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RPAP3 Interacts With Reptin to Regulate UV-Induced Phosphorylation of H2AX and DNA Damage

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ABSTRACT

We have previously reported that Monad, a novel WD40 repeat protein, potentiates apoptosis induced by tumor necrosis factor-α and cycloheximide. By affinity purification and mass spectrometry, RNA polymerase II-associated protein 3 (RPAP3) was identified as a Monad binding protein and may function with Monad as a novel modulator of apoptosis pathways. Here we report that Reptin, a highly conserved AAA + ATPase that is part of various chromatin-remodeling complexes, is also involved in the association of RPAP3 by immunoprecipitation and confocal microscopic analysis. Overexpression of RPAP3 induced HEK293 cells to death after UV-irradiation. Loss of RPAP3 by RNAi improved HeLa cell survival after UV-induced DNA damage and attenuated the phosphorylation of H2AX. Depletion of Reptin reduced cell survival and facilitated the phosphorylation on H2AX. These results suggest that RPAP3 modulates UV-induced DNA damage by regulating H2AX phosphorylation. J. Cell. Biochem. 106: 920–928, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: RPAP3; MONAD; REPTIN; H2AX; UV; CHROMATIN REMODELING

Genomic instability is a broad term used to denote a propensity for genetic changes, especially within tumor development and progression. In higher eukaryotes, four types of pathways elicited by DNA damage are DNA repair, DNA damage checkpoints, transcriptional response, and apoptosis. Defects in any of the four pathways may induce genomic instability [Zhou and Elledge, 2000]. The signal given by DNA damage leads to the activation of specific checkpoints resulting in the appropriate cell response [Sancar et al., 2004]. Progression into the cell cycle is stopped until damage is repaired, or, if there is too much damage, cells can enter apoptosis.

UV radiation is one of the most important ways to cause DNA damage. UV light is usually divided into A (320–400 nm), B (290–320 nm), and C (240–290 nm) by the wavelengths. UV induces two types of the most abundant mutagenic and cytotoxic DNA lesions: cyclobutane pyrimidine dimers and 6–4 photoproducts, thus severely damaging DNA and RNA molecules. The damaging DNA and RNA molecules causes diverse damage forms, including external damage, replication fork collision, apoptosis, dysfunctional telomeres, and double-stand breaks (DSBs) [Herrlich et al., 2008]. Energy absorbed by molecular oxygen (UVA and less so UVB) also generates reactive oxygen intermediates (ROI) by an ill-defined

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process. ROI oxidize DNA sugar residues, modify bases, and cause single and DSBs [Friedberg et al., 2005].

Histone H2AX, a mammalian histone H2A variant, is rapidly phosphorylated at Ser139 to γ-H2AX after DSBs [Rogakou et al., 1999; Pilch et al., 2003]. Because of the rapid induction and amplification of γ-H2AX, γ-H2AX has been recognized as a gold standard to detect the presence of DSBs [Fernandez-Capetillo et al., 2004]. γ-H2AX is necessary for the retention of DNA repair factors at sites of DNA breaks and when DNA is repaired, the levels of γ-H2AX decrease. Therefore, levels of γ-H2AX serve as a measure of DNA damage and repair. H2AX phosphorylation by the DNA damage sensor Ataxia-telangiectasia mutated (ATM), or possibly by ATM/ATR family members, is one of the earliest events in the DNA damage response [Mukherjee et al., 2006; Rahal et al., 2008]. The ATM/ATR family plays a critical role in the G1/S and G2/M checkpoints induced in response to DNA damage. ATM itself is activated by autophosphorylation in response to DNA damage. Phosphorylated ATM localizes at sites of DNA damage to phosphorylate H2AX and many other targets, including Chk2, p53, and BRCA1 [Banin et al., 1998; Canman et al., 1998; Cortez et al., 1999].

Pontin (Tip49a, Rvb1) and Reptin (Tip49b, Rvb2) are two putative AAA + ATPases that are highly conserved in eukaryotes. They are both components of at least four multiprotein complexes that play roles in chromatin remodeling (SRCAP, hINO80 [Jónsson et al., 2004], TRRAP/TIP60 [Fuchs et al., 2001; Kim et al., 2005; Qi et al., 2006; Fazzio et al., 2008], or Uri/ Prefoldin [Gstaiger et al., 2003; Parusel et al., 2006; Djouder et al., 2007; Kirchner et al., 2008]. The role of Pontin and Reptin within these chromatin-remodeling complexes is still unclear. Recently, it was reported that depletion of Pontin increases the amount and persistence of chromatin-associated H2AX after the exposure of cells to UV-irradiation [Jha et al., 2008]. However, Pontin's partner, Reptin's function in the phosphorylation of H2AX is still unknown.

Monad, a novel WD40 repeat protein [Saeki et al., 2006], has a conserved propeller sequence. We identified RNA polymerase II-associated protein 3 (RPAP3) that contains tetratricopeptide repeat (TPR) domains [Jeronimo et al., 2007] as a binding protein of Monad by affinity purification and mass spectrometry [Itsuki et al., 2008]. In addition to the effect of TPR motif functions on cell cycle regulation [Hirano et al., 1990; Sikorski et al., 1990], it is also involved in processes such as transcriptional control, protein transport, and protein folding [Lamb et al., 1995; D'Andrea and Regan, 2003]. However, the functions of RPAP3 and Monad are still unclear. In this article, we report that Reptin binds to the complex of RPAP3 and Reptin-RPAP3 complex regulates H2AX phosphorylation after UV-induced DNA damage.

MATERIALS AND METHODS

REAGENTS

Anti-V5 monoclonal is from Invitrogen (Carlsbad). Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) monoclonal 23040091 is from Chemicon (Temecula). Anti-glutathione S-transferase (GST) monoclonal, anti-phospho- ATM (Ser1981) 4526, anti-phospho- ATR (Ser428) 2853, anti-phosphor-Chk1 (Ser296) 2349, anti-phospho-Chk2 (Thr68) 2661, and anti-phospho-H2AX (Ser139) 2577 are from Cell Signaling Technology (Beverly). Anti-Histon H2AX 07-627 is from Millipore (Temecula, CA). Anti-Reptin R25920 is from Transduction Laboratory (Lexington). Anti-Monad and Anti-RPAP3 antibodies were generated as described before [Saeki et al., 2006; Itsuki et al., 2008].

CONSTRUCTS

Human RPAP3 and Reptin cDNA were cloned into pENTR/D TOPO vector as described previously [Itsuki et al., 2008] and subcloned into GST-tagged pDEST27 (pDEST27-RPAP3), Biotin-tagged pcDNA6 (pcDNA6-RPAP3), and V5-tagged pcDNA6.2 (pDEST6.2-Reptin), respectively, using the Gateway System (Invitrogen). Full-length cDNA encoding human RPAP3 was cloned into the vector pEGFP-N1 (Clontech, Mountain View) between the *Xho*I and *Eco*RI restriction sites to generate pEGFP-RPAP3. The human Reptin sequence was inserted into the *Bgl*II and *Pst*I restriction sites of pDsRed-express-N1 (Clontech) to generate pDsRed-Reptin.

CELL CULTURE

HEK293, HeLa, and COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 1 μl/ml amphotericin B.

SiRNA KNOCKDOWN EXPERIMENTS

RPAP3-specific and Reptin-specific small interfering RNA (siRNA) were purchased from Qiagen (Valencia) and targeted the following sequences: 5′-GGACTATCTTTGAACATAA-3′ (RPAP3-siRNA), 5′-CCGGAGATCCGTGATGTAACA-3′ (Reptin-siRNA), 5′-ACGGTGGG-AGACAAACATCAA-3′ (Monad-siRNA).

AllStars Negative Control siRNA (Qiagen) was used as a control. The specific nucleotide sequences (5′-CACCGCATGGATGCCGATC-CATATACGAATATATGGATCGGCATCCATGC-3′) corresponding to RPAP3 or LacZ were inserted into pENTR/H1/TO, respectively, to generate short hairpin RNA (shRNA) constructs according to the manufacturer's protocols of BLOCK-iT™ Inducible H1 RNAi Entry Vector kit (Invitrogen). After HeLa cells were transfected with shRNA constructs for 24 h, the expression level of RPAP3 was monitored by Western blotting analysis.

ESTABLISHMENT OF CELL LINES

Tetracycline repressor expressing HeLa cells (Invitrogen) or HEK293 cells were transfected with shRNA vector or pDEST6-RPAP3 and selected with 500 μg/ml Zeosin (for shRNA vector) or 5 μg/ml Blasticidin.

TRANSFECTION, DNA DAMAGE, AND IMMUNOBLOTTING

HeLa cells were seeded onto 60 mm Petri dishes and grown overnight. Plasmids or siRNAs were transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 8 h, transfected cells were returned to growth medium and incubated for 48 or 72 h. With or without UV-irradiation, the cells were harvested after indicated time of UV treatment. Cells were lysed

in extraction buffer [50 mM Tris/HCl (pH 7.5), 140 mM NaCl, 2 mM EGTA, 1% Triton X-100] that included protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and phosphatase inhibitor (Sigma, St. Louis) according to the manufacture's instruction. The cell homogenates were mixed with Laemmli sample buffer and boiled for 3 min, separated by SDS–PAGE and transferred to PVDF membrane. Immunoblotting was carried out as described previously [Saeki et al., 2002].

GST PULL-DOWN ASSAY

HEK293 cells were transfected with either pDEST6.2-Reptin (V5-tagged Reptin) or pDEST27-RPAP3 (GST-tagged RPAP3) using Lipofectamine 2000. After 48 h, cells were harvested and lysed in extraction buffer as described above. The lysate was incubated with glutathione sepharose (Amersham Biosciences). The mixture was washed three times with the buffer [50 mM Tris/HCl (pH 7.5), 140 mM NaCl, 2 mM EGTA, 0.1% Triton X-100] and eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0. The eluted proteins were boiled in Laemmli sample.

IMMUNOPRECIPITATION

HeLa lysates were incubated with 2 μg of antibody against to Reptin or IgG1 for 16 h, followed by incubation with Protein G Sepharose (Amersham Biosciences) for 1 h. The sepharose beads were washed three times with washing buffer [0.1% TritonX-100, 140 mM NaCl, 2 mM EGTA, 50 mM Tris/HCl pH 7.5 including protease inhibitor cocktail], associated proteins were recovered by boiling for 5 min in Laemmli buffer, separated by SDS–PAGE and subjected to immunoblotting with anti-RPAP3 antibody.

IMMUNOFLUORESCENCE MICROSCOPY

COS7 cells were grown on glass coverslides for 24 h and transfected with the indicated vectors. For immunofluorescence microscopy, cells were fixed in 3.7% (w/v) paraformaldehyde for 20 min, and further washed with phosphate-buffered saline (PBS). Analysis and photography were performed on a Carl Zeiss LSM510 confocal laser scanning microscope with a Plan-Apochoromat 100×/1.4 oil DIC objective at excitation wavelengths 488 and 543 nm, respectively. Data acquisition was performed with the Leica Confocal Software Pack (version 4.0.0.157) and data collected from 4× serial sections. Figures were prepared using Adobe Photoshop 6.0 software without any adjustments.

CELL VIABILITY ASSAY

HeLa cells seeded in 48-well plate were transfected with siRNA, 48 h later cells were washed in PBS and exposed to 100 J/m^2 UVB light. After irradiation, cells were returned to culture medium (DMEM with10% FBS). Cell viability was measured by MTT (Sigma, M2128) assay.

STATISTICAL ANALYSIS

Data was expressed as means ± SEM. Statistical differences between groups were determined using Tukey's test after ANOVA and considered significant when $P < 0.05$.

RESULTS

RPAP3 INTERACTS WITH REPTIN

We had previously reported that RPAP3 binds to Monad, a novel WD40 repeat protein, which potentiates apoptosis induced by tumor necrosis factor (TNF)-α and cycloheximide (CHX) [Itsuki et al., 2008]. In addition to RPAP3, Reptin with molecular weight of ~50 kDa was also identified as a protein that specifically interacts with Monad.

To investigate the possibility that RPAP3 binds to Reptin, the full-length cDNAs encoding RPAP3 and Reptin were cloned into eukaryotic expression vectors so that the interaction of Reptin and RPAP3 could be investigated by GST pull-down assay. HEK293 cells were transfected with a plasmid encoding V5-tagged Reptin in the presence or absence of a plasmid encoding GST-tagged RPAP3, and the cell lysates were bound to glutathione beads. Western blot analysis with anti-V5 antibody of the material bound to glutathione beads showed that V5-tagged Reptin was bound when GST-tagged RPAP3 was present, suggesting that RPAP3 interacted with Reptin in the transfected cells (Fig. 1A). Using HeLa lysates, we also confirmed that RPAP3 interacted with endogenous Reptin by immunoprecipitation (Fig. 1B).

REPTIN AND RPAP3 CO-LOCALIZE IN BOTH THE CYTOPLASM AND NUCLEUS

Based on its association with Reptin, RPAP3 would be expected to co-localize with Reptin intracellularly. Confocal fluorescence microscopy was used to investigate whether the predicted co-localization could be observed. COS7 cells were transfected with

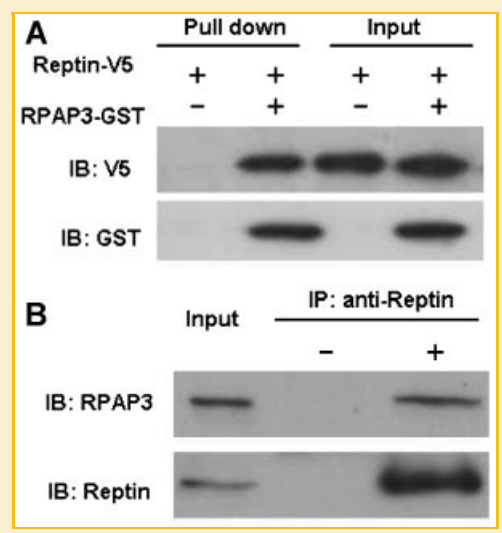

Fig. 1. Interaction of RPAP3 with Reptin (A) HEK293 cells were transfected with expression vectors encoding GST-RPAP3 and Reptin-V5. Lysates were prepared and bound to glutathione beads. Immunoblotting was performed following SDS–PAGE separation, using antibody against V5 which recognizes the transfected Reptin. The input cell lysate is also shown. B: Immunoprecipitation (IP) of RPAP3 and Reptin from HeLa cells. HeLa lysate was immunoprecipitated with control IgG(−) or Reptin antibody(+). Following separation by SDS–PAGE, immunoblotting was performed using anti-RPAP3 antibody.

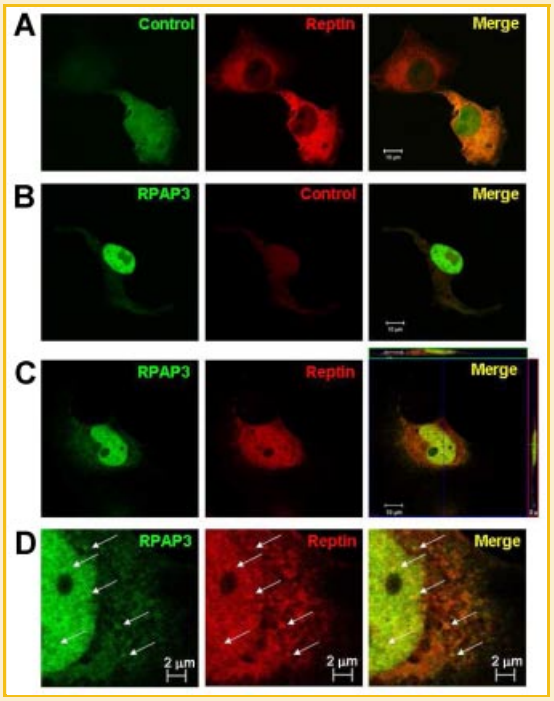

Fig. 2. Intracellular co-localization of the EGFP-RPAP3 and DsRed-Reptin fusion proteins Fluorescent images of COS7 cells co-transfected with either EGFP-tagged RPAP3 or DsRed-tagged Reptin vector or both acquired with a confocal microscope. A: EGFP-control and DsRed-Reptin vectors; B: EGFP-RPAP3 and DsRed-control vectors; C: EGFP-RPAP3 and DsRed-Reptin vectors; D: magnification of C. Arrows indicate co-localization of Reptin and RPAP3 in cytoplasm in speckled structures. A stack of images was taken at every 5 μm to visualize the co-localization. Images were taken as a z-stack using a 100× objective. The merged images are an orthogonal view showing a clear co-localization in x-, y-, and z-planes indicating that Reptin co-localized to RPAP3 in the nucleus. Bars indicate 10 μm in A, B, and C or 2 μm in D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression plasmids encoding RPAP3 fused EGFP (EGFP-RPAP3) and/or Reptin fused DsRed (DsRed-Reptin) at the N-terminal (Fig. 2). Images were acquired as a z-stack using a 100× objective lens. EGFP-RPAP3 showed strong nuclear localization, although faint staining was also detectable in the cytoplasm. DsRed-Reptin was observed in both cytoplasm and nuclear locations, consistent with previous report [Kim et al., 2006]. In cells expressing both proteins, Reptin was concentrated in prominent speckled structures in the cytoplasm in some of which RPAP3 was found to co-localize. The arrows in Figure 2D indicate co-localization of Reptin and RPAP3 in these speckled structures in the cytoplasm. Because of the published interactions between RPAP3 and RNA polymerase II [Jeronimo et al., 2007] and that the strong nuclear localization of RPAP3 suggested that some RPAP3 protein may associate with chromatin. A stack of images was taken every 5 μm in order to visualize the nucleus. Using orthogonal views of the stack, the merged images show a clear co-localization in x-, y-, and z-planes (merged yellow color) indicating that Reptin also co-localized with RPAP3 in the nucleus as well as in the cytoplasm.

Taken together with the biochemical interaction, the co-localization suggests that RPAP3 and Reptin might possess related functions.

OVEREXPRESSION OF RPAP3 DECREASES CELL SURVIVAL OF HEK293 CELLS AFTER UV-IRRADIATION

We generated HEK293 cells stably expressing Biotin-RPAP3 or Biotin-LacZ (control) vector and the overexpression effect was confirmed using Western blotting (Fig. 3A, lower). The UV-irradiation (100 J/m^2, 302 nm, UVB) caused ~50% survival ratio 2–4 days after irradiation. Overexpression of RPAP3 promoted UV-induced cell death (Fig. 3A, upper). We had previously reported that overexpression of RPAP3 potentiates caspase-3 activation and apoptosis induced by TNF-α and CHX, and the depletion of RPAP3 by RNA interference (RNAi) resulted in a significant reduction of apoptosis. Thus, we proposed that *Overexpression of RPAP3 promoted UV-induced cell death.*

KNOCKDOWN OF RPAP3 PROTECTS CELLS AGAINST UV-IRRADIATION

Studies in different organisms have suggested the importance of Pontin and Reptin in the related functions of transcription and DNA repair. RNAi mediated knockdown of Reptin leads to UV hypersensitivity [Wu et al., 2007]. Based on the hypothesis that RPAP3 might prompt cell death but not their survival and these data, we next examined the effect of knockdown of RPAP3 and Reptin on sensitivity to UV-induced DNA damage.

HeLa cells in which RPAP3 and Reptin had been knocked down by use of RNAi, showed opposite responses to UV-irradiation in cell viability assays. The RNAi knockdown efficiencies of the various siRNAs used were confirmed by Western blot of the respective proteins. Knockdown of RPAP3 protects cells against UV-induced DNA damage. Knockdown of RPAP3 and Reptin simultaneously decreased cell survival, which is same to the effect of depletion Reptin only in response to DNA damage (Fig. 3B1). Loss of Monad did not significantly affect cell survival (Fig. 3B2).

To confirm the protective effect of RPAP3 knockdown on UV-induced cell damage, we used HeLa cells, which were transfected with tetracycline-inducible shRNA designed to target endogenous RPAP3 or control LacZ. The efficiency of RNAi knockdown of RPAP3 after doxycycline treatment of these cells was confirmed by Western blotting (Fig. 3C, lower). Cells were exposed to 100 J/m^2 UV-irradiation 48 h after doxycycline treatment. The cells depleted of endogenous RPAP3 showed a higher cell survival rate than control cells (Fig. 3C, upper). This result is consistent with that of Figure 3A that knockdown of RPAP3 by siRNA protects cells against UV-irradiation.

THE LOSS OF RPAP3 RESULTS IN A DECREASE IN γ-H2AX, WHILE THE LOSS OF REPTIN RESULTS IN AN INCREASE IN γ-H2AX

Pontin and Reptin are two putative AAA + ATP binding proteins that are components of various chromatin-remodeling complexes. Phospho-H2AX spans megabases flanking a DNA damage site and helps to recruit proteins involved in DNA repair. Depletion of Pontin increased the amount and persistence of phosphorylation on chromatin-associated H2AX after exposure of cells to UV-irradiation [Jha et al., 2008]. However, it is not known if Reptin affects phosphorylation of H2AX in cells with UV-induced damage. To investigate this, HeLa cells were transfected with siRNAs that knockdown Reptin or RPAP3. After 3 days, the cells were irradiated

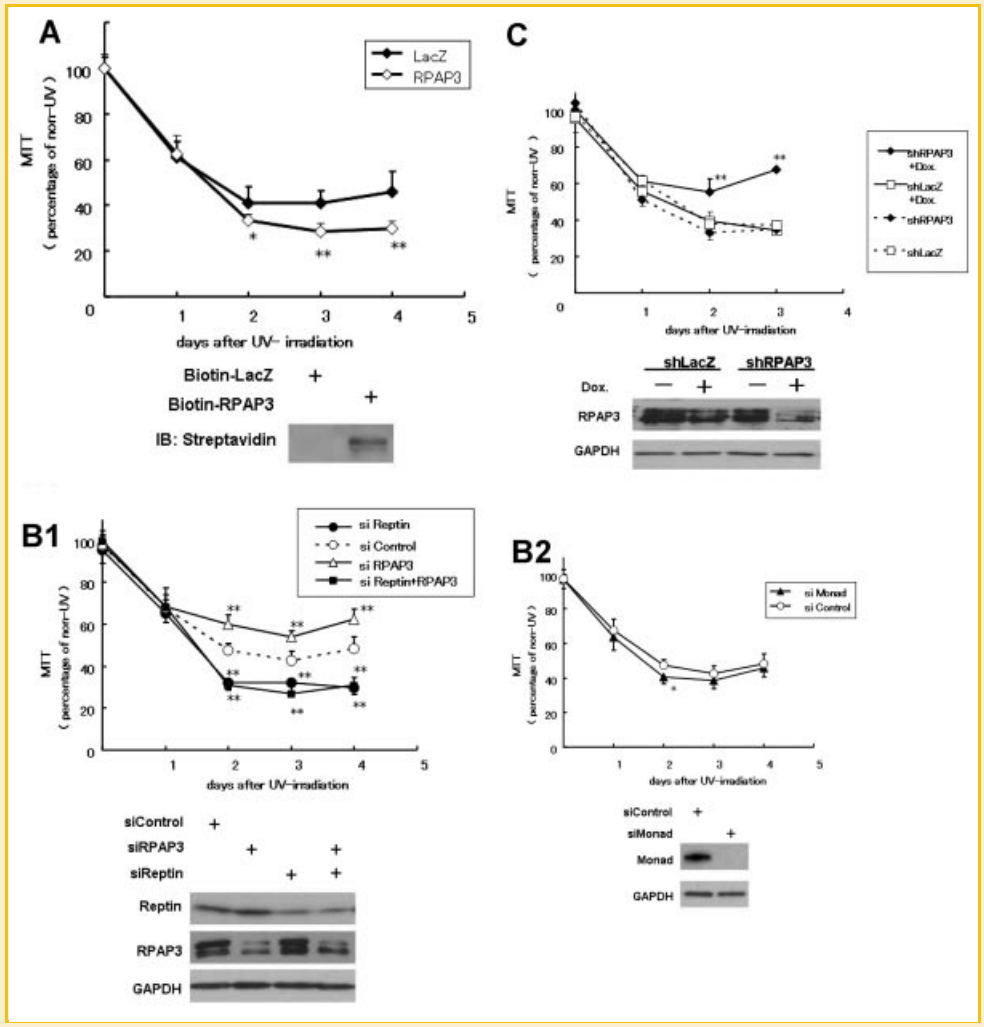

Fig. 3. The role of RPAP3 and Reptin in the regulation of cell survival after UV irradiation. A: Effect of DNA damage in RPAP3 overexpressed cells. HEK293 cell lines stably express Biotin-RPAP3 or its control Biotin-LacZ vector were seeded 10,000 cells per well in 48-well plates. Cell viability was analyzed by MTT assay at the different days after 100 J/m^2 UVB (302 nm) irradiation. Cell survival ratio was expressed as OD 595 nm relative to that of untreated cells. ($n = 8$) B1: Effect of DNA damage on cell survival in RPAP3 and/or Reptin depleted cells. Five thousand HeLa cells were seeded per well in 48-well plates. Cells were transfected with siRNA for RPAP3 and Reptin, respectively or simultaneously. B2: Monad does not affect the UVB-induced cell survival. Cells were transfected with siRNA for Monad. After UVB irradiation, cell viability was analyzed by MTT assay. Cell survival ratio was expressed as OD 595 nm relative to that of untreated cells. ($n = 12$). C: Silencing endogenous RPAP3 by shRNA protected cells against UV-irradiation and increased cell survival rate. HeLa cells that stably express either shRPAP3 or shLacZ (control) were treated with or without doxycycline (Dox.) for 3 days and then exposed to UVB. Five thousand cells were seeded per well in 48-well plates. Cell survival determined by MTT assay was expressed as OD 595 nm relative to that of UV-untreated cells. ($n = 6$) Upper panel of A, B, and C are presented as means $\pm$ SEM, with levels of significance at $^{*}P < 0.05$; $^{**}P < 0.01$ compared to the control group. Lower panels of A, B, and C are the overexpression or depletion effect of RPAP3, Reptin or Monad by siRNA/shRNA monitored by immunoblotting with antibodies against molecules or streptavidine-HRP (for Biotin-tagged RPAP3, Amersham Biosciences).

with UV and then harvested at various time points for analysis by immunoblots. The loss of Reptin dramatically up-regulated γ-H2AX, a marker of DSBs, suggests the presence of unrepaired DNA damage. In contrast, the loss of RPAP3 results in a decrease in the level of γ-H2AX without any change in H2AX levels in UV-treated cells. Interestingly, knockdown of RPAP3 simultaneously with Reptin prevented the decrease in γ-H2AX levels (Fig. 4A, upper and down). Notably, without UV exposure, accumulation of γ-H2AX was observed in the Reptin-knockdown cells, compared with those in control or RPAP3-knockdown cells. Similar experiments were also performed using stable cell lines that conditionally express shRNA vectors against RPAP3 that target different silencing sequence (Fig. 4B, upper and down). Taken together, these data suggest that RPAP3, besides modulating the apoptosis functions of Monad, may also control the UV-induced DNA damage by regulating H2AX phosphorylation.

RPAP3 DEPLETION DECREASES γ-H2AX WHILE REPTIN DEPLETION INCREASES γ-H2AX IN RESPONSE TO UVC IRRADIATION

Maximal absorbance of DNA and RNA occurs at shorter wavelengths (UVC > UVB > UVA), inducing covalent dimer formation of

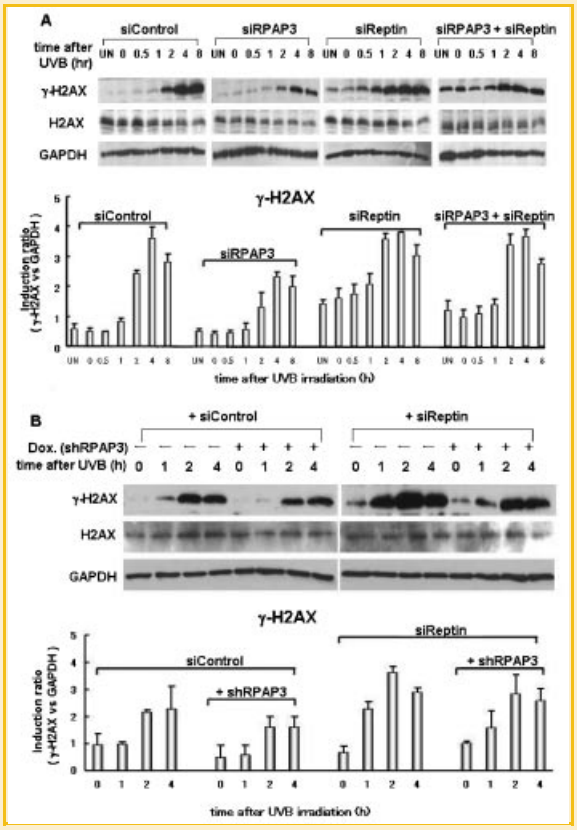

Fig. 4. Knockdown of RPAP3 inhibits the UVB-induced damage. A: The effects of down regulating Reptin, RPAP3, or both on the DNA damage indicator protein H2AX. HeLa cells were transfected with the siRNAs for Reptin and RPAP3, respectively or simultaneously. After 100 J/m^2 UVB-irradiation, cells were harvested at the indicated time (h), and their lysates were immunoblotted with antibodies against molecules in response to DNA damage. Arrow indicates the bands of RPAP3. The lane marked UN is from cells that were not treated with UVB. B: HeLa stable cell line conditionally expresses shRNA vector targeting RPAP3 sequence was transfected with siRNAs for control or Reptin. Cells were treated with doxycyline (Dox.) for 3 days to double silence RPAP3 and Reptin. After UVB-irradiation, cell lysates were harvested at the indicated time. Immunoblotting was performed using same antibodies in A. Upper panel of A and B shown are representative immunoblotting image of at least three independent experiments. Lower panels are quantitation of phospho-H2AX signals in the upper panel (normalized to GAPDH) using FluroChem 8000 (Alpha Innoteck Corp., San Leandro). The experiment was repeated three times, and the means ± SEM are plotted.

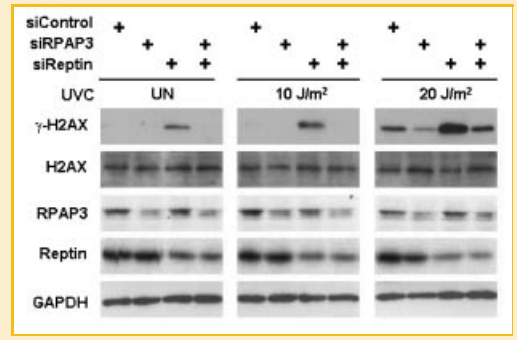

Fig. 5. Knockdown of RPAP3 also protects HeLa cells from the UVC-induced DNA damage. HeLa cells were transfected with the siRNAs for Reptin, RPAP3, or both. Cells were exposed under UVC (10 and 20 J/m^2, 254 nm) and were harvested 4 h after the irradiation. Lysates were immunoblotted with antibodies against molecules in response to DNA damage.

adjacent pyrimidines, thus leading to severe DNA damage [Herrlich et al., 2008]. We therefore checked whether UVC also induced the decrease of γ-H2AX in RPAP3 depleted HeLa cells. Accumulation of γ-H2AX was observed in the Reptin-knockdown cells without or 10 J/m^2 UVC. (Fig. 5) Phosphorylation of H2AX occurred at 20 J/m^2 UVC irradiation. The depletion of RPAP3 results in a decrease in the level of γ-H2AX and the loss of Reptin results in an increase of γ-H2AX after DNA damage. The double knockdown of RPAP3 and Reptin prevented the decrease in γ-H2AX levels caused by Reptin siRNA, which agreed with the effect of UVB irradiation.

RPAP3 AND REPTIN DEPLETION DOES NOT AFFECT THE PHOSPHORYLATION OF PHOSPHATIDYLINOSITOL 3-KINASE (PI3K)-LIKE KINASES AFTER UV-IRRADIATION

ATM and ATR are members of the PI3 K-like kinase family that phosphorylate multiple substrates in response to DNA damage [Deng, 2006; Kranz et al., 2008]. Since it is possible that in the DNA repair process, decreased γ-H2AX may be due to the attenuation of ATM/ATR, we investigated whether the levels of phosphorylated ATM (p-ATM) were altered by immunoblotting after UV-irradiation. The knockdown of RPAP3 results in a slight decrease in phosphorylated ATM (p-ATM) and but not phosphorylated ATR (p-ATM) compared with the control (Fig. 6A). However, the phosphorylation of Chk1 and Chk2, checkpoint kinases that are known targets of ATM and ATR, were not affected when RPAP3 is depleted (Fig. 6A). These results suggest that the decrease of γ-H2AX cannot be elucidated by the phosphorylation by PI3K-like kinase family. The loss of Reptin does not increase the phosphorylation of ATM/ATR, nor the phosphorylation status of Chk1 (p-Chk1) and Chk2 (p-Chk2) (Fig. 6B), suggesting that the increase in γ-H2AX was not due to hyperactivation of ATM or ATR. These results are consistent with the previous report that Pontin depletion does not affect the phosphorylation level of ATM/ATR [Jha et al., 2008].

DISCUSSION

We previously reported the identification and characterization of a novel human WD40 repeat protein, Monad [Saeki et al., 2006]. By affinity purification and mass spectrometry, we identified RPAP3 as a binding partner for Monad. Overexpression of RPAP3 in HEK293 cells also potentiates apoptosis and caspase-3 activation induced by TNF-α and CHX. RPAP3 transcripts are expressed in most tissues, most abundantly in testis [Itsuki et al., 2008]. This distribution pattern is very similar to that of Monad [Saeki et al., 2006]. Notably, Reptin transcripts are also highly expressed in mouse and rat testis and thymus [Kanemaki et al., 1999; Makino et al., 1999; Chauvet et al., 2005; Mizuno et al., 2006]. These data raised the possibility

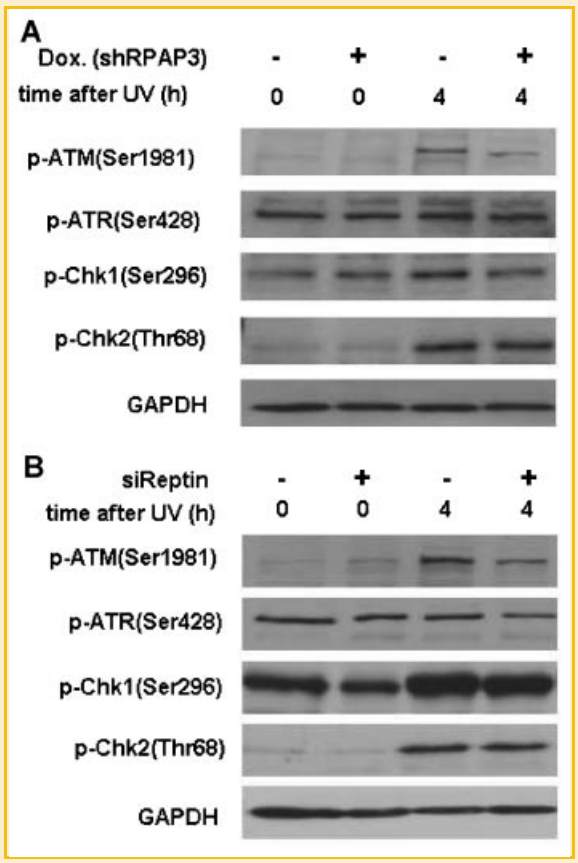

Fig. 6. The effects of down-regulating Reptin or RPAP3 on the major signaling checkpoints molecule in response to DNA damage. After 100 J/m^2 irradiation of UV-B, cells were harvested and lysates were probed with the indicated antibodies. Data shown are representative immunoblotting image of at least three independent experiments. B1: Stable HeLa cell lines containing a shRNA vector that silences RPAP3 expressing after doxycycline (Dox.) treatment for 3 days. B2: HeLa cell transfected with siRNA for Reptin.

that RPAP3 may function as a novel modulator of apoptosis pathway together with Monad.

In this article, we reported that depletion of RPAP3 by RNAi improved survival of HeLa cells after UV-induced DNA damage and reduced phosphorylation of H2AX. In addition, overexpression of RPAP3 in HEK293 cells promoted cell death after UV damage, suggesting that RPAP3 may be a factor related to cell death, in either the apoptosis pathway induced by TNF-α or the pathway activated by UV-induced cell damage.

When UV irradiation induced DNA damage happens, the signal given by DNA damage leads to the activation of specific DNA checkpoints resulting in the appropriate cell response. The transcription response and DNA repair simultaneously work till the damage is repaired, or, if there is too much damage, cells can enter apoptosis. When RPAP3 is overexpressed, cells tend to death either by UV-induced DNA damage or TNF-α induced apoptosis. The apoptosis triggered by TNF-α and CHX is a final phenotype of unrepaired DNA damage.

Reptin, a putative ATPase present in various chromatin-remodeling complexes [Bellosta et al., 2005; Etard et al., 2005; Baek, 2006; Gallant, 2007; Rousseau et al., 2007], also participates in the complex of RPAP3. Pontin and Reptin physically interact and are thought to function together, although there is some evidence that Pontin and Reptin can act independently or in an opposing fashion [Bauer et al., 2000; Rottbauer et al., 2002]. In the present study, the depletion of Reptin inhibited cell survival and facilitated the phosphorylation of H2AX. It is still to be elucidated what the relationship between RPAP3 and Reptin is and how RPAP3 and Reptin impact DNA damage and repair. Our results suggest that RPAP3 positively regulate UV-induced phosphorylation of H2AX and DNA damage by interacting with Reptin. The depletion of both RPAP3 and Reptin has the same effect as depletion of Reptin only, suggest that RPAP3 is a negative regulator of Reptin, although it is still possible that RPAP3 and Reptin act through independent pathways.

Most proteins within a cell reside in a complex with other proteins at least temporarily. A protein of unknown function can be functionally classified if an interaction with another protein of known function can be shown. Our data demonstrated that RPAP3 and Reptin have opposite effects on cell survival and the regulation of γ-H2AX to response the DNA damage. How RPAP3 regulates Reptin's function and how RPAP-3 antagonizes γ-H2AX increase following knockdown of Reptin are still unclear. More detailed observations are being followed up to prove our hypotheses.

PPAP3 is mainly localized in the nucleus. Reptin was observed in both nuclear and cytoplasmic locations whereas SUMO conjugated Reptin resulted in exclusive nuclear localization [Kim et al., 2006]. Does the coincidence of RPAP3 and SUMO-Reptin in nuclear suggest that RPAP3 regulate Reptin by sumoylation? Preliminary data show that Reptin's distribution in subcellular fraction does not change when RPAP3 is knocked down. A series of experiments to check the sumorylation of Reptin using RPAP3 knocked down cell strain are going on.

Both Pontin and Reptin are members of a large superfamily of ATPases that are associated with diverse cellular activities. The chromatin-remodeling complexes containing Reptin include both the ATPase-type remodelers (Ino80, SRCAP, Swr1) and the acetyltransferase remodeler Tip60. We have established the ATPase activity assay using Pontin and Reptin recombinant protein. Does RPAP3 regulate Reptin by modulating its ATPase activity? We hope to get more information of this question by ATPase activity assay using Reptin recombinant protein together with some other cofactors (e.g., RPAP3, Monad) because the ATPase activity of Reptin may depend on the binding of a cofactor or stimulation by a cofactor. It is favorable that Reptin's ATPase activity is essential to chromatin-remodeling by regulating a cofactor.

Nucelosomes at DNA damage sites should be acetylated on histone H4 before γ-H2AX is remodeled and made available for dephosphorylation. Pontin is critical for the dephosphorylation of γ-H2AX, and is necessary to maintain the histone acetyltransferase (HAT) activity of Tip60/NuA4 complex [Jha et al., 2008]. The Tip60 complex associates with the sites of damage DNA and is known to promote opening of the chromatin by the posttranslational modification of the histone tails [Murr et al., 2006]. We propose that Reptin is also an important regulator in the proper assembly and activity of the chromatin-remodeling complex during the response

to DNA damage. It remains to be determined whether Reptin works similarly to Pontin on the HAT activity of Tip60. If Reptin regulates γ-H2AX in a similar mechanism together with Pontin, we will be able to investigate whether RPAP3 regulates phospho-H2AX by antagonizing HAT activity of the TIP60 complex.

Most cancers arise due to genomic instability and malignant conversion induced by mutation accumulation in critical genes (e.g., p53) [Kastan and Bartek, 2004; Feng et al., 2008]. During cancer progression, the incipient tumor experiences ''oncogenic stress,'' which evokes a DNA damage response that delays or eliminates such hazardous cells [Bartkova et al., 2005; Gorgoulis et al., 2005]. According to our study, knockdown of RPAP3 alleviates the UV-induced DNA damage directly or indirectly. Thus, RPAP3 has potential roles in tumor suppression, DNA damage repair, and cell cycle checkpoints.

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