperature-sensitive (*ts*) CHO-K1 mutant cell line, tsTM18, was isolated in a screen to search for mutant cells that exhibit chromosomal instability at non-permissive temperatures [12]. *Smu1* was found to be defective and responsible for the cause of abnormal splicing, increased chromosomal breakage, reduced DNA synthesis and accumulation of single-stranded DNA in tsTM18 cells [13]. In this paper, we characterized the DNA replication and damage responses in siRNA-mediated *Smu1*-knocked down cells. When *Smu1* was silenced, DNA replication was accelerated as the incorporation of BrdU (a dNTP analog) increased per unit time. Upon genotoxic challenges, the ATR-mediated replication checkpoint was over-activated in Smu1-silenced cells. Our data indicate that *Smu1* can negatively regulate DNA replication

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either directly or indirectly. We also showed that a significant amount of *Smu1* protein was associated with chromatin, consistent with the notion that *Smu1* acts as a negative regulator of DNA synthesis. Hence, we conclude that *Smu1* actively participates in the deceleration of DNA synthesis, and loss of this negative control mechanism leads to disruption of genome stability.

2. Materials and methods

2.1. Cell culture and transfection

U2OS and 293T cells were maintained in DMEM (GIBCO) supplemented with 10% FBS. siRNAs were purchased from Ribobio (Guangzhou, China). The target sequence of human *Smu1* siRNA was 5′-GCACGAGAAGGAUGUGAUUdTdT-3′ (sense). Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. MTT assay

Control or *Smu1* knockdown 293T cells were seeded into wells of 96-well plates (5×10^3/well) in quintuplicate and cultured overnight. On the next day, the cells were treated with Camptothecine (CPT), etoposide or ionizing radiation at indicated dosages for specified time period. Ten microliters of freshly prepared MTT solution (5 mg/ml) was added into wells 4 h before the end of incubation. After complete removal of the supernatant, cells in each well were solubilized with 150 μl of DMSO. The absorbance at the wavelength of 490 nm for each well was detected by the use of a Microplate Reader (BIO-RAD).

2.3. Colony formation assay

Transfected 293T cells were treated with or without UV radiation at indicated dose and re-plated in a 100-mm dish at 300 cells per dish. After culture for 14 days, the cells/colonies on the plate were fixed with chilled methanol after complete removal of culture medium. The number of clones was counted after Giemsa stain.

2.4. Cell fraction extraction and Western blot

For whole-cell extraction, the cells were directly lysed in an SDS sample buffer (50 mM Tris–HCl pH 6.8%, 1% SDS, 10% glycerol, 5% β-ME, 0.01% bromophenol blue). Chromatin fraction was prepared using Mendez and Stillman's method [14]. Western blotting was performed using the following antibodies: pS139-H2AX and H2B (Millipore); pS345-Chk1, pS15-p53, pTyr15-Cdc2 (cell signaling); α-tubulin (Sigma); p21 (Abcam); Smu1 (Abnova) and pS33-RPA32 (Novus). Chemiluminescent visualization was performed with horseradish peroxidase-conjugated secondary antibodies (Dako) and detected using ChemiDoc XRS system (Bio-Rad).

2.5. Immunofluorescence (IF) assay

U2Os cells grown on glass coverslips were fixed with 4% paraformaldehyde, and then permeabilized in PBS containing 0.3% triton. Cover slips were incubated with primary antibodies (pS139-H2AX, Millipore; Smu1, Abnova; BrdU, Shanghai Long Island) and detected with secondary antibodies (anti-mouse-FITC, Dako). Cover slips were sealed with mounting medium, which contains DAPI (Vector Laboratories), and the images were obtained using an Olympus fluorescence microscope. For BrdU staining, the cells were pulsed with 10 μM of BrdU for 30 min before fixation, and denatured by 27% of HCl for 25 min before antibody incubation. For TUNEL Assay, apoptotic cells were visualized by the In Situ Cell Death Detection Kit (Roche).

2.6. Flow cytometry

U2OS cells were harvested by trypsinization, and then fixed with ethanol (70%). The cells were then stained with primary and secondary antibodies. Cell pellets were resuspended in 50 μg/ml of propidium iodide (PI), and one- or two-dimensional flow cytometry was performed to detect PI and/or fluorescence.

2.7. [^{3}H]Thymidine incorporation assays

Transfected cells were incubated with 2.5 μCi/ml [^{3}H]Thymidine (Shanghai Institute of Applied Physics, Chinese Academy of Sciences) for 6 h prior to harvest. The cells were then collected on glass fiber filter discs, and radioactivity was measured in counts per minute using a LS 6500 multi-purpose scintillation counter (Beckman Coulter).

3. Results

3.1. Smu1 confers resistance to genotoxic challenges

Transfection of *Smu1*-specific siRNA into either 293T or U2OS cells caused a significantly downregulation of *Smu1* protein expression in both cell types, confirming the effectiveness of the knockdown technique (Fig. 1A). Knockdown of *Smu1* in 293T cells caused moderate growth inhibition (Fig. 1B). To test the role of *Smu1* in protecting cells from genotoxic challenge, we firstly treated control and *Smu1* knockdown 293T cells with agents capable of inducing DNA double-strand breaks (DSBs) such as etopside, camptothecin or ionizing radiation (X ray). As shown in Fig. 1C, knockdown of *Smu1* caused quick loss of viability upon treatment with these three genotoxic agents as determined by MTT assay.

siRNA-transfected cells were also subject to UV irradiation to test their propensities for viability. According to colony formation assay, *Smu1*-defective cells formed less colonies than control cells in response to UV treatment, suggesting that 293T cells with *Smu1* knockdown were more sensitive to UV treatment (Fig. 1D and E). UV-induced reduction of viability correlated to increased rate of apoptosis in *Smu1*-knockdown cells (Fig. 1F and G). Taken together, these data suggest that *Smu1* is potentially required for the protective mechanism against various genotoxic challenges.

3.2. Accumulation of endogenous DNA lesions and G2 arrest after loss of Smu1

To reveal the mechanistic role of *Smu1* in DNA damage responses, we first tested if loss of *Smu1* could increase endogenous DNA lesions by monitoring the phosphorylation event of histone variant (γH2AX), which is used in many studies as a readout for various forms of DNA damage including DSBs and ssDNA breaks. Compared to control cells, *Smu1*-defective cells showed a moderate increase of discrete foci of γH2AX, indicating the accumulation of endogenous DNA lesions (Fig. 2A). Analysis by flow cytometry confirmed that there was a threefold increase in γH2AX positivity in cells losing function of *Smu1* (Fig. 2B). Interestingly, most of the γH2AX-positive cells displayed 2C–4C DNA contents according to the PI staining (Fig. 2B, lower panel), indicating that the cells were mostly in the late S or G2 phase of the cell cycle. Furthermore, we could also detect elevated γH2AX level in *Smu1*-knocked down cells by Western blot (Fig. 2C). Consistent with G2 arrest for the damaged cells, we also detected elevated level of the inhibitory phosphorylation event (Tyrosine 15) of the cell cycle kinase

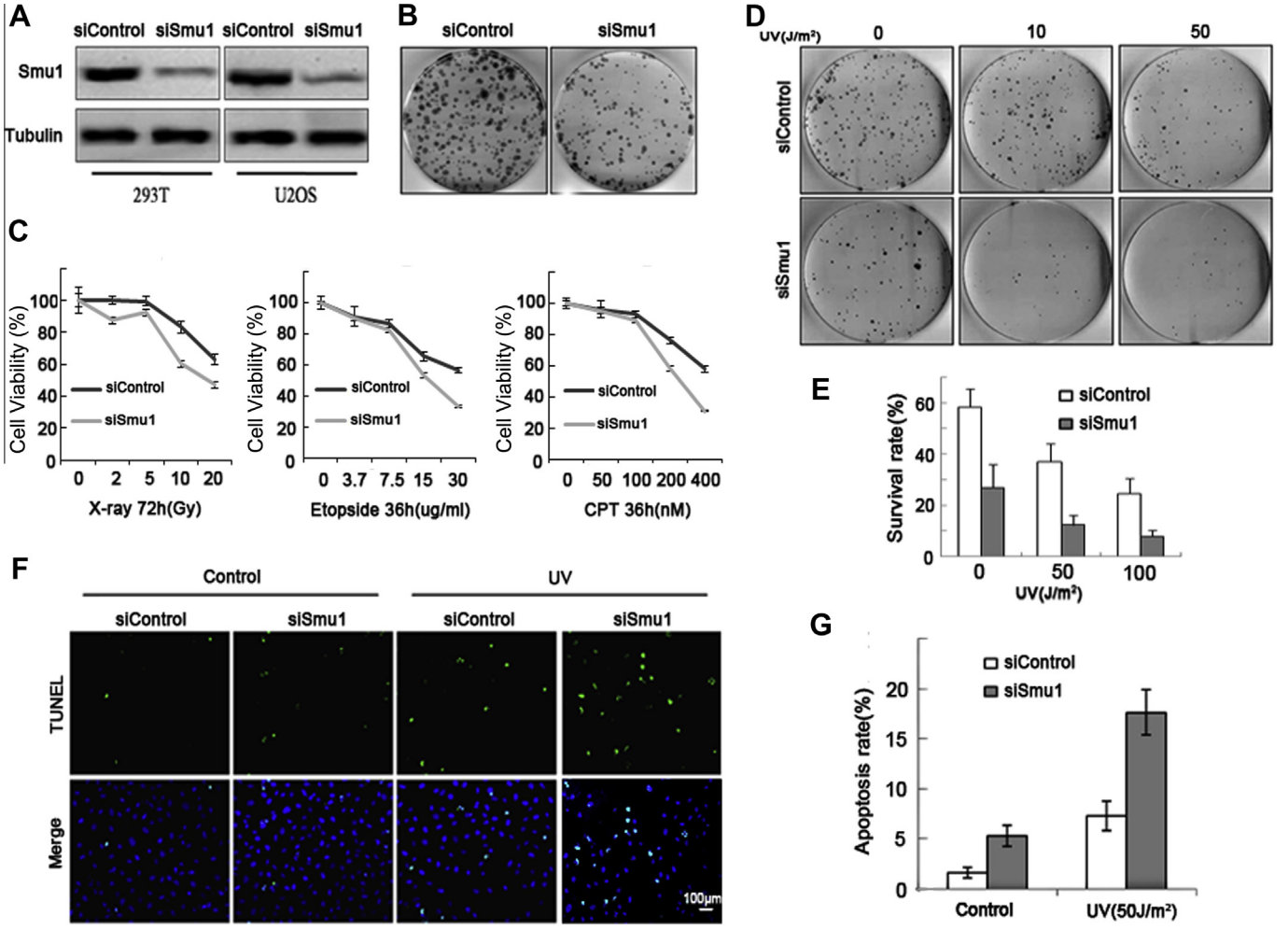

Fig. 1. Knocking down of Smu1 gene expression causes growth defects and hypersensitivity to genotoxic challenges. (A) Knocking down of Smu1 by small interference RNA in 293T and U2OS cells. (B) Colony formation assay of siRNA-treated 293T cells. (C) 293T cells challenged with etopside, X-ray and camptothecin for indicated hours and concentration were subjected to MTT assay to measure viability of each treatment. Error bar, s.e.m. ($n = 5$). (D) and (E) Colony formation assay of siRNA-treated cells after UV treatment. It was clear that reduction of viability was more dramatic in Smu1-silenced cells upon UV treatment than those of mock transfected. Error bar, s.e.m. ($n = 3$). (F) and (G) Increased apoptosis in Smu1-knocked down after UV irradiation detected by TUNEL assay. Error bar, s.e.m. ($n = 3$).

(Cdc2), indicating a moderate activation of DNA damage checkpoint in unperturbed cell cycle of *Smu1*-silenced cells.

The inhibition of Cdc2 activity and G2 arrest in Smu1 defective cells was further confirmed by cell cycle analysis. According to the analysis, a significant increase of G2 and a reduction of S phase populations occurred in *Smu1* siRNA-transfected cells (Fig. 2D).

Hence, we reckon that the DNA lesions (represented by γH2AX) and G2 arrest in *Smu1-silenced* cells were likely caused by pathological DNA structures generated from perturbed DNA replication in S phase and responsible for activating checkpoint at G2/M transition to allow the repair of DNA damage.

3.3. Negative regulation of DNA replication by Smu1

To obtain further insights into the disturbance of DNA metabolism by *Smu1*-depletion, we examined DNA synthesis by two different assays. Firstly, we tested the incorporation of pulse chased [^{3}H]Thymidine into genomic DNA in siRNA-treated cells. Surprisingly, although the ratio of S phase was reduced in *Smu1*-silenced cells according to the previous fluorescence activated cell sorting (FACS) analysis, the incorporation of [^{3}H]Thymidine was significantly higher than that of control cells (Fig. 3A). This result suggested that when the function of *Smu1* was inhibited, DNA synthesis was actually accelerated, even though the population of cells synthesizing DNA at specific time was relatively small.

Secondly, we pulse labeled cells with BrdU and examined the efficiency of dNTP incorporation. According to FACS analysis shown in Fig. 3B, BrdU-positive cells distributed between 2N and 4N DNA contents in both control and *Smu1*-silenced cells, indicating that BrdU-positive cells were progressing through S phase and actively incorporating dNTP into genomic DNA. Most cells transfected with scramble siRNA displayed a typical "hump" pattern in FACS diagram and contained moderate amount of BrdU with fluorescent counts around 11,800 per cell (Fig. 3C). In sharp contrast, although the proportion of BrdU-positive cells was smaller in cells with *Smu1* knockdown (22.8% vs. 29.4%), there were significantly larger population of BrdU-positive cells displayed higher content of BrdU incorporation (fluorescent counts about 16,000 per cell). Therefore the average incorporation efficiency of BrdU in *Smu1*-knocked down cells was 30% higher than those of mock transfection (Fig. 3C).

We also visualized the BrdU incorporation in individual cells by indirect immunofluorescence. When *Smu1* was silenced, the general BrdU signal in individual cells was significantly more intensive than in control cells (Fig. 3D), albeit the percentage of BrdU-positive cells was less in these cells. Thus, the BrdU incorporation experiments reconciled the discrepancy of enhanced potential of

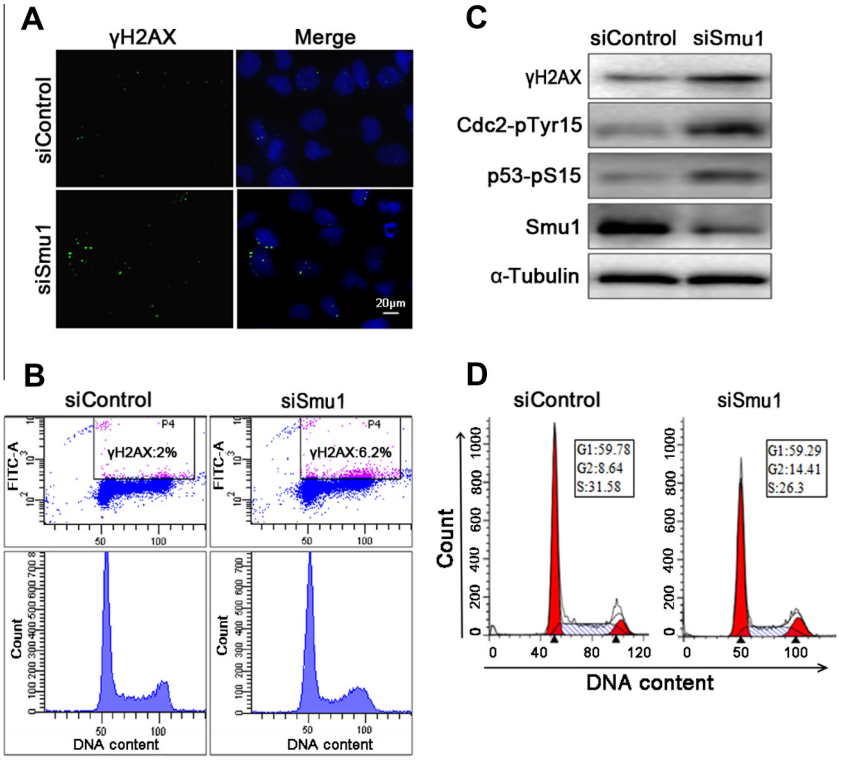

Fig. 2. Endogenous DNA damage and cell cycle arrest in cells with impaired *Smu1* function. (A) siRNA-treated U2OS cells grown on coverslips were immunostained with γH2AX antibody after fixation. Punctuated foci were detected in siSmu1-treated cells. (B) γH2AX-positive cells were detected and quantified by flow cytometry (upper panel). Apparently, γH2AX-positive cells in *siSmu1*-treated U2OS cells were accumulated in late S and G2 phase according to DNA content represented by PI staining (lower panel). (C) Total protein was separated by SDS–PAGE and indicated proteins were detected by specific antibodies. Moderated phosphorylation of H2AX, p53-S15 and Cdc2-Tyr15 were detected. (D) Cell cycle assay by FACS analysis of *Smu1*-knocked down cells. Decrease of S phase and increase of G2 population were indicated in insert boxes.

[^{3}H]Thymidine incorporation in *Smu1*-silenced cells despite that they displayed smaller S phase population. Consequently, we concluded that *Smu1* participates in the regulatory process controlling the velocity of DNA synthesis during S phase, either directly or indirectly.

3.4. Over-activation of ATR-dependent replication checkpoint upon loss of Smu1

To understand the outcome of de-repression of DNA synthesis by *Smu1* interference, we challenged cells with UV and hydroxyurea to examine the activation of DNA integrity checkpoints after DNA damage. We detected excessive phosphorylation events marking the over-activation of ATR-dependent checkpoint pathway in *Smu1*-knocked down cells, including the phosphorylation of Chk1, H2AX (Fig. 4A). This correlated with the de-repression of DNA replication in *Smu1* cells, in which the replication forks might become unstable and liable to collapse upon DNA damage, which, in turn, caused excessive activation of checkpoint to arrest cell cycle in G2 phase. Consistent with this scenario, *Smu1*-silenced cells had less Cdc2 activity upon UV or HU treatment as illustrated by the elevated level of Cdc2 phosphorylation at Tyrosine 15 in comparison with control cells. After UV irradiation, both mock or *siSmu1*-transfected cells dramatically reduced dNTP (BrdU) incorporation (Fig. 4B), indicating that the ATR-dependent checkpoint pathway was activated, resulting in a stall of replication fork and reduction of DNA synthesis [15–17]. Moreover, we also detected increased phosphorylation of p53 at serine 15 in *Smu1*-defective cells (Fig. 4A). P53 is one of the direct phosphorylation targets of the activated ATR kinase that can also trigger the transcription of pro-apoptotic genes including Bax and Bad [18]. This is consistent with increased apoptotic level after acute DNA damage such as UV treatment in *Smu1*-silenced cells (Fig. 1F and G). Thus, the over-activation of ATR pathway could potently inhibit accelerated DNA replication in S phase, promote cell cycle arrest in G2 phase to protect the vulnerable DNA structure caused by excessively accelerated DNA synthesis in *Smu1*-defective cells.

3.5. Association of Smu1 with chromatin

It has been reported that *Smu1* is a splicing factor that co-localizes with ASF-associated nuclear speckles. In our experiments, we also found *Smu1* localized in discrete nuclear sub-domains without apparent re-localization before and after UV irradiation (Fig. 4C), indicating the distribution of *Smu1* is not dynamic in responsive to DNA damages. To obtain clues as to how *Smu1* is involved in DNA synthesis, we proceeded to carry out chromatin fractioning assay. Intriguingly, significant amount of *Smu1* protein was detected not only in soluble fraction of nucleoplasm, but also in the insoluble part of chromatin, using H2B as an indicator of chromatin (Fig. 4D). Although there was no re-distribution or post-translational modification events of chromatin-bound *Smu1* after UV treatment, the detection of *Smu1* protein in chromatin fractions strongly suggested that *Smu1* may be involved in proper functioning of chromatin, including the regulation of DNA synthesis.

4. Discussion

DNA replication is a highly regulated cellular process by a range of surveillance mechanisms in the cell, including the firing of replication origin, stall and re-initiation of replication fork by replicative checkpoint and synthesis of dNTPs. Loss of any of these regulatory mechanisms would cause aberrance of DNA replication

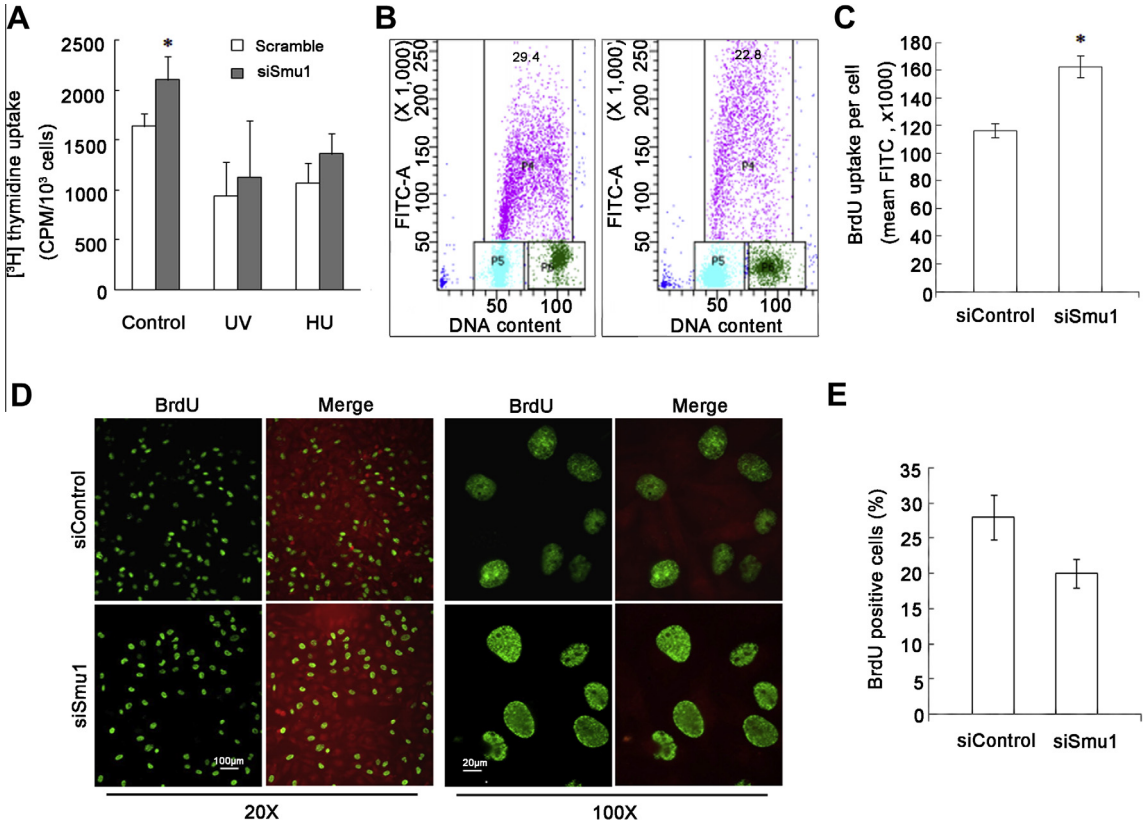

Fig. 3. De-repression of DNA replication in *Smu1*-knocked down cells. (A) siRNA-treated U2OS cells were pulse labeled with [^{3}H]Thymidine and DNA synthesis was measure as counts of radioactivity per minute by scintillator. Error bar, s.e.m. ($n = 3$). Student's two-tailed t-test: $^*P < 0.05$ vs. control. (B) U2OS cells were pulse labeled with BrdU for 30 min. BrdU incorporation was subsequently detected by anti-BrdU antibody and measured by fluorescent flow cytometry. Inserted number indicates the percentages of BrdU-positive cells representing cells undergoing DNA replication at the time of pulse labeling. (C) Average incorporation of BrdU per cell was calculated by total fluorescent intensity divided by cell counts. Error bar, s.e.m. ($n = 3$). Student's two-tailed t-test: $^*P < 0.05$ vs. control. (D) Indirect immunofluorescent staining of BrdU for U2OS cells grown on cover slips. It is apparent that the general intensity of individual BrdU-positive cells treated with *Smu1* siRNA was stronger than mock-treated cells. (E) Counting of BrdU-positive (S phase) cells on the same cover slips from (D). Error bar, s.e.m. ($n = 3$).

[4,19,20]. However, to date, there is no study describing a protein or mechanism negatively regulating the efficiency of dNTP incorporation. Our data demonstrate that *Smu1* is a factor capable of suppressing dNTP incorporation during DNA synthesis based on several lines of evidence through different assays. This notion is further supported by the over-activation of ATR-dependent replicative checkpoint and the hypersensitivity of *Smu1*-silenced cells to genotoxic challenges.

We have performed several different and mutually supportive assays to demonstrate *Smu1's* role in the regulation of DNA replication. Firstly, loss of *Smu1* caused disturbance of cell cycle progression, which led to reduction of cells in S phase and simultaneous increase of cells in G2 phase. However, dNTP incorporation and total DNA synthesis measured by [^{3}H]Thymidine incorporation was increased despite the reduction in numbers of cells in S phase. To explain these contradictory results, it is assumed that the velocity of DNA replication fork and efficacy of dNTP incorporation is de-repressed by the disruption of Smul, which would shorten S phase and lengthen G2 phase due to increased DNA damage and checkpoint activation.

The perturbation of DNA replication in S phase of *Smu1*-silenced cells was further supported by over-activation of the ATR replicative checkpoint pathway upon treatment with genotoxic agents such as UV and HU. It is conceivable to speculate that excessive activation of cell cycle checkpoint could be caused by abnormally fast movement of replication fork that became unstable and was hyper-vulnerable to genotoxic challenges. When cells were treated with replication toxin, replication fork might stall or even collapse upon loss of *Smu1*, which, in turn, caused excessive activation of ATR pathway.

Despite our tremendous efforts to demonstrate the role of Smu1 in DNA replication, the results are still in discrepancy with previous works to some extent. Tsuji and Sugaya showed that the temperature sensitive CHO-1 K1 mutant cell line (tsTM18) exhibited reduced DNA synthesis and cell cycle arrest at S and G2 phase at non-permissive temperature [12,13]. We think the contradiction is due to the different mutant forms of *Smu1* studied in each work. In our work, we used siRNA to knock down the total amount of *Smu1* gene expression whereas in Tsuji and Sugaya's work, *Smu1* in tsTM18 cell line harbored a missense mutation, which casued a change from glycine to arginine at amino acid 498 (G498R). How the G498R mutation causes defects in DNA replication remains to be elucidated, and its cellular behavior would be difficult to determine before full understanding of the function of the wild type *Smu1*. Moreover, although Sugaya's work reported that DNA synthesis was slowed down in tsTM18 cells at restricted temperature, the immunostaining data provided in the experiment showed no reduction in BrdU incorporation in individual tsTM18 cells, an observation closely resembles our results [21].

As for the molecular function of *Smu1*, we are still unable to paint a full picture about its role(s) in gene replication and expression based on the present data. There are several lines of evidence suggesting that *Smu1* is required for RNA splicing for a subset of genes in an evolutionarily conserved manner [10,13,21]. Although the work done in this study demonstated that *Smu1* played an important role in DNA replication by negatively regulating the

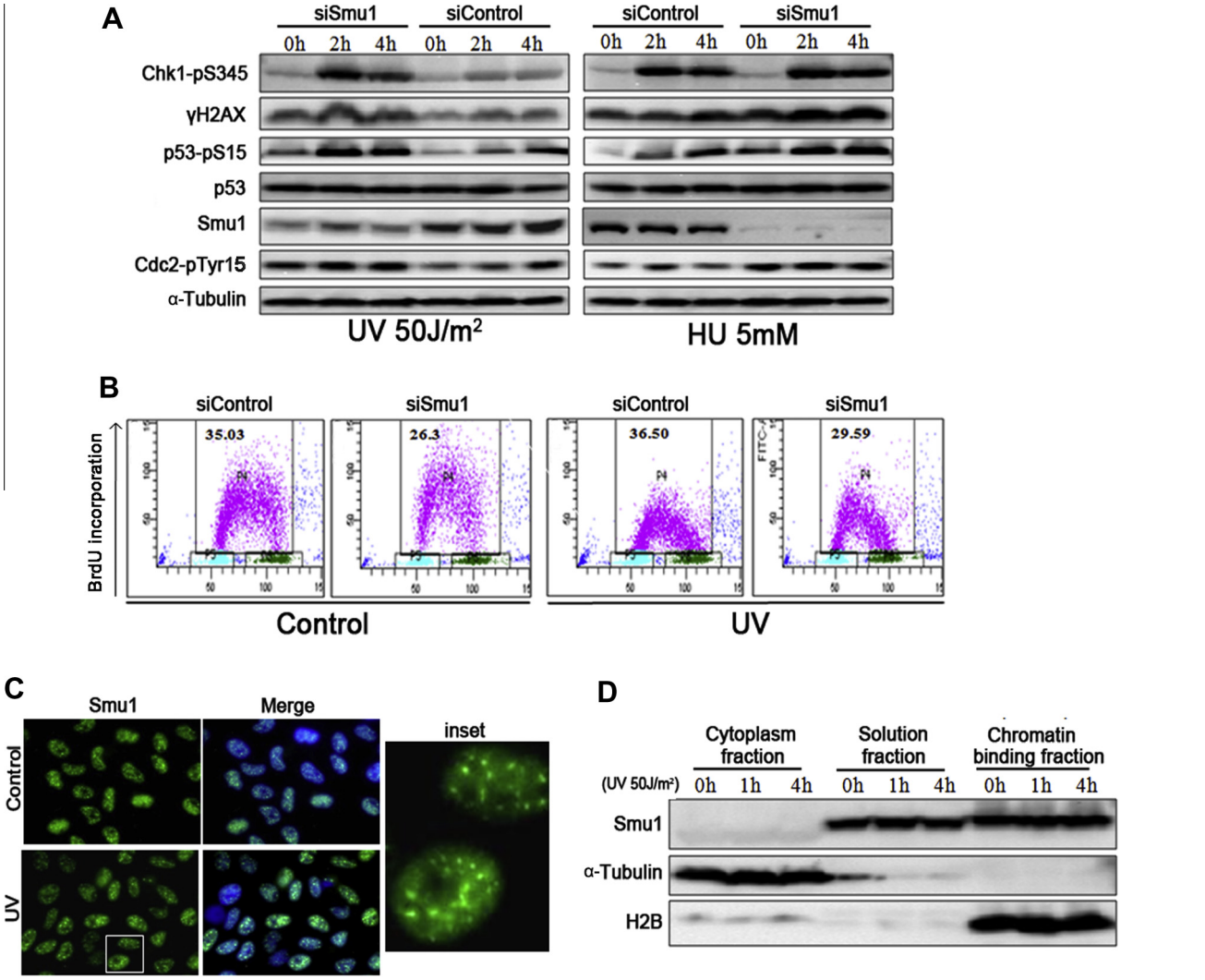

Fig. 4. Over-activation of ATR checkpoint pathway in *Smu1*-depleted cells. (A) Western blot analysis of the downstream targets including phosphorylated Chk1-S345, H2AX-S139, p53-S15 in indicated siRNA-transfected 293T cells. The inhibitory phosphorylation site of Cdc2 (Tyrosine 15) was also excessively phosphorylated in *Smu1*-silenced cells. (B) FACS analysis in U2OS cells for the incorporation of BrdU after UV irradiation for each siRNA-treated group. In both mock and *Smu1* siRNA treated cells, the UV irradiation dramatically reduced the incorporation efficiency of BrdU. Efficacy of BrdU incorporation was measured by FITC intensity and inserted number indicates the percentage of S phase cell represented by BrdU positivity. (C) Speckle-pattern of nuclear localization of Smu1 protein shown by indirect immunofluorescence. There was no re-distribution after UV irradiation. (D) 293T cells with or without UV treatment werefractionated into indicated subcellular compartments. Smu1 protein was detected by Western blot and shown to distribute in both nucleoplasm and chromatin fractions.

velocity of DNA synthesis, it is insufficient to propose a unified model for its function(s) in both RNA splicing and DNA replication. However, Sugaya et al. reported [10] that *Smu1* is functionally associated with the serine/arginine (SR)-rich splicing group of factors (SF2/ASF), which is involved in preventing the formation of R-loop between nascent RNA transcripts and DNA template strands. Furthermore, it has recently been reported that loss-of-function of ASF caused R-loop-mediated genomic instability through impairing of replication fork progression [22].

Although it is not clear how SRSF factors regulate DNA replication and the functional association between SRSF and *Smu1* at this level of regulation of DNA replication, we speculate that it is likely for *Smu1* to play a role at the interface of R-loop and replication. Consequently, it is also worthwile to elucidate the mechanism of *Smu1* in R-loop formation and its effect on the velocity of DNA replication.

Taken together, we have shown more direct and compelling evidence that *Smu1* is a chromatin-bound protein involved in the regulatory circuit of DNA replication and loss of its function would lead to over-activation of replicative checkpoint, cell cycle arrest and cell death.

Acknowledgments

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C.L. and L.R. designed research; L.R. and Y.L. performed experiments; C.L. wrote the manuscript with contributions from all authors.

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