The EMBO Journal (2012), 1–12 | © 2012 European Molecular Biology Organization | All Rights Reserved 0261-4189/12
www.embojournal.org

THE EMBO JOURNAL

SUMOylation of hnRNP-K is required for p53-mediated cell-cycle arrest in response to DNA damage

Seong Won Lee, Moon Hee Lee, Jong Ho Park, Sung Hwan Kang, Hee Min Yoo, Seung Hyun Ka, Young Mi Oh, Young Joo Jeon* and Chin Ha Chung*

Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, Korea

Heterogeneous ribonucleoprotein-K (hnRNP-K) is normally ubiquitinated by HDM2 for proteasome-mediated degradation. Under DNA-damage conditions, hnRNP-K is transiently stabilized and serves as a transcriptional co-activator of p53 for cell-cycle arrest. However, how the stability and function of hnRNP-K is regulated remained unknown. Here, we demonstrated that UV-induced SUMOylation of hnRNP-K prevents its ubiquitination for stabilization. Using SUMOylation-defective mutant and purified SUMOylated hnRNP-K, SUMOylation was shown to reduce hnRNP-K's affinity to HDM2 with an increase in that to p53 for p21-mediated cell-cycle arrest. PIAS3 served as a small ubiquitin-related modifier (SUMO) E3 ligase for hnRNP-K in an ATR-dependent manner. During later periods after UV exposure, however, SENP2 removed SUMO from hnRNP-K for its destabilization and in turn for release from cell-cycle arrest. Consistent with the rise-and-fall of both SUMOylation and stability of hnRNP-K, its ability to interact with PIAS3 was inversely correlated to that with SENP2 during the time course after UV exposure. These findings indicate that SUMO modification plays a crucial role in the control of hnRNP-K's function as a p53 co-activator in response to DNA damage by UV.

The EMBO Journal advance online publication, 23 October 2012; doi:10.1038/emboj.2012.293
Subject Categories: proteins; genome stability & dynamics
Keywords: HDM2; p21; PIAS3; SENP2; ubiquitin

Introduction

The p53 tumour suppressor plays a pivotal role in maintenance of genome integrity under cellular stresses, such as DNA damage (Lane, 1992; Lakin and Jackson, 1999; Kruse and Gu, 2009; Levine and Oren, 2009). Upon DNA damage, ATM, ATR, and DNA-PK are activated for phosphorylation of downstream targets, such as the p53 transcription factor and the checkpoint CHK1 and CHK2 kinases (Abraham, 2001, 2004; Ciccia and Elledge, 2010). This process in turn regulates the functions of downstream effector proteins involved in cell-cycle arrest, DNA repair, and/or apoptosis. A key example is ATM- and ATR-mediated phosphorylation of both p53 and HDM2, which impairs their interaction and thereby prevents HDM2-mediated ubiquitination of p53 for degradation by proteasome, leading to stabilization and activation of p53 (Perry, 2004).

A major consequence of p53 activation in response to DNA damage is the induction of cell-cycle arrest (Vogelstein *et al*, 2000; Bartek and Lukas, 2001; Vousden and Lu, 2002; Horn and Vousden, 2007) at the G1/S or G2/M phase. Cell-cycle arrest at the G1/M phase is primarily achieved by expression of p53-downstream genes, such as *p21*, an inhibitor of cyclin-dependent kinases (CDKs). Notably, p21 also acts as an anti-apoptotic protein. This function of p21 is mediated by its ability to inhibit caspase-3 (Suzuki *et al*, 1998), stabilize the anti-apoptotic cIAP1 (Steinman and Johnson, 2000), or downregulate caspase-2 (Baptiste-Okoh *et al*, 2008). Thus, p21 plays an important role in inhibiting apoptosis as well as in cell-cycle arrest, allowing cells to repair damaged DNA and prevent tumorigenesis.

Small ubiquitin-related modifier (SUMO) is an ubiquitin-like protein that is conjugated to a variety of cellular proteins. Like ubiquitin, SUMO is conjugated to target proteins by a cascade enzyme system consisting of E1 activating enzyme (SAE1/SAE2), E2 conjugating enzyme (Ubc9), and E3 ligases (PIASs) (Kerscher *et al*, 2006; Capili and Lima, 2007; Rytinki *et al*, 2009). Conjugated SUMO can be removed by a family of SUMO-specific proteases (SENPs) (Mukhopadhyay and Dasso, 2007; Yeh, 2009). This reversible SUMOylation process participates in the control of diverse cellular processes, including transcription, nuclear transport, and signal transduction (Kim *et al*, 2002; Johnson, 2004; Hay, 2005; Geiss-Friedlander and Melchior, 2007; Gareau and Lima, 2010). Significantly, many proteins involved in DNA-damage response are modified by ubiquitin and/or SUMO, implicating the role of ubiquitination, SUMOylation, or both in the control of checkpoint responses and DNA-repair pathways (Hoege *et al*, 2002; Lee *et al*, 2006; Bergink and Jentsch, 2009; Altmannova *et al*, 2010; Dou *et al*, 2010; Polo and Jackson, 2011; Cremona *et al*, 2012). For example, Rad52, a mediator of homologous recombination in yeast, is SUMOylated in response to DNA damage, and this modification stabilizes Rad52 for its sustained function (Sacher *et al*, 2006).

Heterogeneous ribonucleoprotein-K (hnRNP-K) is an RNA-binding protein that is associated with various cellular processes, including chromatin remodelling, transcription, mRNA splicing, and translation (Matunis *et al*, 1992; Bomsztyk *et al*, 1997, 2004). Intriguingly, hnRNP-K was shown to be transiently stabilized and function as a

*Corresponding authors. YJ Jeon or CH Chung, Department of Biological Sciences, College of Natural Sciences, Seoul National University, 56-1 Shillim-dong, Gwanak-gu, Seoul 151-742, Korea.
Tel.: +82 2 880 6693; Fax: +82 2 871 9193;
E-mail: gydbs98@snu.ac.kr or chchung@snu.ac.kr

Received: 8 June 2012; accepted: 8 October 2012

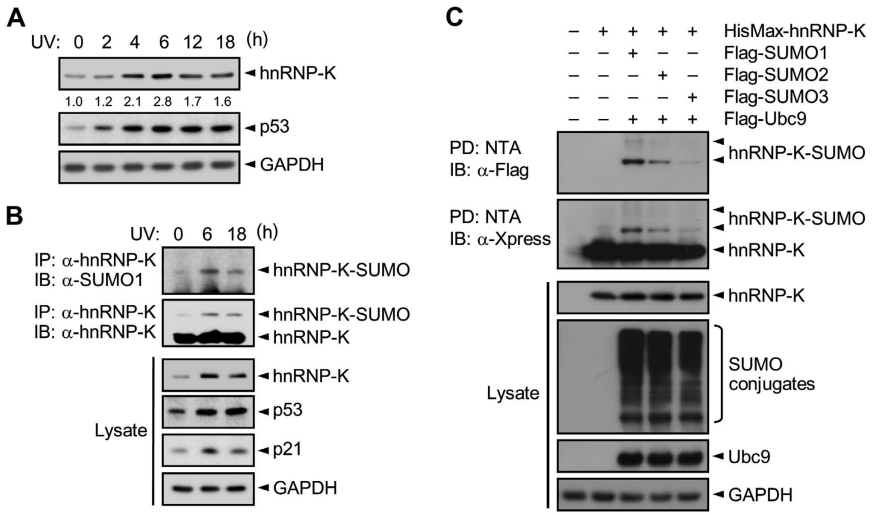

Figure 1 UV induces SUMOylation of hnRNP-K. (**A**) UV increases the cellular level of hnRNP-K. After exposure of HeLa cells to UV (10 J/m^2), cell lysates were subjected to immunoblot with anti-hnRNP-K or anti-p53 antibody. The resulting gels were scanned using a densitometer, and the intensities of hnRNP-K bands were quantified by using 'Image J' program. The intensity of hnRNP-K seen before UV (i.e., 0 h) was expressed as 1.0 and the others as its relative values. (**B**) UV induces SUMOylation of hnRNP-K. After UV treatment, cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-SUMO1 or anti-hnRNP-K antibody. (**C**) Modification of hnRNP-K by SUMO isoforms. Flag-tagged SUMO isoforms were expressed in HEK293T cells with Flag-Ubc9 and HisMax-hnRNP-K. After incubation with 10 μM MG132 for 4 h, cell lysates were subjected to pull down with NTA beads followed by immunoblot with anti-Flag or anti-Xpress antibody. Figure source data can be found with the Supplementary data.

transcriptional co-activator of p53 in response to DNA damage (Moumen *et al*, 2005). However, how the stability and function of hnRNP-K is regulated remained unknown. Here, we showed that UV induces PIAS3-mediated hnRNP-K SUMOylation, which increases hnRNP-K stability, interaction between hnRNP-K and p53, and p21 expression in an ATR-dependent manner, leading to cell-cycle arrest. At later periods after UV treatment, however, SENP2 reversed the SUMOylation-mediated processes by removing SUMO from hnRNP-K, implicating the role of SENP2 in the release of cells from cell-cycle arrest to resume normal growth after DNA repair. These findings indicate that reversible SUMO modification of hnRNP-K by PIAS3 and SENP2 plays a crucial role in the control of hnRNP-K stability and thereby its function as a p53 co-activator in response to DNA damage by UV.

Results

UV-induced SUMOylation increases the stability of hnRNP-K

hnRNP-K has been identified as a candidate for SUMOylation by proteomic analysis (Li *et al*, 2004). Therefore, we first examined whether hnRNP-K could indeed be modified by SUMO and whether this modification is related with DNA damage-induced stabilization of hnRNP-K. UV treatment led to 2- to 3-fold increase in the level of hnRNP-K by 6 h and declined thereafter (Figure 1A). Moreover, the level of SUMOylated hnRNP-K was markedly increased by 6 h and declined by 18 h after UV treatment and this change occurred in parallel with that of hnRNP-K level (Figure 1B), suggesting that UV-induced SUMOylation stabilizes hnRNP-K. Thus, further studies were performed at three time points; prior to, 6 h after, and 18 h after UV treatment, which were henceforth referred to as before UV, 6 h after UV, and 18 h after UV, respectively. We also examined whether hnRNP-K SUMOylation could be induced under other DNA damage conditions. Both SUMOylation and stabilization of hnRNP-K were also induced by treatments with ionizing radiation (IR) and doxorubicin, although the timing of their rise-and-fall was significantly different from that induced by UV (Supplementary Figure S1). Thus, hnRNP-K SUMOylation appears to be a common response to DNA damage for its stabilization.

When SUMO isoforms were overexpressed with hnRNP-K, SUMO1 was more efficiently conjugated to hnRNP-K than SUMO2 or SUMO3 (Figure 1C). Thus, further studies were performed only with SUMO1. Since two SUMOylated hnRNP-K bands appeared under the overexpression conditions, two Lys residues in the sequences closely matched to the consensus motif for SUMOylation (ψ-K-X-D/E) were substituted with Arg (Figure 2A). Replacement of Lys422 alone or together with Lys198 by Arg prevented hnRNP-K SUMOylation, whereas that of Lys198 alone did not (Figure 2B). Similar results were obtained by *in vitro* SUMOylation assay using purified SAE1/SAE2 (E1), Ubc9 (E2), and SUMO1 (Figure 2C), indicating that Lys422 serves as the major SUMOylation site of hnRNP-K. Henceforth, the SUMOylation-defective mutant was referred to as K422R.

We next examined whether UV-induced SUMOylation influences hnRNP-K ubiquitination and in turn its stability. The level of ubiquitinated hnRNP-K was markedly reduced at 6 h after UV and returned almost to the initial level at 18 h after UV (Figure 3A), indicating that the change in the level of ubiquitinated hnRNP-K is inversely correlated with that of SUMO1-conjugated hnRNP-K. However, SUMOylation-defective K422R, unlike wild-type hnRNP-K, remained ubiquitinated at 6 h after UV (Figure 3B). Consistently, UV treatment increased the stability of hnRNP-K, but not K422R (Figure 3C and D). In addition, MG132, a proteasome inhibitor, prevented K422R destabilization under the same conditions.

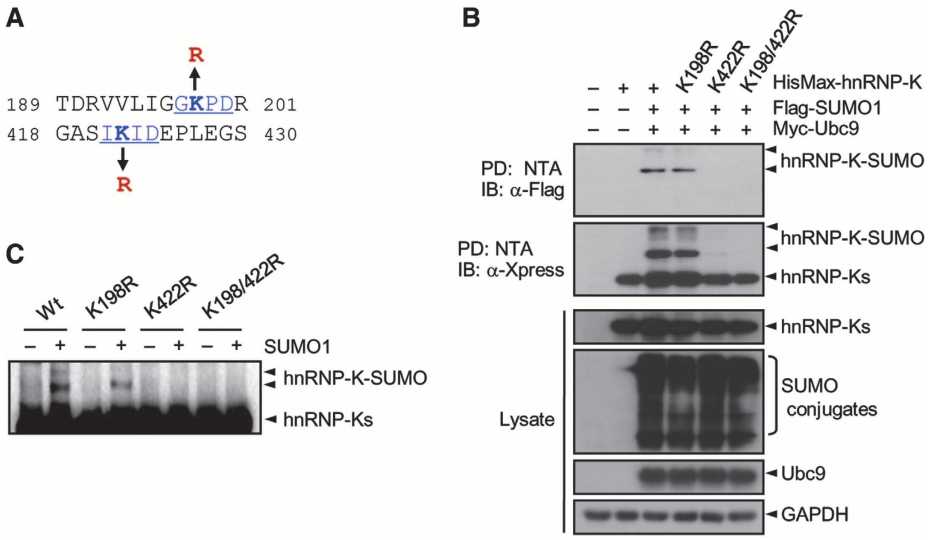

Figure 2 Lys422 is the major SUMO1 acceptor site in hnRNP-K. (**A**) Potential SUMOylation sites in hnRNP-K. The Lys residues in the underlined sequences of hnRNP-K were substituted with Arg by site-directed mutagenesis. (**B**) K422R mutation ablates hnRNP-K SUMOylation *in vivo*. Flag-tagged hnRNP-K, K198R, K422R, and the double mutant (K198R/K422R) were overexpressed in HEK293T cells with HisMax-SUMO1 and Flag-Ubc9. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-Flag or anti-SUMO1 antibody. (**C**) K422R mutation ablates hnRNP-K SUMOylation *in vitro*. SUMOylation was performed using purified proteins followed by immunoblot with anti-His antibody as described under 'Materials and methods'. Figure source data can be found with the Supplementary data.

These results indicate that UV-induced SUMOylation of hnRNP-K is responsible for the increase in its stability.

SUMOylation of hnRNP-K switches its interaction with HDM2 to that with p53

To elucidate the mechanism for SUMOylation-mediated stabilization of hnRNP-K, we first examined the effect of UV treatment on the interaction of hnRNP-K with HDM2. The level of HDM2 co-immunoprecipitated with hnRNP-K was significantly decreased at 6 h after UV and returned to the initial level at 18 h after UV (Figure 4A). Moreover, the ability of hnRNP-K to bind HDM2 was markedly reduced at 6 h after UV, whereas that of K422R remained the same regardless of UV treatment (Figure 4B and C). In addition, purified SUMOylated hnRNP-K (Figure 4D) showed a lower affinity to HDM2 than unmodified hnRNP-K (Figure 4E). Note that the C-terminal region harbouring the SUMOylation site Lys422 overlaps with that for HDM2 binding (see below). These results indicate that UV-induced SUMOylation of hnRNP-K interferes with its interaction with HDM2, leading to hnRNP-K stabilization.

We next examined whether UV-induced SUMOylation also influences the interaction of hnRNP-K with p53. In contrast to HDM2, the amount of p53 co-immunoprecipitated with hnRNP-K was significantly increased at 6 h after UV and returned almost to the initial level at 18 h after UV (Figure 4F). Moreover, the ability of hnRNP-K to bind p53 was markedly increased at 6 h after UV, whereas that of K422R remained decreased regardless of UV treatment (Figure 4G and H). In addition, purified SUMOylated hnRNP-K showed a much higher affinity to p53 than unmodified hnRNP-K (Figure 4I). These results indicate that UV-induced SUMOylation of hnRNP-K promotes its interaction with p53.

To confirm whether SUMOylation of hnRNP-K is responsible for the alterations in its affinity to HDM2 and p53 under *in vivo* conditions, hnRNP-K and K422R were overexpressed with HDM2, p53, and Ubc9. Co-expression of increasing amounts of SUMO1 (i.e., increasing the level of SUMOylated hnRNP-K) led to a gradual increase in the level of hnRNP-K-bound p53 concurrently with a decrease in that of hnRNP-K-bound HDM2 (Figure 4J). On the other hand, the level of K422R-bound p53 and HDM2 remained the same regardless of SUMO1 expression. Although the experiments were performed under overexpression conditions, which could be non-physiological, these results strongly suggest that SUMOylated hnRNP-K preferentially binds p53 whereas its unmodified form binds better to HDM2. Thus, UV-induced SUMOylation of hnRNP-K appears to switch its interaction with HDM2 to that with p53.

Of note was the finding that without UV treatment, hnRNP-K binds p53 better than K422R (see Figure 4G and H), whereas K422R binds HDM2 better than hnRNP-K (see Figure 4B and C). However, *in vitro* binding assays showed that purified K422R interacts with p53 or HDM2 as well as wild-type hnRNP-K (Supplementary Figure S2A and B), indicating that the K-to-R mutation itself has no effect on the binding affinity of hnRNP-K to p53 or HDM2. Since endogenous hnRNP-K can be SUMOylated in the absence of UV although to a basal level (see Figure 1B), it appeared that overexpression of hnRNP-K (i.e., elevation of the substrate concentration for SUMOylation) increases the level of SUMOylated hnRNP-K and this increase alters the binding affinity of hnRNP-K to p53 and HDM2. Indeed, increased expression of hnRNP-K led to an increase in the level of SUMOylated hnRNP-K in the absence of UV treatment (Supplementary Figure S2C). Moreover, when hnRNP-K SUMOylation was prevented by knockdown of Ubc9 by using Ubc9-specific shRNA (shUbc9), both hnRNP-K and K422R bound to p53 or HDM2 to similar extents (Supplementary Figure S3). These results indicate that changes in the binding affinity of hnRNP-K to p53 or HDM2 in the absence of UV treatment are due to an increase in the level of SUMOylated hnRNP-K upon its overexpression.

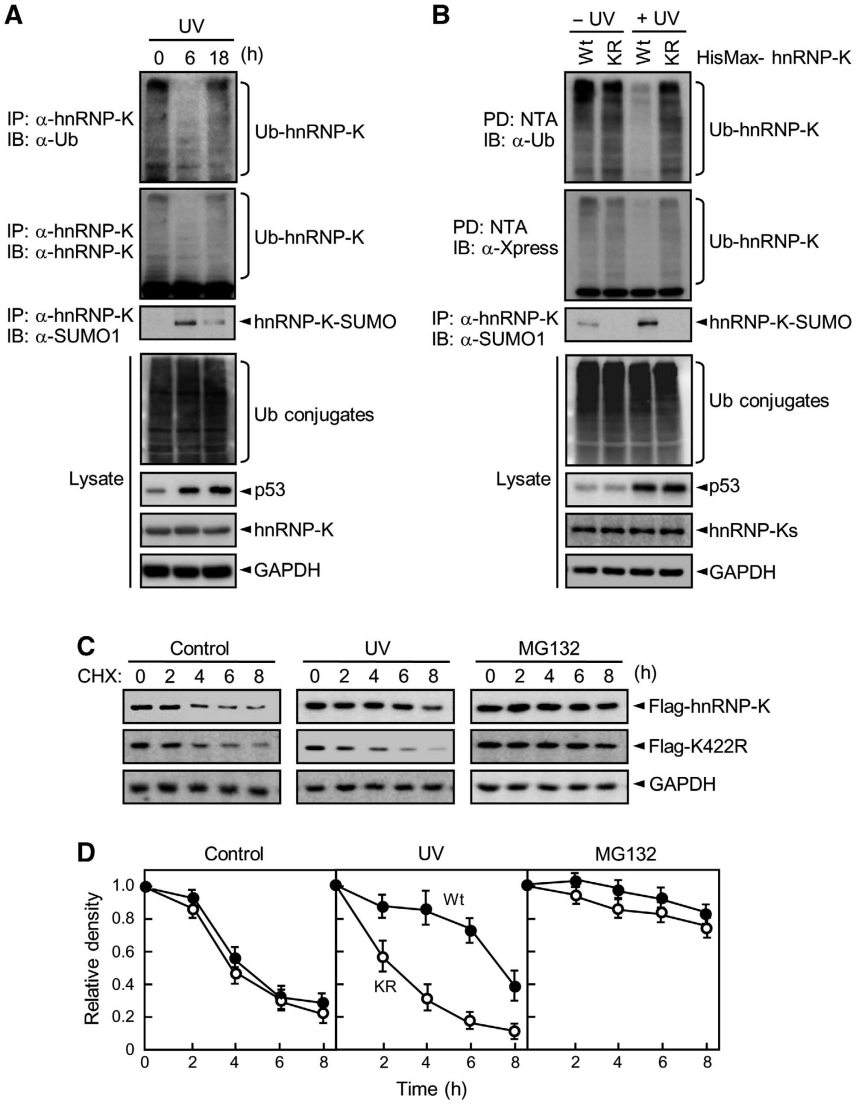

Figure 3 UV-induced SUMOylation increases the stability of hnRNP-K. (**A**) UV blocks hnRNP-K ubiquitination. After exposure to UV, HeLa cells were incubated with 10 μM MG132 for 4 h. Cell lysates were subjected to immunoprecipitation with anti-ubiquitin, anti-hnRNP-K, or anti-SUMO1 antibody followed by immunoblot analysis. (**B**) SUMOylation prevents hnRNP-K ubiquitination. After exposure to UV, cells overexpressing HisMax-tagged hnRNP-K (Wt) or K422R (KR) were incubated for 2 h and then treated with MG132 for the next 4 h. Cell lysates were subjected to pull down with NTA beads followed by immunoblot analysis. (**C**) SUMOylation increases the hnRNP-K stability. Cells overexpressing Flag-tagged hnRNP-K (Wt) or K422R (KR) were treated with 200 μg/ml of cycloheximide. After exposure to UV, they were incubated with and without MG132 followed by immunoblot with anti-Flag antibody. (**D**) Band intensities in (**C**) were quantified by using a densitometer. The data represent the mean ± s.d. of three independent experiments. Figure source data can be found with the Supplementary data.

SUMOylation of hnRNP-K is required for its function as a p53 co-activator

To determine whether UV-induced SUMOylation of hnRNP-K influences its co-activator function, p53 transactivity was measured by using two reporter vectors, *PG13-Luc* and *p21-Luc*. In both cases, UV treatment increased the luciferase activity and this increase was further enhanced by overexpression of hnRNP-K, but not by that of K422R (Figure 5A and B). Under the same conditions, both mRNA and protein levels of p21 were increased and this increase was further enhanced by overexpression of hnRNP-K, but not by that of K422R (Figure 5C and D). hnRNP-K overexpression without UV treatment also increased p53 transactivity, as it could increase the SUMOylated hnRNP-K level. Moreover, chromatin immunoprecipitation (ChIP) analysis revealed that UV treatment increased recruitment of both hnRNP-K and p53 to the *p21* promoter site and this increase could be further enhanced by hnRNP-K overexpression, but not by that of K422R (Figure 5E). These results indicate that UV-induced hnRNP-K SUMOylation promotes p53 transactivity and thereby p21 expression.

Of note was the finding that hnRNP-K overexpression leads to an increase in the level of endogenous p53 in the absence of UV treatment (see Figure 5D), raising a possibility that overexpressed hnRNP-K may stabilize p53 although it has been shown that hnRNP-K knockdown does not affect p53 stability (Moumen *et al*, 2005). However, expression of increasing amounts of hnRNP-K showed little or no effect on HDM2-mediated p53 ubiquitination or HDM2 auto-ubiquitination, indicating that hnRNP-K has no effect on the stability of p53 (Supplementary Figure S4). Since hnRNP-K overexpression causes an increase in the level of

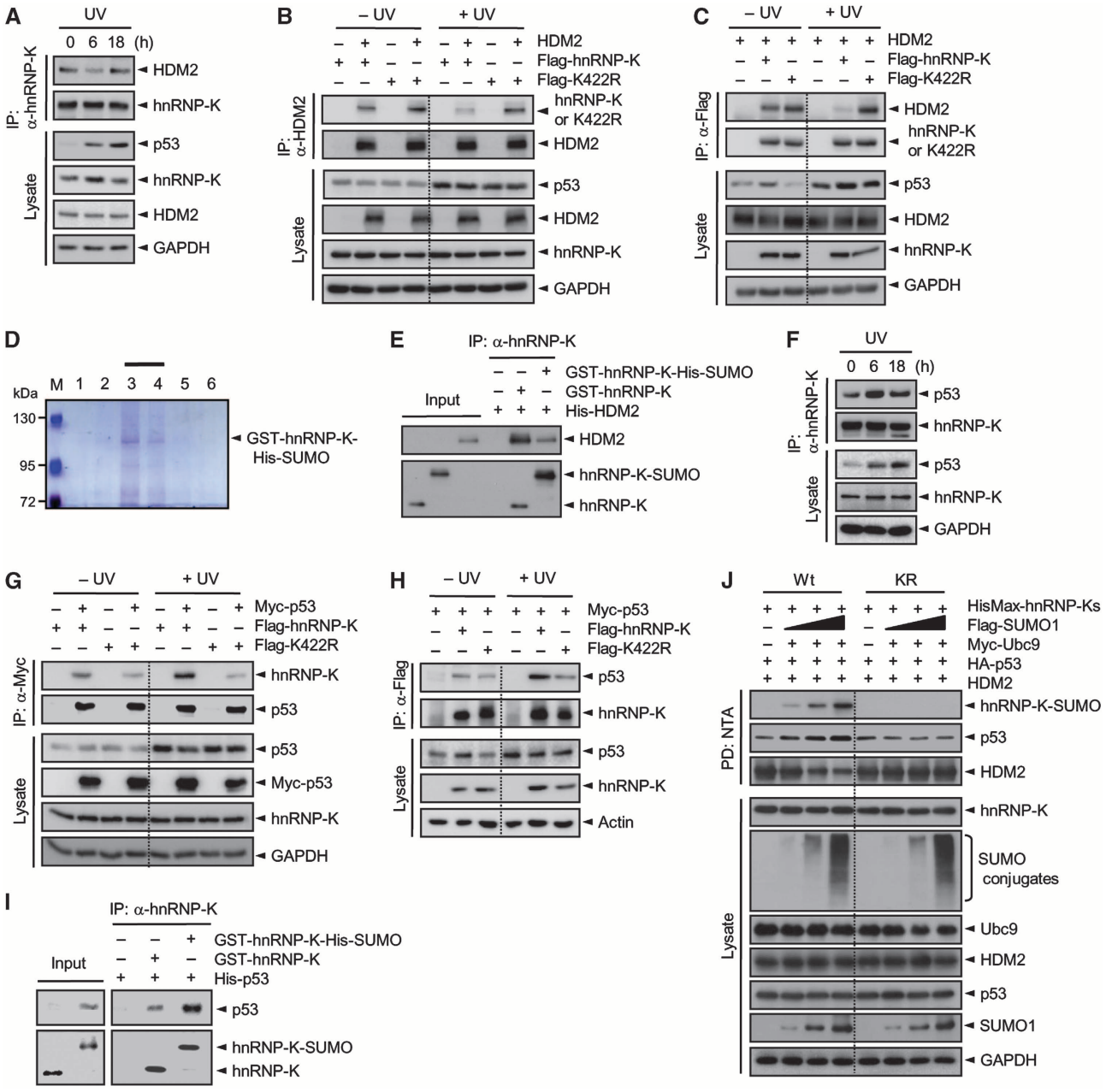

Figure 4 hnRNP-K SUMOylation switches its interaction with HDM2 to that with p53. (**A**) UV inhibits the interaction of hnRNP-K with HDM2. After UV treatment, HeLa cells were incubated for the indicated periods. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-HDM2 and anti-hnRNP-K antibodies. (**B**, **C**) SUMOylation inhibits the interaction of hnRNP-K with HDM2. HDM2 was overexpressed in cells with Flag-tagged hnRNP-K or K422R. After exposure to UV, cells were incubated for 6 h. Cell lysates were subjected to immunoprecipitation with anti-HDM2 (**B**) or anti-Flag antibody (**C**). (**D**) Purification of His-SUMO1-conjugated GST-hnRNP-K. SUMOylated hnRNP-K proteins eluted from NTA-agarose column were subjected to SDS–PAGE followed by staining with Coomassie blue R-250. Fractions under the bar were pooled for further use. (**E**) SUMOylation reduces the affinity of hnRNP-K to HDM2. Purified His-HDM2 was incubated with GST-hnRNP-K-His-SUMO1 or GST-hnRNP-K followed by immunoprecipitation with anti-hnRNP-K antibody. (**F**) UV promotes the interaction of hnRNP-K with p53. Experiments were performed as in (**A**), except that anti-p53 antibody was used in place of anti-HDM2 antibody. (**G**, **H**) SUMOylation increases the affinity of hnRNP-K to p53. Myc-p53 was overexpressed in cells with Flag-tagged hnRNP-K or K422R. After exposure to UV, cells were incubated for 6 h. Cell lysates were subjected to immunoprecipitation with anti-Myc (**G**) or anti-Flag antibody (**H**). (**I**) SUMOylated hnRNP-K shows higher affinity to p53. Experiments were done as in (**E**), except that His-p53 was used in place of His-HDM2. (**J**) SUMOylation inversely affects the binding of hnRNP-K to HDM2 and p53. HisMax-tagged hnRNP-K (Wt) and K422R (KR) were overexpressed in cells with Myc-Ubc9, HA-p53, HDM2, and increasing amounts of Flag-SUMO1. Cell lysates were subjected to pull down with NTA beads followed by immunoblot analysis. Note that 10 μM MG132 was treated 4 h before cell lysis in (**A**–**C**), (**F**–**H**), and (**J**). Figure source data can be found with the Supplementary data.

SUMOylated hnRNP-K even in the absence of UV (see Supplementary Figure S2) and since p53 is known to positively regulate its own expression, it appears likely that the overexpressed hnRNP-K-mediated increase in endogenous p53 level without UV treatment is due to the ability of SUMOylated hnRNP-K in promotion of p53 expression.

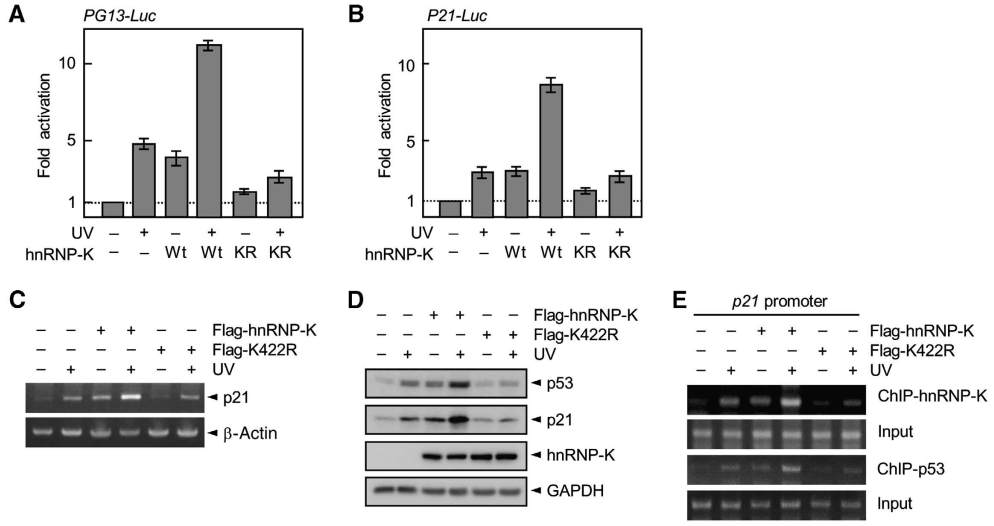

Figure 5 SUMOylation of hnRNP-K is required for its function as a p53 co-activator. (**A**, **B**) SUMOylation of hnRNP-K promotes p53 transactivity. HeLa cells overexpressing Flag-tagged hnRNP-K or K422R were transfected with *PG13-Luc* (**A**) or *P21-Luc* (**B**). After exposure to UV, cells were incubated for 6 h. Cell lysates were assayed for the luciferase activity. The activity seen without hnRNP-K overexpression and UV treatment was expressed as 1.0 and the others were as its relative values. The data represent the mean ± s.d. of three experiments. (**C**) SUMOylation of hnRNP-K increases the level of p21 transcripts. Total RNAs prepared from the same cells used in (**A**) were subjected to RT–PCR to determine p21 mRNA levels. (**D**) SUMOylation of hnRNP-K promotes p21 expression. Cell lysates prepared as in (**A**) were subjected to immunoblot with anti-p53, anti-p21, or anti-hnRNP-K antibody. (**E**) SUMOylation of hnRNP-K promotes recruitment of both hnRNP-K and p53 to the *p21* promoter. Cells prepared as in (**A**) were subjected to ChIP assay by using anti-hnRNP-K or anti-p53 antibody. Precipitated DNAs were subjected to PCR with primers covering the p53-response element in the *p21* gene. Figure source data can be found with the Supplementary data.

PIAS3 and SENP2 counteract on SUMO modification of hnRNP-K

To identify hnRNP-K-specific SUMO E3 ligase, each of PIAS1-4 was overexpressed with hnRNP-K. Among them, PIAS3 specifically interacted with hnRNP-K (Supplementary Figure S5A) and promoted its SUMOylation (Figure 6A). Furthermore, PIAS3 knockdown by shPIAS3 prevented not only hnRNP-K SUMOylation but also p21 expression (Figure 6B), suggesting that PIAS3-mediated SUMOylation of hnRNP-K is required for its function as a p53 co-activator. Interestingly, the amount of PIAS3 co-immunoprecipitated with hnRNP-K was significantly increased at 6 h after UV and returned almost to the initial level at 18 h-after-UV (Figure 6C). Thus, it appears that UV-mediated increase in hnRNP-K SUMOylation is due to an increase in the affinity of hnRNP-K to PIAS3.

We next attempted to identify hnRNP-K-specific deSUMOylating enzyme. Among the enzymes tested, overexpressed SENP1, SENP2, SENP6, and mouse SUSP4 interacted with hnRNP-K (Supplementary Figure S5B). Without overexpression, however, only SENP2 interacted with hnRNP-K and this interaction was markedly decreased at 6 h after UV and recovered at 18 h after UV (Figure 6D and E). Moreover, SENP2, but not its catalytically inactive mutant (in which the active site Cys548 was replaced by Ser), removed SUMO from hnRNP-K (Figure 6F), whereas SENP2 knockdown by shSENP2 promoted hnRNP-K SUMOylation (Figure 6G). Notably, without UV treatment SENP2 knockdown significantly increased the level of SUMOylated hnRNP-K, suggesting that endogenous SENP2 rapidly deSUMOylates hnRNP-K under unstressed conditions. Collectively, these results demonstrate that PIAS3 and SENP2 antagonistically regulate SUMO modification and stability of hnRNP-K during the time course after exposure to UV.

To map the regions within hnRNP-K for binding of HDM2, p53, SENP2, and PIAS3, deletions of hnRNP-K were generated and subjected to pull-down analysis (Supplementary Figure S6A–D). Both p53 and SENP2 bound to the same N-terminal region of hnRNP-K (Supplementary Figure S6E), suggesting that p53 and SENP2 could compete with each other for binding to hnRNP-K. While PIAS3 interacted with the middle region of hnRNP-K, HDM2 bound to its C-terminal region, which includes the SUMO-conjugation site Lys422. The latter data are consistent with the finding that SUMOylated hnRNP-K shows a lower affinity to HDM2 than its unmodified form (see Figure 4C and E). To identify hnRNP-K-binding regions within HDM2, p53, SENP2, and PIAS3, deletions of each protein were generated (Supplementary Figure S7). hnRNP-K bound to the C-terminal regions of p53, SENP2, and PIAS3, while it is interacted with the middle region of HDM2. A map for the interaction between hnRNP-K and HDM2, p53, SENP2, or PIAS3 was shown in Figure 7.

Effect of hnRNP-K SUMOylation on its subcellular localization

Previous studies have suggested that SUMOylation of hnRNP-K is involved in its nucleocytoplasmic transport (Vassileva and Matunis, 2004). Therefore, we examined whether UV-induced SUMOylation influences the subcellular localization of hnRNP-K. Immunocytochemical analysis showed that in the absence of UV treatment, overexpressed hnRNP-K resided in both the nucleus and the cytoplasm in ~30% of cells as well as exclusively in the nucleus in the remaining cells (Supplementary Figure S8A). In its presence, however, the entire hnRNP-K proteins were localized exclusively in the nucleus. In contrast, K422R was localized in both the nucleus and the cytoplasm in ~40% of cells regardless of UV treatment, suggesting that SUMOylation is involved in the nuclear localization of hnRNP-K. Therefore, we next examined whether the nuclear localization of hnRNP-K could be

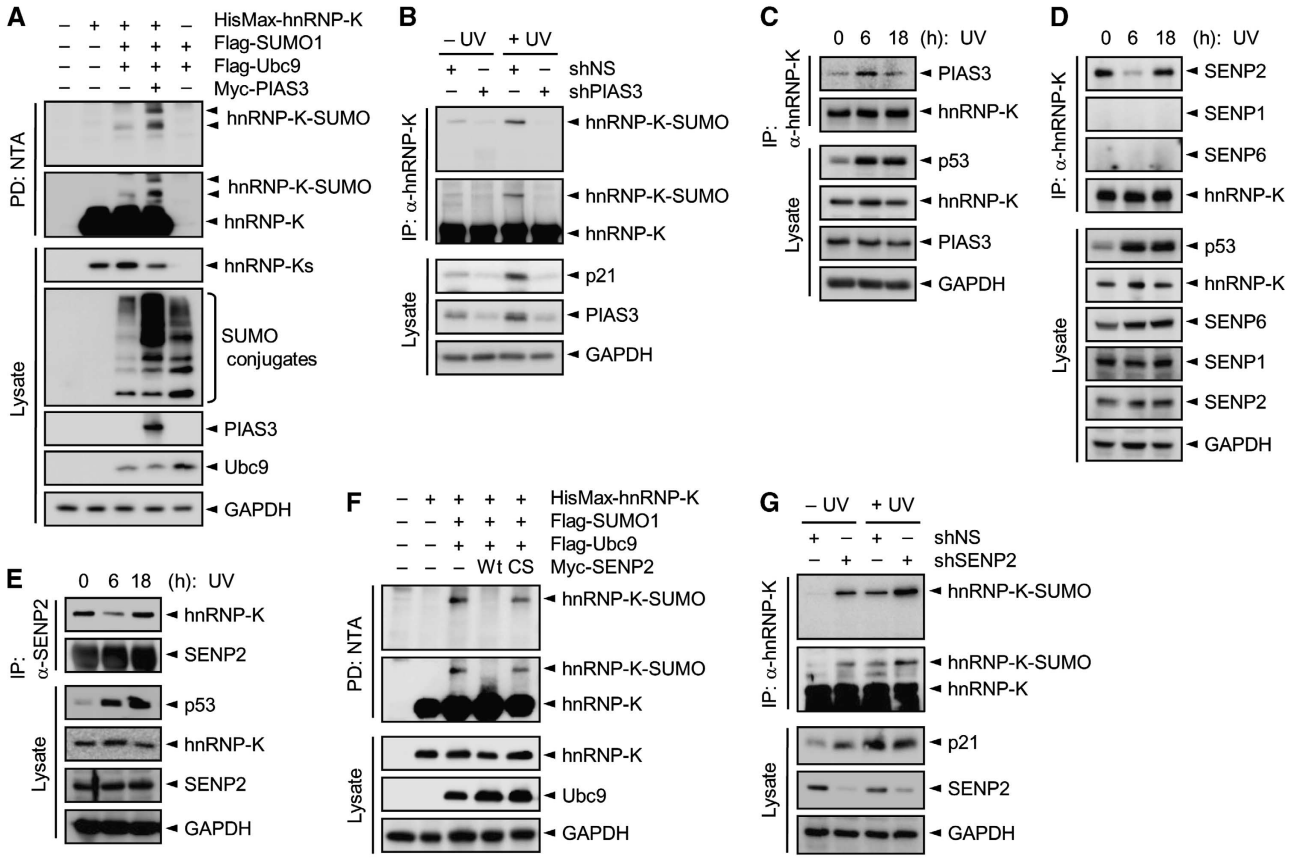

Figure 6 PIAS3 and SENP2 antagonistically regulate hnRNP-K SUMOylation. (**A**) PIAS3 promotes hnRNP-K SUMOylation. HisMax-hnRNP-K was overexpressed in HEK293T cells with Flag-SUMO1, Flag-Ubc9, and Myc-PIAS3. Cell lysates were subjected to pull down with NTA beads followed by immunoblot with anti-SUMO1 or anti-Xpress antibody. (**B**) PIAS3 knockdown blocks hnRNP-K SUMOylation. HeLa cells transfected with shNS or shPIAS3 were exposed to UV and then incubated for 6 h. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-SUMO1 or anti-hnRNP-K antibody. (**C**) UV promotes the interaction of hnRNP-K with PIAS3. After exposure to UV, cells were incubated for the indicated periods. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-PIAS3 or anti-hnRNP-K antibody. (**D**, **E**) UV inhibits the interaction of hnRNP-K with SENP2. Cells treated with UV were subjected to immunoprecipitation with anti-hnRNP-K (**D**) or anti-SENP2 antibody (**E**). Note that MG132 was treated 4 h before cell lysis in (**C**–**E**). (**F**) SENP2 deSUMOylates hnRNP-K. HisMax-hnRNP-K was overexpressed in HEK293T cells with Flag-SUMO1, Flag-Ubc9, and Myc-tagged SENP2 (Wt) or its catalytically inactive form (CS). Cell lysates were subjected to pull down with NTA beads followed by immunoblot with anti-SUMO1 or anti-Xpress antibody. (**G**) SENP2 knockdown promotes hnRNP-K SUMOylation. HeLa cells transfected with shNS or shSENP2 were exposed to UV and incubated for 6 h. Cell lysates were then treated as in (**B**). Figure source data can be found with the Supplementary data.

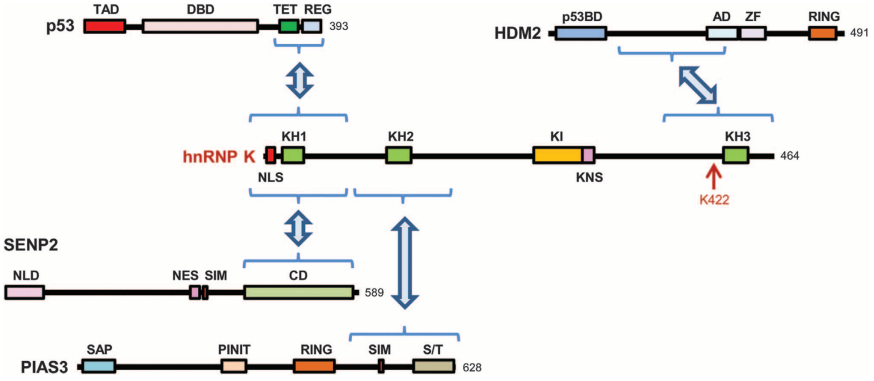

Figure 7 Map for the interaction between hnRNP-K and p53, HDM2, SENP2, or PIAS3. The data obtained from Supplementary Figures S6 and S7 were summarized. In p53: TAD, transcription activation domain; DBD, DNA-binding domain; TET, tetramerization domain; REG, regulatory domain. In HDM2: p53BP, p53 binding domain; AD, acidic domain; ZF, zinc finger; RING, really interesting gene. In SENP2: NLD, nuclear localization domain; NES, nuclear export signal; SIM, SUMO-interacting motif; CD, catalytic domain. In PIAS3: SAP, SAF-A/B, Acinus and PIAS; PINIT, Pro-Ile-Asn-Ile-Thr; S/T, Ser/Thr-rich.

blocked by knockdown of PIAS3. Depletion of PIAS3 prevented UV-induced nuclear localization of hnRNP-K (Supplementary Figure S8B). The nuclear localization of hnRNP-K could also be prevented by overexpression of wild-type SENP2, but not by its catalytically inactive mutant in which the active site Cys548 was replaced by Ser

(Supplementary Figure S8C). These results again suggest that SUMOylation is involved in the nuclear localization of hnRNP-K.

To confirm this finding, we examined whether the localization of endogenous hnRNP-K could be influenced by PIAS3 knockdown in the presence and absence of UV. In contrast to the data obtained by hnRNP-K overexpression, neither UV treatment nor PIAS3 depletion showed any effect on the nuclear localization of hnRNP-K (Supplementary Figure S9). Noteworthy, however, were the findings that in cells having overexpressed hnRNP-K in both the nucleus and the cytoplasm, the portion of hnRNP-K located in the cytoplasm is much lower than that in the nucleus and that ~60% of SUMOylation-defective K422R mutant resided exclusively in the nucleus regardless of UV treatment (see Supplementary Figure S8A). Furthermore, overexpression of SENP2 did not alter the population of cells that have hnRNP-K exclusively in the nucleus, although it can prevent the UV-induced nuclear localization of hnRNP-K in the cytoplasm (see Supplementary Figure S8C). In addition, endogenous hnRNP-K is known to predominantly localize in the nucleus, and this nuclear localization is mediated mainly by the nuclear shuttling sequence (KNS) and in part by the NLS sequence in hnRNP-K (Michael *et al*, 1977). Thus, it appears likely that SUMOylation plays only a minor, auxiliary role in the nuclear localization of hnRNP-K.

UV-induced hnRNP-K SUMOylation is required for cell-cycle arrest

To determine whether SENP2 is involved in the control of hnRNP-K's co-activator function by altering its SUMOylation state, *PG13-Luc* and *p21-Luc* were again used for assaying p53 transactivity. In both cases, UV treatment increased p53 transactivity and this increase was further enhanced by SENP2 knockdown (Figure 8A and B; Supplementary Figure S10A). Without UV treatment, SENP2 knockdown moderately promoted p53 transactivity, since it increases the level of SUMOylated hnRNP-K (see Figure 6G). These stimulatory effects of SENP2 knockdown on p53 transactivity were ablated by simultaneous knockdown of hnRNP-K by shhnRNP-K, indicating that the observed effects are specific to hnRNP-K. These results indicate that SENP2 negatively regulates the function of hnRNP-K as a p53 co-activator. On the other hand, knockdown of PIAS3 alone or together with hnRNP-K completely abrogated UV-induced p53 transactivity (Figure 8C and D; Supplementary Figure S10B), indicating that PIAS3 positively regulates the co-activator function of hnRNP-K. Collectively, these results indicate that SENP2 and PIAS3 antagonistically regulate the role of hnRNP-K as a p53 co-activator.

We next examined whether hnRNP-K SUMOylation is required for UV-induced cell-cycle arrest upon flow cytometry. UV treatment increased cell fractions in G1 phase and this increase was blocked by hnRNP-K knockdown (Figure 8E; Supplementary Figure S11). Supplement of shhnRNP-K-insensitive hnRNP-K to cells that had been depleted of endogenous hnRNP-K, but not that of shhnRNP-K-insensitive K422R, restored accumulation of G1-phase cells. To determine whether the cell-cycle arrest is mediated by p53-induced expression of p21, the same cells used for flow cytometry were subjected to immunoblot analysis. Knockdown of hnRNP-K led to a marked decrease in the expression of p21 as well as in that of p53, and this decrease could be reversed by supplement of shhnRNP-K-insensitive hnRNP-K, but not by that of shhnRNP-K-insensitive K422R (Figure 8F). These results indicate that hnRNP-K SUMOylation is required for UV-induced cell-cycle arrest. Knockdown of SENP2 enhanced UV-mediated increase in cell fractions in G1 phase, whereas that of PIAS3 ablated it (Figure 8G). Knockdown of SENP2 together with hnRNP-K also prevented UV-induced cell-cycle arrest. Figure 8H shows that PIAS3 knockdown decreases the expression of p21 as well as of p53 whereas SENP2 knockdown increases it, and this increase could be ablated by simultaneous knockdown of hnRNP-K. Collectively, these results indicate that reversible SUMO modification of hnRNP-K by SENP2 and PIAS3 plays a key role in the control of p21-mediated cell-cycle arrest in response to UV damage.

UV-induced DNA damage response is at least in part mediated by ATR kinase, which phosphorylates downstream targets, such as p53 and CHK1 (Durocher and Jackson, 2001; Cimprich and Cortez, 2008). Treatment with caffeine, an inhibitor of ATR (in addition to ATM), and knockdown of ATR by shATR abrogated not only UV-induced hnRNP-K SUMOylation and but also the increased interaction of PIAS3 with hnRNP-K at 6 h after UV (Figure 9A and B). They also resulted in sustained interaction of hnRNP-K with SENP2. These results indicate that UV-induced hnRNP-K SUMOylation, which is essential for hnRNP-K's function as a p53 co-activator, is ATR dependent.

Discussion

Based on the findings in this study, we propose a model for the role of hnRNP-K SUMOylation in UV-induced cell-cycle arrest (Figure 9C). Under unstressed conditions, SENP2 removes SUMO from SUMO-conjugated hnRNP-K, if there is any, allowing hnRNP-K to bind HDM2 with high affinity for its ubiquitination and subsequent degradation by proteasome. Although not shown, HDM2 also promotes proteasome-mediated degradation of p53. Upon exposure to UV, PIAS3 binds and ligates SUMO to hnRNP-K, allowing p53 to bind SUMOylated hnRNP-K with high affinity and recruitment of their complex to the *p21* promoter. Thus, hnRNP-K SUMOylation by PIAS3 serves as a critical switch for shifting the interaction of hnRNP-K with HDM2 to that with p53 for its function as a transcriptional co-activator of p53 and in turn for p21 expression and cell-cycle arrest in response to DNA damage by UV.

Of interest was the finding that the level of SUMOylated hnRNP-K increases and then declines during the time course after exposure of cells to UV. This phenomenon is apparently mediated by a rise-and-fall of hnRNP-K's ability to interact with PIAS3 and its inversed ability to bind SENP2 during the same time course. This reversible SUMOylation process that should occur in conjunction with the p53-HDM2 feedback loop is of importance for cells to escape from cell-cycle arrest and to resume normal growth after the repair of damaged DNA. However, it remains unknown how the binding ability of hnRNP-K is shifted to PIAS3 and then to SENP2 after UV treatment. Since ATR knockdown prevents the interaction of hnRNP-K with PIAS3 but promotes that with SENP2 and since UV does not affect the expression of either PIAS3 or SENP2, it seems possible that ATR-mediated phosphorylation of hnRNP-K followed by dephosphorylation by an unknown protein phosphatase(s) might change the affinity of hnRNP-K to PIAS3 and SENP2. Notably, hnRNP-K has the SQ and TQ

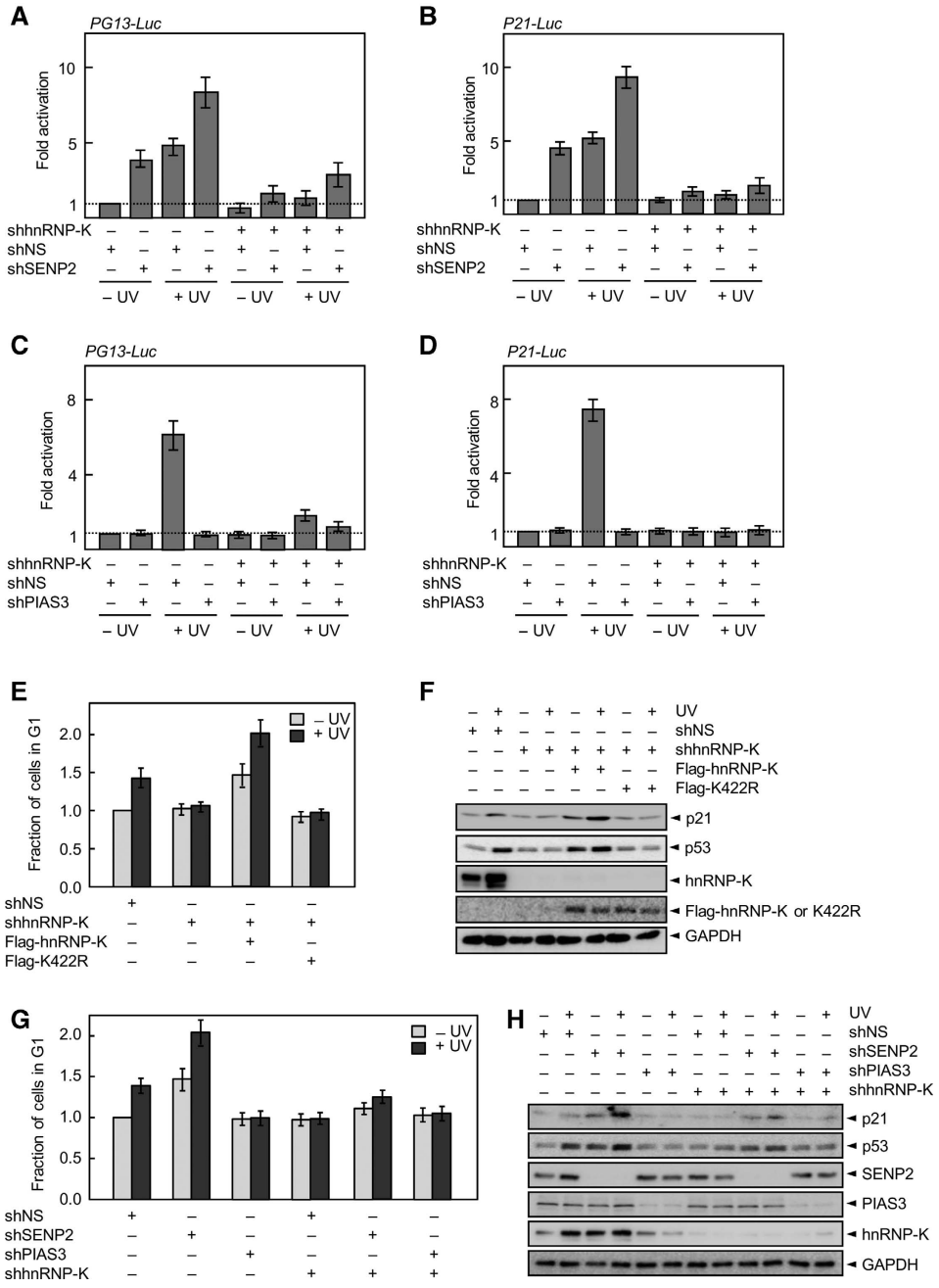

Figure 8 PIAS3 and SENP2 antagonistically regulate cell-cycle arrest. (**A**, **B**) SENP2 knockdown promotes p53 transactivity. HeLa cells transfected with shNS or shSENP2 alone or together with shhnRNP-K were incubated for 48 h. They were then transfected with *PG13-Luc* (**A**) or *P21-Luc* (**B**) and further incubated for the next 24 h. After exposure to UV, cells were incubated for 6 h. Cell lysates were assayed for luciferase. The enzyme activity seen in cells transfected with shNS only but without UV treatment was expressed as 1.0 and the others were as its relative values. (**C**, **D**) PIAS3 knockdown ablates p53 transactivity. Experiments were performed as above, except that cells were transfected with shPIAS3 in place of shSENP2. (**E**, **F**) SUMOylation of hnRNP-K is required for p21-mediated cell-cycle arrest. Cells transfected with shNS or shhnRNP-K were complemented with shhnRNP-K-insensitive Flag-tagged hnRNP-K or K422R. After exposure to UV, they were incubated for 6 h followed by flow cytometry (**E**) or immunoblot analysis (**F**). (**G**, **H**) PIAS3 and SENP2 inversely regulate cell-cycle arrest. Cells were transfected with shNS, shSENP2, or shPIAS3 alone or together with shhnRNP-K. After exposure to UV, cells were incubated for 6 h followed by flow cytometry (**G**) or immunoblot analysis (**H**). The data in (**A**–**E**) and (**G**) represent the mean ± s.d. of four experiments. Figure source data can be found with the Supplementary data.

motifs that can be phosphorylated by ATR (Kim *et al*, 1999). However, replacement of the Ser and Thr residues by Ala or Glu showed little or no effect on UV-induced SUMOylation of hnRNP-K. Nonetheless, we could not exclude a possibility that kinases downstream of ATR, such as CHK1, or other kinase(s) and unknown phosphatases may be involved in reversible phosphorylation of hnRNP-K and in turn in the control of the affinity of hnRNP-K to PIAS3 and SENP2.

PIAS3 serves as an endogenous protein inhibitor of activated signal transducers and activators of transcription 3 (STAT3), in addition to its role as a SUMO E3 ligase (Chung *et al*, 1997; Jackson, 2001; Jang *et al*, 2004). The STAT3 protein, which promotes cell-cycle progression and inhibits apoptosis, has been implicated in the pathogenesis of various human cancers (Niu *et al*, 2002; Wei *et al*, 2003; Levy and Inghirami, 2006). Interestingly, PIAS3 expression is

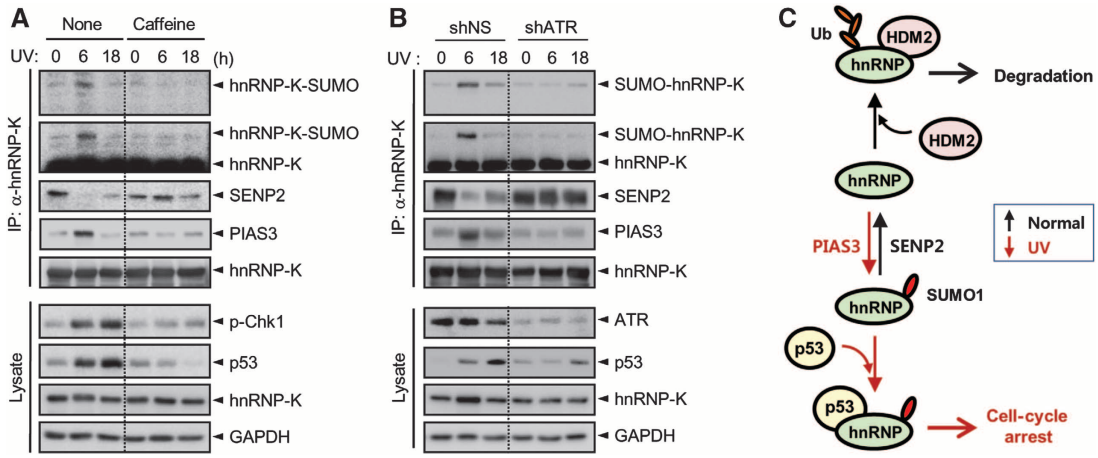

Figure 9 SUMOylation of hnRNP-K is ATR dependent. (**A**) Caffeine inhibits hnRNP-K SUMOylation. After exposure of HeLa cells to UV, they were incubated with and without 5 mM caffeine for the indicated periods. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-SUMO1, anti-SENP2, anti-PIAS3, or anti-hnRNP-K antibody. (**B**) ATR knockdown prevents hnRNP-K SUMOylation. Cells transfected with shNS or shATR were incubated for 48 h. After exposure to UV, cells were incubated for the indicated periods. Cell lysates were subjected to immunoprecipitation as in (**A**). Note that MG132 was treated 4 h before cell lysis in (**A**, **B**). (**C**) A model for the role of hnRNP-K SUMOylation in UV-induced cell-cycle arrest. Figure source data can be found with the Supplementary data.

downregulated in several cancers, such as human gastric carcinoma, glioblastoma, and squamous cell carcinoma of the lung (Brantley *et al*, 2008; Kluge *et al*, 2011; Liu *et al*, 2011). Therefore, it has been suggested that loss or reduction of PIAS3 expression contributes to enhanced STAT3 transcriptional activity, leading to aberrant cell proliferation and tumorigenesis. Here, we showed that PIAS3 promotes hnRNP-K SUMOylation and thereby p53-mediated cell-cycle arrest. Thus, PIAS3 might exert its anti-tumorigenic function in both E3 ligase activity-dependent and -independent manners by promoting cell-cycle arrest.

Targeted disruption of SENP2 in mice was shown to impair cell-cycle progression at the G1/S phase, leading to abnormalities in trophoblast proliferation and differentiation (Chiu *et al*, 2008). During trophoblast development, SENP2 removes SUMO from Mdm2 and thereby promotes Mdm2-mediated ubiquitination of p53 and its subsequent degradation by proteasome, allowing cell-cycle progression. Disruption of the *SENP2* gene, however, results in cytoplasmic localization of Mdm2 and in turn in p53 stabilization in the nucleus, leading to p53-mediated cell-cycle arrest. On the other hand, overexpression of SENP2 makes cells resistant to apoptosis induced by genotoxic stress, such as doxorubicin treatment, indicating that SENP2 plays a critical role in the control of cell-cycle progression (Jiang *et al*, 2011). Here, we showed that SENP2 knockdown increases hnRNP-K SUMOylation, its interaction with p53, and consequently its co-activator function in expression of p53-downstream genes, such as *p21*, for cell-cycle arrest. In addition, it has been shown that under unstressed conditions hnRNP-K is ubiquitinated by HDM2 for degradation by proteasome (Moumen *et al*, 2005). Thus, it appears that SENP2 could regulate cell-cycle progression by targeting two different substrates: one by deSUMOylating HDM2 for HDM2-mediated degradation of p53 and the other by deSUMOylating hnRNP-K for HDM2-mediated degradation of hnRNP-K, which ablates its function as a p53 co-activator.

The p21 protein can function as an anti-apoptotic protein as well as an inhibitor of CDKs for cell-cycle arrest. Recently, it was shown that an anti-cancer drug, RITA (reactivation of p53 and induction of tumour cell apoptosis) releases HDM2 from p53 and the freed HDM2 molecules promote proteasome-mediated degradation of hnRNP-K, which impairs its co-activator function in p53-mediated p21 expression (Enge *et al*, 2009). HDM2 also directly promotes p21 degradation by proteasome, eliminating anti-apoptotic function of p21 and thus switching towards induction of apoptosis. Deregulated cell-cycle progression such as by preventing p21-mediated cell-cycle arrest could also evoke tumorigenesis. Therefore, small molecules that specifically inhibit SENP2 could be used as therapeutic drugs against cancers, as they should prevent hnRNP-K deSUMOylation and in turn induce p53 activation for p21 expression, leading to cell-cycle arrest.

While our work was under revision, another study reported that DNA damage induces hnRNP-K SUMOylation, which in turn enhances the transcriptional activity of p53 (Pelisch *et al*, 2012). However, the major differences between their and our works are the effect of SUMOylation on the stability of hnRNP-K and the identity of hnRNP-K-specific SUMO E3 ligase. While they concluded that SUMOylation does not alter hnRNP-K stability and that Pc2 acts as an E3 ligase, we found that the same modification leads to hnRNP-K stabilization and that PIAS3 serves as an hnRNP-K-specific ligase. These differences might be due to the use of a single time point in analysing the effect of DNA damage on alterations in the level of hnRNP-K and to the use of overexpression system in analysing the role of Pc2 in hnRNP-K SUMOylation rather than that of RNA interference system. However, we could not exclude a possibility that different SUMO E3 ligases may act on hnRNP-K under different DNA damage conditions, since their work mainly used doxorubicin as a DNA damaging agent whereas ours utilized UV.

Materials and methods

Plasmids and antibodies

hnRNP-K cDNA was isolated from a cDNA library of HeLa cells, and cloned into pcDNA-HisMax and pCMV2-Flag. It was also cloned into pET-32b and pGEX-4T3 for bacterial expression. shRNAs were purchased from Open Biosystems. Target sequences for shRNAs are as follows: shhnRNP-K, 5′-ACGATGAAACCTATGATTA-3′; shSENP2, 5′-CCCACAGGATGAAATCCTA-3′; shPIAS3, 5′-GCTGTCGGTCAGACAT

CATTT-3′. Antibodies against Myc (9E10), p53 (DO-1), p21 (C-19), hnRNP-K (D-6), GAPDH (2D4A7), HDM2 (SMP14), Ub (A-5), Ubc9 (N-15), and GST (Z-5) were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Flag M2 (Sigma-Aldrich), anti-Xpress, anti-SUMO-1 (Invitrogen), anti-His (BD Biosciences), anti-SENP1, anti-SENP2, anti-SENP6 (Abgent), and anti-PIAS3 (Cell Signaling) antibodies were also used.

Cell culture and transfection

HEK293T and HeLa cells were grown at 37°C in DMEM supplemented with 100 units/ml penicillin, 1 μg/ml streptomycin, and 10% FBS. MRC5 cells were cultured as above except that the use of MEM in place of DMEM. All transfections were carried out using Metafectene reagent (Biontex) and jetPEI™ DNA Transfection Reagent (Polyplus-transfection).

Assays for SUMO modification

HisMax-hnRNP-K, Flag-SUMO1, and Flag-Ubc9 were overexpressed in HEK293T cells with or without Myc-tagged SENP2 or PIAS3. After culturing for 36 h, cells were lysed by boiling for 10 min in 150 mM Tris–HCl (pH 8), 5% SDS, and 30% glycerol. Cell lysates were diluted 20-fold with buffer A consisting of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM imidazole, 1% Triton X-100, 1 × protease inhibitor cocktail (Roche), and 2 mM NEM. After incubating them with Ni^{2+}-NTA-agarose for 2 h at 4°C, the resins were collected, washed with buffer A containing 20 mM imidazole, and boiled in SDS-sampling buffer. Supernatants were subjected to SDS–PAGE followed by immunoblot analysis. For assaying SUMOylation of endogenous hnRNP-K, HeLa cells without any overexpression were lysed as above. Cell lysates were diluted 20-fold with buffer B consisting of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100, 1 × protease inhibitor cocktail, and 2 mM NEM. The samples were incubated with anti-hnRNP-K antibody for 2 h at 4°C and then with protein-A-Sepharose for the next 2 h. The resins were collected, washed with buffer B containing 1% Triton X-100, and boiled. Supernatants were subjected to SDS–PAGE followed by immunoblot analysis.

For *in vitro* SUMOylation assay, purified His-hnRNP-K (2 μg), SUMO1 (5 μg), SAE1/SAE2 (1.5 μg), and Ubc9 (5 μg) were incubated with an ATP-regenerating system consisting of 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, 5 units/ml of phosphocreatine kinase, and 1 × protease inhibitor cocktail in a total volume of 30 μl. After incubating the mixtures for 2 h at 37°C, they were subjected to SDS–PAGE followed by immunoblot.

Flow cytometry

Cells were washed with PBS, trypsinized, and fixed at 4°C with 70% ethanol. They were washed with PBS and incubated in PBS containing 0.1% Triton X-100, 200 μg/ml of RNase A, and 20 μg/ml of propidium iodide for 30 min at room temperature in the dark. DNA contents were then determined by flow cytometry using FACSCalibur (Becton Dickinson).

UV and γ irradiation

Cells cultured to 50–70% confluence were washed with PBS and irradiated at 254 nm (UV-C) by using TUV lamp (Philips) or at 5 Gy by using Gammacell Low Dose-rate Research Irradiator (GC 3000 Elan). UV dose (10 J/m^2) was determined by using a UVX radiometer (UVP Inc.). They were then incubated for various periods in DMEM supplemented with 100 units/ml of penicillin, 1 mg/ml of streptomycin, and 10% FBS.

RT–PCR

Total RNAs were isolated from cells by using TRIzol (Invitrogen). RT–PCR was performed using RevertAid M–MuLV reverse transcriptase (Invitrogen) and oligo (dT) primer, according to manufacturer's instructions. Primers used in PCR for p21 were 5′-CTTTGTCACCGA GACACCAC-3′ and 5′-GGCGTTTGGAGTGGTAGAAA-3′.

Luciferase assays

HeLa cells transfected with pcDNA-β-Gal and *PG13-Luc* or *p21-Luc* were incubated for 48 h. After UV treatment, cells were cultured for 6 h, harvested, and assayed for luciferase. The enzyme activity was measured in a luminometer and normalized by β-galactosidase expression with a luciferase system (Promega).

ChIP assay

Assays were conducted with an average size of sheared fragments of about 300–1000 bps as described (Jepsen *et al*, 2000; Shang *et al*, 2000). For PCR, 1 μl from 50 μl DNA extraction and 25–30 cycles of amplification were used. Primers used in PCR of p21 promoter sequence were 5′-GTGGCTCTGATTGGCTTTCTG-3′ and 5′-CTGAAA ACAGGCAGCCCAAGG-3′ (Zeng *et al*, 2002).

Purification of recombinant SUMOylated hnRNP-K

For production of SUMOylated hnRNP-K, BL21(DE3) cells were transformed with pGEX4T3-hnRNP-K and pT-E1/E2/His-SUMO1. BL21 colonies carrying both plasmids were selected as described (Zeng *et al*, 2002). Extracts (10 mg) from the cells were loaded onto a glutathione-Sepharose 4B column, and proteins bound to the column were eluted with PBS containing 50 mM glutathione. After dialysis against buffer C consisting of NaH_2PO_4/Na_2HPO_4 (pH 8), 0.5 M NaCl, 50 mM imidazole, 1% Triton X-100, and 2 mM 2-mercaptoethanol, proteins were loaded onto a NTA-agarose column. Bound proteins (i.e., GST-hnRNP-K-His-SUMO1) were eluted with buffer C containing 200 mM imidazole.

Immunocytochemistry

HeLa cells were grown on coverslips. After transfection, they were fixed by incubation with 3.7% paraformaldehyde in PBS for 10 min. Cells were washed three times with PBS containing 0.1% Triton X-100, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 3% BSA in PBS for 30 min, cells were incubated for 1 h with appropriate antibodies. After washing with PBS containing 0.1% Triton X-100, cells were incubated for 1 h with FITC- or TRITC-conjugated secondary antibody in PBS containing 3% BSA. Cells were then observed using a confocal laser scanning microscope (Carl Zeiss-LSM700).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

This work was supported by grants from the National Research Foundation of Korea (NRF-2005-084-C00025 and M10533010001-05N3301). SWL, HMY, and JHP were the recipients of the BK21 fellowship.

Author contributions: SWL, YJJ, and CHC analysed the data, conceived the study, and wrote the paper. SWL and MHL initiated the project and SWL performed all the experiments except purification of p53, hnRNP-K, SUMO1, Sae1/2, and Ubc9, which was done by SHK, JHP, and HMY. Cloning of hnRNP-K cDNA and other plasmids was performed by SHK and SWL, and immunocytochemistry was done by SWL and YMO.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* **15:** 2177–2196

Abraham RT (2004) PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. *DNA Repair (Amst)* **3:** 883–887

Altmannova V, Eckert-Boulet N, Arneric M, Kolesar P, Chaloupkova R, Damborsky J, Sung P, Zhao X, Lisby M, Krejci L (2010) Rad52 SUMOylation affects the efficiency of the DNA repair. *Nucleic Acids Res* **38:** 4708–4721

Baptiste-Okoh N, Barsotti AM, Prives C (2008) Caspase 2 is both required for p53-mediated apoptosis and downregulated by p53 in a p21-dependent manner. *Cell Cycle* **7:** 1133–1138

Bartek J, Lukas J (2001) Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol* **13:** 738–747

Bergink S, Jentsch S (2009) Principles of ubiquitin and SUMO modifications in DNA repair. *Nature* **458:** 461–467

Bomsztyk K, Denisenko O, Ostrowski J (2004) hnRNP K: one protein multiple processes. *Bioessays* **26:** 629–638

Bomsztyk K, Van Seuningen I, Suzuki H, Denisenko O, Ostrowski J (1997) Diverse molecular interactions of the hnRNP K protein. *FEBS Lett* **403:** 113–115

Brantley EC, Nabors LB, Gillespie GY, Choi YH, Palmer CA, Harrison K, Roarty K, Benveniste EN (2008) Loss of protein inhibitors of activated STAT-3 expression in glioblastoma multiforme tumors: implications for STAT-3 activation and gene expression. *Clin Cancer Res* **14:** 4694–4704

Capili AD, Lima CD (2007) Taking it step by step: mechanistic insights from structural studies of ubiquitin/ubiquitin-like protein modification pathways. *Curr Opin Struct Biol* **17:** 726–735

Chiu SY, Asai N, Costantini F, Hsu W (2008) SUMO-specific protease 2 is essential for modulating p53-Mdm2 in development of trophoblast stem cell niches and lineages. *PLoS Biol* **6:** e310

Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, Shuai K (1997) Specific inhibition of Stat3 signal transduction by PIAS3. *Science* **278:** 1803–1805

Ciccia A, Elledge SJ (2010) The DNA damage response: making it safe to play with knives. *Mol Cell* **40:** 179–204

Cimprich KA, Cortez D (2008) ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9:** 616–627

Cremona CA, Sarangi P, Yang Y, Hang LE, Rahman S, Zhao X (2012) Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint. *Mol Cell* **45:** 422–432

Dou H, Huang C, Singh M, Carpenter PB, Yeh ET (2010) Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. *Mol Cell* **39:** 333–345

Durocher D, Jackson SP (2001) DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr Opin Cell Biol* **13:** 225–231

Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A, Selivanova G (2009) MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer Cell* **15:** 171–183

Gareau JR, Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* **11:** 861–871

Geiss-Friedlander R, Melchior F (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8:** 947–956

Hay RT (2005) SUMO: a history of modification. *Mol Cell* **18:** 1–12

Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419:** 135–141

Horn HF, Vousden KH (2007) Coping with stress: multiple ways to activate p53. *Oncogene* **26:** 1306–1316

Jackson PK (2001) A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. *Genes Dev* **15:** 3053–3058

Jang HD, Yoon K, Shin YJ, Kim J, Lee SY (2004) PIAS3 suppresses NF-kappaB-mediated transcription by interacting with the p65/RelA subunit. *J Biol Chem* **279:** 24873–24880

Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW, Rosenfeld MG (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell* **102:** 753–763

Jiang M, Chiu SY, Hsu W (2011) SUMO-specific protease 2 in Mdm2-mediated regulation of p53. *Cell Death Differ* **18:** 1005–1015

Johnson ES (2004) Protein modification by SUMO. *Annu Rev Biochem* **73:** 355–382

Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22:** 159–180

Kim KI, Baek SH, Chung CH (2002) Versatile protein tag, SUMO: its enzymology and biological function. *J Cell Physiol* **191:** 257–268

Kim ST, Lim DS, Canman CE, Kastan MB (1999) Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* **274:** 37538–37543

Kluge A, Dabir S, Vlassenbroeck I, Eisenberg R, Dowlati A (2011) Protein inhibitor of activated STAT3 expression in lung cancer. *Mol Oncol* **5:** 256–264

Kruse JP, Gu W (2009) Modes of p53 regulation. *Cell* **137:** 609–622

Lakin ND, Jackson SP (1999) Regulation of p53 in response to DNA damage. *Oncogene* **18:** 7644–7655

Lane DP (1992) Cancer. p53, guardian of the genome. *Nature* **358:** 15–16

Lee MH, Lee SW, Lee EJ, Choi SJ, Chung SS, Lee JI, Cho JM, Seol JH, Baek SH, Kim KI, Chiba T, Tanaka K, Bang OS, Chung CH (2006) SUMO-specific protease SUSP4 positively regulates p53 by promoting Mdm2 self-ubiquitination. *Nat Cell Biol* **8:** 1424–1431

Levine AJ, Oren M (2009) The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* **9:** 749–758

Levy DE, Inghirami G (2006) STAT3: a multifaceted oncogene. *Proc Natl Acad Sci USA* **103:** 10151–10152

Li T, Evdokimov E, Shen RF, Chao CC, Tekle E, Wang T, Stadtman ER, Yang DC, Chock PB (2004) Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis. *Proc Natl Acad Sci USA* **101:** 8551–8556

Liu LM, Yan MG, Yang DH, Sun WW, Zhang JX (2011) PIAS3 expression in human gastric carcinoma and its adjacent non-tumor tissues. *Clin Res Hepatol Gastroenterol* **35:** 393–398

Matunis MJ, Michael WM, Dreyfuss G (1992) Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. *Mol Cell Biol* **12:** 164–171

Michael WM, Eder PS, Dreyfuss G (1977) The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. *EMBO J* **16:** 3587–3598

Moumen A, Masterson P, O'Connor MJ, Jackson SP (2005) hnRNP K: an HDM2 target and transcriptional coactivator of p53 in response to DNA damage. *Cell* **123:** 1065–1078

Mukhopadhyay D, Dasso M (2007) Modification in reverse: the SUMO proteases. *Trends Biochem Sci* **32:** 286–295

Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, Yu H (2002) Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* **21:** 2000–2008

Pelisch F, Pozzi B, Risso G, Munoz MJ, Srebrow A (2012) DNA damage-induced heterogeneous nuclear ribonucleoprotein K SUMOylation regulates p53 transcriptional activation. *J Biol Chem* **287:** 30789–30799

Perry ME (2004) Mdm2 in the response to radiation. *Mol Cancer Res* **2:** 9–19

Polo SE, Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev* **25:** 409–433

Rytinki MM, Kaikkonen S, Pehkonen P, Jaaskelainen T, Palvimo JJ (2009) PIAS proteins: pleiotropic interactors associated with SUMO. *Cell Mol Life Sci* **66:** 3029–3041

Sacher M, Pfander B, Hoege C, Jentsch S (2006) Control of Rad52 recombination activity by double-strand break-induced SUMO modification. *Nat Cell Biol* **8:** 1284–1290

Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103:** 843–852

Steinman RA, Johnson DE (2000) p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. *Mol Med* **6:** 736–749

Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M (1998) Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* **17:** 931–939

Vassileva MT, Matunis MJ (2004) SUMO modification of heterogeneous nuclear ribonucleoproteins. *Mol Cell Biol* **24:** 3623–3632

Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. *Nature* **408:** 307–310

Vousden KH, Lu X (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer* **2:** 594–604

Wei D, Le X, Zheng L, Wang L, Frey JA, Gao AC, Peng Z, Huang S, Xiong HQ, Abbruzzese JL, Xie K (2003) Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene* **22:** 319–329

Yeh ET (2009) SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem* **284:** 8223–8227

Zeng SX, Dai MS, Keller DM, Lu H (2002) SSRP1 functions as a co-activator of the transcriptional activator p63. *EMBO J* **21:** 5487–5497