BMB
reports

Knock-down of human MutY homolog (hMYH) decreases phosphorylation of checkpoint kinase 1 (Chk1) induced by hydroxyurea and UV treatment

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The effect of human MutY homolog (hMYH) on the activation of checkpoint proteins in response to hydroxyurea (HU) and ultraviolet (UV) treatment was investigated in hMYH-disrupted HEK293 cells. hMYH-disrupted cells decreased the phosphorylation of Chk1 upon HU or UV treatment and increased the phosphorylation of Cdk2 and the amount of Cdc25A, but not Cdc25C. In siMYH-transfected cells, the increased rate of phosphorylated Chk1 upon HU or UV treatment was lower than that in siGFP-transfected cells, meaning that hMYH was involved in the activation mechanism of Chk1 upon DNA damage. The phosphorylation of ataxia telangiectasia and Rad3-related protein (ATR) upon HU or UV treatment was decreased in hMYH-disrupted HEK293 and HaCaT cells. Co-immunoprecipitation experiments showed that hMYH was immunoprecipitated by anti-ATR. These results suggest that hMYH may interact with ATR and function as a mediator of Chk1 phosphorylation in response to DNA damage. [BMB reports 2011; 44(5): 352-357]

INTRODUCTION

Oxidatively damaged DNA lesions are mainly repaired by base excision repair (BER), which is tightly coupled with cell cycle regulation and DNA damage checkpoints (1). Human MutY homolog (hMYH) is a representative BER DNA glycosylase that removes adenine or 2-hydroxyadenine mismatched with guanine or 7,8-dihydroxy-8-oxoguanine (8-oxoG), and their removal prevents G:C to T:A transversion (2). Together with human 8-oxoG glycosylase (hOGG1) (3) and human MutT homolog (hMTH1) (4), hMYH protects cells from the mutagenic effects of 8-oxoG.

Deficiencies in mouse *MYH* and *OGG1* result in increased tumor predisposition, predominantly lung and ovarian tumors and lymphomas (5). The MYH-null mouse generates more spontaneous tumors, especially oxidative stress-induced intestinal tumors compared to wild type (6). Therefore, MYH was suggested to suppress spontaneous tumorigenesis in mammals. Further, deficiencies in mouse *MYH* and *OGG1* have been shown to increase the sensitivity of cells in G2/M phase to oxidants, accompanied by increased centrosome amplification and formation of multiple nuclei (7). hMYH was suggested to play multiple roles in normal cell cycle progression and cell division under oxidative stress and to act as an adaptor molecule that recruits checkpoint proteins to DNA lesions (8). However, the effect of hMYH on the activation of checkpoint proteins upon DNA damage has not been fully investigated.

In this study, we determined for the first time the association of hMYH with phosphorylation of checkpoint kinase 1 (Chk1) and ataxia telangiectasia and Rad3-related protein (ATR) in response to DNA damage in hMYH-disrupted HEK293 and HaCaT cells.

RESULTS

Knock-down of hMYH by siRNA

To investigate the effects of hMYH on DNA damage-induced cell cycle regulation, small interfering RNA (siRNA) was employed in order to deplete endogenous cellular hMYH. Two siRNAs (siMYH782 and siMYH1392) corresponding to nucleotides 782-806 and 1392-1416 of hMYH, respectively, were designed and transfected into HEK293 cells. Western blot analysis showed that after 24 h incubation, approximately 90 and 84% of hMYH were disrupted by siMYH782 and siMYH1392 transfection, respectively (Fig. 1). Maximal disruption of hMYH was obtained after 36 and/or 48 h of transfection (Supple-

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DOI 10.5483/BMBRep.2011.44.5.352

Received 18 December 2010 , Accepted 15 March 2011

Keywords: Ataxia telangiectasia and Rad3-related protein (ATR), Checkpoint kinase 1 (Chk1), Human MutY homolog (hMYH), Hydroxyurea (HU), Ultraviolet (UV)

mentary data 1). Transfection of siMYH782 followed by 36 h incubation was selected for use in subsequent experiments in order to maximize the disruption of endogenous hMYH.

Depletion of endogenous hMYH reduces phosphorylation of Chk1 upon HU or UV treatment

The effect of hMYH on the phosphorylation of Chk1 and Chk2 in response to HU or UV treatment was investigated in siGFP or siMYH-transfected HEK293 cells. After 36 h siRNA transfection, cells were treated with 20 mM HU or 100 J/m^2 UV, followed by incubation for 1 h. HU and UV treatment induced phosphorylation of Chk1 and Chk2 in siRNA-transfected cells (Fig. 2A, lanes 3-6). The phosphorylation of Chk1 by HU and UV treatment was decreased by 33.1 and 72.6%, respectively, in 87-94% hMYH-disrupted cells (lanes 4 and 6), compared to siGFP-transfected cells (lanes 3 and 5). Meanwhile, HU and UV treatment increased Chk2 phosphorylation by 20.1 and 27.6%, respectively (Fig. 2A and C). The total amount of Cdc25A in hMYH-disrupted and HU or UV-treated cells was increased by 73.5 or 84.5%, respectively (Fig. 2A and D).

To further determine the effect of hMYH on the phosphorylation of Chk1, siRNA-transfected cells were treated with HU or UV, incubated for 15, 30, 60, 120, 240, or 360 min, and analyzed by Western blot analysis (Fig. 2E-H and Supplementary data 2). Chk1 phosphorylation in siGFP-transfected cells was rapidly increased, reaching a maximum 60 min after HU or UV treatment and then gradually decreasing thereafter. Chk1 phosphorylation in siMYH-transfected cells was also maximal at 60 min. However, the increase in phosphorylated Chk1 upon HU or UV treatment was slower than that in siGFP-transfected cells. Sixty minutes after HU or UV treatment, siMYH-mediated knock-down of endogenous hMYH resulted in 33 or 58% reduction of Chk1 phosphorylation,

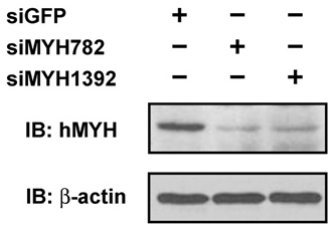

Fig. 1. Knock-down of hMYH using siRNA. HEK293 cells transfected with 500 pmol of siRNAs (siGFP, siMYH782, and siMYH 1392) were incubated for 24 h. Endogenous hMYH proteins presented in siRNA-transfected cells were determined by Western blot analysis.

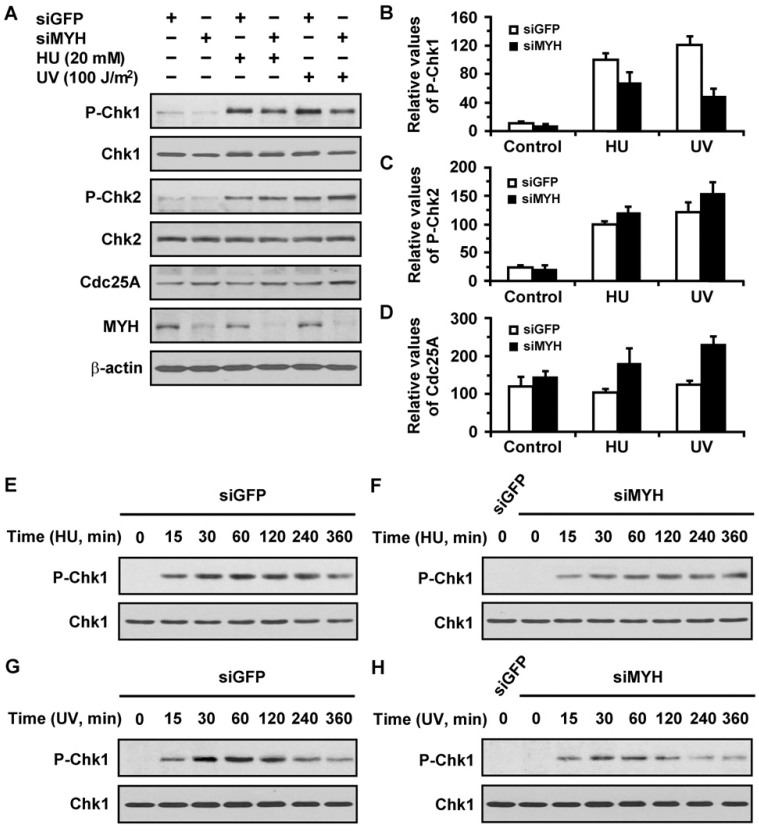

Fig. 2. Knock-down of hMYH reduces phosphorylation of Chk1 upon HU or UV treatment. (A) HEK293 cells were transfected with 500 pmol of siGFP or siMYH 782. After 36 h incubation, the cells were treated with 20 mM HU or 100 J/m^2 UV light for 1 h. Total proteins were extracted and analyzed by Western blot analysis. (B-D) The contents of phospho-Chk1, phospho-Chk2, and Cdc25A obtained from three independent experiments of (A) were quantified and are represented as bar diagrams. The value of phospho-Chk1, phopho-Chk2, and Cdc25A obtained from siGFP-transfected and HU-treated cells was estimated as 100. (E-H) HEK293 cells were transfected with 500 pmol of siGFP or siMYH782. After 36 h incubation, the cells were treated with 20 mM HU (E, F) or 100 J/m^2 UV light (G, H) for the indicated times. Total proteins were extracted from cells, and phospho-Chk1 and Chk1 were analyzed by Western blot analysis.

respectively. Taken together, these results show that hMYH is involved in the activation of Chk1 upon DNA damage.

Depletion of endogenous hMYH reduces the phosphorylation of ATR upon HU or UV treatment

To determine the phosphorylation of ATR upon DNA damage, cell lysates were prepared from UV-treated cells and immunoprecipitated with goat IgG or anti-ATR. The presence of phospho-ATR in the immunoprecipitates was determined by Western blot analysis. Phospho-ATR was only detected in the immunoprecipitate of UV-treated cell lysates using anti-ATR (Fig. 3A lane 4). To determine the effect of hMYH on the phosphorylation of ATR upon DNA damage, siRNA-transfected cells were treated with 20 mM HU or 100 J/m^2 UV, followed by incubation for 1 h. Cell lysates pre-cleared with protein A/G-Sepharose beads were immunoprecipitated with anti-ATR, and the presence of ATR and phospho-ATR in the immunoprecipitates was determined by Western blot analysis (Fig. 3C). Immunoprecipitated ATR was constant regardless of siGFP or siMYH transfection and HU or UV treatment. Phospho-ATR was detected in HU and UV-treated immunoprecipitate (lanes 3-6). Phospho-ATR was decreased by 48 and 32% in immunoprecipitates of siMYH-transfected cells treated with HU and UV, respectively (lanes 4 and 6). The contents of phospho-Chk1, Chk1, hMYH, and ATR in cell lysates were also determined by Western blot analysis (Fig. 3D).

To further determine the effect of hMYH on the phosphorylation of ATR, siRNA-transfected HaCaT cells were treated with HU or UV, after which the phosphorylation of ATR was determined by Western blot analysis (Fig. 4A and B). Endogenous hMYH levels in siMYH-transfected cells were decreased by about 62%. The phosphorylation of Chk1 by HU and UV treatment was decreased by 64 and 55% in siMYH-transfected cells, respectively, compared to siGFP-transfected cells. HU or UV-induced phosphorylation of ATR was also determined in hMYH-disrupted HaCaT cells. HU or UV treatment induced the phosphorylation of ATR, which was dramatically diminished in hMYH-disrupted HaCaT cells. Taken together, these results indicate that the disruption of hMYH reduces activation of ATR upon HU or UV treatment, thereby reducing the activation of Chk1.

Endogenous hMYH is co-immunoprecipitated with ATR

The interaction between endogenous hMYH and ATR was determined by co-immunoprecipitation using anti-ATR or anti-hMYH. Western blot analysis showed that hMYH was present in the immunoprecipitate of anti-ATR (Fig. 4C lane 2). ATR was also co-immunoprecipitated with anti-hMYH (lane 3). hMYH and ATR were not detected in the immunoprecipitate of goat IgG, which used as a negative control. The presence of ATR and hMYH in input cell lysates were determined by Western blot analysis (Fig. 4D). This result indicates that hMYH may interact with ATR.

DISCUSSION

Genotoxic stress activates checkpoint signaling pathways that block cell cycle progression, trigger apoptosis, and regulate DNA repair. Replication stress or other types of DNA damage

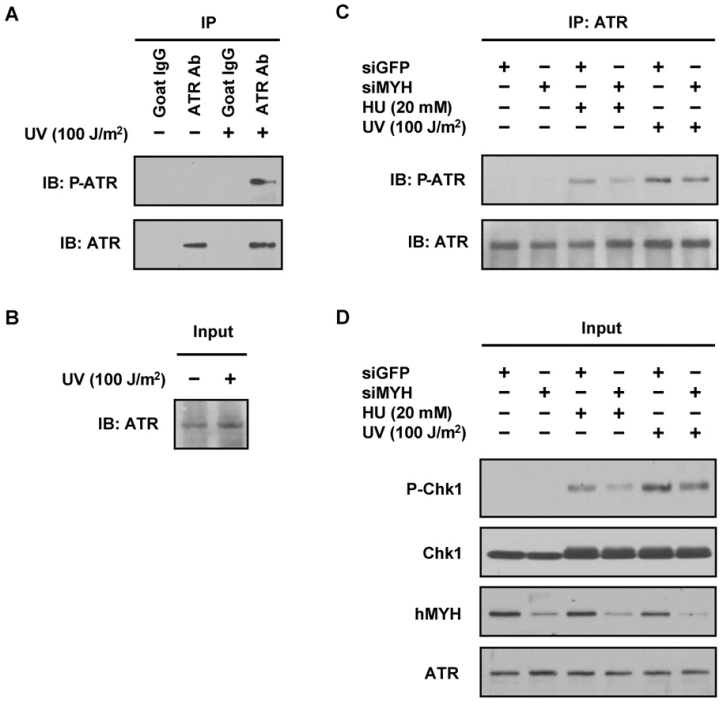

Fig. 3. Knock-down of hMYH decreases phosphorylation of ATR upon HU or UV treatment. (A) Endogenous ATR from UV-treated (100 J/m^2, 1 h) or non-treated HEK293 cells was immunoprecipitated with goat IgG or anti-ATR. The presence of ATR or phospho-ATR in immunoprecipates was analyzed by Western blot analysis. (B) Input samples used in (A) experiment were determined by Western blot analysis. (C) HEK293 cells were transfected with siGFP or siMYH782. After 36 h incubation, the cells were treated with 20 mM HU or 100 J/m^2 UV for 1 h. After immunoprecipitation of endogenous ATR using anti-ATR, the contents of phospho-ATR and ATR in immunoprecipates were analyzed by Western blot analysis. (D) Input samples used in (C) experiment were determined by Western blot analysis. IP and IB represent immunoprecipitation and immunoblotting, respectively.

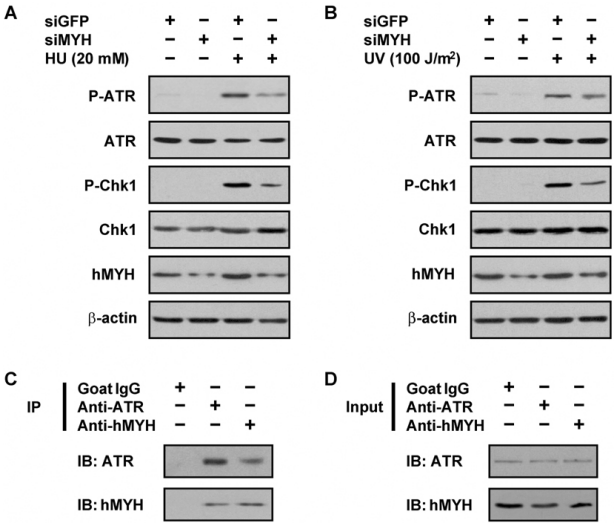

Fig. 4. hMYH disruption decreases phosphorylation of ATR in HU or UV-treated HaCaT cells. (A, B) HaCaT cells were transfected with 500 pmol of siGFP or siMYH782. After 36 h incubation, the cells were treated with 20 mM HU (A) or 100 J/m^2 UV (B) for 1 h, respectively. Total proteins were extracted and analyzed by Western blot analysis. (C, D) HaCaT cell lysates were immunoprecipitated with goat IgG, anti-ATR, or anti-hMYH. The presence of ATR or hMYH in immunoprecipitates was determined by Western blot analysis (C). Endogenous ATR and hMYH levels in cell lysates used in (C) experiment were determined by Western blot analysis (D).

during S phase promotes the phosphorylation and activation of Chk1 through ATR (9). Activated Chk1 plays a critical role in the cellular checkpoint response by stabilizing stalled replication forks, blocking the firing of late origins of replication forks, and arresting cells in S or G2/M phase. In this work, the phosphorylation of Chk1 in response to HU and UV treatment was decreased in hMYH-disrupted cells. Activation of Chk1 upon DNA damage is induced through the phosphorylation of Ser317 and Ser345 by ATR (9). The phosphorylation of Ser317 was also decreased in hMYH-disrupted cells, similar to Ser345 (data not shown). Otherwise, HU or UV-induced phosphorylation of Chk2 was slightly increased in hMYH-disrupted cells. The inhibition of ATR-Chk1 signaling is known to elevate Chk2 phosphorylation (10). Therefore, this seems to be the result of interference in ATR/Chk1 signaling upon hMYH depletion. A major target of Chk1 at cell cycle checkpoints is Cdc25 phsophatase, which dephosphorylates and activates cyclin-dependent kinases (Cdks), thereby promoting cell progression (11). Regulation of Cdc25 by activated Chk1 eventually inhibits Cdks, thereby delaying or arresting the cell cycle at specific stages (12). Vertebrates contain three phosphatases, Cdc25A, B, and C, all of which can remove the phosphate groups from phosphotyrosine and phosphothreonine residues for activation of their Cdk substrates (13). Cdc25A plays a more general role, being involved in both early (G1/S) and late (G2/M) cell-cycle transitions, whereas Cdc25B and Cdc25C only regulate the G2/M transition. Cdc25A is also tightly regulated at the protein level, being periodically synthesized and degraded via ubiquitin-mediated proteolysis induced by Chk1 phosphorylation (14). Here, Cdc25A in hMYH-disrupted, HU and UV-treated cells was increased, whereas Cdc25C did not show any change regardless of HU or UV treatment and hMYH disruption (data not shown). These results suggest that hMYH may be involved in early (G1/S) and late (G2/M) cell-cycle transitions. Endogenous hMYH is known to be increased 3- to 4-fold during the cell cycle, reaching maximum levels during S phase and remaining throughout mitosis (15). In our preliminary experiment, endogenous hMYH in HEK293 cells was increased during early and/or late G1/S phase and decreased in G2/M phase, although a basal level of hMYH was maintained during G2/M phase (Supplementary data 3B and D). The knockdown of endogenous hMYH by siMYH in asynchronied, early G1/S, late G1/S, and G2/M phase cells resulted in 26.7, 22.2, 46.5, and 65.7% reduction of Chk1 phosphorylation, respectively (Supplementary data 3B and C). Although the highest reduction was observed in G2/M phase of hMYH-disrupted cells, activation of Chk1 by HU was maximal in late G1/S phase cells. Although further study is necessary to evaluate the rule of hMYH in cell cycle regulation and Chk1 activation by DNA damage, these results suggest that hMYH is mainly involved in Chk1 activation by DNA damage during G1/S phase. The activation of Chk1 can also be induced during G2/M phase and reduced by hMYH disruption.

In hMYH-disrupted cells, the increased rate of activated Chk1 upon HU and UV treatment was lower than that in siGFP-transfected cells (Fig. 2E-H). This means that hMYH is involved in the activation mechanism of Chk1 upon DNA damage, but not in stability or inactivation. Phospho-ATR (Ser428), which can be used to confirm the activation of ATR upon DNA damage and growth inhibition (16), was decreased in immunoprecipitates of siMYH-transfected cells treated with HU and UV irradiation. HU and UV-induced ATR phosphorylation were also dramatically diminished in hMYH-disrupted HaCaT cells (Fig. 4A, B). This strongly implies that the disruption of hMYH reduces ATR activation upon HU or UV treatment, thereby reducing the activation of Chk1.

The activation and recruitment of ATR to DNA lesions can be regulated by replication protein A (RPA), ATR interacting protein (ATRIP), topoisomerase binding protein 1 (TopBP1), and the Rad9-Rad1-Hus1 (9-1-1) complex (17). However, no interactions between hMYH and these proteins were observed, except for the RPA and 9-1-1 proteins (data not shown). Although a direct interaction between ATR and hMYH was not obvious, hMYH was shown to interact with ATR. This suggests that hMYH interacting with ATR may be involved in the activation of ATR. hMYH physically and functionally interacts with several proteins for its role in replication and DNA repair, such as apurinic/apyrimidinic (AP) endonuclease (APE), RPA, and mutY homolog 6 (MSH6) (18,19). During checkpoint signaling

in response to DNA methylation in S phase, ATR-ATRIP is preferentially recruited to O6-MeG/T mismatches in a MutSα (a heterodimer of MSH2 and MSH6)- and hMutLα (a heterodimer of MLH1 and PMS2)-dependent manner. Therefore, MMR proteins have been suggested to act as direct sensors of methylation damage, helping to recruit ATR-ATRIP to sites of cytotoxic O6-meG adducts for the initiation of ATR checkpoint signaling (20). In addition, MYH was shown to interact with the cell cycle checkpoint Rad9-Rad1-Hus1 (9-1-1) complex in *Schizosaccharomyces pombe*, *Xenopus*, and human (8, 21 and 22). Human Rad9, Rad1, Hus1, and Rad17 serve as DNA damage sensors by forming DNA damage-responsive complexes that clamp around the damaged DNA and transduce damage signals to downstream effectors (23), thereby facilitating the activation of Chk1 and ATR-mediated phosphorylation (24). The fact that MYH can interact with the MSH6 and 9-1-1 complex suggests that MYH may play a role in the recognition of DNA lesions and signal transduction to checkpoint factors.

In conclusion, we demonstrated that hMYH is involved in the activation mechanism of Chk1 in response to HU or UV treatment, but not stability or inactivation of Chk1. Our results suggest that hMYH functions as a mediator of Chk1-associated cell cycle checkpoint pathways upon DNA damage.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney HEK293 and human keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO, USA) at 37°C in a 5% CO_2 incubator.

siRNA construction and transfection into cells

The optimum siRNA sequences for the knock-down of endogenous hMYH were designed and purchased from the Stealth™ RNAi program of Invitrogen. siRNA corresponding to nucleotides 415-439 of green fluorescence protein (GFP) was used as a negative control. The sequences of siRNA constructed for the experiments were: siGFP, 5'-GGGCACAAGCUGGAGUACAACUACA-3'; siMYH782, 5'-CAGGAGAUUUCAACCAAGCAGCCAU-3'; siMYH1392, 5'-GGAGGAAUUUCACACCGCAGCUGUU-3'. Double-stranded siRNA was prepared by annealing with complementary RNA oligonucleotides. Five hundred picomoles of double-stranded siRNA was transfected into 50-60% confluent HEK293 or HaCaT cells in 6-well plates using Lipofectamine™ 2000 reagent according to the manufacturer's protocol.

Protein extraction and Western blot analysis

HEK293 or HaCaT cells were harvested, washed with PBS, and lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, 10 μg/ml PMSF, protease, and phosphatase inhibitor cocktail (Sigma)] for 1 h at 4°C with occasional vortexing. Protein extracts were collected after centrifugation at 16,000 × g for 20 min. Protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA). Protein extracts resolved on 8 or 10% SDS-polyacrylamide gel were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Princeton, NJ, USA). The membranes were blocked with 5% non-fat dried milk in TBS-T (TBS with 0.05% Tween-20) and then incubated with antibodies against ATR, Chk1, Chk2, phospho-Chk1 (Ser345), phospho-Chk2 (Thr68), Cdc25A, β-actin (all from Santa Cruz Biotech. Santa Cruz, CA, USA), phospho-ATR (Ser428; Cell Signaling Technology, Beverly, MA, USA), or hMYH (Abnova, Taiwan), followed by incubation with horse-radish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotech.). Protein bands were detected using ECL Pico Western blotting detection reagents (Pierce, Rockford, IL, USA).

Immunoprecipitation of ATR

HEK293 cell lysates (2 mg proteins) were pre-cleared with 50 μl of protein A/G-Sepharose beads (Santa Cruz Biotech.) in order to remove non-specific proteins. After 1 h incubation, cell lysates were separated from the beads by centrifugation. Pre-cleared supernatants were incubated for 3 h with 4 μg of goat IgG (Sigma) or anti-ATR, followed by 12 h incubation with 50 μl of protein A/G-Sepharose beads, all at 4°C with gentle rotation. Protein-bead complexes were precipitated by centrifugation at 600 × g for 5 min, washed five times with 1 : 1 mixture of lysis buffer and PBS, and mixed with 2× SDS polyacrylamide gel loading buffer. After boiling for 5 min, immunoprecipitated samples were resolved on SDS-polyacrylamide gel and subjected to Western blot analysis.

Co-immunoprecipitation

Pre-cleared HaCaT cell lysates (2 mg poteins) with 30 μl of protein G-Sepharose beads were incubated for 3 h with 2 μg of goat IgG, anti-ATR, or anti-hMYH, followed by 12 h incubation with 30 μl of protein A/G-Sepharose beads, all at 4°C under gentle rotation. Protein-bead complexes were precipitated by centrifugation at 600 × g for 5 min, washed five times with washing buffer (1 : 1 mixture of lysis buffer and PBS), and mixed with 2× SDS polyacrylamide gel loading buffer. After boiling for 5 min, immunoprecipitated samples were resolved on SDS polyacrylamide gel and subjected to Western blot analysis.

Statistical analysis

Experiments were performed three times, and statistical analysis was conducted using Student's *t*-test. Data are expressed as means ± SD.

Acknowledgements

This work was supported by Basic Science Research Program through National Research Foundation of Korea (NRF, 2010-

0016187), a grant from Kyung Hee University in 2009 (KHU-20100159), the Real Time Molecular Imaging Project of the Korean Ministry of Science and Technology, and the WCU (World Class University, R33-2008-000-1071) program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology.

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