HIPK2 inhibits both MDM2 gene and protein by, respectively, p53-dependent and independent regulations

Valeria Di Stefano[a], Marina Mattiussi[a], Ada Sacchi[a], Gabriella D'Orazi[a,b,*]

[a] *Department of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Via della Messi d'Oro 156, 00158 Rome, Italy*
[b] *Department of Oncology and Neurosciences, University "G. D'Annunzio", 66013 Chieti, Italy*

Received 12 July 2005; revised 18 August 2005; accepted 7 September 2005

Available online 27 September 2005

Edited by Varda Rotter

Abstract We address here the involvement of the homeodomain-interacting protein kinase 2 (HIPK2)/p53 complex on MDM2 regulation following apoptotic DNA damage. Our results provide a plausible transcriptional (p53-dependent) and posttranscriptional (p53-independent) double mechanism by which HIPK2 accomplishes MDM2 downmodulation. First, in wtp53-carrying cells HIPK2-dependent p53Ser46 phosphorylation selectively inhibits MDM2 at transcriptional level. Secondly, HIPK2 interacts with MDM2 in vitro and in vivo and promotes MDM2 nuclear export and proteasomal degradation, in p53-null cellular context. This p53-independent effect is likely mediated by HIPK2 catalytic activity and we found that HIPK2 phosphorylates MDM2 in vitro. In response to DNA damage, depletion of HIPK2 by RNA-interference abolishes MDM2 protein degradation. We propose that HIPK2 contributes to drug-induced modulation of MDM2 activity at transcriptional (through p53Ser46 phosphorylation) and posttranscriptional (through p53-independent subcellular re-localization and proteasomal degradation) levels.

Keywords: HIPK2; p53; Phosphorylation; Proteasomal degradation; MDM2; Nuclear export

1. Introduction

P53 occupies a central position in an intricate stress' signalling network [1,2]. P53 is normally silent and kept at low concentration. DNA damage provokes p53 stabilization and activation as transcription factor for specific target genes [3]. P53 accumulation and activation are regulated through specific posttranslational modifications including phosphorylation, acetylation, ubiquitination, sumoylation, and protein/protein interaction [4]. Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase [5] whose functional importance was estimated by means of its interacting proteins and downstream phosphorylation targets, including p53. HIPK2 binds and phosphorylates p53 at Ser46 selectively inducing apoptosis in tumour cells [6,7]. Moreover, HIPK2 prevents the MDM2-mediated p53 cytoplasmic shuttling and ubiquitination, in vitro and in vivo, neutralizing p53 degradation and recovering its apoptotic function [8].

MDM2 is an oncoprotein whose transforming potential is activated by overexpression [9,10]. It is a negative regulator of p53, induces p53 nuclear export and proteasomal degradation, thus restraining its oncosuppressor functions [11–16]. The MDM2-negative regulation of p53 can be neutralized by partner proteins and by specific protein modifications. Several mechanisms to block MDM2 activity have been described, including the interaction with proteins such as ARF [17] or posttranslational modifications of either p53 or MDM2 [18,19]. DNA-damage activated kinases have been shown to contribute to destabilization of MDM2 and subsequent activation of p53 [20] and DNA-damage induced phosphorylation of N-terminus-p53 contributes to p53 stability by preventing MDM2 from degrading it [19,21,22].

Here, we show that HIPK2 regulates MDM2 at transcriptional and posttranscriptional levels, in p53-dependent and -independent ways, respectively. We found that (i) HIPK2-dependent p53Ser46 phosphorylation favours p53 detachment from MDM2 and inhibits p53-mediated MDM2 gene transcription; (ii) HIPK2 and MDM2 form a complex in vitro and in vivo that remains stable in response to DNA damage; (iii) the capacity of HIPK2 to phosphorylate MDM2 may underlie the observed translocation of MDM2 into the cytoplasm and its proteasomal degradation, in p53-null cellular context. These findings suggest that HIPK2 can act through different but overlapping routes with the same principal outcome, i.e., the interruption of the p53/MDM2 loop leading to induction of p53 oncosuppressor function.

2. Materials and methods

2.1. Cell culture, reagents, and transfection

Human embryonal kidney (Hek)293 (wtp53) cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technology, Grand Island, NY), RKO human colon cancer (wtp53), modified RKO-pSuper, and RKO-HIPK2-interfered (HIPK2i) [23], H1299 lung adenocarcinoma (p53 null) and H1299–HIPK2-IND (HIPK2-inducible) [24] cells were cultured in RPMI-1640 (Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL).

For DNA damage, subconfluent cells were irradiated with UV light at 50 J/m^2 and total cell extracts prepared at the indicated time after irradiation. Cisplatin was purchased from TEVA Pharma-Italia and used at 1.7 and 5 μg/ml for 12 h. Doxurubicin (adriamycin: ADR) was diluted into the medium to a final concentration of 2 μg/ml. To induce HIPK2 expression in H1299–HIPK2-IND cell line, Ponasterone A (PonA), a synthetic analog of ecdysone (Alexis Biochemicals, San

*Correspondending author. Fax: +0039 06 52662505.
E-mail address: dorazi@ifo.it (G. D'Orazi).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.09.008

Diego, CA) was added to the medium to a final concentration of 2.5 μm. Leptomycin B (LMB) was used to a final concentration of 10 nM, for 18 h.

Proteasome inhibitors MG132 (Biomol, Research Laboratories, Plymouth Meeting, PA, USA) and Epoxomicin (Biomol) [25] were prepared as, respectively, 50 mM and 1 mM stocks in DMSO, stored at −20 °C and diluted into the medium at the indicated concentrations.

Transient transfection assays were performed using the BES method as described earlier [7]. The amount of plasmid DNA was equalized in each sample by supplementing with empty plasmids.

2.2. Antibodies and plasmids

The antibodies used were: anti-p53 mouse monoclonal (Ab1801, DO1) (Santa Cruz Biotechnology), anti-phospho-p53Ser46 rabbit polyclonal (Cell Signaling Technology), anti-p53 sheep polyclonal (Ab7) (Oncogene Science), anti-PARP mouse monoclonal (BD PharMingen), anti-Flag mouse monoclonal (M5) (SIGMA, BIO-Sciences), anti-tubulin mouse monoclonal (SIGMA, BIO-Sciences), anti-HIPK2 rabbit antiserum (kindly provided by M.L. Schmitz, University of Bern, Switzerland), anti-MDM2 mouse monoclonal (Ab2, Santa Cruz), anti-GFP rabbit polyclonal antiserum (Amersham Corp., Arlington Heights, IL), anti-HA rat monoclonal (Roche Diagnostics, Monza, Italy), and anti-NFYB rabbit polyclonal (kindly provided by R. Mantovani, University of Milan, Italy). Immunoreactivity was detected by ECL chemiluminescence reaction kit (Amersham).

The expression vectors used in this study were: pCAG3.1-wtp53, p53S46A, and p53S15A (kindly provided by E. Appella, NIH, Bethesda, MD, USA); Flag2B, Flag–HIPK2, Flag–K221R, pGEX4T1, GST–HIPK2, pEGFP-C2, GFP-HIPK2, and GFP–K221R-kinase dead (KD) [7]; Flag–HIPK2ΔC, Flag–HIPK2ΔN [6] (kindly provided by T.G. Hofmann, Heinrich-Pette Institute for Experimental Virology and Immunology, University of Hamburg, Germany); human GST–Flag–MDM2 (kindly provided by A.L. Haas, Health Science Center, New Orleans, LA, USA); human HA-MDM2 (kindly provided by M. Oren, Weizmann Institute of Science, Rehovot, Israel). GFP–MDM2 fusion protein was obtained by subcloning human MDM2 into *Eco*RI and *Bam*HI sites of the pEGFP-C2 vector (Clontech).

2.3. GST pull downs

GST–HIPK2, GST–MDM2 and GST were expressed in *Escherichia coli* BL21 cells and purified on glutathione–sepharose beads (Amersham Biosciences) following standard procedures. For the GST pull-down assay, 293 cells transfected with HA-MDM2, Flag–HIPK2, Flag–HIPK2ΔC and Flag–HIPK2ΔN expression vectors were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 400 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.025% SDS) plus protease inhibitors (Complete, Roche), and centrifugated to precipitate cellular debris. Equal amounts of GST fusion proteins were incubated with 1.5 mg of total cell extracts for 2 h at 4 °C. Unbound proteins were removed by washing five times with HNET buffer (20 mM HEPES, 150 mM Na Cl, 0.1% Triton X-100, 10% glycerol) and the precipitates were resolved by SDS–PAGE. The immunoblots were probed with the indicated antibodies.

2.4. Immunoprecipitation, Western blot analysis and kinase assay

For co-immunoprecipitation (co-IP) of overexpressed proteins, 293 cells were transiently transfected with Flag–HIPK2, Flag–HIPK2ΔC, Flag–HIPK2ΔN (8 μg) and HA-MDM2 (6 μg) and harvested 36 h after transfection. Cells were then lysed as described above. Following preclearing for 1 h at 4 °C, immunoprecipitation was performed by incubating 1.5 mg of total cell extracts with anti-Flag antibody, rocking for 2 h at 4 °C. Immunocomplexes were collected by incubating with protein G–agarose (Pierce). The beads were then resuspended in 5× Laemmli buffer and subjected to Western blot with the indicated primary antibodies.

For subcellular fractionation, cells were trypsinized, rinsed with PBS and collected by centrifugation. Cells were then suspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) and placed on ice for 15 min. NP40 was added to a final concentration of 0.5%. Cells were spun top speed for 30 s before the supernatant (cytoplasmic fraction) was collected. The remaining pellet was washed with hypotonic buffer, resuspended in RIPA buffer, sonicated and spun at $15000 \times g$ for 15 min to remove debris and collect the supernatant (nuclear fraction). We confirmed the separation of the cytoplasmic and nuclear fractions by Western immunoblotting of tubulin (cytoplasmic marker) and NFYB (nuclear marker), respectively.

Kinase assay was performed essentially as described [7]. Immunocomplexes were incubated in kinase buffer in the presence of 5 μCi [γ-^{32}P] ATP and 2 μg MBP as substrate, for 30 min at 30 °C. Reaction products were resolved by SDS–PAGE and [γ-^{32}P]-labeled proteins were detected by autoradiography. Gels were then hydrated and stained with Comassie for proteins detection.

2.5. RNA extraction and RT-PCR analysis

RT-PCR analysis was performed as described [23]. 293 and H1299 cells were transfected with the indicated plasmids and total mRNA extracted using the RNeasy mini kit (Qiagen S.P.A., Milan, Italy). Reverse-transcription reactions and PCR assays were performed using the MuLV reverse transcriptase and the AmpliTaq DNA Polymerase (Gene Amp RNA PCR kit, Perkin–Elmer, Roche Molecular System, Brachburg, NJ, USA). cDNA was amplified using primers for *Mdm2* and *GAPDH*.

2.6. Chromatin immunoprecipitation analysis

RKO cells were treated with cisplatin (1.7 and 5 μg/ml) for 12 h. DNA and protein complexes were cross-linked in living cells for chromatin immunoprecipitation (ChIP) analysis as described previously [24]. Cell lysates were incubated with anti-p53 antibody (Ab7) and DNA bound to immunoprecipitates amplified with promoter-specific primers for *Mdm2*, *p21* and *AIP1*.

3. Results

3.1. HIPK2-mediated p53Ser46 phosphorylation downmodulates MDM2 gene expression

Others and we have recently found that, in the presence of p53, HIPK2 overexpression downmodulates MDM2 levels [8,26]. It is known that MDM2 levels are reduced after apoptotic DNA damage by different transcriptional and posttranscriptional mechanisms [18,20]. Here, we propose to study the molecular mechanisms mediating this regulation knowing that, following DNA damage, HIPK2-dependent p53-post-translational modifications select gene targeting [24]. We first performed Western blot of RKO cells treated with apoptotic dose of ADR. As shown in Fig. 1A, MDM2 levels strongly declined 24 h after treatment in control pSuper cells concomitantly to induction of p53Ser46 phosphorylation and PARP cleavage. On the contrary, MDM2 was not downmodulated in HIPK2i cells (Fig. 1A), confirming that HIPK2 plays a role in MDM2 regulation. To test whether MDM2 downmodulation was at the transcriptional level, depending on p53Ser46 phosphorylation, we transfected HIPK2 and the K221R (KD) expression vectors in 293 cells bearing endogenous wtp53. By RT-PCR analysis we observed a reduction of MDM2 mRNA only following HIPK2 expression (Fig. 1B), suggesting that the kinase activity of HIPK2 might play a role in MDM2 downmodulation. To evaluate whether HIPK2-mediated p53Ser46 phosphorylation was involved in this regulation, we transfected p53-null H1299 cells with HIPK2 in combination with wtp53, S46A, and S15A expression vectors. MDM2 mRNA levels were increased only by wtp53 itself and S46A transfected with HIPK2 (Fig. 1C) whereas they were not induced by wtp53 and S15A mutant in combination with HIPK2; in addition co-expression of HIPK2 and p53 did not reduce MDM2 mRNA expression as clearly as in 293 cells whereas the endogenous p53 can be more efficiently activated

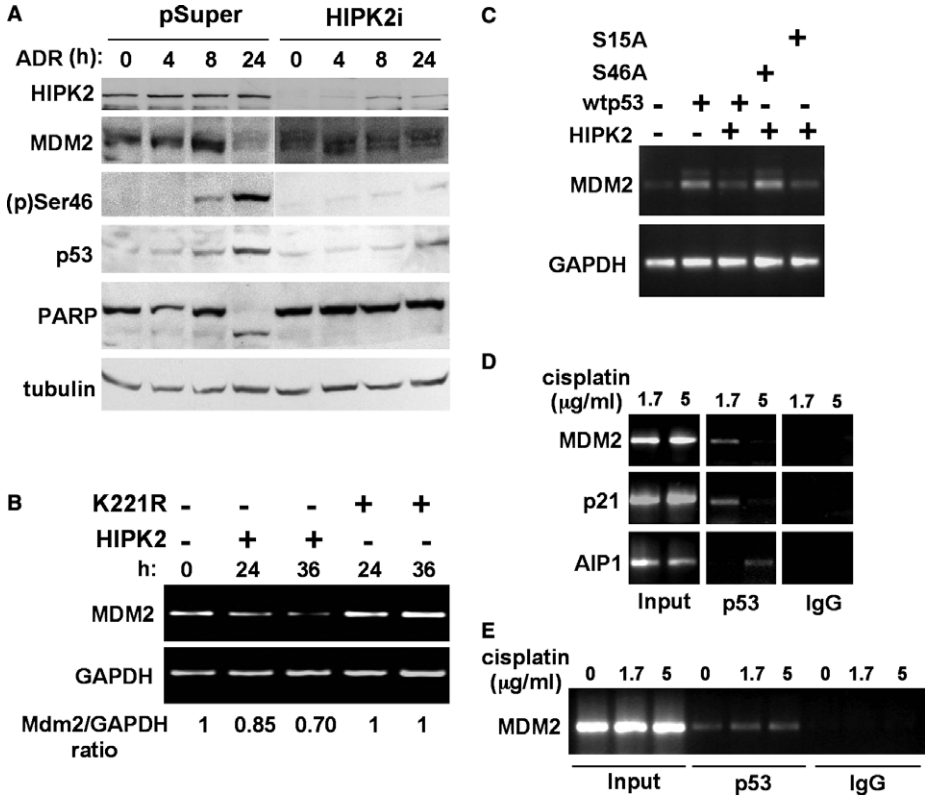

Fig. 1. MDM2 levels are transcriptionally regulated by HIPK2-dependent p53Ser46 phosphorylation. (A) Time-course Western blot analysis of RKO-pSuper and HIPK2i cells following ADR treatment (2 μg/ml). Total cell extracts were immunoblotted with the indicated antibodies. Tubulin expression is verified as protein loading control. (B) 293 cells were transfected with Flag–HIPK2 and Flag–K221R (8 μg) expression vectors. Total mRNA was isolated at the indicated time and analysed for MDM2 gene expression by RT-PCR. GAPDH expression was used as loading control. Densitometric analysis was performed and MDM2/GAPDH ratio is shown below. (C) H1299 cells were transfected with the indicated combinations of Flag–HIPK2 (8 μg), wtp53, p53S46A and p53S15A (2 μg) expression vectors. Total mRNA was isolated 36 h after transfection and analysed for MDM2 gene expression by RT-PCR. GAPDH expression was used as loading control (D) RKO cells were treated with cisplatin (1.7 and 5 μg/ml) for 12 h. Total cell extracts were subjected to ChIP analysis by using specific anti-p53 antibody (Ab7) and no specific IgG as control. Immunoprecipitates from each sample were analysed by PCR using specific primers for *Mdm2*, *p21*, and *AIP1* promoters. A sample representing linear amplification of the total input chromatin was included. (E) RKO-HIPK2i cells were treated as in (D) and subjected to ChIP analysis for p53 binding to *Mdm2* promoter.

by HIPK2 transfection. We have recently shown that HIPK2-mediated modifications (i.e., p53Ser46 phosphorylation or Lys320 acetylation) in different DNA damage conditions (i.e., cytostatic and cytotoxic doses of cisplatin), contribute to the selective activation of p53 target promoters (i.e., $p21^{Waf1}$ and *AIP1* genes) for, respectively, growth arrest and apoptosis [24]. Here, we further explored the functional significance of p53 promoter selection by ChIP analyses in RKO cells treated with cytostatic and cytotoxic doses of cisplatin. We show that p53 is bound to *Mdm2* promoter preferentially in cytostatic conditions, when the $p21^{Waf1}$ promoter is bound (Fig. 1D). On the contrary, p53 levels are strongly reduced on *Mdm2* promoter in cytotoxic conditions, when the *AIP1* promoter is bound. Furthermore, the relevance of HIPK2 for the differential levels of p53 binding to the *Mdm2* promoter was explored in HIPK2i cells. As shown in Fig. 1E, comparable levels of p53 binding to the *Mdm2* promoter were found following both conditions of DNA damage.

These results show that MDM2 downmodulation following apoptotic DNA damage is mediated, at least in part, at the transcriptional level and that HIPK2-mediated p53 phosphorylation is involved in this regulation.

3.2. HIPK2 and MDM2 interact in vitro and in vivo

In order to verify whether HIPK2 associates with MDM2, we performed GST pull-down assay followed by in vitro co-IPs. 293 cells were transfected with a vector encoding HA-tagged-MDM2. Cell lysates were mixed with either GST or HIPK2 fused to GST proteins. MDM2 was retained by GST–HIPK2 whereas no significant binding to GST alone was detected (Fig. 2A). The reciprocal experiment, transfecting 293 cells with Flag-full-length, C-terminal (ΔC), and N-terminal (ΔN) truncated HIPK2 expression vectors, showed that HIPK2 was efficiently retained by GST–MDM2 and that MDM2 binds to the C-terminal domain of HIPK2, but not to its N-terminal domain (Fig. 2B). This interaction was also confirmed by co-IP of overexpressed MDM2 and HIPK2 proteins whereas the HIPK2ΔN form bound MDM2 while the HIPK2ΔC form did not (Fig. 2C).

To gain insight into the mechanistic basis for the functional link between MDM2 and HIPK2, co-IP experiments were performed in vivo in control and HIPK2i cells. 293 cells were treated with UV at a dose (50 J/m^2) that allows HIPK2 binding to p53 and phosphorylation at Ser46 for apoptotic commitment [6,7] and harvested 16 h thereafter. As shown in Fig. 3A,

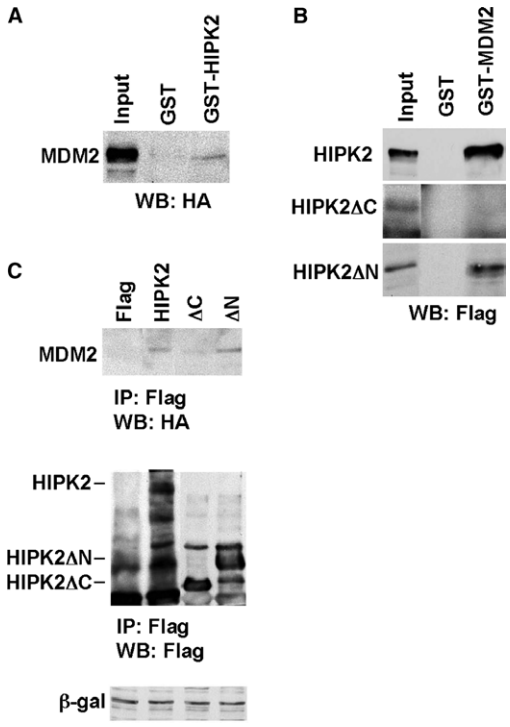

Fig. 2. Physical interaction between HIPK2 and MDM2. Bacterially expressed GST–HIPK2 (A), GST–MDM2 (B) and GST were incubated with 293 total cell extracts transfected with HA-MDM2 (A), Flag–HIPK2, Flag–HIPK2ΔC and Flag–HIPK2ΔN (B) expression vectors for in vitro pull-down assay. Protein/protein interaction was analysed by Western blot detection of HA-MDM2 and Flag–HIPK2 proteins. Input lanes: total cell extracts. (C) Co-IP of overexpressed proteins. 293 cells were transiently transfected with Flag–HIPK2, Flag–HIPK2ΔC, Flag–HIPK2ΔN (8 μg) and HA-MDM2 (6 μg) and harvested 24 h after transfection. Immunoprecipitation was performed by incubating 1.5 mg of total cell extracts with anti-Flag antibody, rocking for 2 h at 4 °C. The immunocomplexes were subjected to Western blot analysis with HA antibody for MDM2 detection. Comparable amounts of immunopecipitated HIPK2 proteins were ensured by anti-Flag immunoblotting. Anti-β-gal antibody was used on total cell extracts as control of transfection.

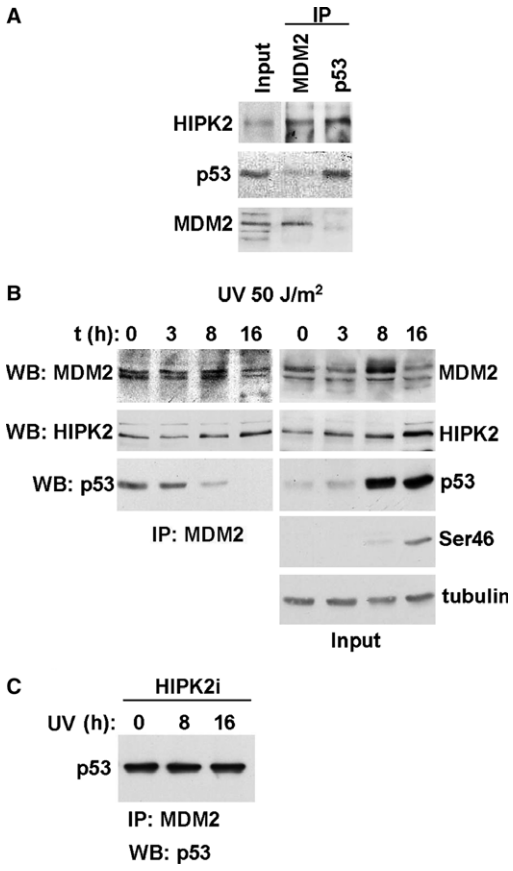

Fig. 3. Functional interaction between HIPK2 and MDM2. (A) Co-IP of endogenous proteins. 293 cells were irradiated with UV light at 50 J/m^2. Sixteen hours after irradiation, 500 μg of total cell extracts were immunoprecipitated with anti-MDM2 (Ab2) and anti-p53 (DO-1) antibodies. Immunocomplexes were separated by SDS–PAGE, blotted onto nitrocellulose membranes and probed with the indicated antibodies. (B) Same 293 cells as in (A) were lysed at the indicated time points after irradiation. 500 μg of total cell extracts were immunoprecipitated with anti-MDM2 (Ab2) and the immunocomplexes were subjected to Western blot with the indicated antibodies. Input lanes represent Western immunoblotting of total cell extracts. Anti- tubulin was used as protein loading control. (C) 293 cells, transiently transfected with HIPK2-interfering vector were irradiated with UV light at 50 J/m^2 and total cell extracts immunoprecipitated with anti-MDM2 (Ab2) 8 and 16 h thereafter. Immunocomplexes were separated by SDS–PAGE, blotted onto nitrocellulose membranes and probed with anti-p53 (Ab7) antibody.

IP-Western blot assay readily detected HIPK2 in both anti-MDM2 and anti-p53 immunocomplexes. Interestingly, MDM2 was no longer bound to p53. In order to verify whether p53, HIPK2, and MDM2 can exist as components of a multiprotein complex in vivo, we treated 293 cells with UV (50 J/m^2) and immunoprecipitated endogenous MDM2 at different time points after treatment. As shown in Fig. 3B, MDM2 was bound to both HIPK2 and p53 in basal condition; interestingly, MDM2 remained bound to HIPK2 during the entire treatment but it was no longer bound to p53 when the protein was phosphorylated at Ser46. Furthermore, in the absence of HIPK2 the amount of MDM2/p53 complexes remained unchanged upon UV irradiation (Fig. 3C).

These data indicate that HIPK2 and MDM2 can form a stable complex in vitro and in vivo and that, following apoptotic DNA damage, p53Ser46-phosphorylation coincides with the abolishment of p53/MDM2 physical interaction.

3.3. HIPK2 regulates MDM2 nuclear export

MDM2 consistently shuttles between the nucleus and the cytoplasm [14–16]. In order to verify whether HIPK2 was involved in that nuclear/cytoplasmic shuttling, we monitored exogenously expressed GFP–MDM2 fusion protein in p53-null H1299–HIPK2-IND cell line. GFP–MDM2 was found primarily within nuclei (Fig. 4A, *left panel*) while PonA treatment (Fig. 4B) caused efficient delocalization and degradation of MDM2 into the cytoplasm (Fig. 4A, *right panel*). The proportion of cells with MDM2-GFP nuclear and cytoplasmic staining was scored in the absence and in the presence of PonA treatment and plotted in Fig. 4C. Biochemical analysis of subcellular fractionation confirmed that HA-MDM2 protein was primarily detected in the nuclear compartment and that HIPK2 induction accumulated MDM2 into the cytoplasm (Fig. 4D). To find out whether HIPK2 kinase activity was involved in MDM2 cytoplasmic delocalization 293 cells were transfected with GFP–MDM2 expression vector along with Flag–HIPK2 or K221R mutant. We found that MDM2 was

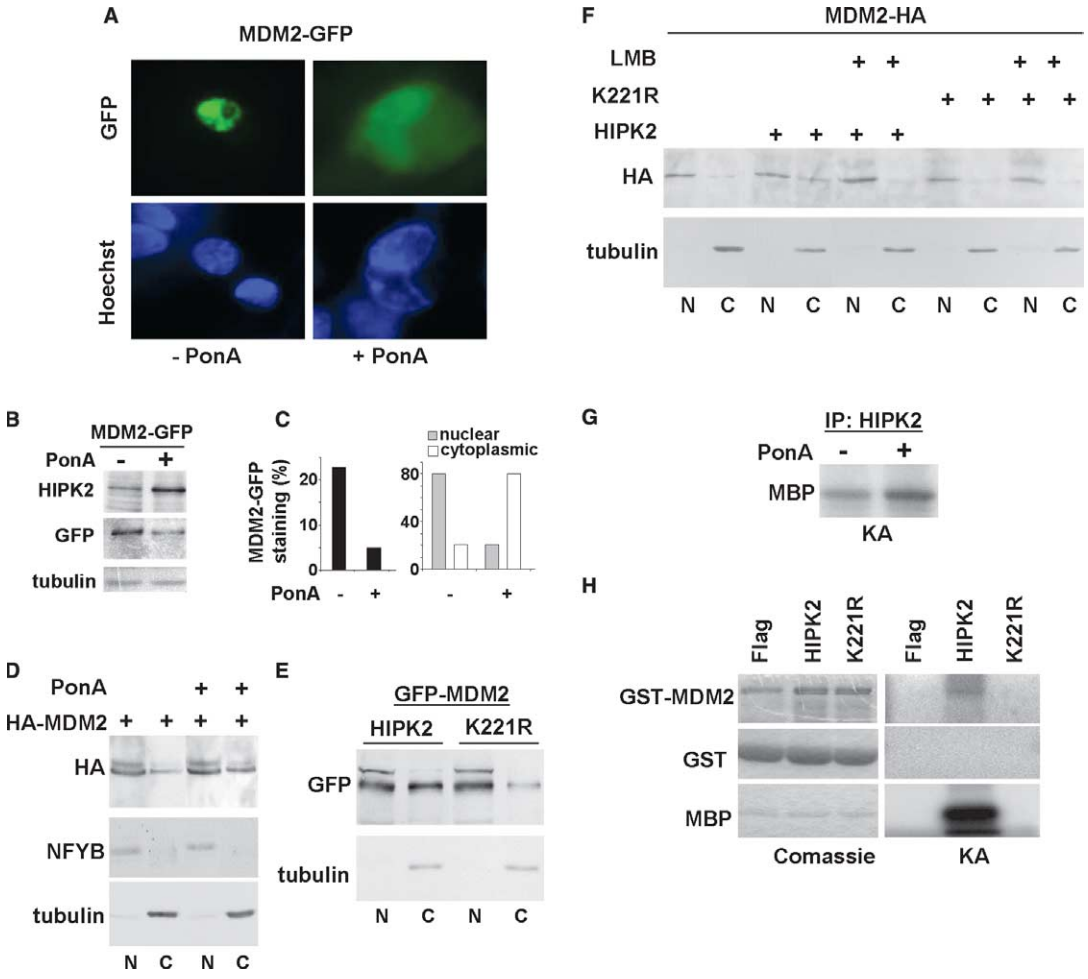

Fig. 4. HIPK2 regulates MDM2 nuclear export through its kinase domain. (A) P53-null H1299–HIPK2-IND cells (10^5) were plated in 35 mm Petri dishes and 24 h later transfected with GFP–MDM2 (4 μg) expression vector. Soon after transfection cells were trypsinized and replated in duplicate. Twenty-four hours after transfection, HIPK2 was induced by PonA (2.5 μM for 8 h) treatment. Cells were then fixed in 2% formaldehyde and stained with Hoechst. Subcellular localizzation of GFP–MDM2 fusion protein was analysed with a fluorescent microscopy. (B) H1299–HIPK2-IND cells were transfected with MDM2-GFP expression vector and 24 h later treated with PonA as in (A). Total cell extracts were subjected to Western blot analysis using anti-HIPK2 and anti-GFP antibodies. (C) Summary of MDM2-GFP staining and nuclear and cytoplasmic localization from three independent experiments. (D) H1299–HIPK2-IND cells plated in 60 mm Petri dishes were transfected with HA-MDM2 (6 μg) expression vectors. Twenty-four hours after transfection, HIPK2 was induced by PonA (2.5 μM for 8 h) treatment. The cells were collected, subjected to nuclear (N) and cytoplasmic (C) fractionation and analysed by Western blot with anti-HA antibody. Anti-tubulin and anti-NFYB antibodies were used to, respectively, control the cytoplasmic and nuclear fractions. (E) 293 cells were transfected with GFP–MDM2 (6 μg) in combination with Flag–HIPK2 and Flag–K221R (8 μg) expression vectors. Cells were harvested 24 hours after transfecion and nuclear (N) and cytoplasmatic (C) cell extracts analysed by Western blot of exogenous MDM2 protein using anti-GFP antibody. (F) 293 cells were transfected with HA-MDM2 (6 μg), in combination with Flag–HIPK2 and Flag–K221R (8 μg). The day after transfection LMB was added for 18 h at final concentration of 10 nM. Cells were then lysed for nuclear (N) and cytoplasmic (C) fractionation and analysed by Western blot using anti-HA antibody. (G) Kinase assay of endogenous HIPK2 catalytic activity in HIPK2-IND cells in the presence or absence of PonA. Equal amount of total cell extracts were subjected to immunoprecipitation using with anti-HIPK2 antibody and assayed for kinase activity using MBP protein as substrate. (H) 293 cells were transfected with 8 μg of plasmids encoding Flag–HIPK2 or Flag–K221R. Equal amount of total cell extracts were subjected to immunoprecipitation using anti-Flag monoclonal antibody. The immunocomplexes were incubated with bacterially expressed GST or GST–MDM2 fusion proteins and assayed for kinase activity in the presence of [γ-^{32}P] ATP (*right panel*). MBP was used as control substrate. Equal expression of each protein was confirmed on comassie-staining of kinase assay gel (*left panels*).

localized within both nuclear and cytoplasmic compartments following co-transfection with HIPK2, while co-transfection with K221R retained MDM2 into the nucleus (Fig. 4E). The role of HIPK2 catalytic activity in MDM2 subcellular distribution was confirmed by using the drug LMB that blocks the export of NES-containing proteins from the nucleus [27–29]. Consistent with the above results, Flag–HIPK2 over-expression moved exogenously expressed HA-MDM2 protein within the cytoplasm and LMB addition blocked this nuclear export; on the contrary K221R did not change MDM2 nuclear localization neither in the presence or absence of LMB (Fig. 4F). Moreover, HIPK2 was immunoprecipitated from H1299–HIPK2-IND cells in the presence or absence of PonA treatment and tested for in vitro kinase assay. As shown in Fig. 4G, HIPK2 phosphorylates its substrate MBP. We then tested the ability of HIPK2 to phosphorylate MDM2. 293 cells were transfected with Flag–HIPK2 and K221R expression vectors and anti-Flag immunoprecipitates incubated with GST and GST–MDM2 protein for in vitro kinase assay. Fig. 4H shows that HIPK2 phosphorylates GST–MDM2 in vitro.

These results suggest that the capacity of HIPK2 to regulate MDM2 expression, including subcellular distribution and degradation, might depend on its kinase activity.

3.4. HIPK2 downmodulates MDM2 via proteasomal degradation in p53-independent manner

It has been shown that HIPK2 overexpression, in the presence of p53, reduces MDM2 levels [8,26]. We here wanted to investigate whether HIPK2 promotes MDM2 proteasomal degradation. The effect of HIPK2 on endogenous MDM2 protein was first analysed in p53-null H1299–HIPK2-IND cells. Following PonA treatment, time-dependent MDM2 drop off was assessed concomitantly to HIPK2 upregulation (Fig. 5A). The MDM2 mRNA was not affected, as assessed by RT-PCR (Fig. 5B), indicating that, in this experimental condition (i.e., in the absence of p53), MDM2 downmodulation occurred at posttranslational level. To test whether the reduced MDM2 levels depended on proteasomal machinery, we used different specific proteasome inhibitors, including MG132 and Epoxomicin, in the presence or absence of PonA treatment. Both proteasome inhibitors increased MDM2 levels in greater extent after HIPK2 induction, as shown by densitometric analysis (Fig. 5C). In agreement with the above results, HIPK2 co-transfection induced HA-MDM2 degradation, compared to K221R mutant co-transfection, depending on proteasomal machinery as shown by MG132 treatment (Fig. 5D). This finding suggests that HIPK2 catalytic activity is involved in MDM2 proteasome degradation. To establish HIPK2 contribution to MDM2 proteasomal degradation following apoptotic DNA damage, we treated 293 cells, transiently transfected with HIPK2-interfering vector, with ADR. This treatment induced MDM2 downmodulation that was rescued at the protein level by MG132 proteasome inhibitor only in pSuper control cells (Fig. 5E).

Altogether, these results suggest that HIPK2 plays a role in MDM2 protein regulation also independently from p53 and that HIPK2 is essential for the drug-induced MDM2 downmodulation.

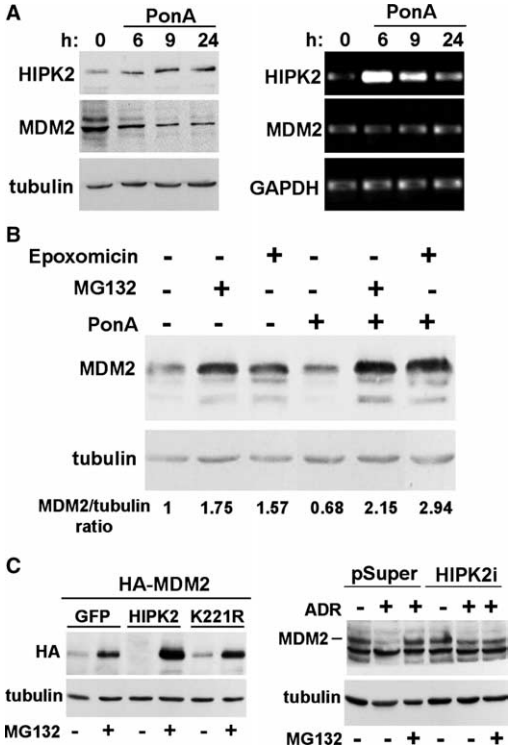

Fig. 5. HIPK2 downmodulates MDM2 protein levels via proteasomal degradation. (A) H1299–HIPK2-IND cells were treated with PonA and collected at the indicate time points. Equal amount of total cell extracts was separated on SDS–PAGE and immunoblotted with the indicated antibodies. Anti-tubulin was used as protein loading control. (B) Total mRNA from cells treated as in (A) was isolated and screened for expression of MDM2 and HIPK2 mRNAs by RT-PCR. GAPDH expression was used as control. (C) H1299–HIPK2-IND cells were transfected with p53 (2 μg) and 24 h later treated with PonA for 4 hours before adding proteasome inhibitors MG132 (25 μM) and Epoxomicin (10 nM) for 4 h. Equal amount of total cell extracts was subjected to Western immunoblotting with anti-MDM2 antibody. Anti-tubulin was used as protein loading control. MDM2/tubulin ratio, after densitometric analysis, is shown below. (D) 293 cells were transfected with HA-MDM2 (6 μg) in combination with GFP, GFP–HIPK2 and GFP–K221R (8 μg) expression vectors. Twenty-four hours after transfection MG132 (25 μM) was added for 4 h. Equal amount of total cell extracts was analysed by Western immunoblotting using anti-HA antibody. Anti-tubulin was used as protein loading control. (E) 293-pSuper and HIPK2i cells were treated with ADR (2 μg/ml) for 16 h before adding MG132 for 4 h. Equal amount of total cell extracts were analysed by Western blot with anti-MDM2 antibody. Anti-tubulin was used as protein loading control.

4. Discussion

The MDM2 protein is overexpressed in a significant number of human tumours underscoring its involvement in the development of this human disease [30,31]. The principal function of MDM2 is that of mediating p53 proteasomal degradation [12,13]. For this reason, the destabilization of MDM2 is required for p53 activation therefore inhibiting p53/MDM2 interaction provides a potentially significant approach for therapy in oncology [32]. Recent studies point out an important role for HIPK2 in apoptosis exerting its action on p53 oncosuppressor [6,7], p73 [33], and possibly other molecules involved in apoptosis in p53-independent manner [34,35]. We have previously shown that HIPK2 is able to rescue p53-transcriptional activity and apoptotic outcome overcoming MDM2-mediated proteasomal degradation [8]. Here, we improve this connection between p53 and HIPK2 by demonstrating that HIPK2 physically and functionally associates with MDM2 in vitro and in vivo. Although HIPK2 has been proposed to have many potential targets, an association with MDM2 has not previously been identified. We found that HIPK2 and MDM2 form a stable complex that, in response to apoptotic DNA damage, allows MDM2 detachment from phosphorylated p53Ser46. The end result of this action is HIPK2-dependent MDM2 destabilization obtained in two different but overlapping ways, i.e., MDM2 downmodulation by means of transcriptional and posttranscriptional control (Fig. 6). Previous studies have observed that diverse stresses can reduce MDM2 levels through both transcriptional and posttranscriptional mechanisms [36–39]. We found here that MDM2 gene is downmodulated at the transcriptional level only following HIPK2/p53Ser46 activation. These data further strengthen the significance of Ser46 site in p53 in promotion of promoters' specificity [40–43]. Indeed, p53Ser46phosphorylation by HIPK2 activates genes of the apoptotic program

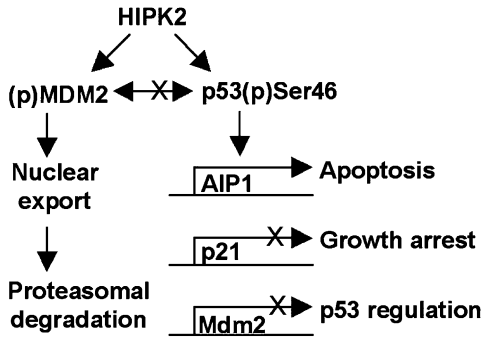

Fig. 6. Transcriptional (p53Ser46-dependent) and posttranscriptional (p53-independent) double mechanism by which HIPK2 accomplishes MDM2 downmodulation. HIPK2 (i) phosphorylates p53 at Ser46 and induces downmodulation of MDM2 trancription and (ii) binds and targets MDM2 for nuclear export and proteasomal degradation.

(i.e., AIP1), downmodulating its regulatory gene MDM2, as we show here.

Additionally, we found that HIPK2 promotes MDM2 nuclear export and proteasomal degradation. Because MDM2 shuttling to the cytoplasm regulates p53 protein levels in the cells [14–16], these data provide another mechanistic explanation of how HIPK2 regulates p53 oncosuppressor functions, maintaining it active in the nucleus as transcription factor [8].

Involvement of HIPK2 in MDM2 proteasomal degradation was supported by experiments using specific proteasome inhibitors and HIPK2-interfered cells. We found that MDM2 clearance was blocked both after HIPK2 induction and apoptotic DNA damage. MDM2 destabilization is likely to be controlled by phosphorylation at multiple sites and by multiple DNA-damage activated kinases [18,20]. Here, we show that HIPK2 phosphorylates MDM2 in vitro and that HIPK2 catalytic activity is important for MDM2 cytoplasmic translocation and proteasomal degradation. Thus, MG132 rescued MDM2 levels in greater extent after HIPK2 co-transfection. MDM2 is degraded by ubiquitin/proteasome pathway and it is also auto-ubiquitinated [18,44,45], whether HIPK2 accelerates MDM2 auto-ubiquitination needs to be elucidated. In similar manner, HIPK2 has been shown to promote apoptosis through phosphorylation and proteasomal degradation of CtBP corepressor [46]. Furthermore, HIPK2 binding to c-Myb, together with NLK, also results in c-Myb phosphorylation followed by its ubiquitination and proteasome-dependent degradation playing an important role in a variety of developmental steps [47]. The role of phosphorylation in ubiquitin-mediated protein degradation has been well established, thus ubiquitin E3 ligases specifically recognize phosphorylated target proteins [48,49]. In this regard, whether HIPK2 directly or indirectly phosphorylates MDM2 in vivo to target it for proteasomal degradation remains to be elucidated.

These findings indicate that the stabilization of p53 in response to apoptotic DNA damage is not likely to occur through a single pathway, but instead may involve multiple mechanisms. In this regard HIPK2 appears to be a promising target molecule for cancer therapy since it favours the interruption of the p53/MDM2 loop in both transcriptional (through p53Ser46) and posttranscriptional ways (through p53-independent subcellular re-localization and proteasomal degradation), leading to induction of apoptosis.

Acknowledgements: We thank all the people cited in the text for their generous gifts. We thank S. Soddu and P. Trivedi for critical reading of the manuscript. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), FIRB-MIUR, and Fondi Ateneo MIUR.

References

[1] Vogelstein, B., Lane, D. and Levine, A. (2000) Surfing the p53 network. Nature 408, 307–310.
[2] Giaccia, A.J. and Kastan, L.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev. 12, 2973–2983.
[3] Oren, M. (2003) Decision making by p53: life, death and cancer. Cell Death Diff. 10, 431–442.
[4] Appella, E. and Anderson, C.W. (2001) Post-translational modifications and activation of p53 by genotoxic stresses. Eur. J. Biochem. 268, 2764–2772.
[5] Kim, Y.H., Choi, C.Y., Lee, S., Conti, M.A. and Kim, Y. (1998) Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J. Biol. Chem. 273, 875–879.
[6] Hofmann, T.G., Moller, A., Sirma, H., Zentgraf, H., Taya, Y., Droge, W., Will, H. and Schmitz, M.L. (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat. Cell Biol. 4, 1–10.
[7] D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M. and Soddu, S. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser46 and mediates apoptosis. Nat. Cell Biol. 4, 11–19.
[8] Di Stefano, V., Blandino, G., Sacchi, A., Soddu, S. and D'Orazi, G. (2004) HIPK2 neutralizes MDM2 inhibition rescuing p53 transcriptional activity and apoptotic outcome. Oncogene 23, 5185–5192.
[9] Fakharzadeh, S.S., Trusko, S.P. and George, D.L. (1991) Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumour cell line. EMBO J. 19, 1565–1569.
[10] Finlay, C.A. (1993) The mdm2-oncogene can overcome wild-type p53 suppression of transformed cell growth. Mol. Cell. Biol. 13, 301–306.
[11] Momand, J., Zambetti, G.P., Olson, D.C., Gorge, D. and Levine, A.J. (1992) The Mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69, 1237–1245.
[12] Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) MDM2 promotes the rapid degradation of p53. Nature 387, 296–299.
[13] Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by MDM2. Nature 387, 299–303.
[14] Roth, J., Dobbelstein, M., Freedman, D.A., Shenk, T. and Levine, A.J. (1998) Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO J. 17, 554–564.
[15] Freedman, D.A. and Levine, A.J. (1998) Nuclear export is required for degradation of endogenous p53 by MDM2 and human papilloma virus E6. Mol. Cell. Biol. 18, 7288–7293.
[16] Tao, W. and Levine, A.J. (1999) Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. Proc. Natl. Acad. Sci. 96, 3077–3080.
[17] Michael, D. and Oren, M. (2003) The p53-MDM2 module and the ubiquitin system. Sem. Cancer Biol. 13, 49–58.
[18] Meek, D.W. and Knippschild, U. (2003) Posttranslational modification of MDM2. Mol. Cancer Res. 1, 1017–1026.
[19] Shieh, S.-Y., Ikeda, M., Taya, Y. and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 9, 325–334.
[20] Stommel, J.M. and Wahl, G.M. (2004) Accelerated MDM2 auto-degradation by DNA-damage kinases is required for p53 activation. EMBO J. 23, 1547–1556.
[21] Siliciano, J.D., Canman, C.E., Taya, Y., Sakaguchi, K., Appella, E. and Kastan, M.B. (1997) DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev. 11, 3471–3481.

[22] Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M. and Haupt, Y. (1999) Critical role for Ser20 of human p53 in the negative role of p53 by MDM2. EMBO J. 18, 1805–1814.
[23] Di Stefano, V., Rinaldo, C., Sacchi, A., Soddu, S. and D'Orazi, G. (2004) Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. Exp. Cell Res. 293, 311–320.
[24] Di Stefano, V., Soddu, S., Sacchi, A. and D'Orazi, G. (2005) HIPK2 contributes to PCAF-mediated p53 acetylation and selective transactivation of p21Waf1 after non-apoptotic DNA damage. Oncogene 24, 5431–5442.
[25] Meng, L., Mohan, L., Kwok, B.H.B., Elofsson, M., Sin, N. and Crews, C.M. (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc. Natl. Acad. Sci. USA 86, 10403–10408.
[26] Wang, Y., Bedatin, K.-M. and Hug, H. (2001) HIPK2 overexpression leads to stabilization of p53 protein and increased p53 transcriptional activity by decreasing Mdm2 protein. BMC Mol. Biol. 2, 8–16.
[27] Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 90, 1051–1060.
[28] Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature 390, 308, 311.
[29] Ossareh-Nazari, B., Bachelerie, F. and Dargemont, C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science 278, 141–144.
[30] Leach, F.S., Tokino, T., Meltzer, P., Burrell, M., Oliner, J.D., Smith, S., Hill, D.E., Sidransky, D., Kinzler, K.W. and Vogelstein, B. (1993) P53 mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res. 53, 2231–2234.
[31] Reifenberger, G., Liu, L., Ichimura, K., Schmidt, E.E. and Collis, V.P. (1993) Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Res. 53, 2736–2739.
[32] Chene, P. (2004) Inhibition of the p53-MDM2 interaction: targeting a protein/protein interaction. Mol. Cancer Res. 2, 20–28.
[33] Kim, E.-J., Park, J.-S. and Um, S.-J. (2002) Identification and characterization of HIPK2 interacting with p73 and modulating functions of p53 family in vivo. J. Biol. Chem. 277, 32020–32028.
[34] Zhang, Q., Yoshimatsu, Y., Hildebrand, J., Frisch, S.M. and Goodman, R.H. (2003) Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP. Cell 115, 177–186.
[35] Doxakis, E., Huang, E.J. and Davies, A.M. (2004) Homeodomain-interacting protein kinase-2 regulates apoptosis in developing sensory and sympathetic neurons. Curr. Biol. 14, 1761–1765.
[36] Alarcon, R., Koumenis, C., Geyer, R.K., Maki, C.G. and Giaccia, A.J. (1999) Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. Cancer Res. 59, 6046–6051.
[37] Ashcroft, M., Kubbutat, M.H. and Vousden, K.H. (1999) Regulation of p53 function and stability by phosphorylation. Mol. Cell. Biol. 19, 1751–1758.
[38] Inoue, T., Geyer, R.K., Yu, Z.K. and Maki, C.G. (2001) Downregulation of MDM2 stabilizes p53 by inhibiting p53 ubiquitination in response to specific alkylating agents. FEBS Lett. 490, 196–201.
[39] Wang, X., Michael, D., de Murcia, G. and Oren, M. (2002) Activation by nitric oxide involves down-regulation of Mdm2. J. Biol. Chem. 277, 15697–15702.
[40] Friedlander, P., Haupt, Y., Prives, C. and Oren, M. (1996) A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. Mol. Cell. Biol. 16, 4961–4971.
[41] Ryan, K.M. and Vousden, K.H. (1998) Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Mol. Cell. Biol. 18, 3692–3698.
[42] Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y. and Taya, Y. (2000) P53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102, 849–862.
[43] Mayo, L.D., Rok Seo, Y., Jackson, M.W., Smith, M.L., Rivera Guzman, J.R., Koegaonkar, C.K. and Donner, D.B. (2005) Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J. Cell. Biol., Apr 20 [Epub ahead of print].
[44] Chang, Y.C., Lee, Y.S., Tejima, T., Tanaka, K., Omura, S., Heintz, N.H., Mitsui, Y. and Magae, J. (1998) Mdm2 and Bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway. Cell Growth Diff. 9, 79–84.
[45] Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H. and Weissman, A.M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. 275, 8945–8951.
[46] Zhang, Q., Nottke, A. and Goodman, R.H. (2005) Homeodomain-interacting protein kinase-2 ediates CtBP phosphorylation and degradation inUV-triggered apoptosis. Proc. Natl. Acad. Sci. USA 102, 2802–2807.
[47] Kanei-Ishii, C., Ninomiya-Tsuji, J., Tanikawa, J., Nomura, T., Ishitani, T., Kishida, S., Kokura, K., Kurahashi, T., Ichikawa-Iwata, E., Kim, Y., Matsumoto, K. and Ishii, S. (2004) Wnt-1 signal induces phosphorylation and degradation of cMyb protein via TAK1, HIPK2, and NLK. Genes Dev. 18, 816–829.
[48] Busino, L., Chiesa, M., Draetta, G.F. and Donzelli, M. (2004) Cdc25A phosphatase: combinatorial phosphorylation, ubiquitylation and proteolysis. Oncogene 23, 2050–2056.
[49] Shmueli, A. and Oren, M. (2005) Life, death, and ubiquitin: taming the mule. Cell 121, 963–965.