이학박사학위논문

DNA 손상시 유도되는 hnRNP-K의 수모화에 의한 세포 주기 중지 조절에 관한 연구

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A dissertation submitted in partial
Fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

To the Faculty of
School of Biological Sciences
at
SEOUL NATIONAL UNIVERSITY
By
Seong Won Lee

Date Approved:

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ABSTRACT

Studies on the control of p53 mediated cell-cycle arrest by hnRNP-K sumoylation in response to DNA damage

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p53 tumor supprresor can integrate numerous signals that control cell life and death. The p53 protein is a transcription factor that regulates the expression of a large number of target genes under cellular stress, and induces a number of different responses, including the induction of cell cycle arrest, DNA repair, and cell death. A major consequence of p53 activation in response to DNA damage is the induction of cell-cycle arrest at the G1/S or G2/M phase. Cell-cycle arrest at the G1/S phase is primarily achieved by expression of p21, an inhibitor of cyclin-dependent kinases.

Small ubiquitin-related modifier (SUMO) is a 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). Conjugated SUMO can be removed by a family of SUMO-specific proteases (SENPs). This reversible sumoylation process participates in the control of diverse cellular processes, including transcription, nuclear transport, and signal transduction.

Heterogeneous ribonucleoprotein-K (hnRNP-K) is an RNA-binding protein that is associated with various cellular processes, including chromatin remodeling, transcription, mRNA splicing, and translation. 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 coactivator of p53 for cell-cycle arrest. However, how the stability and function of hnRNP-K is regulated remains unknown.

In this study, I demonstrated that UV-induced sumoylation of hnRNP-K prevents its ubiquitination for stabilization. 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. The level of ubiquitinated hnRNP-K was

markedly reduced and then returned almost to the initial level during the same time couse after UV treatment, indicating that the level of sumoylated hnRNP-K is inversely correlated with that of ubiquitinated hnRNP-K. These results also suggest that sumoylation leads to stabilization of hnRNP-K. Since hnRNP-K could be sumoylated under different types of DNA damage conditions, such as treatment with IR and doxorubicin, hnRNP-K sumoylation appears to be a common response to DNA damage for its stabilization.

Using sumoylation-defective mutant and purified sumoylated hnRNP-K, sumoylation was found to reduce hnRNP-K's affinity to HDM2 with an increase in that to p53. In addition, 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. Moreover, UV-induced hnRNP-K sumoylation promoted p53 transactivity and thereby p21 expression for cell cycle arrest. Consistently, hnRNP-K, but not its sumoylation-defective mutant form, was recuited to the p21 promoter upon UV treatment. These results indicate that sumoylation of hnRNP-K not only increases its stability but also promotes the

transcriptional activity of p53.

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 results demonstrate that PIAS3 and SENP2 antagonistically regulate SUMO modification and stability of during the time course after exposure to UV.

Immunocytochemical analysis showed that sumoylation is involved in the nuclear localization of hnRNP-K. Wild-type hnRNP-K proteins were localized exclusively in the nucleus after UV exposure. In contrast, K422R was localized in both the nucleus and the cytoplasm in ~40% of cells regardless of UV treatment. In addition, depletion of PIAS3 or overexpression of SENP2 prevented UV-induced nuclear localization of wild-type hnRNP-K. However, neither UV treatment nor PIAS3 depletion showed any effect on the nuclear localization of endogenous hnRNP-

K, indicating that sumoylation plays only a minor, auxiliary role in the nuclear localization of hnRNP-K. Collectively, the present findings indicate that SUMO modification plays a crucial role in the control of hnRNP-K's function as a p53 co-activator for cell cycle arrest in response to DNA damage, such as by UV.

Key word: p53, HDM2 (human double minute 2), p21, PIAS3 (protein inhibitor of activated STAT 3), SENP2 (sentrin/sumo-specific protease 2), ubiquitin, hnRNP-K (heterogenous nuclear ribonucleoprotein-K), cell-cycle regulation

Student Number : 2005-20476

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BACKGROUND

1. p53 pathway

The p53 pathway is a network play of genes and their products that are targeted to respond to a variety of cellular stress signals that impact upon DNA replication, chromosome segregation, and cell division (Vogelstein *et al.*, 2000). In response to a stress signal, the p53 protein is activated in a specific manner by post-translational modifications, and this leads to either cell cycle arrest, a program that induces cell senescence or cellular apoptosis (Vogelstein B, 2001). In normal unstressed cells, the level of p53 protein is downregulated via the binding of proteins, such as MDM2, COP1 or PIRH2 that promote p53 degradation via the ubiquitin- proteasome pathway. As the expression of most of these proteins are up-regulated by p53, this process forms a negative feedback loop that will keep p53 level very low in a normal cells (Dornan et al, 2004; Leng et al 2003; Perry, 2004).

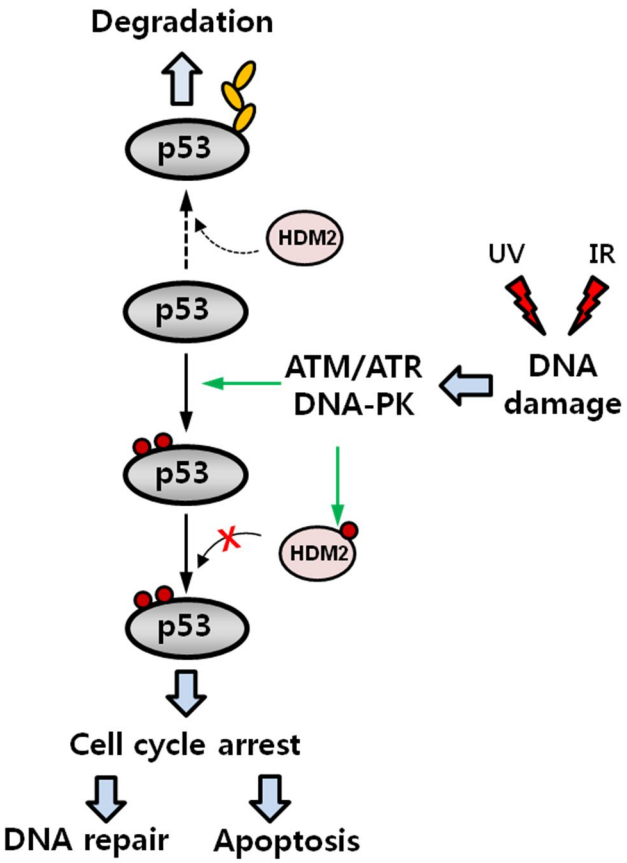
After genotoxic or non-genotoxic stresses, activation of p53 occurs as a two-step process. First, p53 protein level is increased via the inibition of its interaction

with Mdm2 and the other negative regulators. Second, a series of modulator will activates p53 transcriptional activity. Different types of DNA damage activate different enzyme activities that modify the p53 protein at different amino-acid residues, and so the nature of the stress signal is transmitted to the protein, and presumably its activity, by a code inherent to the posttranslational modifications that reflect the different types of stress (Colman et al., 2000). The regulation of p53 acitivity in response to cellular stresses is controlled by the complex regulatory mechanisms that control the p53 posttranslational modification, the regulation of p53 DNA binding activity by other transcription factors, and the cooperation of p53 with transcriptional coactivators (Coutts and La Thangue, 2005) (Figure 1).

Once the p53 protein is activated, it initiates a transcriptional program that reflects the nature of the stress signal, the protein modifications, and proteins associated with the p53 protein. The activated p53 protein binds to a specific DNA sequence, termed the p53-responsive element (RE), composed of RRRCWWGYYY (spacer of 0–21 nucleotides) RRRCWWGYYY, where R is a purine, W is A or T, and Y is a pyrimidine (El-Deiry et al., 1992). The genes in this p53 network initiate one of

Figure 1. p53 pathway

In Normal conditions, p53 is ubiquitinated by HDM2 for degradation by proteasome. In Stress conditions, DNA damage activates various kinds of kinases which can phosphorylate p53. Phosphorylated p53 can be dissociated from HDM2 for stabilization. Stabilized p53 can activate p21 which can induce cell cycle arrest for DNA repair in mild stress conditions, in strong stress conditions, p53 activate apoptotic protein which can induce cell death.


Degradation
p53
HDM2
p53
UV
IR
ATM/ATR
DNA-PK
DNA
damage
p53
HDM2
p53
Cell cycle arrest
DNA repair
Apoptosis

three programs, resulting in cell cycle arrest, DNA repair, and cellular senescence or apoptosis.

A major consequence of p53 activation in response to DNA damage is the induction of cell-cycle arrest (Bartek & Lukas, 2001; Horn & Vousden, 2007; Vogelstein et al, 2000; Vousden & Lu, 2002) at the G1/S or G2/M phase. The p21 is a major player in the p53-mediated G1 arrest that inhibits cyclin E-Cdk2. This cyclin-dependent kinase acts upon the Rb protein to derepress the E2F1 activity that promotes the transcription of genes involved in preparing a cell to progress from G1 to S phase in the cell cycle. The p53-induced G2 arrest is mediated in part by the synthesis of 14-3-3 sigma, a protein that binds to CDC25C, and keeps it in the cell cytoplasm. Keeping CDC25C in the cytoplasm prevents it from activating cyclin B-CDC2 in the nucleus and these cells are blocked in the G2 phase of the cell cycle. Thus, p53 plays an important role in inhibiting apoptosis as well as in cell-cycle arrest, allowing cells to repair damaged DNA and prevent tumorigenesis.

2. Ubiquitin

Ubiquitin, a 76-amino-acid polypeptide, is covalently attached to target proteins by a cascade system consisting of Ub-activating (E1), conjugating (E2), and ligating (E3) enzymes (Yeh et al., 2000). Many cellular processes are controlled by ubiquitin modification to target proteins, including protein degradation by the 26S proteasome (Conaway et al., 2002; Hershko et al., 2000; Hochstrasser, 1996). The length and linkage type of the Ub chain has the potential to alter the fate of ubiquitinated proteins. The substrates modified with four or more Ub molecules linked through Lys48 are targeted for degradation by the 26S proteasome (Wilkinson et al., 1995). Lys63-linked ubiquitination, however, is implicated in nonproteolytic signaling, such as postreplicational DNA repair, endocytosis, ribosome function, and kinase activation (Deng et al., 2000; Hofmann and Pickart, 1999; Spence et al., 2000). On the other hand, deubiquitination, a removal process of ubiquitin from ubiquitin-conjugated protein substrates, is mediated by deubiquitinating enzymes, which controls the cellular levels of substrate proteins (Amerik and Hochstrasser, 2004). This reversible ubiquitin modification process plays a key role in the regulation of a number of cellular processes, including transcriptional activation, signal transduction, antigen

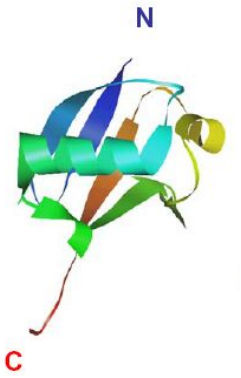
presentation, oncogenesis, and preimplantation (Canning et al., 2004; Wilkinson, 1997; Yeh et al., 2000).

3. Small Ubiquitin-like Modifier (SUMO)

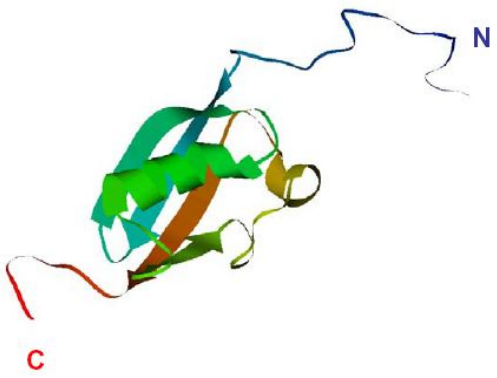
SUMO modification (sumoylation) is a covalent modification process that leads to attachment of the SUMO (small ubiquitin-related modifier) protein to specific lysine residues of target proteins (Kim et al, 2002; Melchior, 2000; Yeh et al, 2000). SUMO is a small polypeptide that shows a significant structural homology to ubiquitin. SUMO and ubiquitin are only 18% identical, but they share a similar three-dimensional structure, the ββαββαβ ubiquitin-fold (Figure 2). Members of the SUMO protein family are present in protozoa, yeast, plants, and metazoa. The SUMO family in metazoa consist of three related proteins, SUMO1 (also known as PIC1, Ubl1, sentrin, GMP1, Smt3c or hSmt3), SUMO2 (sentrin2 or Smt3a), and SUMO3 (sentrin3 or Smt3b). SUMO2 and SUMO3 are 95% identical, but SUMO1 shares about 50% sequence identity with SUMO2/3. The least conserved region of the SUMO proteins is the N terminus. It is highly flexible, protrudes from the core of the proteins, and is

Figure 2. Comparison of the structures of ubiquitin and SUMO1

Both proteins share a characteristic tightly packed fold, and a C-terminal di-glycine motif. SUMO is distinguished from ubiquitin by a long and flexible N-terminal extension.



Ubiquitin



SUMO-1

absent in ubiquitin (Bayer et al, 1998). Both ubiquitin and SUMO have the two conserved Gly residues in their C termini that are essential for conjugation to target proteins. Before conjugation, the last four, eleven, or two amino acids of SUMO1, 2 or 3, respectively, have to be proteolytically cleaved to expose the C-terminal Gly residues. SUMO1 and SUMO2/3 probably have distinct regulatory roles, as SUMO2/3 conjugation has been suggested to play a role in the cellular response to environmental stress (Saitoh & Hinchey, 2000).

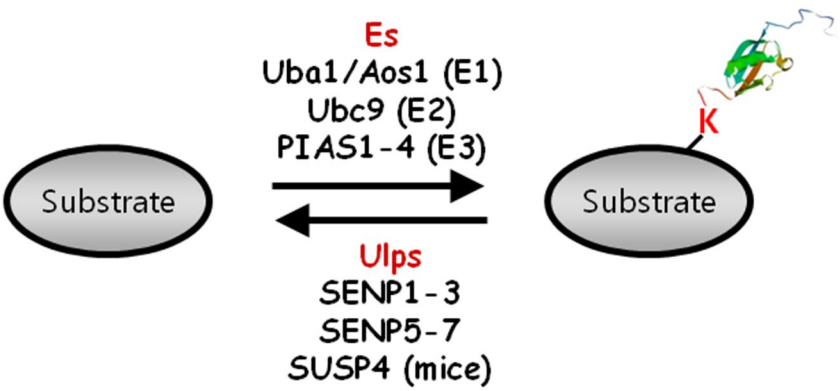
There are striking similarities between the machinery that attaches SUMO to substrate proteins and the enzymes that participate in ubiquitination (Kim et al, 2002). The E1 activating and E2 conjugating enzymes involved in sumoylation are highly related to the E1 and E2 enzymes that participate in ubiquitination. In contrast to the E1 of ubiquitin, which is a single polypeptide (Uba1), the E1 enzymes for SUMO are heterodimeric proteins related to the N- and C-terminal domains of Uba1. While 11 E2 enzymes have been identified for ubiquitin so far, E2 for SUMO is Ubc9 only. Several SUMO E3 ligases have been identified that promote transfer of SUMO from E2 to specific substrates. Although not required for sumoylation in vitro, E3 ligases

may be important in regulating substrate selection in vivo, particularly for substrates that lack consensus SUMO acceptor motifs. To date, three unrelated proteins have been shown to have SUMO E3 ligase activity; RanBP2, the PIAS family, and the polycomb group protein Pc2. These E3s most likely function as adaptors. RanBP2 and the PIAS proteins interact with the SUMO E2 conjugating enzyme Ubc9 and promote transfer of SUMO from Ubc9 to substrate proteins (Johnson & Gupta, 2001; Kahyo et al, 2001; Pichler et al, 2004) (Figure 3).

Conjugated SUMO can be removed by a family of SUMO-specific proteases (SENPs). SUMO-specific proteases are C48 cysteine proteases that possess a conserved catalytic domain characterized by the catalytic triad (histidine, aspartate, and cysteine) and a conserved glutamine residue required for the formation of the oxyanion hole in the active site (Mukhopadhyay & Dasso, 2007; Yeh, 2009). Yeast has a single SUMO-like modifier, Smt3, and two Smt3-specific proteases, Ulp1 and Ulp2. The human SENPs can be divided into three families. The first family consists of SENP1 and SENP2, which have broad specificity for the three mammalian SUMOs (SUMO1–3). The second family includes SENP3 and SENP5, which favor

Figure 3. Summary of SUMO modification pathway

The C-terminal amino acids have to be cleaved off by SUMO-specific protease to expose the Gly-Gly motif for conjugation. Attachment of SUMO to lysine residues of target proteins is catalyzed by E1, E2, and E3 enzymes related to the enzymes in ubiquitination pathway. Sumoylation is reversible, and SUMO can also be removed from target proteins by SUMO-specific proteaes.


Es
Uba1/Aos1 (E1)
Ubc9 (E2)
PIAS1-4 (E3)
Substrate
K
Substrate
Ulps
SENP1-3
SENP5-7
SUSP4 (mice)

SUMO2/3 as substrates and are localized in the nucleolus. The third family contains SENP6 and SENP7, which have an additional loop inserted in the catalytic domain and also appear to prefer SUMO2/3. From an evolutionary standpoint, SENP1–3 and SENP5 are more closely related to Ulp1 whereas SENP6 and SENP7 are related to Ulp2. This reversible sumoylation process participates in the control of diverse cellular processes, including transcription, nuclear transport, and signal transduction (Geiss-Friedlander & Melchior, 2007; Hay, 2005; Johnson, 2004).

4. Heterogeneous nuclear ribonucleoprotein K (hnRNP-K)

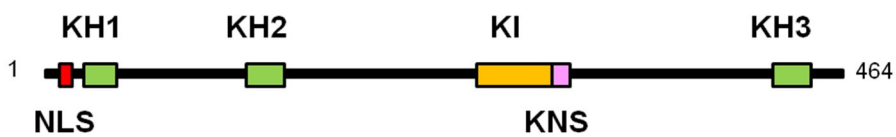
hnRNP-K is a component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex, and found not only in the nucleus but also in the cytoplasm and mitochondria. hnRNP-K is an RNA-binding protein that is associated with various cellular processes, including chromatin remodeling, transcription, mRNA splicing, and translation (Bomsztyk et al, 2004; Bomsztyk et al, 1997; Matunis et al, 1992). The hnRNP-K protein (65 kDa) is structurally related to four other poly(C)-binding proteins (PCBP) which contain three K homology (KH) domains that enable RNA

and DNA binding with a high affinity towards polycytosine tracts. hnRNP-K also carries a nuclear localisation signal (NLS) and a nuclear shuttling domain (KNS), together which allow it to translocate between the cytoplasm and nucleus. It also contains a segment called the K protein interactive (KI) region, located between the KH2 and KH3 domain, which has an intrinsically disordered structure. (Bomsztyk et al., 2004) (Figure 4).

The functions of hnRNP-K are defined by its modular structure that allows it to interact with both nucleic acids and proteins. It has been suggested that hnRNP-K serves as a docking platform that facilitates the interaction between the molecular partners involved in the processes that compose gene expression, such as transcription and translation regulation, mRNA processing and chromatin remodeling (Bomsztyk et al., 2004). With regard to the factors implicated in the process of tumorgenesis, hnRNP-K acts as a transcription activator for the CT element in the human c-myc promoter (Michelotti et al., 1996), the BRCA1 promoter (Thakur et al., 2003), and the basal promoter of the eukaryotic translation initiation factor 4E (eIF-4E) (Lynch et al., 2005). In response to DNA damage, hnRNP-K was shown to be transiently stabilized

Figure 4. Domains of hnRNP-K protein

KH (K-homology) mediate RNA & DNA binding, KI (K-interactive) mediate interaction with proteins, and NLS (nuclear localization signal) and KNS (K-protein nuclear shuttling) mediate translocation between the nucleus and the cytoplasm .


KH1
KH2
KI
KH3
1
464
NLS
KNS

and function as a transcriptional coactivator of p53 (Moumen et al., 2005). A recent study identified K protein as a regulator of androgen receptor (AR) expression levels; it represses AR expression and androgen-induced prostate cancer cell growth through translational regulation of AR mRNA (Mukhopadhyay et al., 2009). In a loss-of-function screening system based on intracellular expression of single domain antibodies, hnRNP-K was found as a potential target for cell migration and metastasis of human cancerous cells (Inoue et al., 2007).

5. Purpose of thesis work

As described above, mammalian cells trigger the p53-dependent transcriptional induction of factors that regulate DNA repair, cell-cycle progression, or cell survival in response to DNA damage. The phosphatidylinositol 3-kinase-like kinases (PIKKs) ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are activated following DNA damage, and then phosphorylate downstream targets, such as transcription factor p53 and checkpoint kinases CHK1 and CHK2 (Abraham, 2004; Shiloh et al., 2004). These

in turn regulate the activities of downstream effector proteins controlling DNA repair, cell-cycle progression, or the initiation of apoptotic or senescence programs. The pivotal role of p53 in cellular stress responses is reflected by the complex regulatory mechanisms that control its activity which include the existence of many forms of p53 posttranslational modification, the regulation of p53 DNA binding activity by other transcription factors, and the cooperation of p53 with transcriptional coactivators that modify chromatin structure and/or facilitate transcription-complex formation (Coutts and La Thangue, 2005).

hnRNP-K is an evolutionarily conserved factor found in the nucleus and cytoplasm that was initially discovered as a component of hnRNP complexes (Matunis et al., 1992). Since then, work has implicated hnRNP-K in processes including chromatin remodeling and transcription as well as mRNA splicing, export, and translation (Bomsztyk et al., 2004). The involvement of hnRNP-K in these events appears to reflect its ability to interact with a range of molecular partners, including DNA, RNA, protein kinases, and proteins involved in chromatin remodeling (Bomsztyk et al., 1997; Bomsztyk et al., 2004). Perhaps the most characterized

function of hnRNP-K is its role in transcription. For example, it has been reported to associate with the κB enhancer motif (Ostrowski et al., 1994); to enhance the expression of the c-*myc*, *EGR*, and *BRCA1* genes (Michelotti et al., 1996; Ostrowski et al., 2003; Thakur et al., 2003); to activate or repress RNA polymerase II transcription in a context-dependent manner (Lee et al., 1996; Michelotti et al., 1996; Tomonaga and Levens, 1996); and to stimulate transcription by purified RNA polymerase II in vitro (Gaillard et al., 1994).

Intriguingly, heterogeneous nuclear ribonucleoprotein K (hnRNP-K) was shown to be transiently stabilized and function as a transcriptional coactivator of p53 in response to DNA damage (Moumen et al, 2005). hnRNP-K is identified as being rapidly induced by DNA damage in a manner that requires the DNA-damage signaling kinases ATM or ATR. Induction of hnRNP-K ensues through the inhibition of its ubiquitin-dependent proteasomal degradation mediated by the ubiquitin E3 ligase HDM2. Furthermore, in response to DNA damage, p53 and hnRNP-K are recruited to the promoters of p53-responsive genes in a mutually dependent manner.

Finally, hnRNP-K is a new HDM2 target and by serving as a cofactor for p53, plays key roles in coordinating transcriptional responses to DNA damage.

However, how the stability and function of hnRNP-K is regulated remained unknown. Therefore, the purpose of this study is to elucidate the molecular mechanism(s) that controls the stability of hnRNP-K in response to DNA damage, such as UV irradiation. Transient stabilization of hnRNP-K is critically required for cells to survive under DNA damage conditions, because hnRNP-K-mediated activation of p53 is essential for p21-induced cell cycle arrest and thereby for providing the time window of DNA repair. Notably, 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 (Altmannova et al, 2010; Bergink and Jentsch, 2009; Cremona et al, 2012; Dou et al, 2010; Hoege et al, 2002; Lee et al, 2006; Polo and Jackson, 2011). Furthermore, proteomic analysis has previously revealed that hnRNP-K is one of the candidate target proteins for sumoylation (Li et al, 2004), although the role SUMO modification of hnRNP-K in DNA damage response has not been studied yet.

Therefore, it is of importance to determine whether SUMO modification is involved in the control of hnRNP-K stability. In summary, the purpose of this study is to clarify the mechanism by which SUMO modification is involved in transient stabilization of hnRNP-K and in turn in the p53-mediated cell cycle arrest under DNA damage conditions.

INTRODUCTION

The p53 tumor suppressor plays a pivotal role in maintenance of genome integrity under cellular stresses, such as DNA damage (Kruse & Gu, 2009; Lakin & Jackson, 1999; Lane, 1992; Levine & 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; Abraham, 2004; Ciccia & 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 (Bartek & Lukas, 2001; Horn & Vousden, 2007;

Vogelstein et al, 2000; Vousden & Lu, 2002) at the G1/S or G2/M phase. Cell-cycle arrest at the G1/S 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 & Johnson, 2000), or down-regulate 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) (Capili & Lima, 2007; Kerscher et al, 2006; Rytinki et al, 2009). Conjugated SUMO can be removed by a family of SUMO-specific proteases (SENPs) (Mukhopadhyay & Dasso, 2007; Yeh, 2009). This reversible sumoylation process participates in the control of

diverse cellular processes, including transcription, nuclear transport, and signal transduction (Geiss-Friedlander & Melchior, 2007; Hay, 2005; Johnson, 2004). 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 (Altmannova et al, 2010; Bergink & Jentsch, 2009; Cremona et al, 2012; Dou et al, 2010; Hoege et al, 2002; Lee et al, 2006; Polo & Jackson, 2011). 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).

hnRNP-K is an RNA-binding protein that is associated with various cellular processes, including chromatin remodeling, transcription, mRNA splicing, and translation (Bomsztyk et al, 2004; Bomsztyk et al, 1997; Matunis et al, 1992). Intriguingly, hnRNP-K was shown to be transiently stabilized and function as a transcriptional coactivator of p53 in response to DNA damage (Moumen et al, 2005). However, how the stability and function of hnRNP-K is regulated remained unknown.

In this study, I 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 coactivator in response to DNA damage by UV.

MATERIALS AND METHODS

1. 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'-CCCACAGGATGAAATCCTA3'; shPIAS3, 5'-GCTGTCGGTCA GACATCATTT-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), HA (Y-11), ATR (H-300) and GST (Z-5) were purchased from Santacruz (Santa Cruz, CA). Anti-Flag M2 (Sigma-Aldrich), anti-Xpress, anti-SUMO1 (Invitrogen), anti-His (BD Biosciences), anti-SENP1, anti-SENP2, anti-SENP6 (Abgent), anti-p-Chk1 and anti-PIAS3 (Cell Signaling) antibodies were also used.

2. Cell culture and transfection

HEK293T and HeLa cells were grown at 37°C with 5% CO2 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. Cells were usually grown about 70~80% confluency in 100mm cell culture dish.

For transfection, cells were plated about 30%-50% confluency in 24 well plates or 60mm dish. After 24h, All transfections were carried out using Metafectene reagent (Biontex) and jetPEI™ DNA Transfection Reagent (Polyplus-transfection). After incubation for 24~48 hr, cells were washed by PBS and subjected to lysis buffer.

3. 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, 1X 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, 1X 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 1X 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.

4. Flow cytometry

Cells were washed with PBS and trypsinized by TE (Trypsin/EDTA). Collected cells by scrapper were fixed at 4ºC with 70% ethanol for 2 hr. 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).

5. 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.

6. 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 the manufacturer's instructions. Primers used in PCR for p21 were: 5'-CTTTGTCACCGAGACACCAC-3' and 5'-GGCGTTTGGAGTGGTAGA AA-3'.

7. 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 -galactosidase expression with a luciferase system (Promega).

8. ChIP assay

Assays were conducted with an average size of sheared fragments of about 300-1,000 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'-CTGAAAACAGGCAGCCCAAGG-3' (Zeng et al, 2002).

9. 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.

10. 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 3 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).

RESULTS

1. 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, I 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 5A). 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 5B), 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.

I 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 (Figure 6). 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 7). 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 8A). Replacement of Lys422 alone or together with Lys198 by Arg prevented hnRNP-K sumoylation, whereas that of Lys198 alone did not (Figure 8B). Similar results were obtained by in vitro sumoylation assay using purified SAE1/SAE2 (E1), Ubc9 (E2), and SUMO1 (Figure 8C), indicating that Lys422 serves as the major sumoylation site of hnRNP-K.

Henceforth, the sumoylation-defective mutant was referred to as K422R.

I 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 9A), 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 9B). Consistently, UV treatment increased the stability of hnRNP-K, but not K422R (Figure 10A and B). In addition, MG132, a proteasome inhibitor, prevented K422R destabilization under the same conditions. These results indicate that UV-induced sumoylation of hnRNP-K is responsible for the increase in its stability.

2. Sumoylation of hnRNP-K switches its interaction with HDM2 to that with p53

To elucidate the mechanism for sumoylation-mediated stabilization of hnRNP-K,

I 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 11A). 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 11B and C). In addition, purified sumoylated hnRNP-K (Figure 12A) showed a lower affinity to HDM2 than unmodified hnRNP-K (Figure 12B). Note that the C-terminal region harboring 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.

I 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 13A). 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 13B and C). In addition, purified sumoylated hnRNP-K showed a much higher affinity to p53 than unmodified hnRNP-K (Figure 14). 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 15). On the other hand, the level of K422R-bound p53 and HDM2 remained the same regardless of SUMO1 expression. Collectively, these results demonstrate 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 13B and C), whereas K422R binds HDM2 better than hnRNP-K (see Figure 12B and C). However, in vitro binding assays showed that purified K422R interacts with p53 or HDM2 as well as wild-type hnRNP-K (Figure 16A 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 5B), 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 (Figure 16C). 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 (Figure 17). 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.

3. Sumoylation of hnRNP-K is required for its function as a p53 coactivator

To determine whether UV-induced sumoylation of hnRNP-K influences its coactivator 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 18A 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 19A and B). 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 19C). 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 19B), 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 (Figure 20). Since hnRNP-K overexpression causes an increase in the level of sumoylated hnRNP-K even in the absence of UV (see Figure 16) 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.

4. 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 (Figure 21A) and promoted its sumoylation (Figure 21B). Furthermore, PIAS3 knockdown by shPIAS3 prevented not only hnRNP-K sumoylation but also p21 expression (Figure 22A), suggesting that PIAS3-mediated sumoylation of hnRNP-K is required for its function as a p53 coactivator. 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 22B). Thus, it appears that UV-mediated increase in hnRNP-K sumoylation is due to an increase in the affinity of hnRNP-K to PIAS3.

I next attempted to identify hnRNP-K-specific desumoylating enzyme. Among the enzymes tested, overexpressed SENP1, SENP2, SENP6, and mouse SUSP4 interacted with hnRNP-K (Figure 23). 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 24A and B). Moreover, SENP2, but not its catalytically inactive mutant (in which the active site Cys548 was replaced by Ser), removed SUMO from hnRNP-K (Figure 25A), whereas SENP2 knockdown by shSENP2 promoted hnRNP-K sumoylation (Figure 25B). 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 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 (Figure 26 and Figure 27). Both p53 and SENP2 bound to the same N-terminal region of hnRNP-K (Figure 27C), 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 is consistent with the finding that sumoylated hnRNP-K shows a lower affinity to HDM2 than its unmodified form (see Figure 11C and 12B).

To identify the hnRNP-K-binding regions within HDM2, p53, SENP2, and PIAS3, deletions of each protein were generated (Figure 28 and 29). hnRNP-K bound to the C-terminal regions of p53, SENP2, and PIAS3, while it 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 30.

5. Effect of hnRNP-K sumoylation on its subcellular localization

Previous studies have suggested that sumoylation of hnRNP-K is involved in its nucleocytoplasmic transport (Steinman & Johnson, 2000). Therefore, I 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 (Figure 31). 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, I next examined whether the nuclear localization of hnRNP-K could be blocked by knockdown of PIAS3. Depletion of PIAS3 prevented UV-induced nuclear localization of hnRNP-K (Figure 32). 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 (Figure 33). These results again suggested that sumoylation is involved in the nuclear localization of hnRNP-K.

To confirm this finding, I 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 (Figure 34).

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 Figure 31). 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 Figure 33). 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 (Bomsztyk et al, 2004; Bomsztyk et al, 1997; Matunis et al, 1992). Thus, it appears likely that sumoylation plays only a minor, auxiliary role in the nuclear localization of hnRNP-K.

6. UV-induced hnRNP-K sumoylation is required for cell-cycle arrest

To determine whether SENP2 is involved in the control of hnRNP-K's coactivator 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 35). Without UV treatment, SENP2 knockdown moderately promoted p53 transactivity, since it increases the level of sumoylated hnRNP-K (see Figure 25B). 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 coactivator. On the other hand, knockdown of PIAS3 alone or together with hnRNP-K completely abrogated UV-induced p53 transactivity (Figure 36), indicating that PIAS3 positively regulates the coactivator function of hnRNP-K. Collectively, these results indicate that SENP2 and PIAS3 antagonistically regulate the role of hnRNP-K as a p53 coactivator.

I 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 37A and 38). 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 decreased 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 37B). These results indicate that hnRNP-K sumoylation is required for UV-induced cell-cycle arrest.

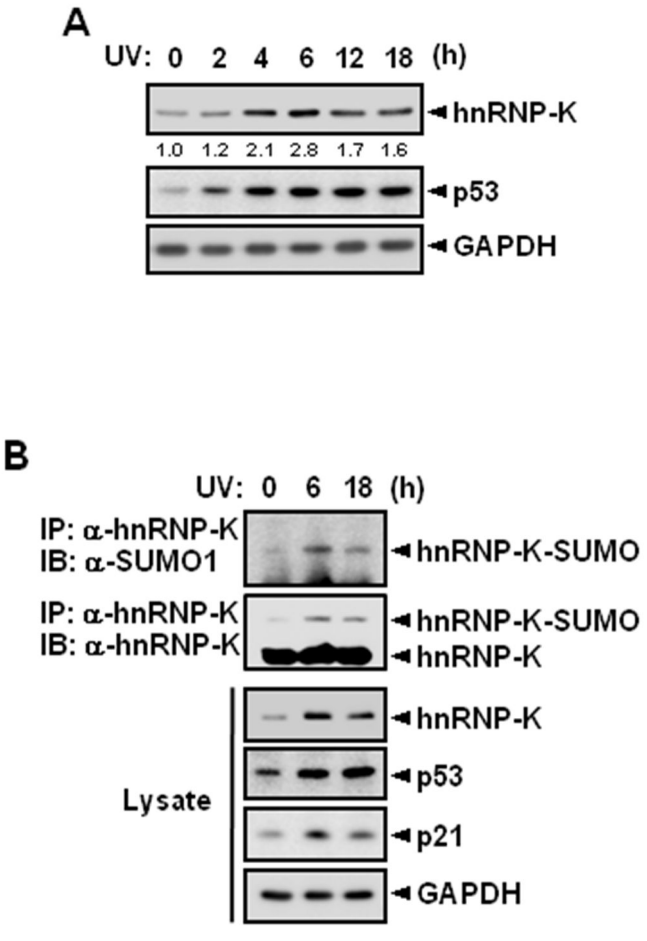
Knockdown of SENP2 enhanced UV-mediated increase in cell fractions in G1 phase, whereas that of PIAS3 ablated it (Figure 39A). Knockdown of SENP2 together with hnRNP-K also prevented UV-induced cell-cycle arrest. Figure 39B shows that

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 (Cimprich & Cortez, 2008; Durocher & Jackson, 2001). 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 40A 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 coactivator, is ATR-dependent.

Figure 5. 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.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hnRNP-K
1.0 1.2 2.1 2.8 1.7 1.6
p53
GAPDH
B
UV: 0 6 18 (h)
IP: α-hnRNP-K
IB: α-SUMO1
hnRNP-K-SUMO
IP: α-hnRNP-K
IB: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
p53
p21
GAPDH

Figure 6. Induction of hnRNP-K sumoylation by IR and doxorubicin.

After exposure of MRC5 cells to IR (5 Gy) (**A**) or HeLa cells to doxorubicin (0.4 g/ml) (**B**), cell lysates were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-SUMO1 or anti-hnRNP-K antibody

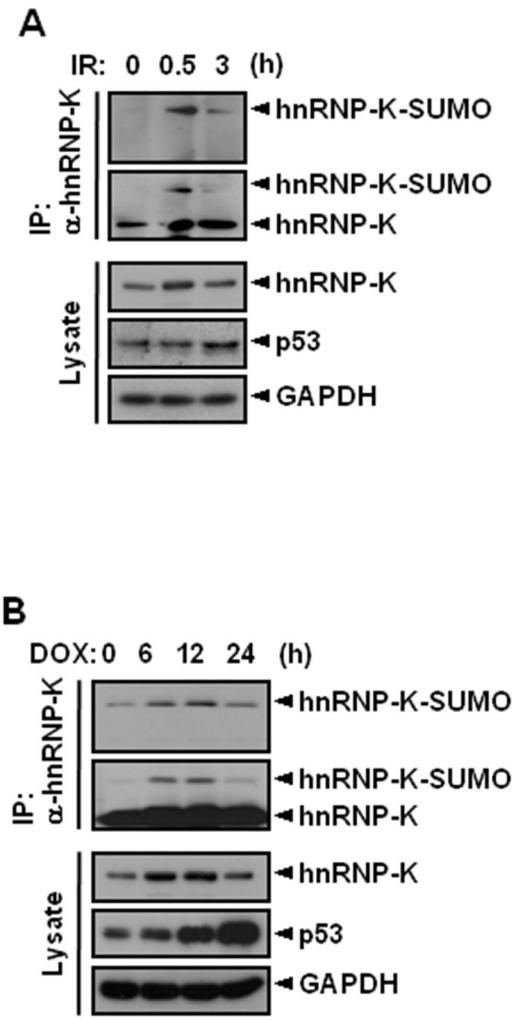

A
IR: 0 0.5 3 (h)
IP: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
p53
GAPDH
B
DOX: 0 6 12 24 (h)
IP: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
p53
GAPDH

Figure 7. 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 M MG132 for 4 h, cell lysates were subjected to pull-down with NTA-beads followed by immunoblot with anti-Flag or anti-Xpress antibody.

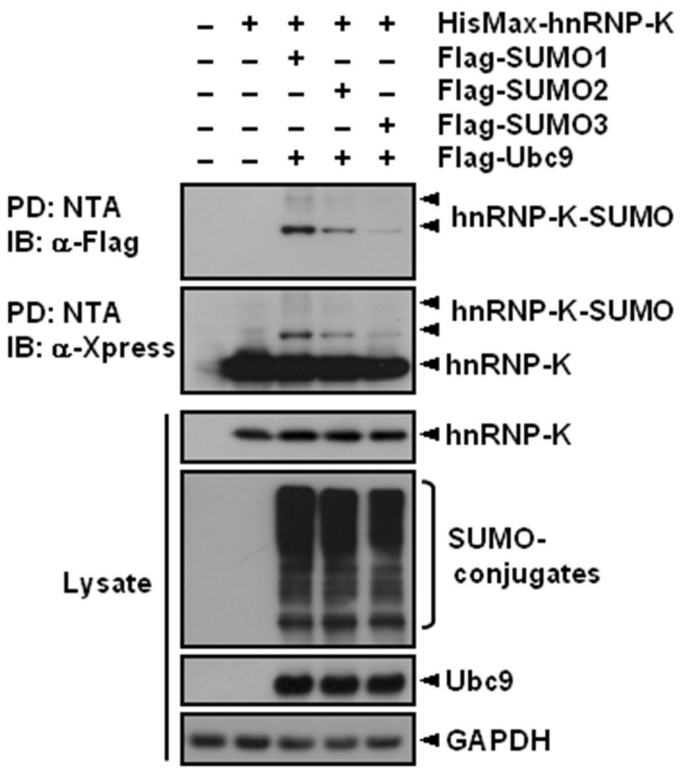

– + + + + HisMax-hnRNP-K
– – + – – Flag-SUMO1
– – – + – Flag-SUMO2
– – – – + Flag-SUMO3
– – + + + Flag-Ubc9
PD: NTA
IB: α-Flag
hnRNP-K-SUMO
PD: NTA
IB: α-Xpress
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
SUMO-
conjugates
Ubc9
GAPDH

Figure 8. 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."

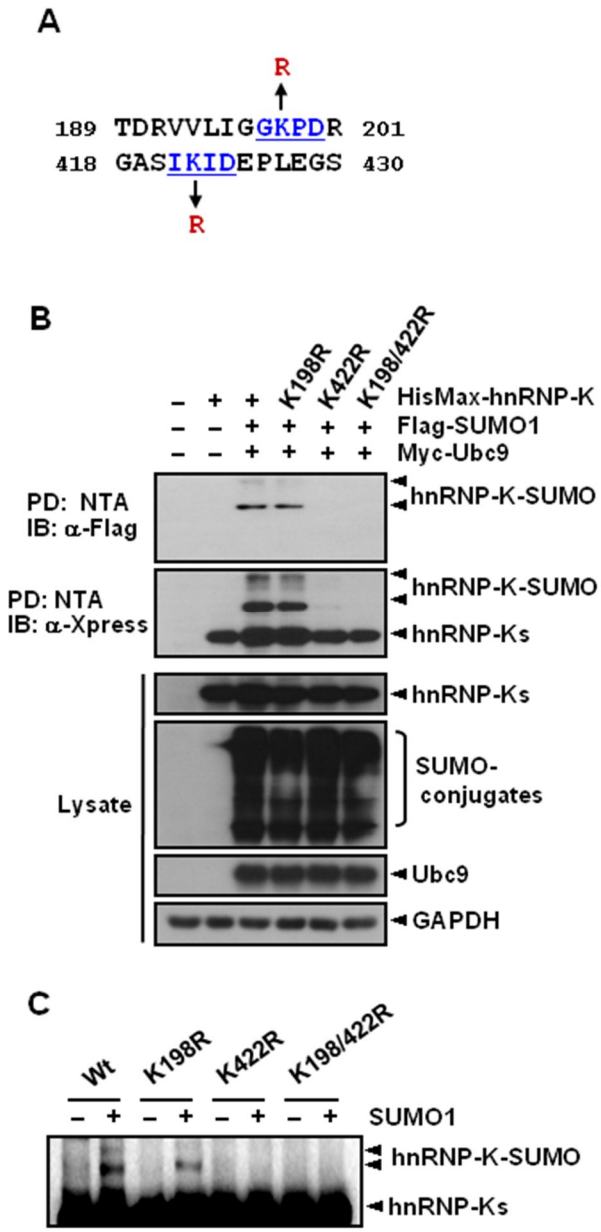

A
R
189 TDRVVLIGGKPDR 201
418 GASIKIDEPLEGS 430
R
B
K198R
K422R
K198/422R
HisMax-hnRNP-K
Flag-SUMO1
Myc-Ubc9
PD: NTA
IB: α-Flag
hnRNP-K-SUMO
PD: NTA
IB: α-Xpress
hnRNP-K-SUMO
hnRNP-Ks
hnRNP-Ks
Lysate
SUMO-
conjugates
Ubc9
GAPDH
C
Wt
K198R
K422R
K198/422R
SUMO1
hnRNP-K-SUMO
hnRNP-Ks

Figure 9. UV-induced sumoylation prevent hnRNP-K ubiquitination

(**A**) UV blocks hnRNP-K ubiquitination. After exposure to UV, HeLa cells were incubated with 10 M 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.

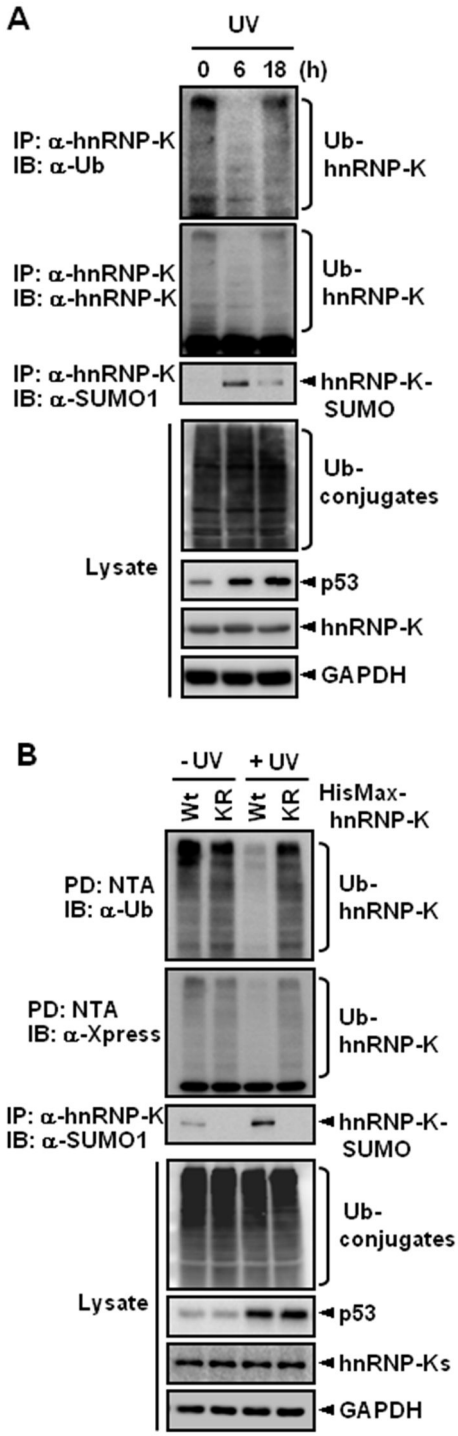

A
UV
0 6 18 (h)
IP: α-hnRNP-K
IB: α-Ub
Ub-
hnRNP-K
IP: α-hnRNP-K
IB: α-hnRNP-K
Ub-
hnRNP-K
IP: α-hnRNP-K
IB: α-SUMO1
hnRNP-K-
SUMO
Ub-
conjugates
Lysate
p53
hnRNP-K
GAPDH
B
- UV
+ UV
Wt
KR
Wt
KR
HisMax-
hnRNP-K
PD: NTA
IB: α-Ub
Ub-
hnRNP-K
PD: NTA
IB: α-Xpress
Ub-
hnRNP-K
IP: α-hnRNP-K
IB: α-SUMO1
hnRNP-K-
SUMO
Ub-
conjugates
Lysate
p53
hnRNP-Ks
GAPDH

Figure 10. Sumoylation increases hnRNP-K stability

(**A**) Cells overexpressing Flag-tagged hnRNP-K (Wt) or K422R (KR) were treated with 200 g/ml of cycloheximide. After exposure to UV, they were incubated with and without MG132 followed by immunoblot with anti-Flag antibody. (**B**) Band intensities in (A) were quantified by using a densitometer. The data represents the mean ± s.d. of three independent experiments.

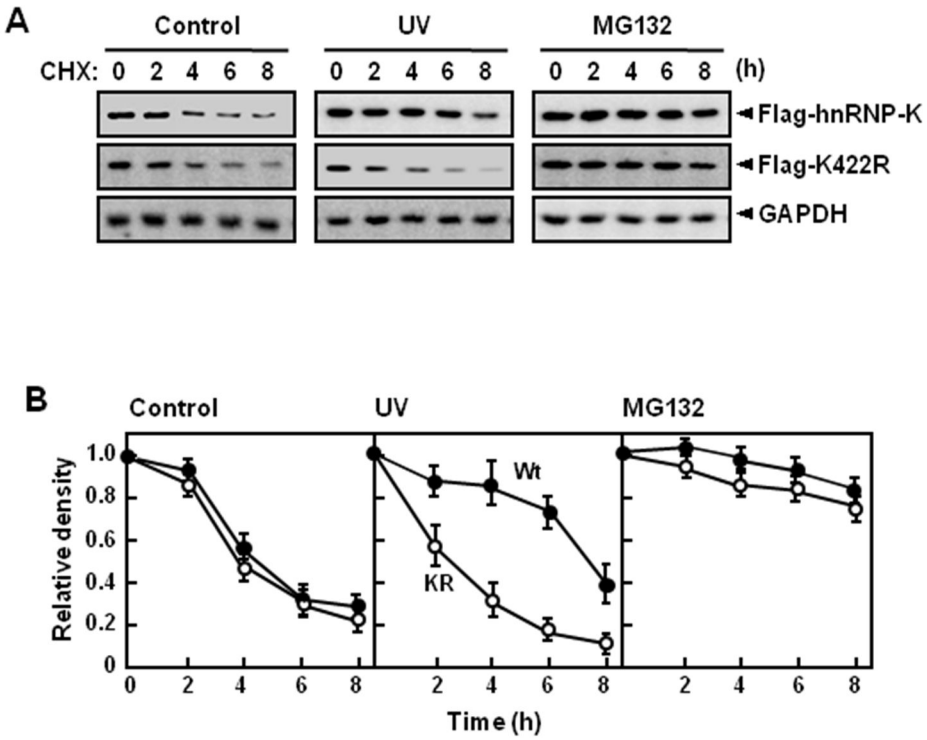

A
Control
UV
MG132
CHX: 0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 (h)
Flag-hnRNP-K
Flag-K422R
GAPDH
B
Control
UV
MG132
Relative density
1.0
0.8
0.6
0.4
0.2
0
Wt
KR
0 2 4 6 8 2 4 6 8 2 4 6 8
Time (h)

Figure 11. UV inhibits the interaction of hnRNP-K with HDM2

(**A**) After UV treatment, HeLa cells were incubated for the indicated periods. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K followed by immunoblot with anti-HDM2 and anti-hnRNP-K antibodies. (**B** and **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**). 10 M MG132 was treated 4 h before cell lysis.

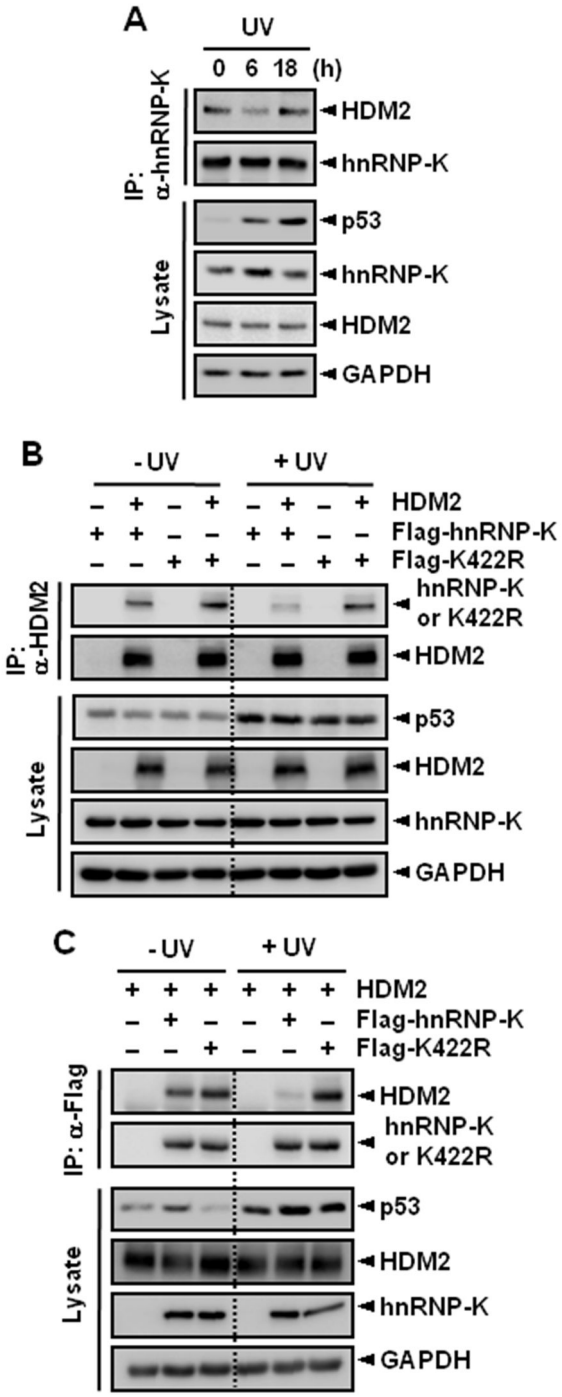

A
UV
0 6 18 (h)
IP: α-hnRNP-K
HDM2
hnRNP-K
Lysate
p53
hnRNP-K
HDM2
GAPDH
B
- UV
+ UV
HDM2
Flag-hnRNP-K
Flag-K422R
IP: α-HDM2
hnRNP-K or K422R
HDM2
Lysate
p53
HDM2
hnRNP-K
GAPDH
C
- UV
+ UV
HDM2
Flag-hnRNP-K
Flag-K422R
IP: α-Flag
HDM2
hnRNP-K or K422R
Lysate
p53
HDM2
hnRNP-K
GAPDH

Figure 12. Sumoylation reduces the affinity of hnRNP-K to HDM2

(**A**) 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. (**B**) Purified His-HDM2 was incubated with GST-hnRNP-K-His-SUMO1 or GST-hnRNP-K followed by immunoprecipitation with anti-hnRNP-K antibody.

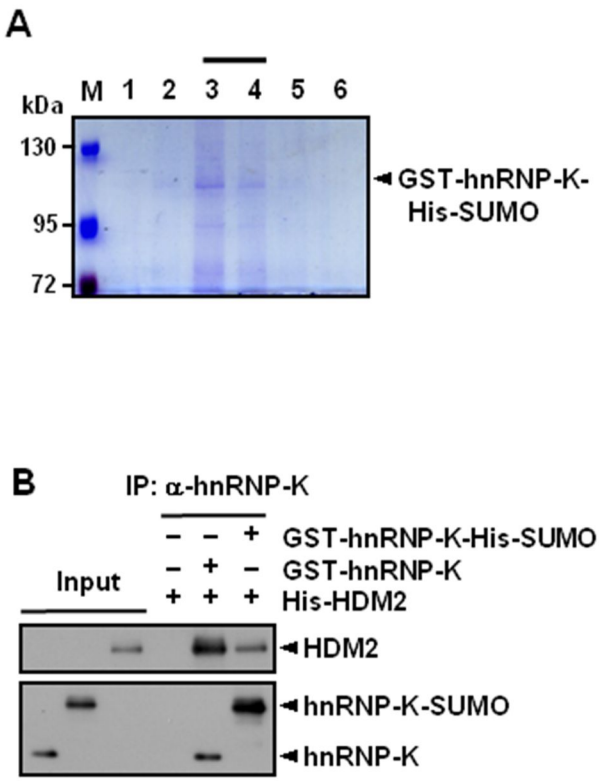

A
kDa
M 1 2 3 4 5 6
130
95
72
GST-hnRNP-K-
His-SUMO
B
IP: α-hnRNP-K
– – + GST-hnRNP-K-His-SUMO
– + – GST-hnRNP-K
+ + + His-HDM2
Input
HDM2
hnRNP-K-SUMO
hnRNP-K

Figure 13. UV promotes the interaction of hnRNP-K with p53

(**A**) After UV treatment, HeLa cells were incubated for the indicated periods. Cell lysates were subjected to immunoprecipitation with anti-hnRNP-K followed by immunoblot with anti-p53 and anti-hnRNP-K antibodies. (**B** and **C**) 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 (**B**) or anti-Flag antibody (**C**). 10 M MG132 was treated 4 h before cell lysis.

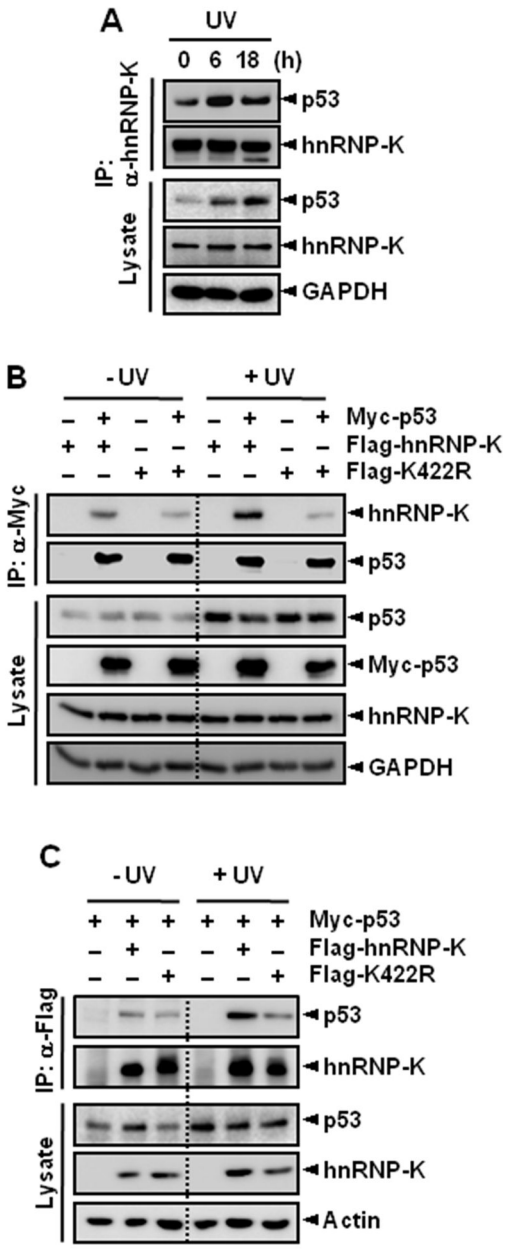

A
UV
0 6 18 (h)
IP: α-hnRNP-K
p53
hnRNP-K
Lysate
p53
hnRNP-K
GAPDH
B
- UV
+ UV
Myc-p53
Flag-hnRNP-K
Flag-K422R
IP: α-Myc
hnRNP-K
p53
Lysate
p53
Myc-p53
hnRNP-K
GAPDH
C
- UV
+ UV
Myc-p53
Flag-hnRNP-K
Flag-K422R
IP: α-Flag
p53
hnRNP-K
Lysate
p53
hnRNP-K
Actin

Figure 14. Sumoylated hnRNP-K shows higher affinity to p53

Purified His-P53 was incubated with GST-hnRNP-K-His-SUMO1 or GST-hnRNP-K followed by immunoprecipitation with anti-hnRNP-K antibody.

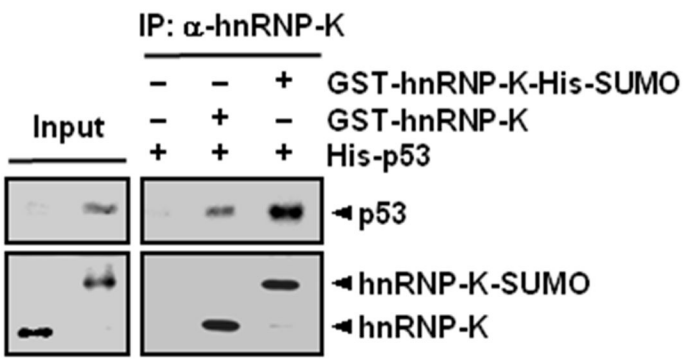

IP: α-hnRNP-K
– – + GST-hnRNP-K-His-SUMO
Input – + – GST-hnRNP-K
+ + + His-p53
p53
hnRNP-K-SUMO
hnRNP-K

Figure 15. 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. 10 M MG132 was treated 4 h before cell lysis.

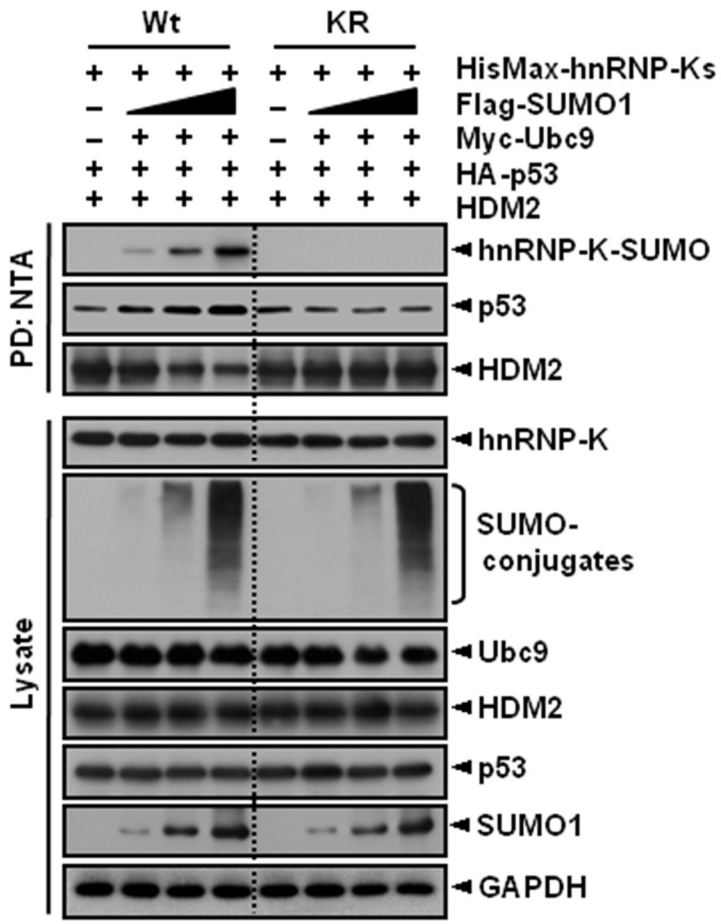

Wt
KR
HisMax-hnRNP-Ks
Flag-SUMO1
Myc-Ubc9
HA-p53
HDM2
PD: NTA
hnRNP-K-SUMO
p53
HDM2
Lysate
hnRNP-K
SUMO-
conjugates
Ubc9
HDM2
p53
SUMO1
GAPDH

Figure 16. K-to-R mutation itself has no effect on the ability of hnRNP-K to bind p53 or HDM2 in vitro

Purified His-p53 (**A**) or His-HDM2 (**B**) was incubated with increasing amounts of GST-tagged hnRNP-K or K422R followed by pull-down with glutathione-Sepharose (GSH-resin). (**C**) HeLa cells were transfected with increasing amounts of pcDNA-hnRNP-K. Cell lysates were subjected to immunoprecipitation followed by immunoblot with anti-hnRNP-K antibody.

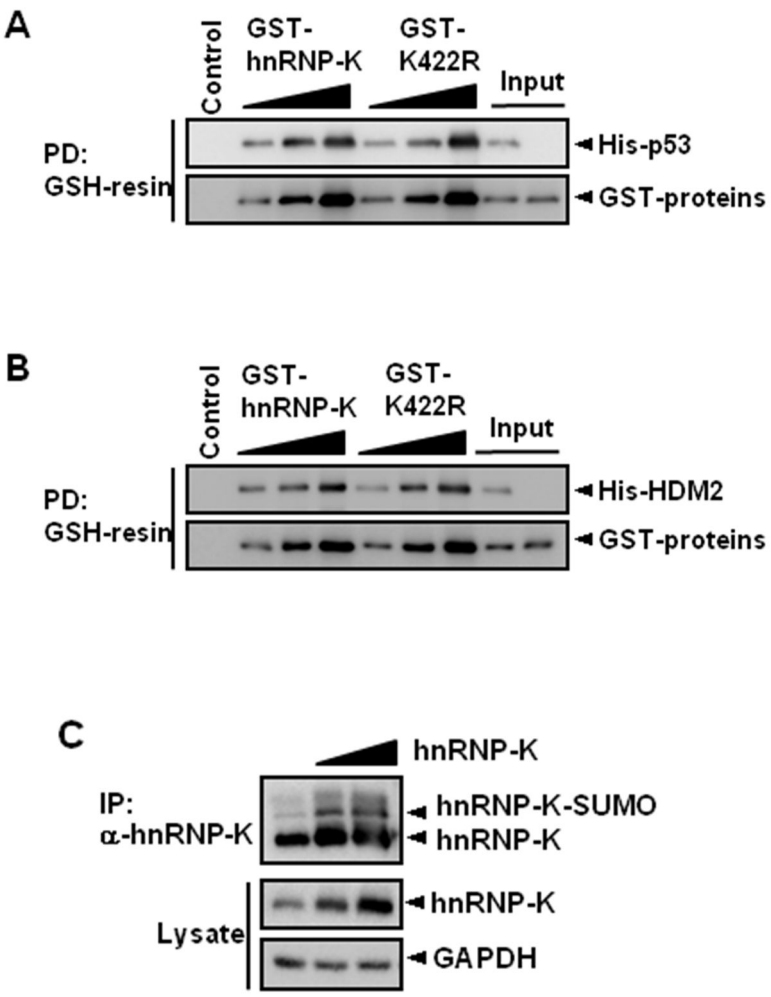
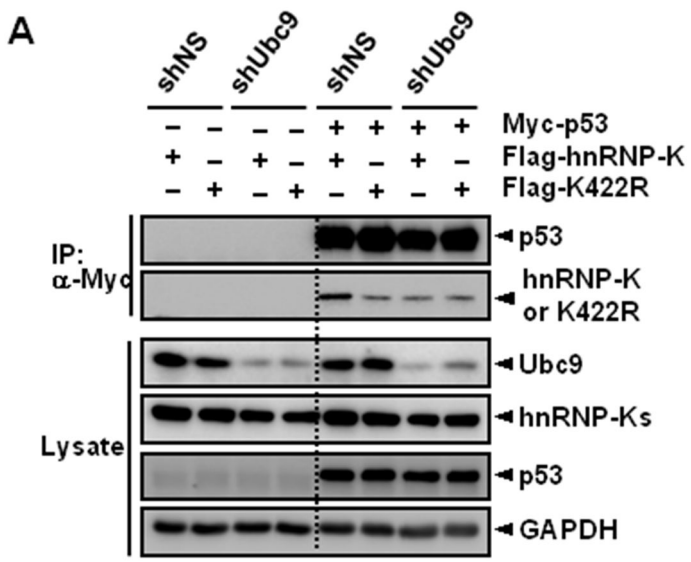

A
Control
GST-
hnRNP-K
GST-
K422R
Input
PD:
GSH-resin
His-p53
GST-proteins
B
Control
GST-
hnRNP-K
GST-
K422R
Input
PD:
GSH-resin
His-HDM2
GST-proteins
C
hnRNP-K
IP:
α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
GAPDH

Figure 17. Effect of Ubc9 knockdown on the abilities of hnRNP-K and K422R to bind p53 and HDM2

HeLa cells transfected with shNS or shUbc9 were incubated for 48 h. Flag-tagged hnRNP-K or K422R was overexpressed in the cells with Myc-p53 (**A**) or HDM2 (**B**). After incubation for 24 h, they were subjected to immunoprecipitation with anti-Myc in (**A**) or anti-HDM2 antibody in (**B**). Note that MG132 was treated 4 h before cell lysis.


A
shNS
shUbc9
shNS
shUbc9
Myc-p53
Flag-hnRNP-K
Flag-K422R
IP:
α-Myc
p53
hnRNP-K
or K422R
Ubc9
hnRNP-Ks
Lysate
p53
GAPDH

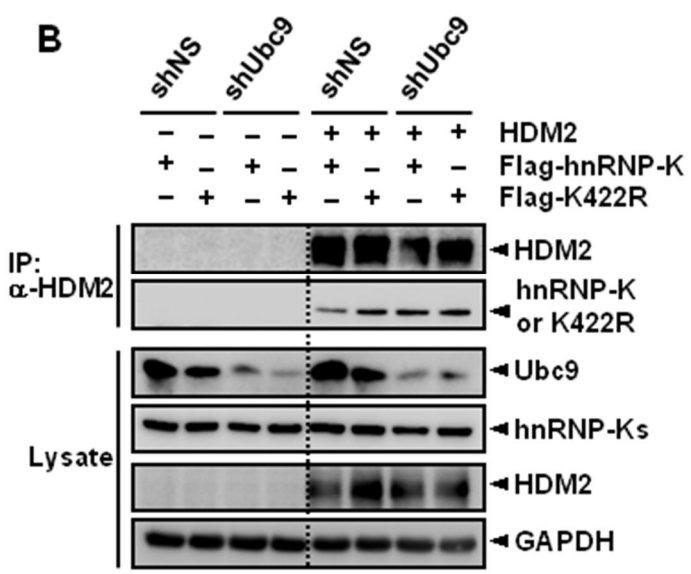
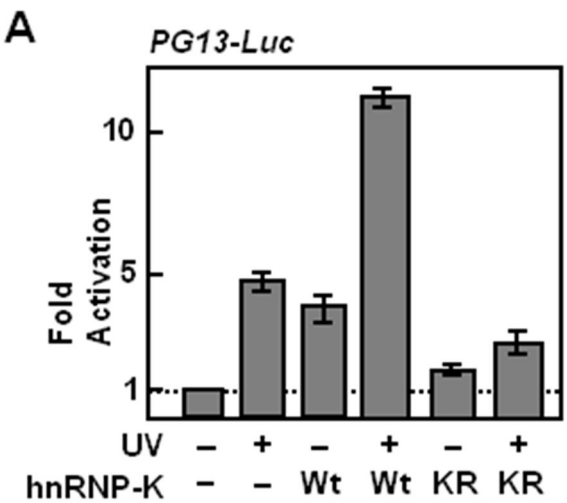

B
shNS
shUbc9
shNS
shUbc9
HDM2
Flag-hnRNP-K
Flag-K422R
IP:
α-HDM2
HDM2
hnRNP-K
or K422R
Ubc9
hnRNP-Ks
Lysate
HDM2
GAPDH

Figure 18. 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 represents the mean ± s.d. of three experiments.


A
PG13-Luc
Fold Activation
10
5
1
UV – + – + – +
hnRNP-K – – Wt Wt KR KR

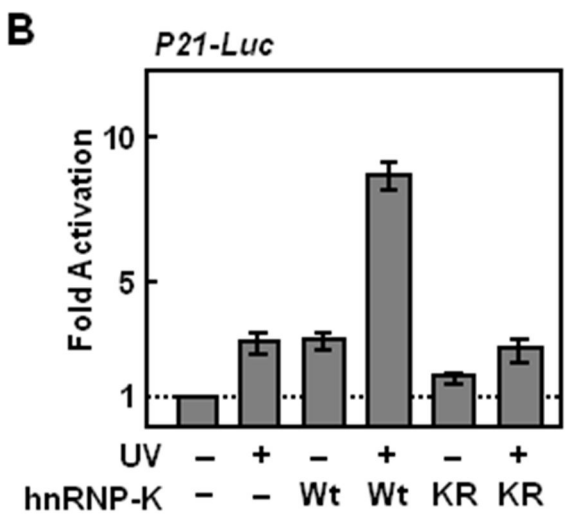
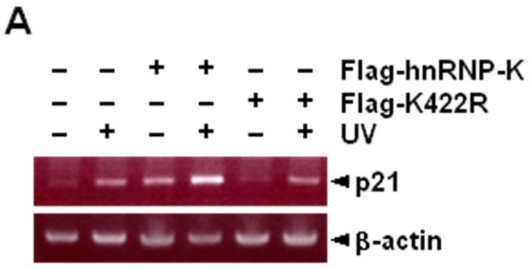
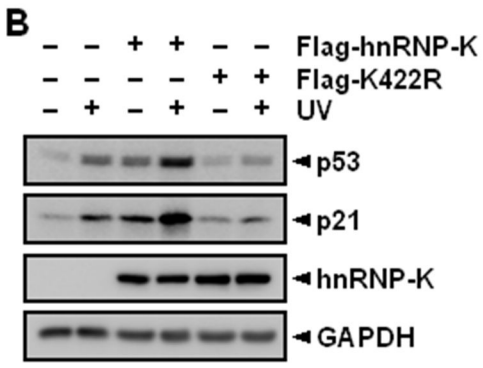

B
P21-Luc
Fold Activation
10
5
1
UV – + – + – +
hnRNP-K – – Wt Wt KR KR

Figure 19. UV-induced hnRNP-K SUMOylation promotes p53 transactivity and thereby p21 expression

(**A**) Sumoylation of hnRNP-K increases the level of p21 transcripts. Total RNAs prepared from the same cells used in (**Figure 14A**) were subjected to RT-PCR to determine p21 mRNA levels. (**B**) Sumoylation of hnRNP-K promotes p21 expression. Cell lysates prepared as in (**Figure 14A**) were subjected to immunoblot with anti-p53, anti-p21, or anti-hnRNP-K antibody. (**C**) Sumoylation of hnRNP-K promotes recruitment of both hnRNP-K and p53 to the *p21* promoter. Cells prepared as in (**Figure 14A**) 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.


A
– – + + – – Flag-hnRNP-K
– – – – + + Flag-K422R
– + – + – + UV
p21
β-actin


B
– – + + – – Flag-hnRNP-K
– – – – + + Flag-K422R
– + – + – + UV
p53
p21
hnRNP-K
GAPDH

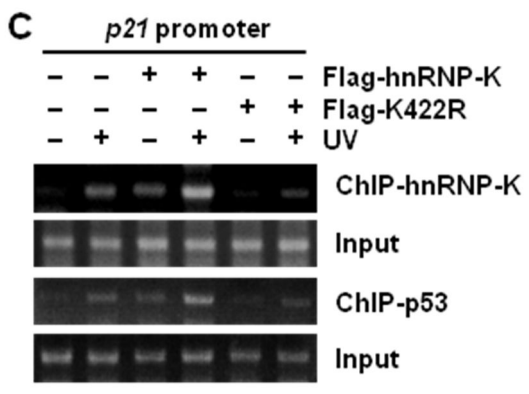

C
p21 promoter
– – + + – – Flag-hnRNP-K
– – – – + + Flag-K422R
– + – + – + UV
ChIP-hnRNP-K
Input
ChIP-p53
Input

Figure 20. Effect of hnRNP-K overexpression on HDM2-mediated p53 ubiquitination and HDM2 auto-ubiquitination

(**A**) HisMax-p53, HA-Ub, and HDM2 were expressed in HEK293T cells with increasing amounts of Flag-hnRNP-K. Cell lysates were subjected to pull-down with NTA-beads followed by immunoblot with anti-HA or anti-Xpress antibody. (**B**) HDM2 and HA-Ub were expressed in cells with increasing amounts of Flag-hnRNP K. Cell lysates were subjected to immunoprecipitation with anti-HDM2 antibody followed by immunoblot with anti-HA or anti-HDM2 antibody.

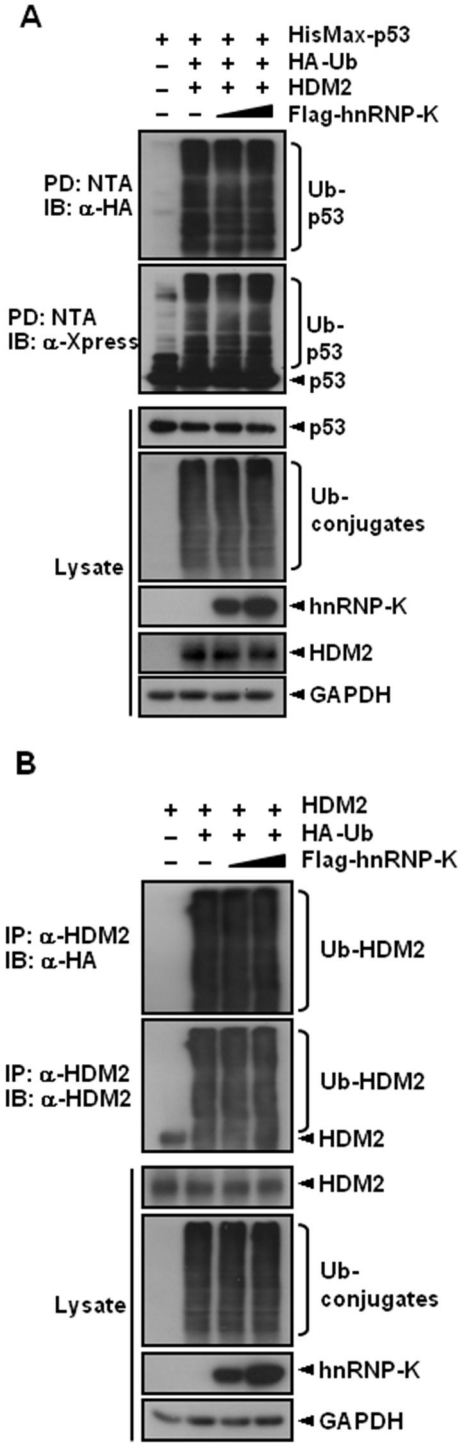
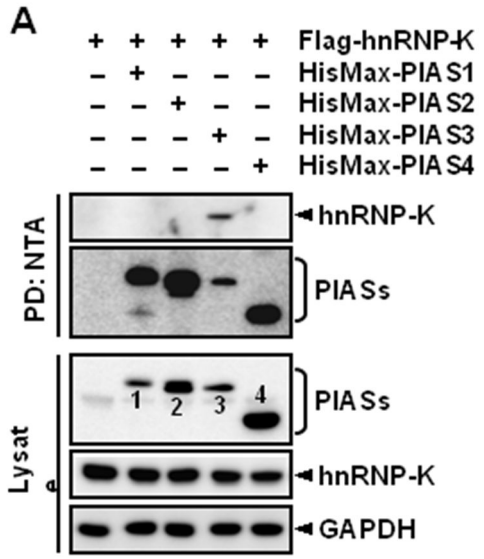

A
+ + + + HisMax-p53
– + + + HA-Ub
– + + + HDM2
– – Flag-hnRNP-K
PD: NTA
IB: α-HA
Ub-
p53
PD: NTA
IB: α-Xpress
Ub-
p53
p53
p53
Ub-
conjugates
Lysate
hnRNP-K
HDM2
GAPDH
B
+ + + + HDM2
– + + + HA-Ub
– – Flag-hnRNP-K
IP: α-HDM2
IB: α-HA
Ub-HDM2
IP: α-HDM2
IB: α-HDM2
Ub-HDM2
HDM2
HDM2
Ub-
conjugates
Lysate
hnRNP-K
GAPDH

Figure 21. PIAS3 specifically interacted with hnRNP-K and promoted its sumoylation

(A) Identification of PIAS interacting with hnRNP-K. Flag-hnRNP-K was overexpressed in HEK293T cells with HisMax-tagged PIAS1-4. Cell lysates were subjected to pull-down with NTA-beads followed by immunoblot with anti-Flag or anti-Xpress antibody. (**B**) 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.


A
+ + + + + Flag-hnRNP-K
– + – – – HisMax-PIAS1
– – + – – HisMax-PIAS2
– – – + – HisMax-PIAS3
– – – – + HisMax-PIAS4
PD: NTA
hnRNP-K
PIASs
Lysate
1 2 3 4
PIASs
hnRNP-K
GAPDH

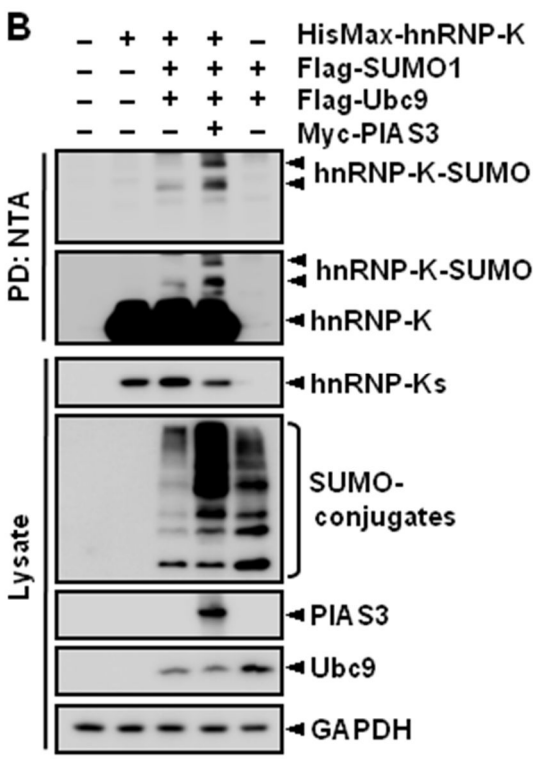
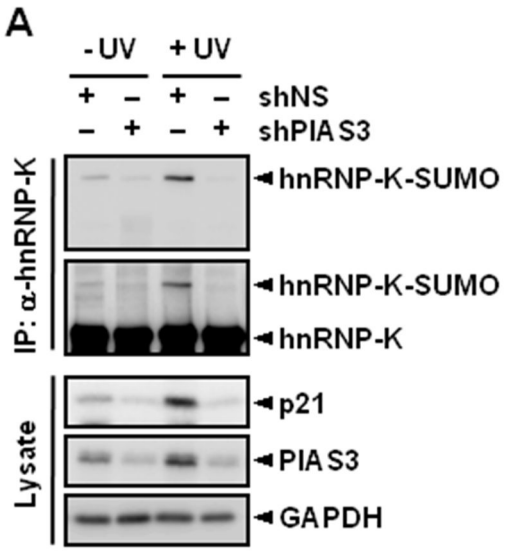

B
– + + + – HisMax-hnRNP-K
– – + + + Flag-SUMO1
– – + + + Flag-Ubc9
– – – + – Myc-PIAS3
PD: NTA
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-Ks
SUMO-
conjugates
PIAS3
Ubc9
GAPDH

Figure 22. UV-mediated increase in hnRNP-K sumoylation is due to an increase in the affinity of hnRNP-K to PIAS3

(**A**) 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. (**B**) 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. MG132 was treated 4 h before cell lysis.


A
- UV
+ UV
+ – + – shNS
– + – + shPIAS3
IP: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
p21
PIAS3
GAPDH

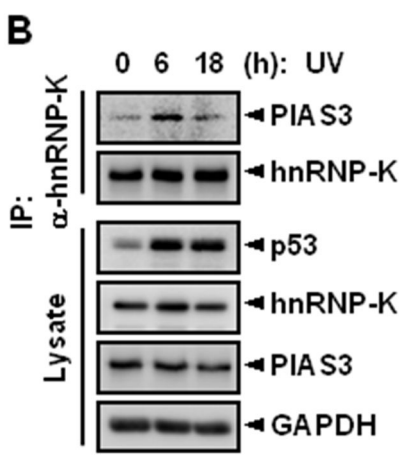

B
0 6 18 (h): UV
IP: α-hnRNP-K
PIAS3
hnRNP-K
Lysate
p53
hnRNP-K
PIAS3
GAPDH

Figure 23. Identification of SENP interacting with hnRNP-K

Flag-hnRNP-K was overexpressed in HEK293T cells with HisMax-tagged SENP1-3, SUSP4, and SENP6 and Myc-tagged SENP5 and 7. Cell lysates were subjected to pull-down with NTA-beads followed by immunoblot with anti-Flag or anti-Xpress antibody.

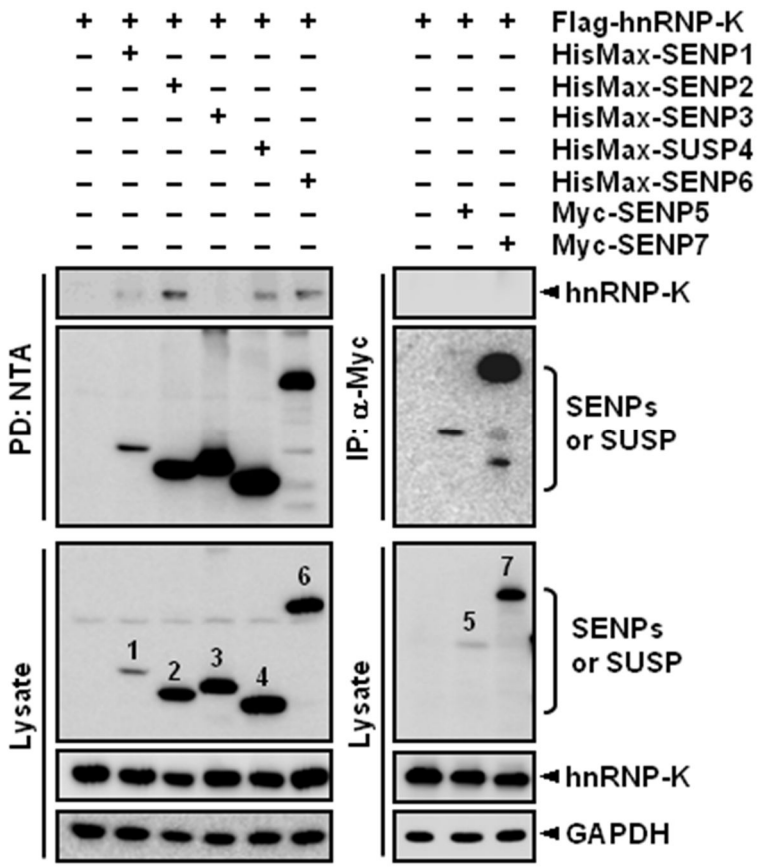

+ + + + + + + + + Flag-hnRNP-K
– + – – – – – – – HisMax-SENP1
– – + – – – – – – HisMax-SENP2
– – – + – – – – – HisMax-SENP3
– – – – + – – – – HisMax-SUSP4
– – – – – + – – – HisMax-SENP6
– – – – – – – + – Myc-SENP5
– – – – – – – – + Myc-SENP7
PD: NTA
IP: α-Myc
hnRNP-K
SENPs
or SUSP
Lysate
Lysate
1
2
3
4
5
6
7
SENPs
or SUSP
hnRNP-K
GAPDH

Figure 24. UV inhibits the interaction of hnRNP-K with SENP2

(A) 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-SENP1, SENP2, and SENP6 or anti-hnRNP-K antibody. MG132 was treated 4 h before cell lysis. (**B**) Cells treated with UV were subjected to immunoprecipitation with anti-SENP2 antibody followed by immunoblot with anti hnRNP K or anti-SENP2 antibody. MG132 was treated 4 h before cell lysis.

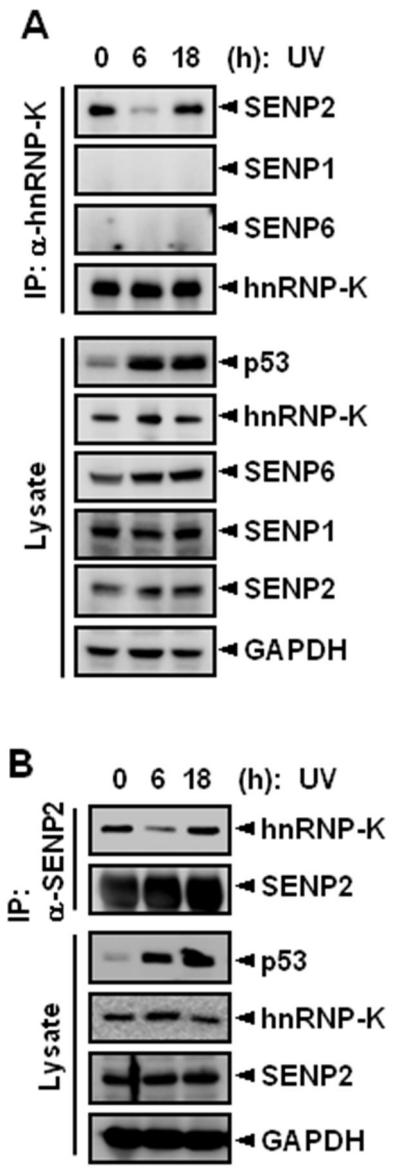
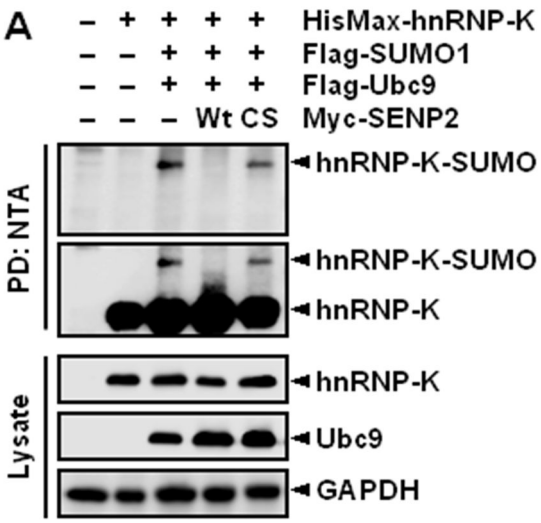

A
0 6 18 (h): UV
IP: α-hnRNP-K
SENP2
SENP1
SENP6
hnRNP-K
Lysate
p53
hnRNP-K
SENP6
SENP1
SENP2
GAPDH
B
0 6 18 (h): UV
IP: α-SENP2
hnRNP-K
SENP2
Lysate
p53
hnRNP-K
SENP2
GAPDH

Figure 25. UV-mediated increase in hnRNP-K sumoylation is due to a decrease in the affinity of hnRNP-K to SENP2

(**A**) 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. (**B**) 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 subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblot with anti-SUMO1 or anti-hnRNP-K antibody


A
– + + + + HisMax-hnRNP-K
– – + + + Flag-SUMO1
– – + + + Flag-Ubc9
– – – Wt CS Myc-SENP2
PD: NTA
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
hnRNP-K
Ubc9
GAPDH

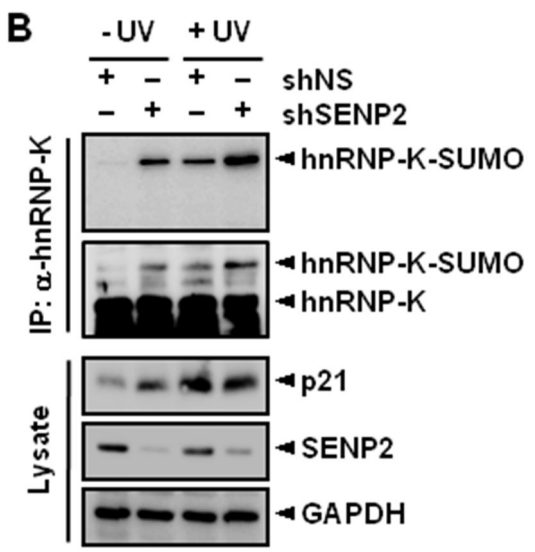

B
- UV + UV
+ – + – shNS
– + – + shSENP2
IP: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
Lysate
p21
SENP2
GAPDH

Figure 26. Identification of the regions within hnRNP-K for binding of HDM2 and p53

Deletions of hnRNP-K were generated and tagged with HisMax to their N-termini. They were overexpressed in HEK293T cells with Flag-tagged HDM2 (**A**) and p53 (**B**). MG132 was treated 4 h before cell lysis. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot with anti-Flag or anti-Xpress antibody.

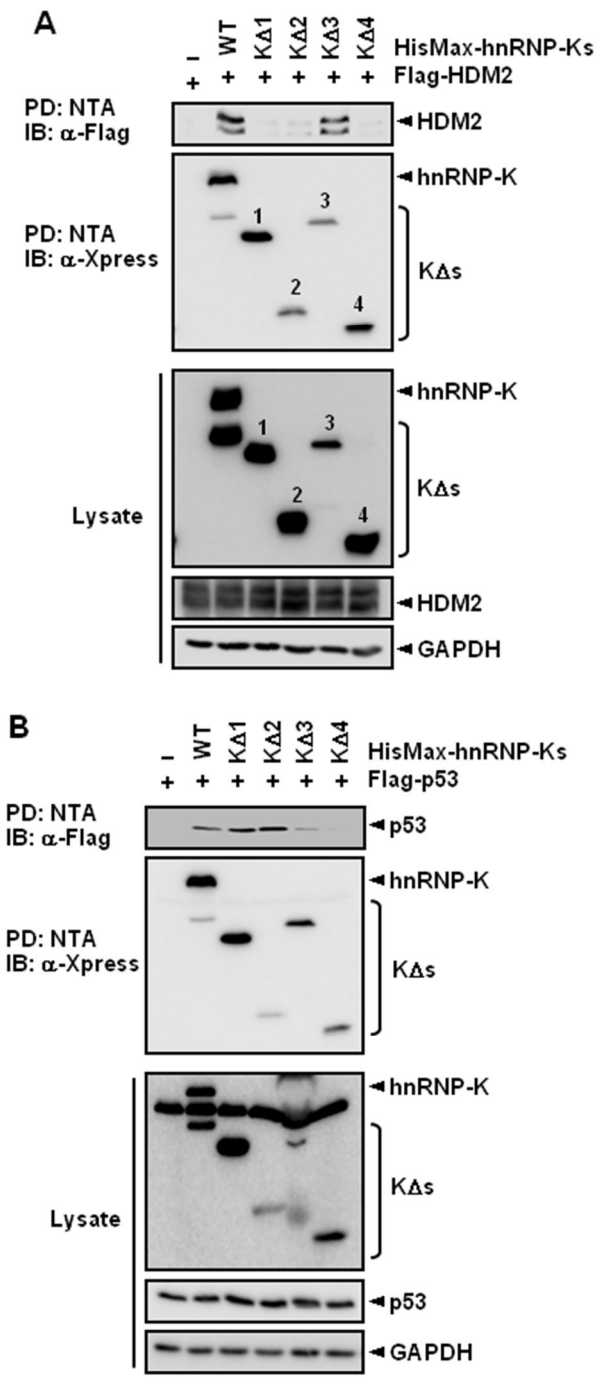

A
HisMax-hnRNP-Ks
Flag-HDM2
– + WT KΔ1 KΔ2 KΔ3 KΔ4
PD: NTA
IB: α-Flag
HDM2
hnRNP-K
PD: NTA
IB: α-Xpress
KΔs
1
2
3
4
Lysate
HDM2
GAPDH
B
HisMax-hnRNP-Ks
Flag-p53
PD: NTA
IB: α-Flag
p53
hnRNP-K
PD: NTA
IB: α-Xpress
KΔs
Lysate
p53
GAPDH

Figure 27. Identification of the regions within hnRNP-K for binding of SENP2 and PIAS3

Deletions of hnRNP-K were generated and tagged with HisMax to their N-termini. They were overexpressed in HEK293T cells with Flag-tagged SENP2 (**A**) and PIAS3 (**B**). MG132 was treated 4 h before cell lysis. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot with anti-Flag or anti-Xpress antibody. (**C**) Whether the proteins bind to each of hnRNP-K deletions or not was shown as "+" or "-" and their binding region within hnRNP-K were indicated by the arrows.

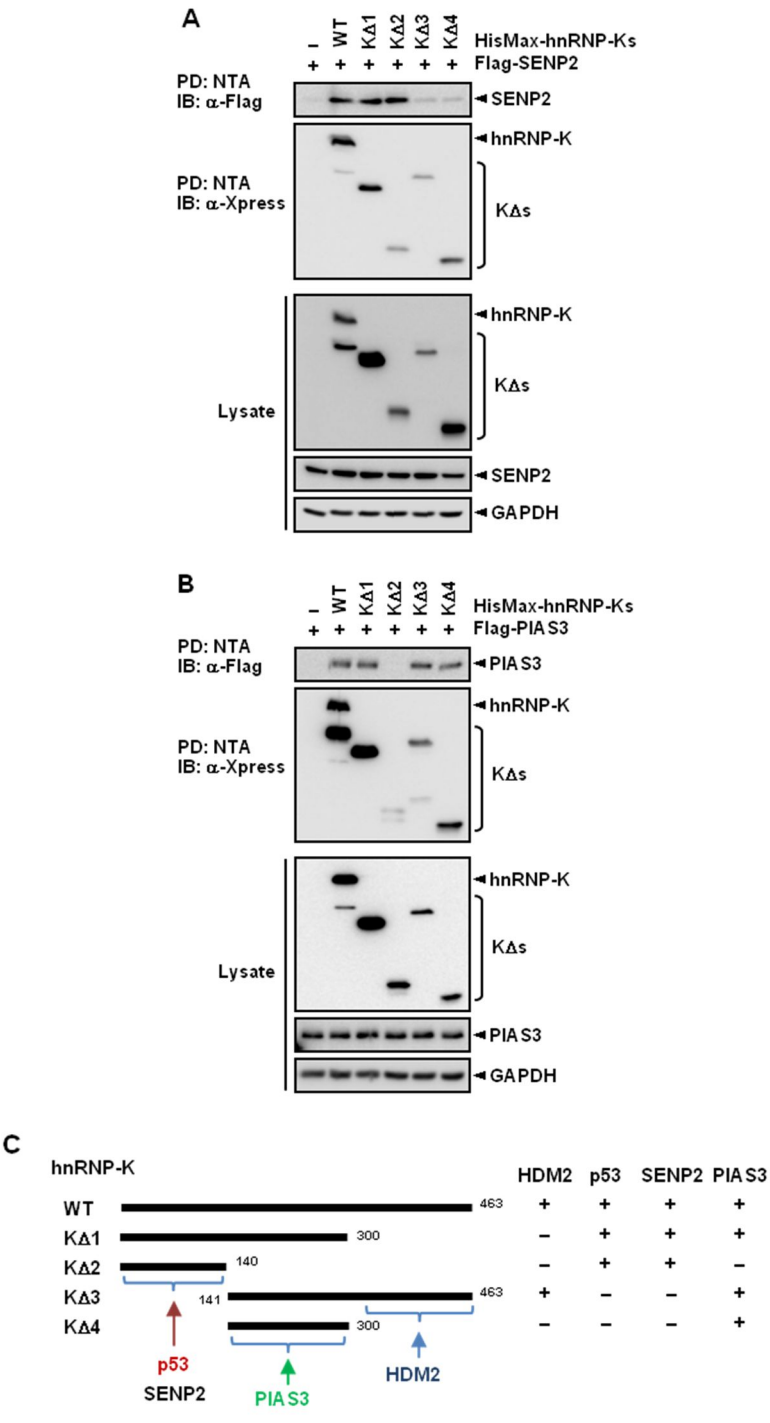

A
HisMax-hnRNP-Ks
Flag-SENP2
– WT KΔ1 KΔ2 KΔ3 KΔ4
PD: NTA
IB: α-Flag
SENP2
hnRNP-K
PD: NTA
IB: α-Xpress
KΔs
hnRNP-K
Lysate
KΔs
SENP2
GAPDH
B
HisMax-hnRNP-Ks
Flag-PIAS3
– WT KΔ1 KΔ2 KΔ3 KΔ4
PD: NTA
IB: α-Flag
PIAS3
hnRNP-K
PD: NTA
IB: α-Xpress
KΔs
hnRNP-K
Lysate
KΔs
PIAS3
GAPDH
C
hnRNP-K
HDM2 p53 SENP2 PIAS3
WT 463 + + + +
KΔ1 300 – + + +
KΔ2 140 – + + –
KΔ3 141 463 + – – +
KΔ4 300 – – – +
p53
SENP2
PIAS3
HDM2

Figure 28. Identification of hnRNP-K-binding regions within HDM2 and p53

(**A**) Deletions of HDM2 were generated and ligated with HisMax-tag to their N-termini. MG132 was treated 4 h before cell lysis. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot with anti-Flag or anti-Xpress antibody. (**B**) Deletions of p53 were generated and ligated with Myc-tag to their N-termini. MG132 was treated 4 h before cell lysis. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblot with anti-Flag or anti-Myc antibody. Whether hnRNP-K binds to the deletions of each protein or not was shown as "+" or "-" and its binding region within each protein was indicated by the arrow.

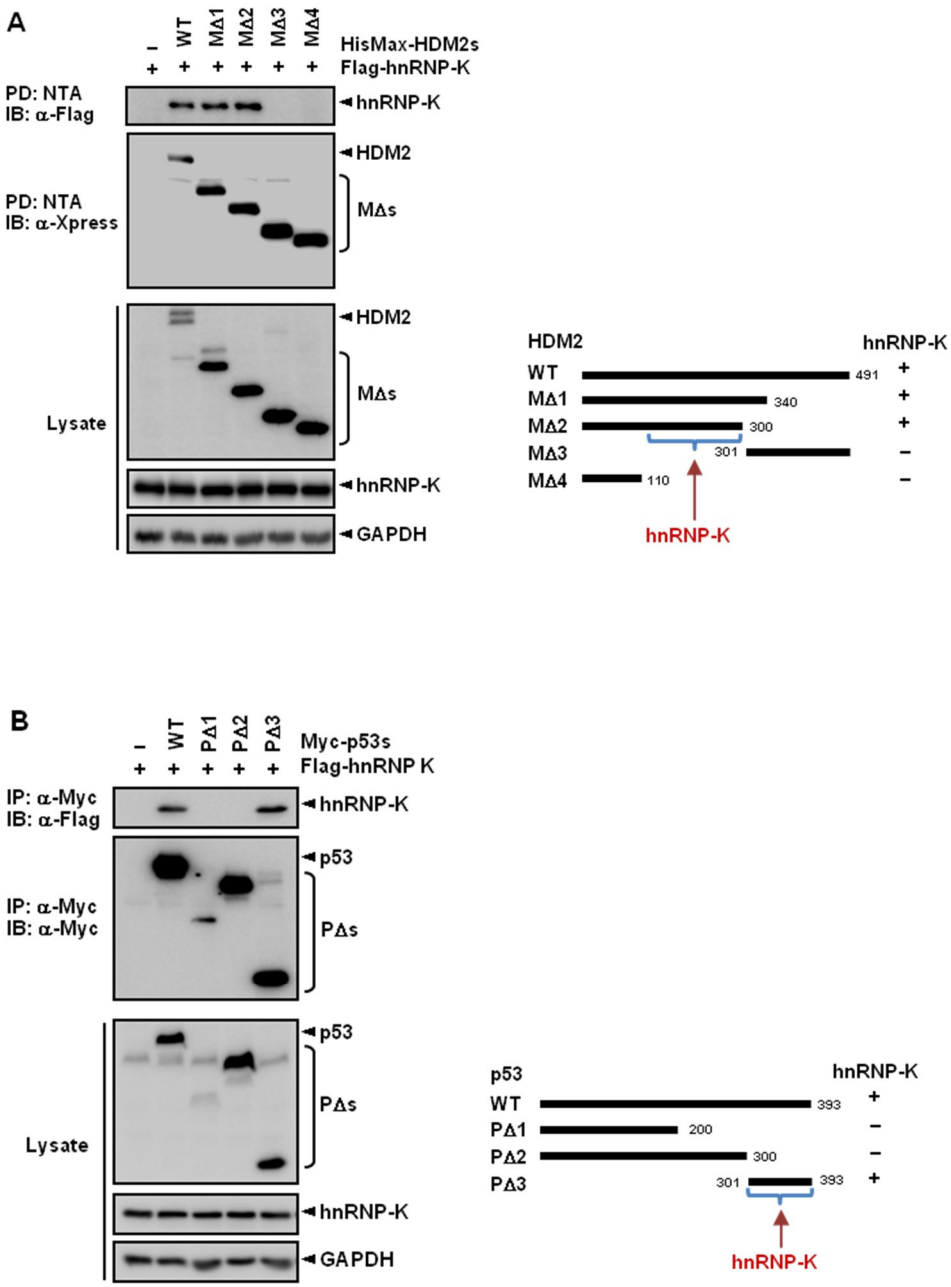

A
HisMax-HDM2s
Flag-hnRNP-K
– WT MΔ1 MΔ2 MΔ3 MΔ4
+ + + + + +
PD: NTA
IB: α-Flag
hnRNP-K
HDM2
PD: NTA
IB: α-Xpress
MΔs
HDM2
MΔs
Lysate
hnRNP-K
GAPDH
HDM2
hnRNP-K
WT 491 +
MΔ1 340 +
MΔ2 300 +
MΔ3 301 –
MΔ4 110 –
hnRNP-K
B
Myc-p53s
Flag-hnRNP K
– WT PΔ1 PΔ2 PΔ3
+ + + + +
IP: α-Myc
IB: α-Flag
hnRNP-K
p53
IP: α-Myc
IB: α-Myc
PΔs
p53
PΔs
Lysate
hnRNP-K
GAPDH
p53
hnRNP-K
WT 393 +
PΔ1 200 –
PΔ2 300 –
PΔ3 301 393 +
hnRNP-K

Figure 29. Identification of hnRNP-K-binding regions within SENP2 and PIAS3

(**A**) Deletions of SENP2 were generated and ligated with Flag-tag to their N-termini. MG132 was treated 4 h before cell lysis. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-Xpress or anti-Flag antibody. (**B**) Deletions of PIAS3 were generated and ligated with HisMax-tag to their N-termini. MG132 was treated 4 h before cell lysis. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot with anti-Flag or anti-Xpress antibody. Whether hnRNP-K binds to the deletions of each protein or not was shown as "+" or "-" and its binding region within each protein was indicated by the arrow.

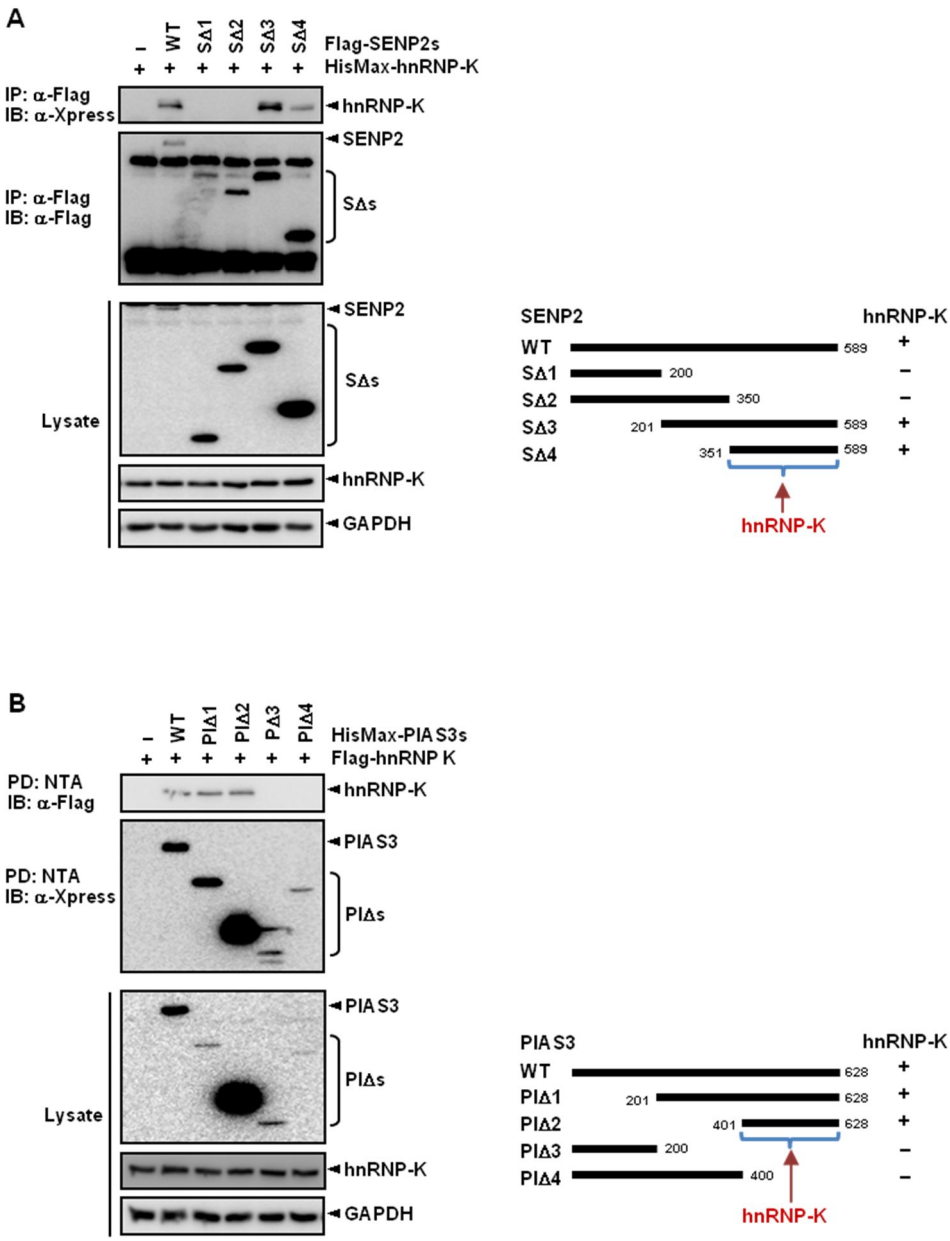

A
Flag-SENP2s
HisMax-hnRNP-K
- WT SΔ1 SΔ2 SΔ3 SΔ4
+ + + + + +
IP: α-Flag
IB: α-Xpress
hnRNP-K
SENP2
IP: α-Flag
IB: α-Flag
SΔs
SENP2
SΔs
Lysate
hnRNP-K
GAPDH
SENP2
hnRNP-K
WT 589 +
SΔ1 200 –
SΔ2 350 –
SΔ3 201 589 +
SΔ4 351 589 +
hnRNP-K
B
HisMax-PIAS3s
Flag-hnRNP K
- WT PIΔ1 PIΔ2 PΔ3 PIΔ4
+ + + + + +
PD: NTA
IB: α-Flag
hnRNP-K
PIAS3
PD: NTA
IB: α-Xpress
PIΔs
PIAS3
PIΔs
Lysate
hnRNP-K
GAPDH
PIAS3
hnRNP-K
WT 628 +
PIΔ1 201 628 +
PIΔ2 401 628 +
PIΔ3 200 –
PIΔ4 400 –
hnRNP-K

Figure 30. Map for the interaction between hnRNP-K and p53, HDM2, SENP2, or PIAS3

The data obtained from figure 25, 26, 27, and 28 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.

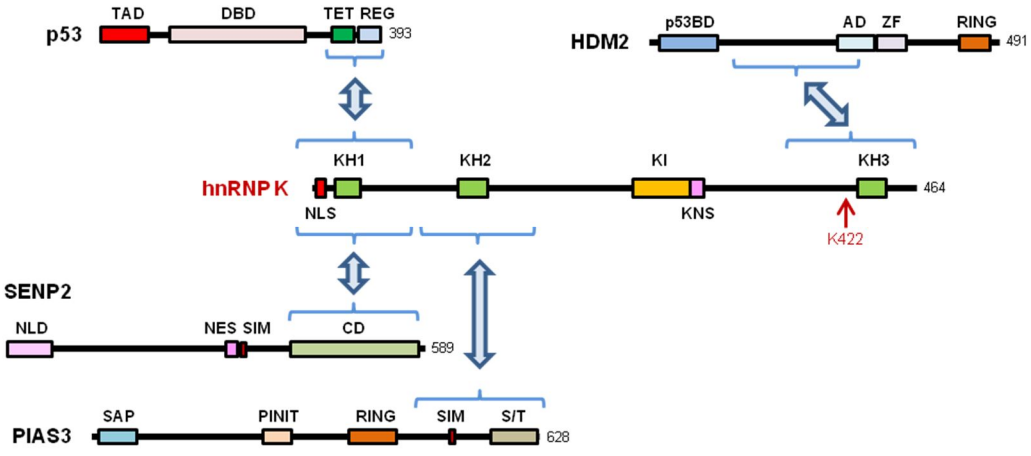

p53
TAD
DBD
TET REG
393
HDM2
p53BD
AD
ZF
RING
491
KH1
KH2
KI
KH3
hnRNP K
464
NLS
KNS
K422
SENP2
NLD
NES SIM
CD
589
PIAS3
SAP
PINIT
RING
SIM
S/T
628

Figure 31. Effect of UV on subcellular localization of overexpressed hnRNP-K

HeLa cells were overexpressed with Flag-tagged hnRNP-K (Wt) or K422R (KR). After incubation for 48 h, cells were exposed to UV, incubated for 6 h, and stained with anti-Flag antibody or DAPI.

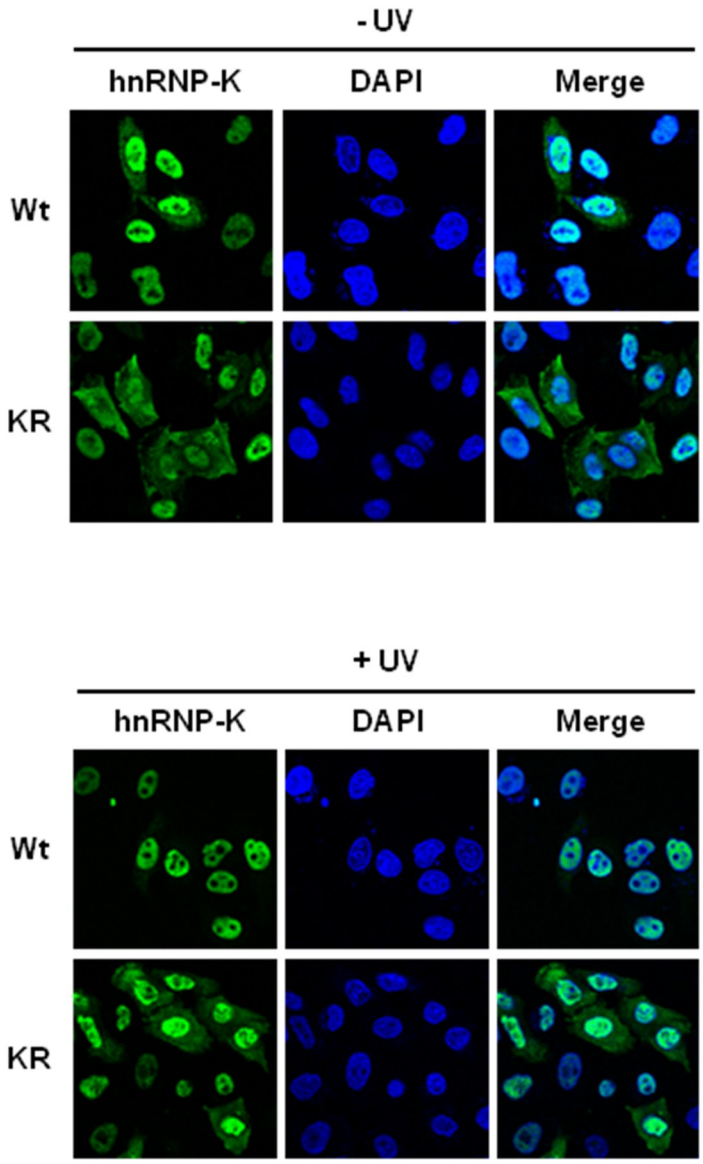

- UV
hnRNP-K
DAPI
Merge
Wt
KR
+ UV
hnRNP-K
DAPI
Merge
Wt
KR

Figure 32. Depletion of PIAS3 prevented UV-induced nuclear localization of overexpressed hnRNP-K

HeLa cells transfected with shNS or shPIAS3 were incubated for 48 h followed by overexpression of Flag-hnRNP-K. After exposure to UV, cells were stained with anti-Flag and anti-PIAS3 antibodies.

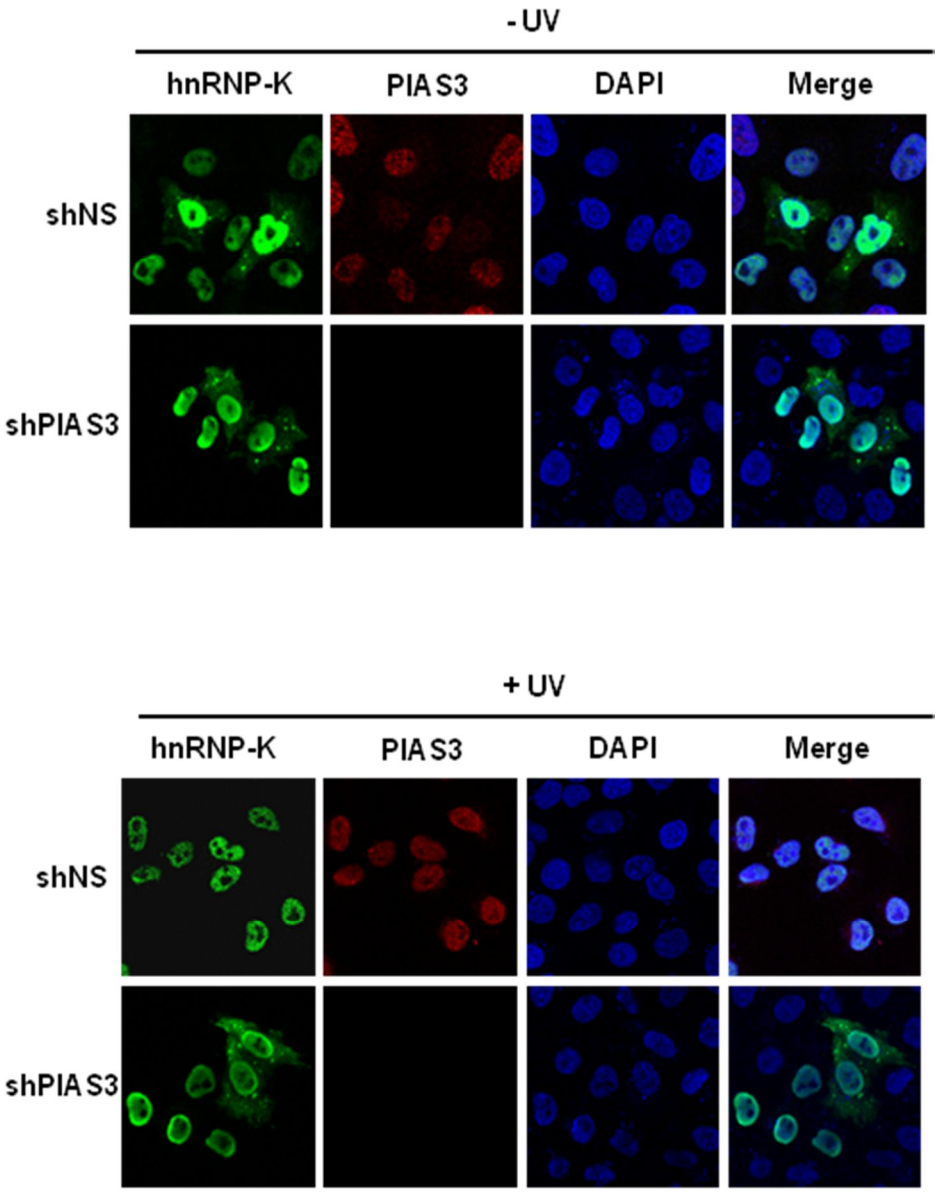

- UV
hnRNP-K
PIAS3
DAPI
Merge
shNS
shPIAS3
+ UV
hnRNP-K
PIAS3
DAPI
Merge
shNS
shPIAS3

Figure 33. Nuclear localization of hnRNP-K could be prevented by overexpression of SENP2

Flag-hnRNP-K was overexpressed in cells with Myc-tagged wild-type SENP2 (Wt) or its inactive mutant (CS). After exposure to UV, cells were stained with anti-Flag and anti-Myc antibodies.

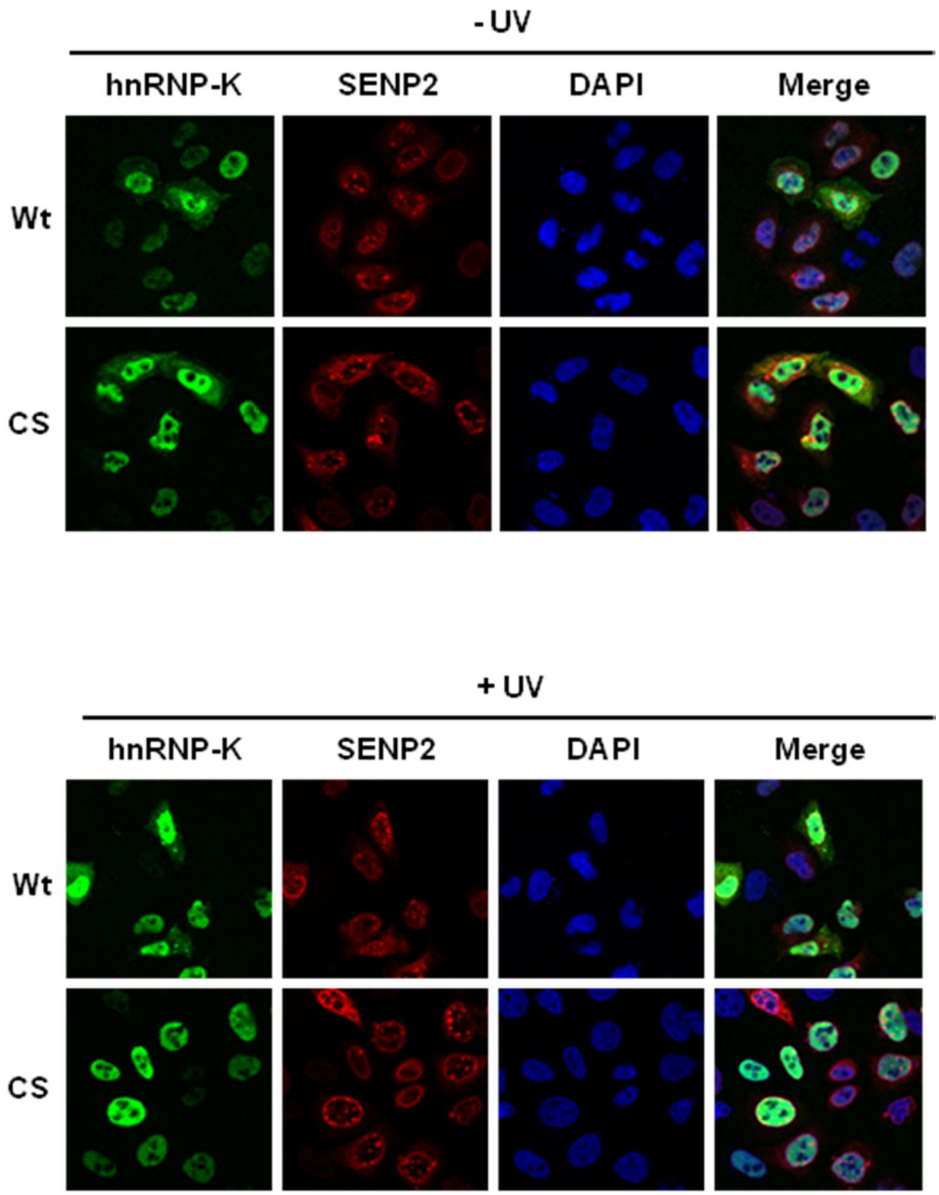

- UV
hnRNP-K
SENP2
DAPI
Merge
Wt
CS
+ UV
hnRNP-K
SENP2
DAPI
Merge
Wt
CS

Figure 34. Effect of UV on subcellular localization of endogenous hnRNP-K

HeLa cells were transfected with shNS or shPIAS3. After incubation for 48 h, cells were exposed to UV, incubated for 6 h, and stained with anti-hnRNP K and anti-PIAS3 antibodies. Note that the intensity of endogenous hnRNP-K is much lower in the absence of UV than in its presence and that the intensity is also significantly lowered upon PIAS3 knockdown even in the presence of UV, since hnRNP-K is unstable without sumoylation.

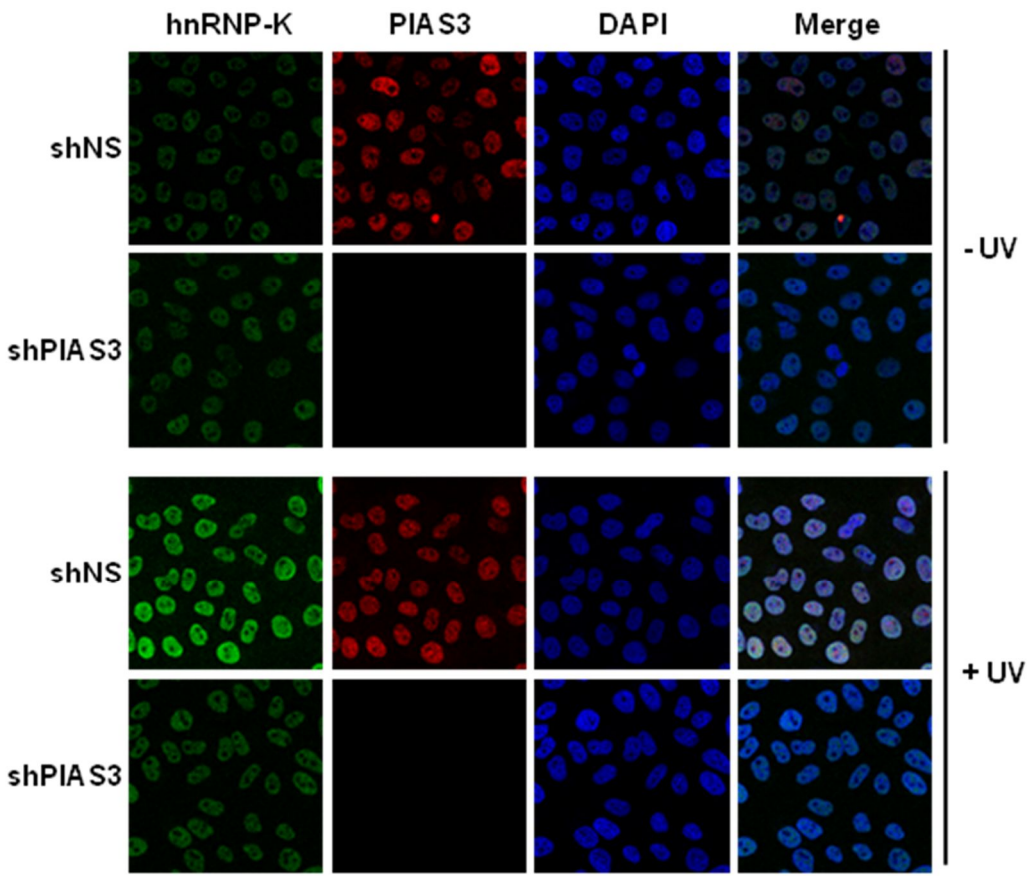

hnRNP-K
PIAS3
DAPI
Merge
shNS
shPIAS3
- UV
shNS
shPIAS3
+ UV

Figure 35. 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. The data in (**A** and **B**) represent the mean ± s.d. of four experiments. (**C**) Knockdown of SENP2 or together with hnRNP-K. Cell lysates prepared as in Figure 31B were subjected to immunoblot analysis.

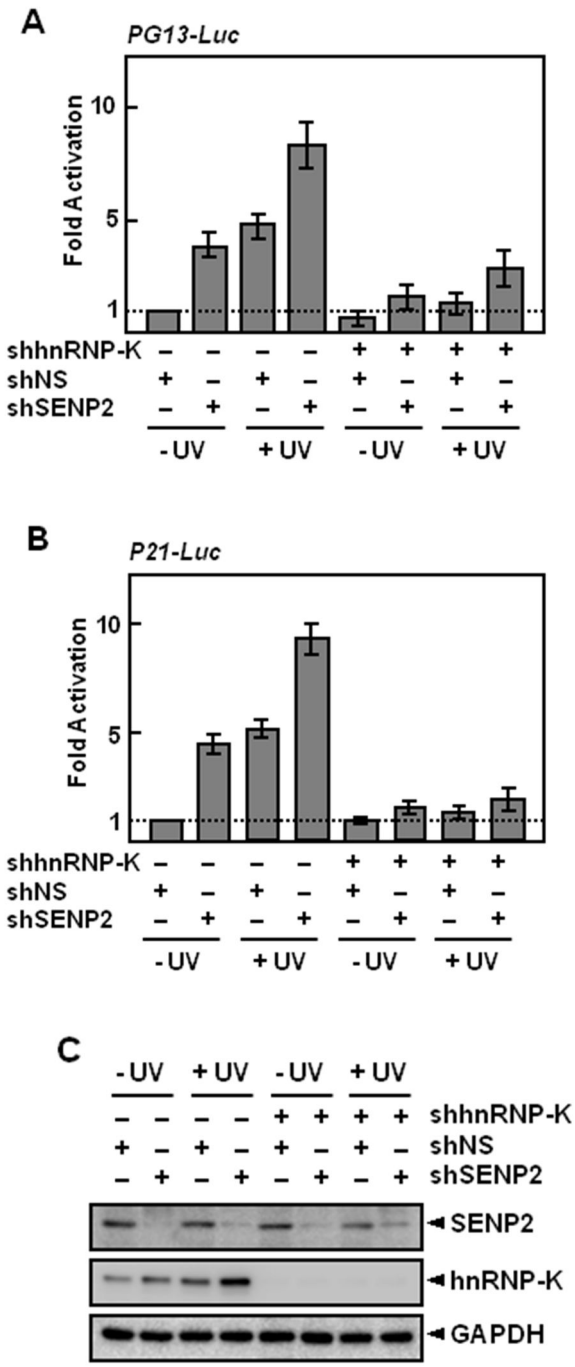

A
PG13-Luc
Fold Activation
10
5
1
shhnRNP-K – – – – + + + +
shNS + – + – + – + –
shSENP2 – + – + – + – +
- UV
+ UV
- UV
+ UV
B
P21-Luc
Fold Activation
10
5
1
shhnRNP-K – – – – + + + +
shNS + – + – + – + –
shSENP2 – + – + – + – +
- UV
+ UV
- UV
+ UV
C
- UV
+ UV
- UV
+ UV
– – – – + + + + shhnRNP-K
+ – + – + – + – shNS
– + – + – + – + shSENP2
SENP2
hnRNP-K
GAPDH

Figure 36. PIAS3 knockdown ablates p53 transactivity

HeLa cells transfected with shNS or shPIAS3 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. The data in (**A** and **B**) represent the mean ± s.d. of four experiments. (**C**) Knockdown of PIAS3 alone or together with hnRNP-K. Cell lysates prepared as in Figure 32B were subjected to immunoblot analysis.

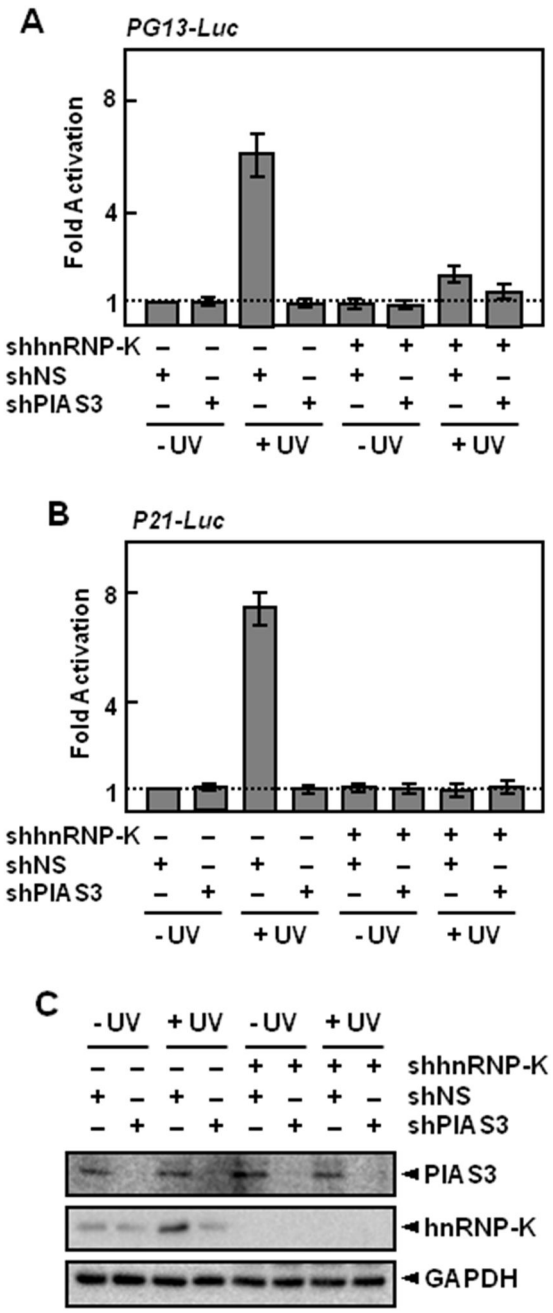

A
PG13-Luc
Fold Activation
8
4
1
shhnRNP-K – – – – + + + +
shNS + – + – + – + –
shPIAS3 – + – + – + – +
-UV +UV -UV +UV
B
P21-Luc
Fold Activation
8
4
1
shhnRNP-K – – – – + + + +
shNS + – + – + – + –
shPIAS3 – + – + – + – +
-UV +UV -UV +UV
C
-UV +UV -UV +UV
– – – – + + + + shhnRNP-K
+ – + – + – + – shNS
– + – + – + – + shPIAS3
PIAS3
hnRNP-K
GAPDH

Figure 37. 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 (**A**) or immunoblot analysis (**B**). The data in (**A**) represent the mean ± s.d. of four experiments.

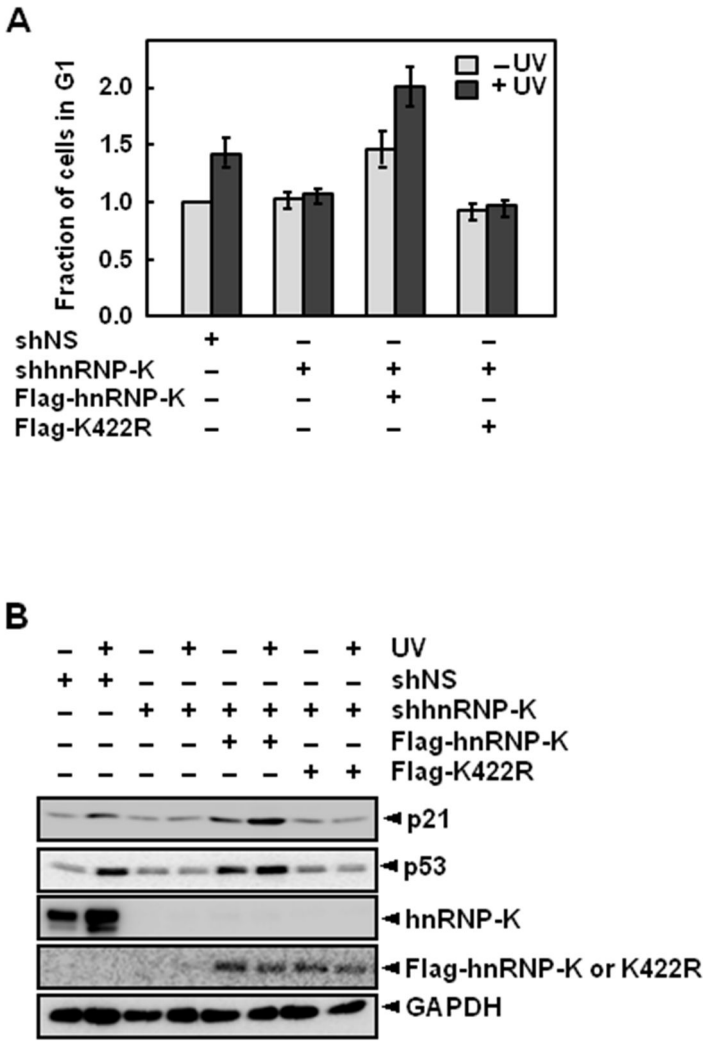

A
Fraction of cells in G1
2.0
1.5
1.0
0.5
0.0
–UV
+UV
shNS + – – –
shhnRNP-K – + + +
Flag-hnRNP-K – – + –
Flag-K422R – – – +
B
– + – + – + – + UV
+ + – – – – – – shNS
– – + + + + + + shhnRNP-K
– – – – + + – – Flag-hnRNP-K
– – – – – – + + Flag-K422R
p21
p53
hnRNP-K
Flag-hnRNP-K or K422R
GAPDH

Figure 38. Effect of complementation of hnRNP-K or K422R to hnRNP-K-depleted cells on 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.

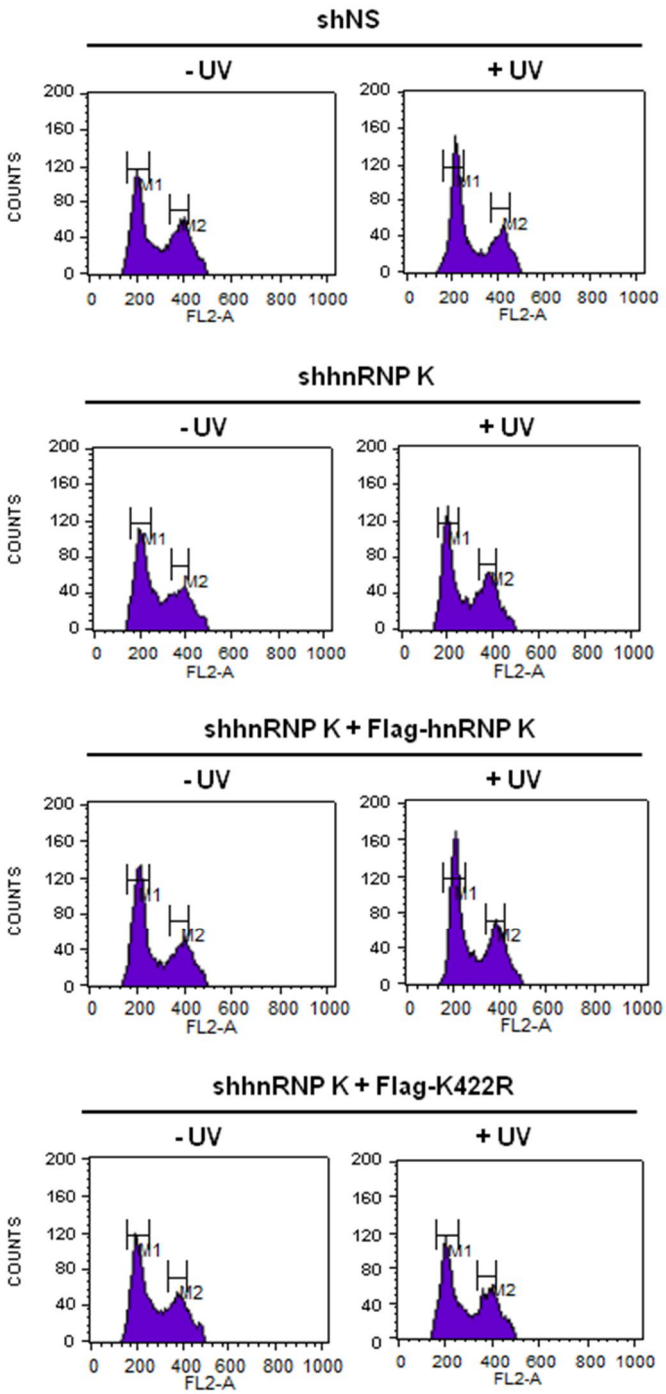
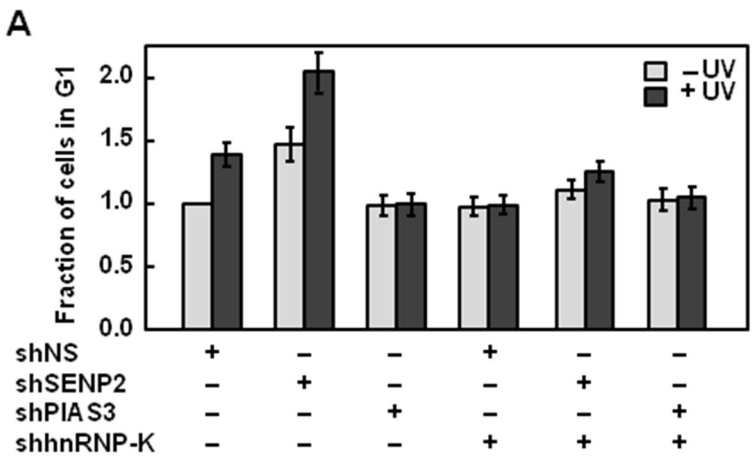

shNS
- UV
+ UV
COUNTS
FL2-A
M1
M2
shhnRNP K
shhnRNP K + Flag-hnRNP K
shhnRNP K + Flag-K422R

Figure 39. 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 (**A**) or immunoblot analysis (**B**). The data in (**A**) represent the mean ± s.d. of four experiments.


A
Fraction of cells in G1
2.0
1.5
1.0
0.5
0.0
– UV
+ UV
shNS + – – + – –
shSENP2 – + – – + –
shPIAS3 – – + – – +
shhnRNP-K – – – + + +

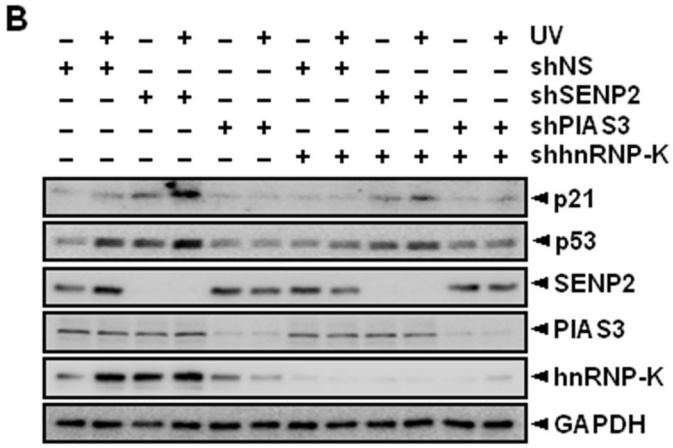
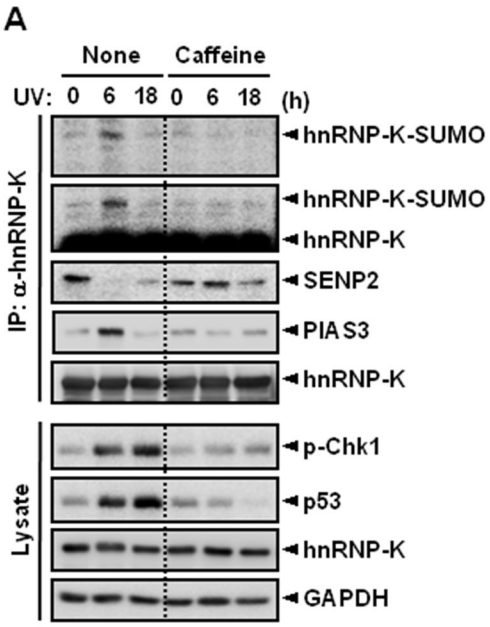
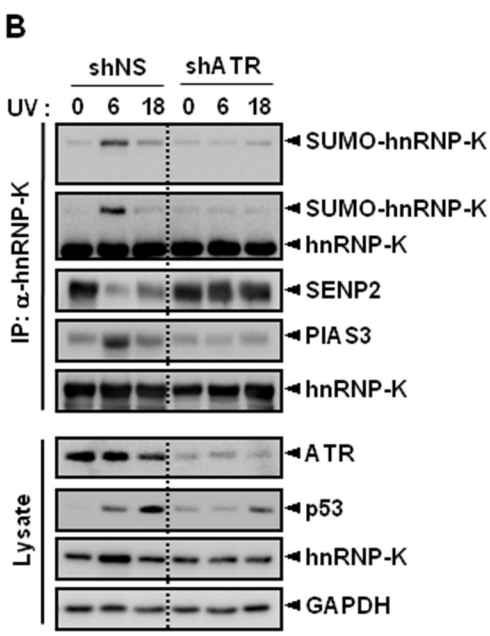

B
– + – + – + – + – + – + UV
+ + – – – – + + – – – – shNS
– – + + – – – – + + – – shSENP2
– – – – + + – – – – + + shPIAS3
– – – – – – + + + + + + shhnRNP-K
p21
p53
SENP2
PIAS3
hnRNP-K
GAPDH

Figure 40. 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.


A
None
Caffeine
UV: 0 6 18 0 6 18 (h)
IP: α-hnRNP-K
hnRNP-K-SUMO
hnRNP-K-SUMO
hnRNP-K
SENP2
PIAS3
hnRNP-K
Lysate
p-Chk1
p53
hnRNP-K
GAPDH

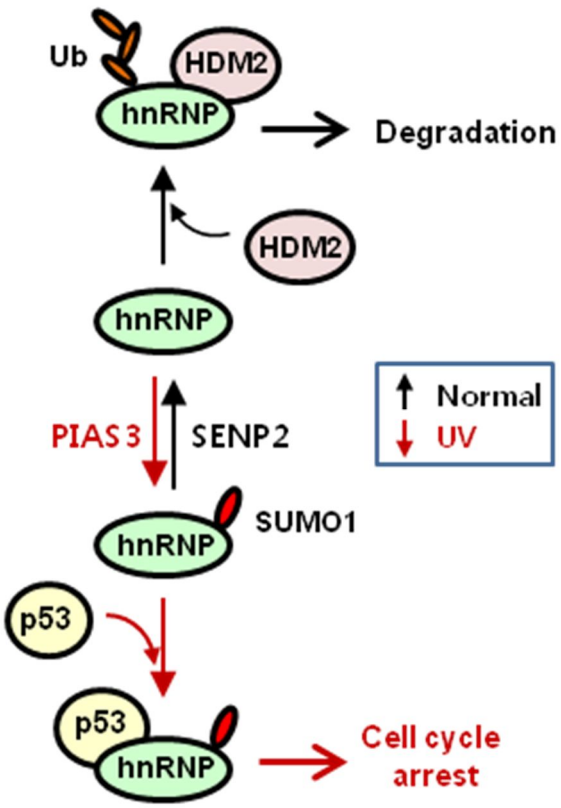

B
shNS
shATR
UV: 0 6 18 0 6 18
IP: α-hnRNP-K
SUMO-hnRNP-K
SUMO-hnRNP-K
hnRNP-K
SENP2
PIAS3
hnRNP-K
Lysate
ATR
p53
hnRNP-K
GAPDH

DISCUSSION

Based on the findings in this study, I propose a model for the role of hnRNP-K sumoylation in UV-induced cell-cycle arrest (Figure 41). 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 coactivator 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

Figure 41. A model for the role of hnRNP-K sumoylation in UV-induced cell cycle arrest


Ub
HDM2
hnRNP
Degradation
HDM2
hnRNP
Normal
UV
PIAS 3
SENP2
hnRNP
SUMO1
p53
p53
hnRNP
Cell cycle
arrest

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 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 (unpublished observation). Nonetheless, I 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 (Levy & Inghirami, 2006; Niu et al, 2002; Wei et al, 2003). Interestingly, PIAS3 expression is down-regulated 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 I 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 I showed that SENP2 knockdown increases hnRNP-K sumoylation, its interaction with HDM2, and consequently its coactivator 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 coactivator.

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 the anti-cancer drug RITA (reactivation of p53 and induction of tumor cell apoptosis) releases HDM2 from p53 and the freed HDM2 molecules promote proteasome-mediated degradation of hnRNP-K, which impairs its coactivator 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 toward induction of apoptosis. Since deregulated cell-cycle progression likely evokes tumorigenesis, small molecules that specifically inhibit SENP2 could also be used as therapeutic drugs against cancers.

While this work was under revision, another study reported that DNA damage

induces hnRNP-K sumoylation, which in turn enhances the transcriptional activity of p53 (Enge et al, 2009). However, the major differences between their and my 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, I 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 analyzing the effect of DNA damage on alterations in the level of hnRNP-K and to the use of overexpression system in analyzing the role of Pc2 in hnRNP-K sumoylation rather than that of RNA interference system. However, I could not exclude a possibility that different SUMO E3 ligase may act on hnRNP-K under different DNA damage conditions, since their work mainly used doxorubicin as a DNA damaging agent whereas my work utilized UV.

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국문 초록

P53은 세포의 삶과 죽음에 관련된 다양한 신호전달 과정에 관여한다. P53은 전사인자로써 세포 손상시 다양한 표적 유전자의 발현을 조절하고 동시에 세포 주기 중지, DNA 복구 그리고 세포 사멸과 같은 다양한 반응을 유도한다. DNA 손상에 반응하여 활성화된 p53은 결과적으로 G1/S 혹은 G2/M 시기의 세포 주기 정지를 유도하는데, G1/S 시기의 정지는 cyclin 의존적 활성효소 억제인자인 p21에 의해서 주요하게 유도된다.

Small Ubiquitin-related modifier (SUMO, 수모)는 유비퀴틴 유사 단백질로써 다양한 세포내 단백질에 결합한다. 수모는 유비퀴틴과 마찬가지로 E1 활성효소 (SAE1/SAE2), E2 접합효소 (Ubc9), E3 결합효소 (PIASs)에 의한 연쇄 반응 효소 작용에 의해 표적 단백질에 결합한다. 결합된 수모는 수모 특이적 단백질 분해효소에 의해 제거된다. 이런 가역적 수모화는 전사과정, 핵내 이동과정, 신호전달체계와 같은

다양한 세포내 과정을 조절할 수 있다.

Heterogeneous nuclear ribonucleoprotein-K (hnRNP-K)는 RNA 결합 단백질로써 염색질 재형성, 전사과정, mRNA 스플라이싱, 번역과정과 같은 다양한 세포내 과정과 연관되어 있다. hnRNP-K는 정상적인 상태에서는 HDM2에 의해서 유비퀴틴화되어 proteosome에 의해 분해된다. 그러나 세포내에 DNA 손상이 일어나면 hnRNP-K는 안정화되고 p53의 전사 활성 인자로 작용하여 세포 주기 정지를 일으킨다. 그러나 어떻게 hnRNP-K의 안정화와 그 기능이 조절되는지는 밝혀지지 않았다.

본 연구에서는 UV에 의해 hnRNP-K의 수모화가 유도되고, 이는 유비퀴틴화를 억제하여 hnRNP-K가 안정화됨을 밝히었다. UV 처리 후 6시간이 되면 hnRNP-K의 수모화는 현저하게 증가하고 이는 18시간만에 다시 감소하는데 이런 변화는 hnRNP-K 자체의 발현 정도와 같은 패턴으로 나타난다. 그러나 hnRNP-K의 유비퀴틴화는 UV 처리 후 6시간때에 감소했다가 다시 처음 상태로 증가한다. 이는 hnRNP-K의

수모화와 유비퀴틴화가 서로 반대적으로 관련이 있다고 할 수 있으며 결과적으로 hnRNP-K의 수모화는 자체의 안정화를 유도한다고 할 수 있겠다. 또한 IR이나 doxorubicin에 의한 다른 종류의 DNA 손상이 일어나는 상태에서도 hnRNP-K의 수모화가 유도됨을 확인함으로써, hnRNP-K의 수모화에 의한 안정화는 DNA 손상에 따라 나타나는 일반적인 반응이라 할 수 있겠다.

수모화가 되지 않는 돌연변이체와 수모화가 되어 있는 hnRNP-K 단백질을 이용하여 비교한 결과, hnRNP-K의 수모화는 HDM2와의 결합을 떨어뜨리고 p53과의 결합을 증가시키는 것을 확인할 수 있었다. 즉, 수모화된 hnRNP-K는 p53과 우선적으로 결합하는 반면, 수모화 되지 않은 hnRNP-K는 HDM2와 더 결합한다. 이처럼 hnRNP-K의 수모화는 p53이나 HDM2와의 결합에 있어서 스위치 역할을 할 수 있다. 게다가 UV에 의해 유도되는 hnRNP-K의 수모화는 p53의 전사활성능력을 증가시켜 p21의 발현을 높임으로써 세포 주기 정지를 유도한다. 이는 수모화가 되지 않는 돌연변이체와는 달리 수모화가 될 수 있는 hnRNP-

K는 UV에 의해 p21의 프로모터에 동원되는 결과와 일치한다. 결론적으로 hnRNP-K의 수모화는 자체의 안정화를 증가시킬 뿐만 아니라 p53의 전사활성능력 또한 증진시킬 수 있다.

PIAS3는 ATR 의존적 경향으로 hnRNP-K의 수모 E3 결합 효소로써 작용한다. 그러나 수모 특이적 단백질 분해효소인 SENP2는 UV 처리후 늦은 시기에 hnRNP-K로부터 수모를 제거하고 이는 hnRNP-K의 불안정화를 가져옴으로써 세포 주기 정지로부터 빠져나오게 한다. UV 처리 후 hnRNP-K의 수모화와 안정화가 증가했다가 감소하는 것과 마찬가지로 hnRNP-K와 PIAS3간의 결합 역시 UV 처리 후 같은 시간대에서 증가했다가 감소하며 이는 SENP2와의 결합 정도와 반대 경향으로 나타난다. 결과적으로, PIAS3와 SENP2는 UV 처리 이후 hnRNP-K의 수모화와 안정화를 시간 과정별로 서로 상반되게 조절한다.

면역 세포 화학 분석에 의하면 수모화는 hnRNP-K의 핵내로의 위치 결정에 관여한다. 정상적인 hnRNP-K는 UV에 의해 오로지 핵에만 위치하지만 수모화가 되지 않는 돌연변이체의 경우 40% 정도가 UV

처리에도 불구하고 여전히 핵과 세포질에 위치한다. 게다가 PIAS3의 감소나 SENP2의 과다발현은 UV에 의한 과다발현된 hnRNP-K의 핵내 위치를 저해한다. 그러나 세포내 hnRNP-K의 핵내 위치의 경우, UV 처리나 PIAS3 감소가 어떠한 영향도 미치지 않는 것으로 보아 수모화는 hnRNP-K의 핵내 위치에 보조적인 역할을 하는 것으로 여겨진다. 요약하여 DNA의 손상에 의해 유도되는 hnRNP-K의 수모화는 p53에 의해 야기되는 세포 주기 정지 조절에 중요한 역할을 한다.

Key word: p53, HDM2 (human double minute 2), p21, PIAS3 (protein inhibitor of activated STAT 3), SENP2 (sentrin/sumo-specific protease 2), ubiquitin, hnRNP-K (heterogenous nuclear ribonucleoprotein-K), cell-cycle regulation

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