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Protein phosphatase 5 is necessary for ATR-mediated DNA repair

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ABSTRACT

Several recent studies have shown that protein phosphatase 5 (PP5) participates in cell cycle arrest after DNA damage, but its roles in DNA repair have not yet been fully characterized. We investigated the roles of PP5 in the repair of ultraviolet (UV)- and neocarzinostatin (NCS)-induced DNA damage. The results of comet assays revealed different repair patterns in UV- and NCS-exposed U2OS-PS cells. PP5 is only essential for Rad3-related (ATR)-mediated DNA repair. Furthermore, the phosphorylation of 53BP1 and BRCA1, important mediators of DNA damage repair, and substrates of ATR and ATM decreased in U2OS-PS cells exposed to UV radiation. In contrast, the cell cycle arrest proteins p53, CHK1, and CHK2 were normally phosphorylated in U2OS and U2OS-PS cells exposed to UV radiation or treated with NCS. In view of these results, we suggest that PP5 plays a crucial role in ATR-mediated repair of UV-induced DNA damage.

1. Introduction

DNA damage induces a range of cellular responses ranging from growth arrest to the induction of senescence or apoptosis, and several mammalian models of chronic DNA damage and genomic instability are characterized by tumor-prone and accelerated aging phenotypes [1–3]. To prevent genomic instability caused by DNA lesions being transmitted to their offspring, cells have developed an elaborate system that integrates DNA damage detection and checkpoint mechanisms to coordinate repair and cell cycle progression [4,5]. The key players in this system have been loosely classified as DNA-damage sensors, mediators, transducers, and effectors [6]. Sensors are defined as factors that detect DNA lesions. Although the nature of these sensors and the mechanism of DNA-damage detection remain unclear, during the early stages of the response to DNA damage, two closely related kinases – ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) – are thought to control multiple cellular processes including cell cycle arrest, DNA repair, and apoptosis [7]. From ATM and ATR, the DNA-damage signal is transmitted to transducer kinases such as checkpoint kinase 1 (CHK1) and CHK2. These transducer kinases function in signal transduction cascades that target downstream DNA damage response (DDR) components and amplify the DDR signal [8]. This signaling between sensors and transducers is thought to be facilitated by mediator proteins such as mediator of DNA-damage checkpoint 1 (MDC1), p53-binding protein 1 (53BP1), and breast cancer 1 early-onset (BRCA1) [9].

Protein phosphatase 5 (PP5) is a member of the protein serine/threonine phosphatase family, which also includes PP1, PP2A, PP2B, PP4, PP6, and PP7 [10,11]. PP5 is unique among these phosphatases in that it contains tetratricopeptide repeat (TPR) motifs that together serve as a protein–protein interaction domain. It differs from most PPP-family phosphatases in that the principal substrate-targeting regulatory and catalytic domains are contained in a single polypeptide chain. Recently, several lines of evidence have shown that PP5 interacts with ATM, ATR, 53BP1, and DNA-dependent protein kinase catalytic subunits (DNA-PKcs) following DNA damage. Studies involving the suppression and overexpression of PP5 suggest that its interaction with DNA-PKcs and 53BP1 results in their dephosphorylation, indicating that PP5 acts as a negative regulator of DNA-PKcs and 53BP1 [12,13]. In contrast, it appears to enhance the activity of ATM and ATR [14–16].

In this study, we investigated the roles of PP5 in the repair of ultraviolet (UV)- and neocarzinostatin (NCS)-induced DNA damage. Our results showed that PP5 is required for the repair of UV-induced DNA damage but not that of NCS-induced DNA damage.

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2. Materials and methods

2.1. Cell culture and NCS and UV treatment

The following cell lines were used in the present study: U2OS, U2OS-PS (stable cell line transfected with a siPP5 construct), and U2OS-PO (stable cell line transfected with the full-length human *PP5* gene) [13]. U2OS cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 10 μg/ml streptomycin, and 10 U/ml penicillin. U2OS-PS and -PO cells were grown in the same medium further supplemented with 800 μg/ml G418 (Sigma). To induce DNA damage, exponentially growing cells were treated with 100 ng/ml NCS (Sigma) or exposed to UV radiation (5 J/m^2) and then harvested at different times following treatment.

2.2. Antibodies and Western blot analysis

Cells were lysed with M-PER (Pierce) supplemented with proteinase inhibitors (Complete Mini, Roche). Proteins were separated by SDS–PAGE and then transferred to PVDF membranes (Millipore). Membranes were blocked for 1 h with TBS-T [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween20] containing 5% nonfat dry milk. They were then incubated with primary antibodies followed by the appropriate secondary antibody. The bound antibody was visualized by chemiluminescence (Intron).

Antibodies raised against CHK1, CHK2, p53 (pSer15), CHK2 (pSer317), ATR (pSer428), 53BP1 (pSer1778), and BRCA1 (pSer1524) were purchased from Cell Signaling Technology; anti-ATM and -ATR antibodies were obtained from Abcam; anti-p53 and -53BP1 antibodies were acquired from Santa Cruz Biotechnology; an anti-PP5 antibody was purchased from BD Biosciences; a phospho-specific anti-ATM antibody (pSer1981) was obtained from Lockland; an anti-BRCA1 antibody was acquired from Calbiochem; and an anti-α-tubulin antibody was purchased from NeoMarkers.

2.3. Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described [17]. Cells were grown on glass slides, rinsed with phosphate-buffered saline (PBS), and fixed through incubation in a freshly prepared 3.7% solution of paraformaldehyde in PBS for 15 min. Slides were either immediately processed or transferred to 70% EtOH and stored at 4 °C. After washing with PBS, the glass slides were blocked in 5% bovine serum albumin in PBS for 1 h at room temperature (RT) and then incubated overnight at 4 °C with a mouse monoclonal anti-γ-H2AX (ser-139) antibody (1:200; Upstate Biotechnology) or a rabbit polyclonal anti-53BP1 antibody (ser 1778) (1:100; Cell Signaling Technology). Next, Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (1:200; Molecular Probes) were applied for 1 h at RT. The slides were finally mounted using mounting solution containing DAPI (Vector Laboratories). Images were acquired using a Nikon ELIPSE 80i microscope.

2.4. Alkaline comet assays

Single-cell gel electrophoresis assays (comet assays) were performed under alkaline conditions. Briefly, cells were treated with NCS (100 ng/ml) or irradiated with UV (5 J/m^2) and then incubated in culture medium at 37 °C for the indicated periods of time. Cells were lysed through immersion in lysis solution (2.5 N NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 °C. The slides were then incubated with alkali buffer (300 mM NaOH, 1 mM EDTA, pH 12) for 30 min at RT and then electrophoresed in alkali buffer at 1 V/cm for 20 min at 4 °C. Following electrophoresis, the slides were stained with ethidium bromide and viewed under a fluorescence microscope. Analysis of the percentage of DNA that was tail DNA in each cell was performed using CometScore software.

3. Results and discussion

3.1. PP5 participates in UV-induced DNA damage repair by regulating ATR activity

To investigate the roles of PP5 in DNA repair, we analyzed DNA repair patterns in U2OS and U2OS-PS cells using comet assays. The comet assay is regarded to be a versatile and sensitive method for measuring single- and double-strand breaks (DSBs) in DNA [18]. In U2OS cells, the percentage of tail DNA was 46% at 1 h post-NCS treatment. It then decreased to 26% and 21% at 3 and 6 h posttreatment, respectively (Fig. 1A and B). In U2OS-PS cells, the percentage of tail DNA decreased gradually from 43% to 29% and then to 26%. Cells exposed to UV radiation exhibited different patterns of DNA damage from those treated with NCS. As shown in Fig. 1C and D, the amount of DNA damage 10 min after UV irradiation was similar in U2OS and U2OS-PS cells (39.3% and 41.4%, respectively). However, we noted a significant difference in repair activity in U2OS and U2OS-PS cells following UV treatment. The percentage of tail DNA fell from 39.3% to 14.8% by 6 h postirradiation in U2OS cells. In contrast, repair activity was very low in U2OS-PS cells, indicating that PP5 is needed for ATR-mediated repair.

After NCS treatment, initial DNA repair activity was similar in U2OS and U2OS-PS cells, which suggests that ATM activity in DNA repair was not affected by the absence of PP5. If ATM activity were influenced by the absence of PP5, the phenotypes of ATM- and PP5-deficient mice should be similar. However, Yong et al. (2007) reported that while ATM-deficient mice displayed growth retardation, male and female infertility due to meiotic failure and abnormal chromosomal synapsis, defects in lymphocyte maturation, and extreme sensitivity to radiation, PP5-deficient mice did not display these features [15]. Therefore, ATM activity during the DNA damage response appears not to depend on the presence of PP5. In contrast, UV-induced DNA damage has been shown to be only slowly repaired in ATR-suppressed cells [19]. Our results from U2OS-PS cells exposed to UV irradiation were comparable to the comet data from this previous study.

3.2. Downregulation of PP5 influences ATR activity in cells with UV-induced DNA damage

Several studies have reported that PP5 interacts with ATM and ATR and influences cell cycle arrest after DNA damage [14–16]. However, no direct evidence exists to show that these interactions also influence DNA repair activities. In this study, we investigated whether PP5 contributes to DNA repair by analyzing the phosphorylation of 53BP1 and BRCA1, substrates of ATR and ATM and important mediators of DDRs. Although 53BP1 is known to contribute to regulation of the G2/M phase checkpoint [20,21], as well as the repair of DNA DSBs, acting via NHEJ [22], whether the phosphorylation of 53BP1 is essential for its repair and recombination activities is not clear [13,23]. Nevertheless, 53BP1 is still considered to be an important mediator in DDRs. Unlike 53BP1, the functions of BRCA1 in DNA repair in terms of homologous recombination (HR) [24], interactions with other proteins [25,26], cell cycle arrest [27], transcriptional regulation [28], and X chromosome inactivation [29] are well characterized.

Moreover, the phosphorylation patterns of both 53BP1 and BR differ in UV-exposed and NCS-treated cells under conditions of PP5-suppression. After the induction of DSBs through treatment

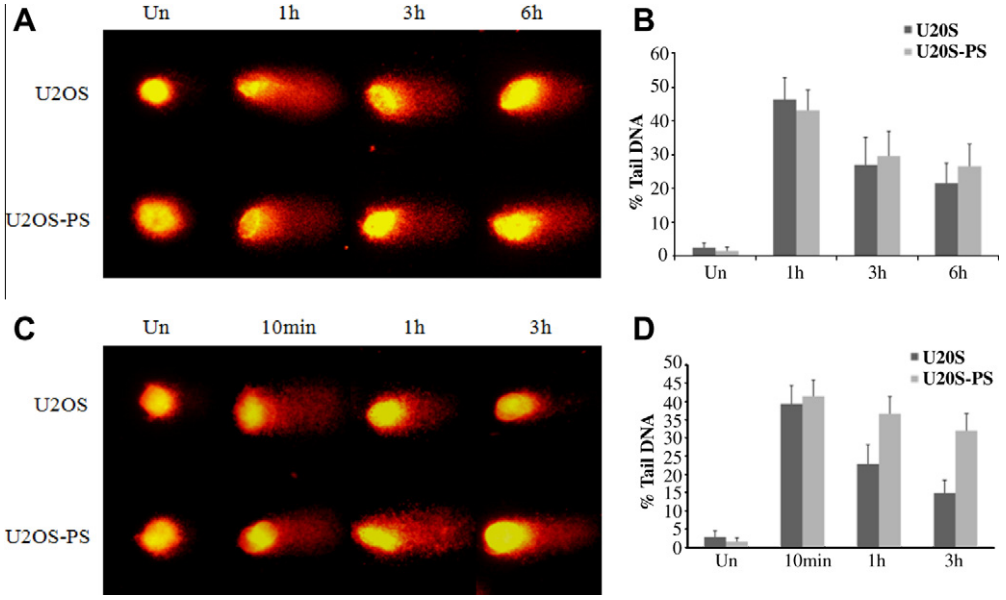

Fig. 1. DNA damages were not repaired in U2OS-PS cells after UV irradiation. DNA fragmentation in U2OS and U2OS-PS cells was quantified by single-cell electrophoresis following 100 ng/ml NCS treatment (A) and 5 J/m^2 UV irradiation (C). DNA was stained with EtBr and all images were acquired with fixed exposure times. DNA fragmentation was quantified by determining the tail DNA percentage at least 100 cells per condition using Comet Score software. (B and D) Graphs show the quantification of the tail DNA percentage shown in A and C at the indicated time point.

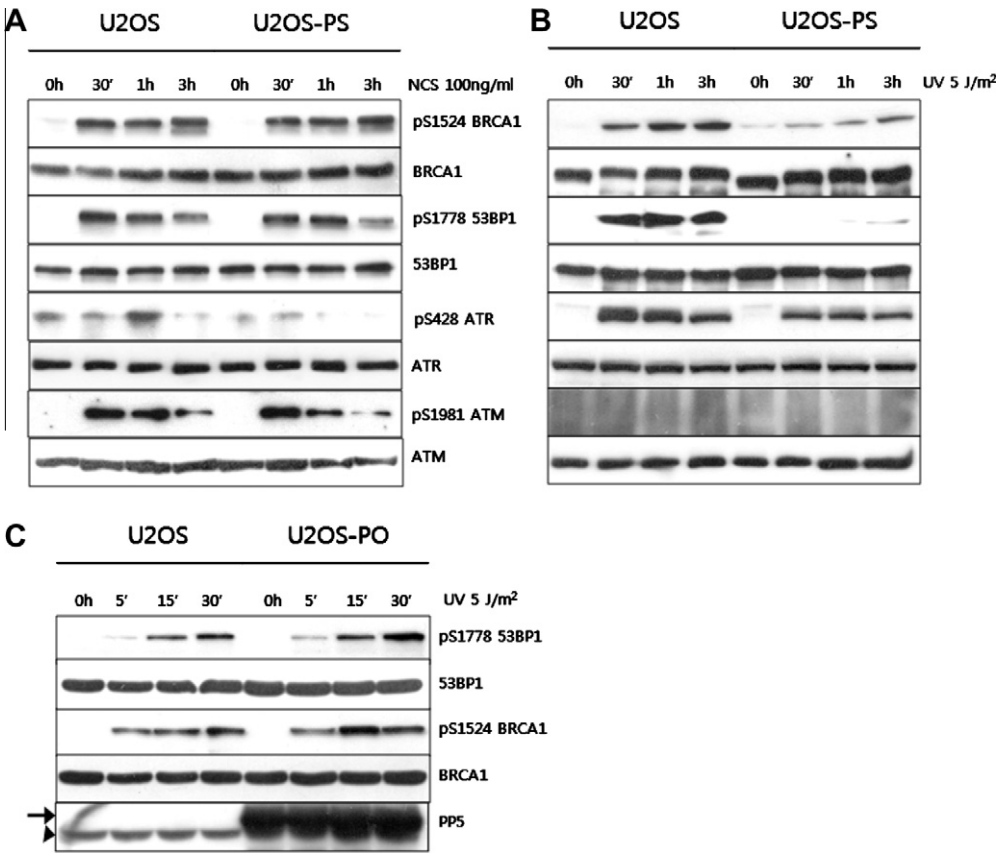

Fig. 2. The phosphorylation status of BRCA1 and 53BP1 were altered in the absence of PP5 after UV but not NCS treatment. (A) PP5 regulates the phosphorylation of 53BP1 and BRCA1 after UV-induced DNA damage. U2OS and U2OS-PS cells were irradiated with 5 J/m^2 and then harvested at an indicated times after UV irradiation. (B) PP5 is not related with the phosphorylation of 53BP1 and BRCA1 after NCS treatment. Cells were lysed with M-PER buffer and extracted proteins were resolved on 6% SDS–PAGE gel. Western blotting was performed to analyze the phosphorylation patterns of 53BP1 and BRCA1 after NCS (100 ng/ml) treatment. (C) The phosphorylation of 53BP1 and BRCA1 is not influenced by PP5over-expression. U2OS and U2OS-PO cells were irradiated with 5 J/m^2 UV and then harvested at the indicated times. Arrow indicates the V5-His tagged PP5 and arrow head indicates the endogenous PP5.

with NCS (100 ng/ml), the phosphorylation patterns of 53BP1 and BRCA1 were very similar in U2OS and U2OS-PS cells. Additionally, we could not detect a difference in the level of ATM phosphorylated at Ser1981, which has been linked to ATM activity [30] (Fig. 2A). However, the phosphorylation patterns of 53BP1 and BRCA1 following UV irradiation were different in U2OS and U2OS-PS cells (Fig. 2B). While high levels of phospho-53BP1 and -BRCA1 were detected at 30 min in U2OS cells, their induction was much weaker in U2OS-PS cells exposed to UV radiation. Together, this result and the results of the comet assays presented in Fig. 1 suggest that the DNA repair activity of ATR is dependent on PP5.

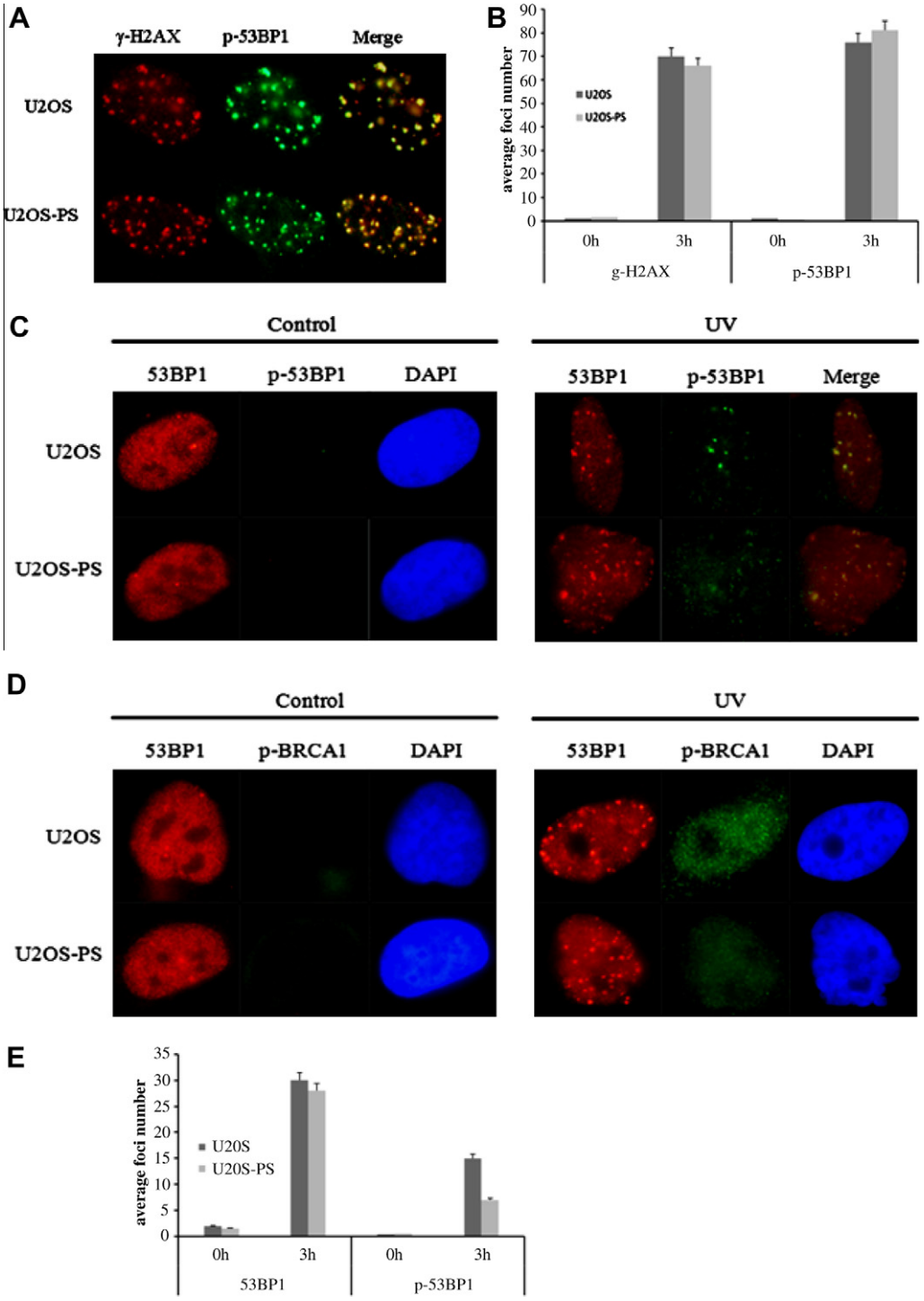

Fig. 3. PP5 does not influence the foci formation of phosphor-53BP1 after DNA damage. (A) The foci formation of γ-H2AX and phosphor-53BP1 were analyzed by immunofluorescence staining after NCS (100 ng/ml) treatment. 3 h after NCS treatment, U2OS and U2OS-PS cells were fixed with 3.7% PFA and then stained with γ-H2AX and pSer1778-53BP1 specific antibodies. (C and D) U2OS and U2OS-PS cells were grown on cover slides and then irradiated with 5 J/m^2 UV. At 3 h after UV irradiation, the cells were fixed and immunostained with antibodies directed against 53BP1, pSer1778-53BP1, and pSer1524-BRCA1. (B and E) Statistical evaluation of the experiments showed in A and C, respectively. The graph shows the average number of each focus based on ~100 nuclei per sample. The values represent the mean ± S.D. of three separate experiments.

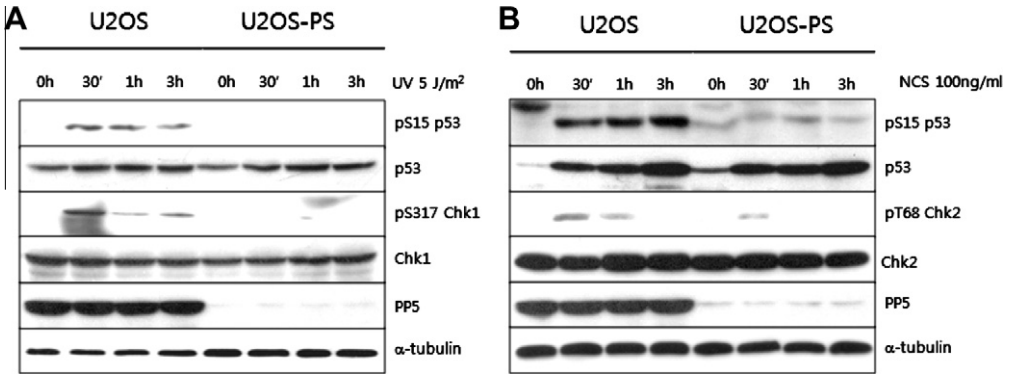

Fig. 4. PP5 is required to phosphorylate the cell cycle arrest proteins in U2OS and U2OS-PS cells after DNA damage. (A) To investigate the relationship between PP5 and cell cycle arrest we irradiated U2OS and U2OS-PS cells with UV (5 J/m^2) then harvested the cells at the indicated times (30 min, 1 h, and 3 h). Western blotting was preformed to check the influence of PP5 in checkpoint activation using antibodies specific to pSer15-p53 and pSer317-Chk1, which are well known checkpoint proteins. (B) U2OS and U2OS-PS cells were treated with NCS (100 ng/ml) to investigate the effect of PP5 on G2/M-phase arrest and harvested at 30 min, 1 h, and 3 h after NCS treatment. Levels of phosphorylated p53 (pSer15-p53), phosphorylated Chk2 (pThr68-Chk2), and total p53 and Chk2 were determined by immunoblotting with specific antibodies. PP5 expression was confirmed with specific antibody and β-tubulin was used as internal loading control.

To evaluate the relationship between PP5 levels and ATR activity, we analyzed the phosphorylation of 53BP1 and BRCA1 in U2OS and U2OS-PO cells, the latter of which overexpress PP5 protein and have been used in a previous study [13]. As shown in Fig. 2C, the levels of phosphorylated 53BP1 and BRCA1 were similar in U2OS and U2OS-PO cells following UV irradiation. This suggests that while PP5 is required for ATR-mediated DNA repair, the relationship is not linear.

4. 53BP1 focus formation is not influenced by ATR

Because they reflect the accumulation of repair proteins at DNA breaks, observing the formation of nuclear foci after DNA damage is important. Since the Tudor domain of 53BP1 is important for the recruitment of 53BP1 following DNA damage [31–33], and the recruitment of 53BP1 is not required for ATM activity [34], we were able to determine the relationship between the recruitment and phosphorylation of 53BP1.

We examined the formation of phospho-53BP1 and -BRCA1 nuclear foci in the presence and absence of PP5. After NCS treatment, we detected no differences in pSer1778 53BP1 and γ-H2AX focus formation between U2OS and U2OS-PS cells (Fig. 3A). However, a slightly different pattern of phospho-53BP1 focus formation was noted after UV treatment (Fig 3B): the number of pSer1778 53BP1foci decreased in U2OS-PS cells while numbers of 53BP1 foci remained unchanged in both cell types. Additionally, the immunofluorescent signal for phospho-BRCA1 also differed between U2OS and U2OS-PS cells following UV treatment (Fig. 3D). We suggest, based on these results, that the phosphorylation of 53BP1 and BRCA1 is closely linked to DNA repair or the recruitment of repair proteins to DNA break sites.

4.1. PP5 is involved in the regulation of checkpoint protein phosphorylation in response to DNA damage

Previous studies have reported that PP5 plays an essential role in cell cycle arrest following DNA damage by interacting with ATM and ATR [14–16]. To confirm the role of PP5 in cell cycle arrest following DNA damage, we analyzed the phosphorylation patterns of the checkpoint proteins p53, CHK1, and CHK2 following UV irradiation and NCS treatment. As shown in Fig. 4A, the phosphorylation of p53 at Ser15 and of CHK1 at Ser317 was induced between 30 min and 3 h post-UV irradiation in U2OS cells, but not in U2OS-PS cells, in which PP5 expression is downregulated [13]. Treatment with NCS produced patterns of p53 and Chk2 phosphorylation similar to those resulting from UV irradiation (Fig. 4B).

After exposure to IR, human BJ and mouse MEF cells with reduced levels of PP5 fail to arrest at the G2/M checkpoint and have decreased ATM activity [14,15]. PP5 is reportedly necessary for ATR-mediated checkpoint control: its suppression prevented the phosphorylation of p53, CHK1, and Rad17 in HeLa cells [16]. Our results are in agreement with those of other reports describing impaired phosphorylation of p53, CHK1, and CHK2 after DNA damage, and reports stating that PP5 is required for the phosphorylation of checkpoint proteins following NCS- and UV-induced DNA damage.

5. Conclusions

We showed that PP5 plays an essential role in the phosphorylation of the checkpoint proteins p53, CHK1, and CHK2 following DNA damage by regulating ATR and ATM activity. However, our results show that it plays different roles in ATM- and ATR-mediated DNA repair. Our comet assay and Western blotting data reveal that PP5 is essential for the activity of ATR, but not that of ATM, during DNA repair, but the mechanisms underpinning the roles of PP5 in response to different types of DNA damage remain unclear. Future studies are needed to unravel the different roles of PP5 in DNA damage responses.

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