Oncogene (2005) 24, 5431–5442

www.nature.com/onc

HIPK2 contributes to PCAF-mediated p53 acetylation and selective transactivation of p21^{Waf1} after nonapoptotic DNA damage

Valeria Di Stefano[1], Silvia Soddu[1], Ada Sacchi[1] and Gabriella D'Orazi*[,1,2]

[1]*Department of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy;* [2]*Department of Oncology and Neurosciences, University 'G. D'Annunzio', 66013 Chieti, Italy*

The p53 tumor suppressor gene is activated in response to DNA damage resulting in either growth arrest or apoptosis. We previously demonstrated the specific involvement of homeodomain interacting protein-kinase 2 (HIPK2), a nuclear serine/threonine kinase, in inducing p53-dependent apoptosis through selective p53 phosphorylation at serine 46 after severe genotoxic damage. Here we show that HIPK2 contributes to p53 regulation, independently from serine 46 phosphorylation upon nonapoptotic DNA damage such as that induced by cytostatic doses of cisplatin. We show that HIPK2 depletion by RNA interference inhibits p53 binding to the *p21^{Waf1}* promoter affecting its p53-induced transactivation thereby allowing cell proliferation. We found that nonapoptotic DNA damage induces p53 acetylation mediated by the HAT protein PCAF and this p53 post-translational modification is abolished by HIPK2 depletion. In this regard, we found that HIPK2 cooperates with PCAF to induce selectively p53 transcriptional activity toward the *p21^{Waf1}* promoter while depletion of either HIPK2 or PCAF abolished this function. These data show that HIPK2 regulates the p53 growth arrest function through its PCAF-mediated acetylation.
Oncogene (2005) **24,** 5431–5442. doi:10.1038/sj.onc.1208717; published online 9 May 2005

Keywords: HIPK2; p53; acetylation; chromatin immunoprecipitation; DNA damage; PCAF

Introduction

The type and dose of stress within a cell appears to dictate the outcome of cellular response, which is channeled into complex pathways mediating cell-cycle arrest or apoptosis (Gottlieb and Oren, 1996; Vogt Sionov and Haupt, 1999; Ashcroft *et al*., 2000). Both processes are major tumor suppressor functions of p53, which is induced by a variety of DNA-damaging agents as well as many other forms of cellular stress (Giaccia and Kastan, 1998; Ashcroft *et al*., 2000). DNA damage is widely believed to activate p53 as transcription factor through post-translational modifications and the modulation of specific target genes mediates most of the p53 oncosuppressor functions (Prives and Hall, 1999). Thus, p53-induced cell cycle arrest is mediated by the p21 cyclin-dependent kinase inhibitor (el Deiry *et al*., 1993), whereas p53-induced apoptosis is mediated, among others, by the Bcl2 family member Bax (Miyashita and Reed, 1995), and the mitochondrial proteins p53AIP1 (Oda *et al*., 2000a) and Noxa (Oda *et al*., 2000b). Despite the increasing number of p53 downstream target genes identified, it is still unclear how different post-translational modifications imposed by DNA damage on p53 may affect the selection of either cell cycle arrest or apoptosis target genes and therefore the choice between cytostasis and death.

A complex pattern of post-translational modifications including phosphorylation and acetylation was shown to occur and influence p53 functions in response to genotoxic damage (Sakaguchi *et al*., 1998; Appella and Anderson, 2000, 2001). Nevertheless, the comprehensive role of p53 phosphorylation and acetylation in response to DNA damage is still incompletely understood. After DNA damage, several kinases including Chk2 (Chehab *et al*., 2000; Hirao *et al*., 2000; Shieh *et al*., 2000), Cdk-activated kinase CAK (Ko *et al*., 1997), the phosphoinositol 3 kinase family members ATM (Banin *et al*., 1998; Canman *et al*., 1998), ATR (Tibbetts *et al*., 1999), and DNA-PK (Lees-Miller *et al*., 1992; Shieh *et al*., 1997) phosphorylate p53 at serine and threonine residues in the N-terminal and C-terminal domains and strongly contribute to its activation.

Acetylation occurs at the carboxyl terminus of p53, specifically in the regulatory regions surrounding the tetramerization domain (Gu and Roeder, 1997; Sakaguchi *et al*., 1998; Liu *et al*., 1999). The level of acetylation may be an important regulator of p53 function, since p53 deacetylation by overexpressed histone deacetylase-associated proteins compromises p53-induced cell cycle arrest and apoptosis (Luo *et al*., 2000). Furthermore, acetylation of p53 facilitates the recruitment of coactivators/HATs onto promoters of p53 responsive genes (Barlev *et al*., 2001). Phosphorylation of p53 N-terminal residues also permits the interaction of p53 with histone acetyl-transferases CBP/p300 and PCAF, which leads to acetylation of

*Correspondence: G D'Orazi; E-mail: dorazi@ifo.it
Received 22 November 2004; revised 25 February 2005; accepted 20 March 2005; published online 9 May 2005

the p53 C-terminus (Sakaguchi *et al*., 1998). This is followed by an increase in p53 stability and sequence-specific DNA-binding activity, both *in vitro* and *in vivo*, possibly due to conformational changes (Gu and Roeder, 1997; Sakaguchi *et al*., 1998; Espinosa and Emerson, 2001; Luo *et al*., 2003).

Others and we have recently shown that the homeodomain interacting protein-kinase 2 (HIPK2) specifically phosphorylates p53 at residue serine 46 (Ser46) thereby activating the p53 apoptotic function after severe DNA damage (D'Orazi *et al*., 2002; Hofmann *et al*., 2002; Di Stefano *et al*., 2004a). This finding has revealed a role for HIPK2 in the post-translational modification and selective regulation of p53 apoptotic function. Indeed, HIPK2 gene silencing efficiently reduced p53-mediated transcriptional activation of apoptotic target genes after severe DNA damage. The involvement of Ser46 phosphorylation in specifically regulating p53 to induce apoptosis has recently become evident. Thus, p53Ser46 phosphorylation induces a subtle change in p53 conformation and a stronger affinity for promoters of apoptosis-related genes such as Bax, PIG3, and p53AIP1 (Bulavin *et al*., 1999; Oda *et al*., 2000a). Furthermore, HIPK2 neutralizes the MDM2-mediated inhibition of p53 (Di Stefano *et al*., 2004b) recovering its transcriptional activity and apoptotic function, *in vitro* and *in vivo*, in agreement with a previously proposed model (Shieh *et al*., 1997). Interestingly, Ser46 phosphorylation does not participate in the intramolecular phosphorylation site interdependences of p53 protein, confirming the hypothesis that post-translational modifications of p53-specific sites, as well as the involvement of different kinases, may serve to integrate signals from multiple stress pathways (Saito *et al*., 2003).

Despite the HIPK2 role in the activation of p53-dependent apoptotic target genes, it was shown that HIPK2 might also induce the p53-dependent, growth arrest-related, *p21* target gene (Hofmann *et al*., 2002; Moller *et al*., 2003). Therefore, it is possible that HIPK2 is involved in the regulation of different p53-mediated cellular processes likely through different p53 post-translational modifications. In this regard, it was shown that, following UV damage, HIPK2-induced p53Ser46 phosphorylation is required for CBP-mediated acetylation at residue Lys382 and subsequent p53 transcriptional activity (Hofmann *et al*., 2002). Here we found that, in response to nonapoptotic genotoxic damage, such as that induced by cytostatic doses of cisplatin that do not phosphorylate p53 at Ser46, HIPK2 is required to selectively direct p53 recruitment onto the *p21* promoter. This recruitment is associated with p53 acetylation by the HAT protein PCAF and HIPK2 cooperates with PCAF to increase specifically p53 transcriptional activity toward the *p21* gene, while the proapoptotic *p53AIP1* gene is not affected. Consistent with a functional role of acetylation in this process, the p21 transcriptional activity of a p53 mutant in the PCAF target Lys320 (Liu *et al*., 1999) cannot be modified by either cisplatin treatment or PCAF overexpression. In addition, abrogation of the PCAF acetylase activity by expression of HAT-defective PCAF or PCAF depletion by specific small interfering RNAs (siRNA) abolishes the cisplatin-induced activation of the *p21* promoter. These results support a role for HIPK2 in regulating p53 response to nonapoptotic DNA damage that is independent from p53Ser46 phosphorylation and requires PCAF-mediated acetylation, driving p53 to transactivate selectively the growth arrest-related *p21* gene.

Results

Cellular response to different concentrations of genotoxic damage

The cellular response of RKO cells to increasing doses of cisplatin was compared to determine the drug concentration responsible for growth arrest or apoptosis. As shown in Figure 1a (upper panel), cell proliferation was inhibited starting from the lowest dose of cisplatin (1.75 μg/ml) used, whereas cell viability was strongly reduced starting from 5 μg/ml of cisplatin (lower panel). TUNEL assay confirmed that 5 μg/ml of drug markedly induced apoptosis (Figure 1b), while 1.75 μg/ml of drug failed to induce cell death even after 72 h of treatment, as shown by viability assays (Figure 1c).

Next, we evaluated HIPK2 expression and enzymatic activity. Western blot analysis shows that HIPK2 expression increased in a similar manner upon exposure to both concentrations of cisplatin (Figure 1d), as well as its catalytic activity (Figure 1e).

Taken together, these data indicate that the low (1.75 μg/ml) and high (5 μg/ml) concentrations of cisplatin chosen promote, respectively, cytostatic or cytotoxic effect, although they induce similar HIPK2 protein expression and enzymatic activity.

DNA damage-dependent selective activation of p53 target genes

To study the p53 biochemical pathways in response to cytostatic and cytotoxic doses of cisplatin, we performed immunoblot analysis of several p53 target genes. We found that 1.75 μg/ml of drug led to a strong induction of p21, while Bax and PARP were not affected, and p53 was slightly induced but not phosphorylated at Ser46 (Figure 2a). In contrast, 5 μg/ml of drug strongly induced Bax expression, p53 phosphorylation at Ser46, and PARP cleavage, whereas expression of p21 was slightly reduced (Figure 2a). Consistent with these observations, the transcription of the *p21* promoter was preferentially induced by the cytostatic dose of drug (Figure 2b, upper panel), whereas the apoptotic gene promoter p53AIP1 was efficiently induced only by the cytotoxic dose of drug (Figure 2b, lower panel).

To evaluate the selective *in vivo* recruitment of p53 onto target gene promoters, chromatin immunoprecipitation assay (ChIP) was performed and immunoprecipitated DNA was analysed by PCR using

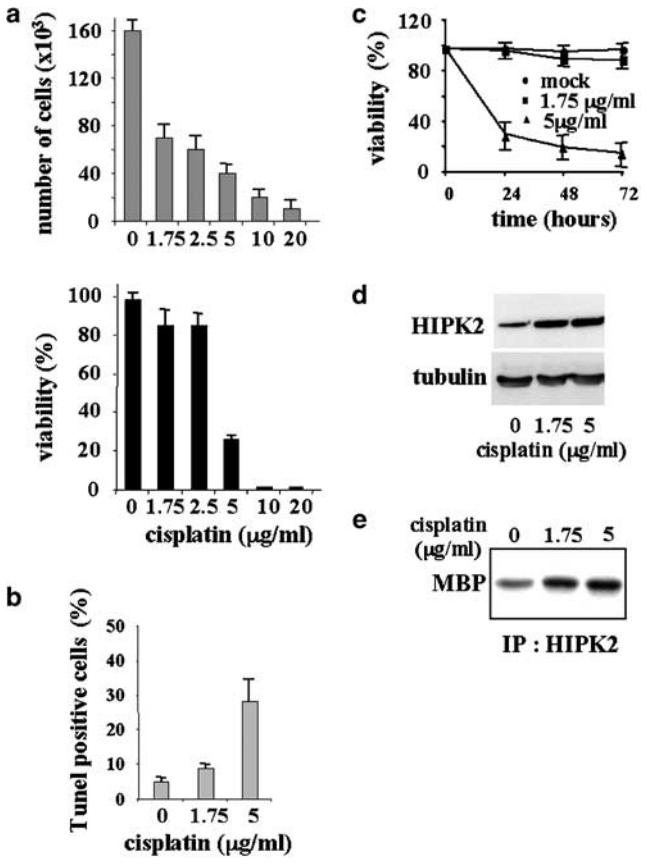

Figure 1 Cellular response to cisplatin treatment is dose-dependent. (**a**) Analysis of proliferation rate (upper panel) and cell viability (lower panel) of RKO cells treated with the indicated doses of cisplatin for 24 h. Data are presented as the mean of three independent experiments ±s.d. (**b**) Apoptosis analysed by TUNEL staining of RKO cells treated with the low (1.75 μg/ml) and high (5 μg/ml) doses of cisplatin for 36 h. The figure shows the percentage of TUNEL-positive cells presented as the mean of three independent experiments ±s.d. (**c**) Analysis of RKO cell viability after treatment with cisplatin for 24, 48, and 72 h, compared to the mock-treated control cells. Data are presented as the mean of three independent experiments ±s.d. (**d**) Expression of HIPK2 levels. RKO cells were treated for 24 h with cisplatin and total cell extracts were analysed by immunoblotting with specific anti-HIPK2 antibody. Anti-tubulin was used as protein loading control. (**e**) Equal amounts of total RKO cell extracts, treated with cisplatin (1.75 and 5 μg/ml for 24 h), were immunoprecipitated with polyclonal anti-HIPK2 antibody and assayed for kinase activity in the presence of [^{32}P]-ATP and MBP as substrate

promoter-specific primers for *p21*, *p53AIP1*, *Tk*, and *GAPDH* genes under conditions of linear amplification (Figure 2c). *GAPDH* and *Tk* promoters were used as control for specificity of p53 binding. We found that p53 was preferentially bound *in vivo* to the *p21* promoter in response to the cytostatic dose of drug and to the *p53AIP1* promoter in response to the cytotoxic dose of drug (Figure 2d).

These results indicate that the doses of cisplatin we selected for their functional outcomes (i.e., growth arrest or apoptosis) elicit distinct p53 biochemical pathways.

HIPK2 is required for cisplatin-induced p53 binding to p21 promoter

We evaluated whether HIPK2 plays a role in the p53-dependent p21 regulation upon nonapoptotic genotoxic damage. To this end, we used tumor cells interfered for HIPK2 function (HIPK2i) as confirmed by RT–PCR (Figure 3a, left panel) and Western blot (right panel) analyses. Next, luciferase assays showed that the induction of the *p21* promoter in response to cisplatin was strongly impaired by HIPK2 depletion in both RKO (Figure 3b, left panel) and 2008 (right panel) cells. In agreement, HIPK2 depletion resulted in a strong reduction of the p53 levels onto *p21* promoter, as compared to the pSuper control cells (Figure 3c). Importantly, HIPK2-depletion did neither affect p53 basal levels and stabilization after drug treatment nor the p53Ser15 phosphorylation that was induced by the cytostatic dose of cisplatin (Figure 3d). Furthermore, p21 mRNA and protein levels were analysed in HIPK2i cells after exposure to cisplatin. These results show that p21 mRNA (Figure 3e) and proteins (Figure 3f) were induced only in RKO-pSuper control cells, indicating that HIPK2 is required for this regulation.

To next evaluate whether HIPK2 was required for p53-dependent growth inhibition, HIPK2-interfered cells and their relative controls were treated with cisplatin and their colony-forming efficiency was evaluated. As shown in Figure 4a, a strong reduction in colony formation was found only in pSuper control cells. Accordingly, the pSuper cells showed a strong inhibition of cell proliferation in response to cisplatin compared to the mock-treated counterparts while the HIPK2-depleted cells continued to proliferate with a rate that almost reached that of the mock-treated cells (Figure 4b). Altogether, these findings underscore an involvement of HIPK2 in the regulation of growth arrest, following nonapoptotic DNA damage, through the impairment of p53 binding to the *p21* promoter and its transcriptional activation.

HIPK2-dependent PCAF-mediated acetylation of p53 specifically regulates p21^{Waf1} transcription

Together with p53 phosphorylation at Ser46, HIPK2 was shown to modulate p53 acetylation through CBP (Hofmann *et al*., 2002). Here, we investigated whether acetylation could affect HIKP2-mediated p53 activation in response to the cytostatic dose of drug. To this aim, endogenous p53 was immunoprecipitated from HIPK2-interfered cells and analysed by Western blotting with anti-acetyl-lysine antibody. As shown in Figure 5a, p53 was acetylated only in pSuper control cells treated with cisplatin, compared to the HIPK2-depleted ones, as also confirmed by densitometric analysis to quantify the ratio between the signal of acetylated p53 over total p53. In order to identify the p53 sites involved in this post-translational modification, we first performed a luciferase assay overexpressing mutant p53 proteins. H1299 cells were cotransfected with expression vectors encoding for wtp53, and nonacetylatable p53K320Q

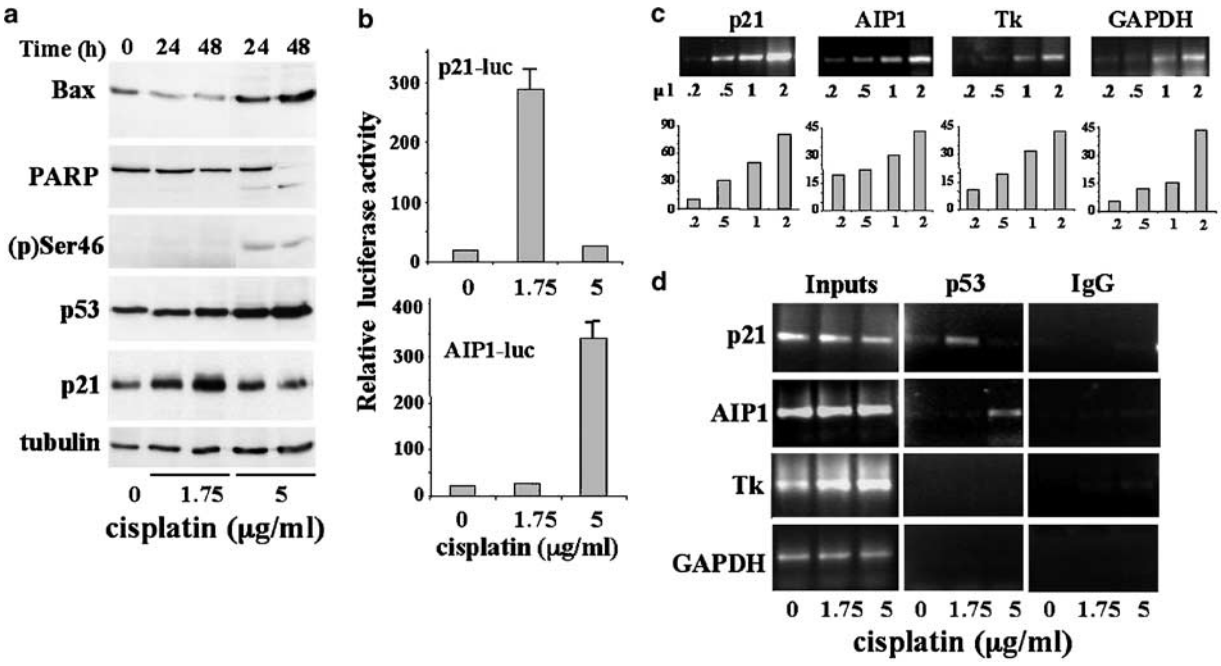

Figure 2 p53 target genes are activated dose-dependently after cisplatin treatment. (**a**) RKO cells were treated with the indicated doses of cisplatin for 24–48 h. Total cell extracts were separated on denaturing SDS–PAGE and analysed by immunoblotting with the indicated antibodies. Anti-tubulin was used as protein loading control. (**b**) p53 transactivation of stably transfected p21- and p53AIP1-luc reporters was compared in RKO cells, left untreated, or exposed to cisplatin for 24 h. Results are representative of three independent experiments performed in duplicate $\pm$s.d. (**c**, **d**) Lysates from RKO cells exposed to cisplatin for 12 h were subjected to ChIP using specific polyclonal anti-p53 antibody (Ab7) and no specific IgGs as control. (**c**) Increasing amounts (0.2, 0.5 1, 2 μl) of input samples were used as template in PCR amplifications with specific primers for *p21*, *p53AIP1*, *Tk*, and *GAPDH* promoters. (**d**) Immunoprecipitates from each sample were analysed by PCR using specific primers for the indicated promoters. A sample representing linear amplification (0.5–1 μl) of the total input chromatin was included

(Liu *et al*., 1999) and p53K382R (Gu and Roeder, 1997) proteins, along with p21-luc reporter. We found that p53K320Q mutant was not able to further activate the p21-luc reporter in response to cisplatin, compared to both the wtp53 and the p53K382R mutant (Figure 5b). We next overexpressed wtp53 and both mutants in H1299 cells to look at the extent of p53 acetylation after cisplatin treatment. P53 proteins were immunoprecipitated and analysed by Western blotting with anti-acetyl-lysine antibody. As shown in Figure 5c, an increase in acetylation was present in wtp53 and K382R mutant after treatment with cisplatin, while no changes in acetylation levels were observed in the K320Q mutant, suggesting that Lys320 is the target of acetylation in this experimental condition.

In vitro, p53Lys320 is acetylated by PCAF-HAT (Liu *et al*., 1999) and p53Lys382 is acetylated, among others residues such as lysine 373, by p300-HAT (Gu and Roeder, 1997). To determine whether PCAF affects p53-induced transactivation in response to cisplatin, H1299 cells were cotransfected with p21-luc reporter along with selected plasmids. As shown in Figure 6a, coexpression of PCAF with p53 further enhanced the reporter activity in the presence of cisplatin, while a histone acetyltransferase (HAT)-defective PCAF mutant (PCAFΔHAT) was unable to cooperate with p53 to increase transcription from the *p21* promoter. In agreement, the K320Q p53 mutant was not able to respond transciptionally to cotransfection with PCAF (Figure 6a). Interestingly, p53/PCAF cooperation was highly specific for the *p21* promoter since *p53AIP1* promoter was not induced (Figure 6b). The apoptotic dose of cisplatin (5 μg/ml, lane 6) was used as control of the p53-induced *AIP1* promoter activation. These results show that PCAF is a coactivator of p53-dependent transcription and that the HAT domain of PCAF is important for p53-induced transactivation. To directly support this finding, we abrogated PCAF acetylase activity by transfecting PCAF-specific or control siRNA together with p21-luc reporter in the presence or absence of drug. As shown in Figure 6c, PCAF siRNA abolished the cisplatin-induced activation of the *p21* promoter.

To investigate the role played by HIPK2 in the PCAF-mediated acetylation of p53, H1299 cells were first transfected with pSuper control and HIPK2-interfering vectors and then with the p21-luc reporter along with selected plasmids in the presence of cisplatin. We first found that HIPK2 depletion abolished the p53-dependent p21 transcription (Figure 6d). Furthermore, PCAF cotransfection could not overcome this effect, suggesting that HIPK2 acts upstream of PCAF and modulates its activity in the p53-mediated p21 transcription. To test whether p53 phosphorylation at Ser46 plays a role in the effect of HIPK2 on activation of p21

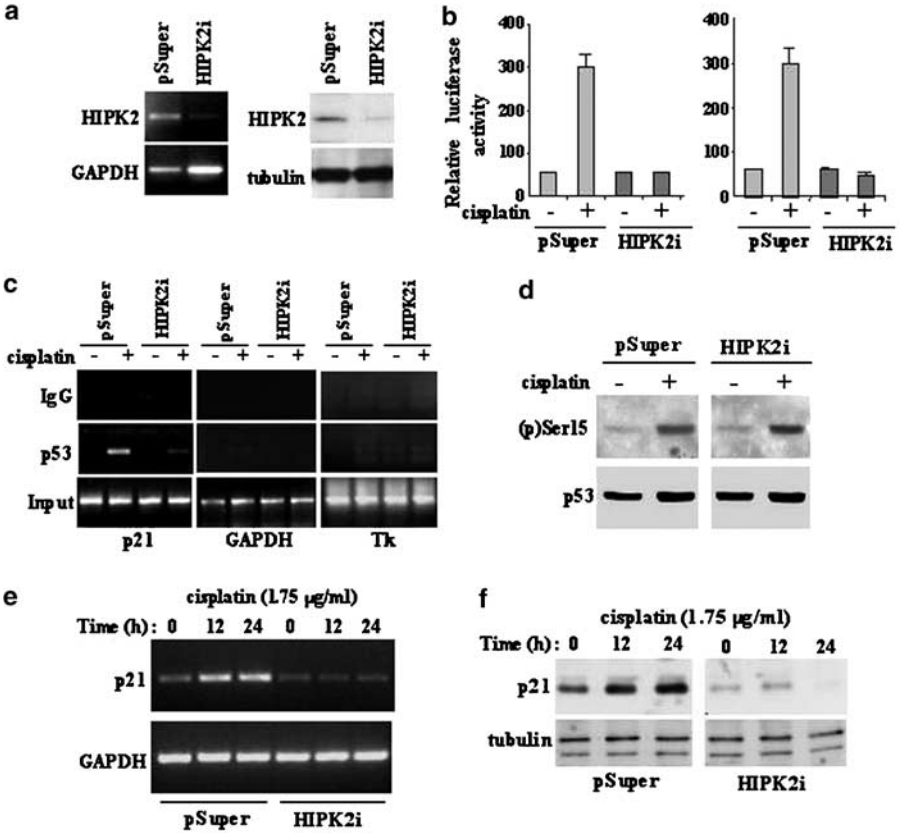

Figure 3 Inhibition of p21 transactivation by HIPK2-depletion. (**a**) RKO cells were transfected with pSuper control (pSuper) or HIPK2-interfering vectors (HIPK2i) and 72 h later RNA was isolated. Equal amounts of RNA were analysed by RT–PCR for expression of HIPK2 gene. GAPDH was amplified as control (left panel). Total cell extracts from 2008 ovarian cancer cells, transfected as above, were subjected to immunoblot blot analysis using specific anti-HIPK2 antibody to verify protein expression (right panel). Anti-tubulin was used as protein loading control. (**b**) HIPK2 depletion inhibits p21 transcription. RKO (left panel) and 2008 (right panel) cells, interfered for HIPK2 as above, were transfected with the p21-luc reporter, and treated with cisplatin (1.75 μg/ml). At 24 h after treatment, luciferase activity was determined following normalization to β-gal activity. Data are representative of three independent experiments performed in duplicate $\pm$s.d. (**c**) RKO-pSuper and HIPK2-interfered cells were treated with cisplatin (1.75 μg/ml) for 12 h and subjected to ChIP using polyclonal anti-p53 antibody (Ab7) and no specific IgGs as control. Immunoprecipitates from each sample were analysed by PCR using specific primers for *p21*, *GAPDH*, and *Tk* promoters. A sample representing linear amplification (0.5–1 μl) of the total chromatin was included as control. (**d**) Equal amounts of total RKO pSuper and HIPK2-interfered cell extracts, treated with the cytostatic dose of cisplatin (1.75 μg/ml for 24 h), were subjected to Western blot analysis to detect p53 and phospho-Ser15 protein levels. (**e**) RKO-pSuper and HIPK2-interfered cells were treated with cisplatin for 12 and 24 h and subjected to RT–PCR analysis to detect the levels of p21 gene expression. GAPDH was amplified as control. (**f**) Equal amounts of total cell extracts from the same cells as in (**e**) were subjected to Western blot analysis to detect p21 protein levels. Anti-tubulin was used as protein loading control

promoter, we performed a similar luciferase assay as above using p53Ser46A mutant instead of wtp53. We found that, in this experimental condition, Ser46A mutant was transcriptionally active as wtp53 and that HIPK2 depletion abolished this activation (Figure 6e).

Finally, we performed ChIP analysis using specific anti-p53Lys320 antibody. We found higher levels of p53 acetylated at Lys320 bound to the *p21* promoter in pSuper control cells compared to the HIPK2-interfered counterparts (Figure 6f, left panel). We also found that, p53 becomes selectively acetylated on Lys320 after growth-inhibitory dose of cisplatin, but not apoptotic dose of cisplatin (Figure 6f, right panel). These findings support our proposed model that acetylation on lysine 320 is responsible for the selective binding and activation of the p21 promoter only after nonapoptotic dose of cisplatin.

To further analyse the involvement of HIPK2 in regulating PCAF/p53 cooperation, we generated cell clones carrying transcriptionally inducible HIPK2 protein. This system, together with the expression of low levels of exogenous p53, allows the induction of growth arrest rather than of apoptosis, which we usually detect upon exogenous HIPK2 expression. H1299 cells, stably transfected with the pVgRXR vector (Wang *et al*., 1998), were transfected with the pIND-HIPK2 vector followed by G418 selection. Several clones were isolated and Figure 7a shows the induced HIPK2 expression in two different selected clones, compared to the endogenous HIPK2 basal levels without transcriptional

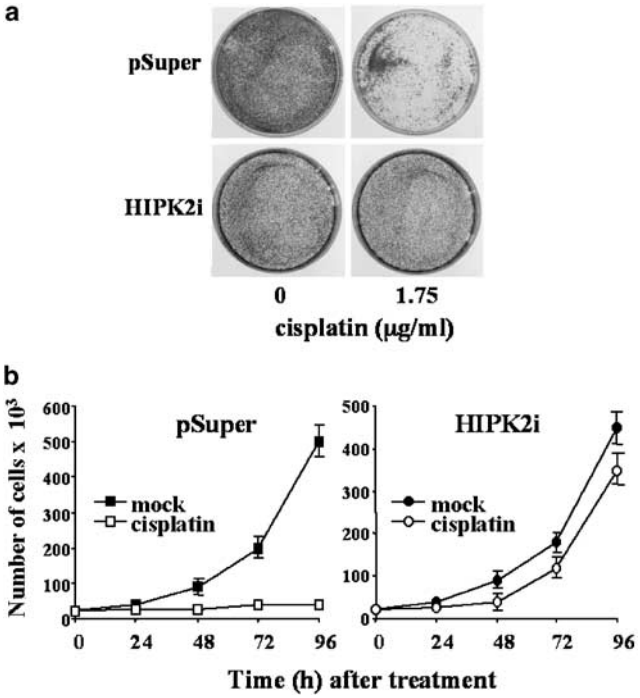

Figure 4 HIPK2 is required for cisplatin-mediated growth arrest. (**a**) RKO-pSuper and HIPK2-interfered cells were treated with cisplatin for 2 h and subsequently reefed with fresh medium. Death-resistant colonies were stained with crystal violet one week later. (**b**) Analysis of proliferation rate of the same cells treated with the cytostatic dose of cisplatin (1.75 μg/ml), for the indicated periods of time, compared to mock-treated controls

induction. Next, these clones were cotransfected with p21- and p53AIP1-luc reporters, along with expression vectors encoding for p53, PCAF, and PCAFΔHAT proteins, with or without HIPK2 induction. As shown in Figure 7b, HIPK2 cooperates with PCAF and p53 in increasing the transcriptional activity of the *p21* promoter, whereas the PCAF mutant devoid of acetylase activity (PCAFΔHAT) fails to do so. The effect was highly specific because neither PCAF nor HIPK2 cooperate with p53 to activate the proapototic p53AIP1-luc promoter (Figure 7c).

To get insight into the mechanisms leading to HIPK2-mediated, PCAF-induced acetylation of p53, we started investigating whether HIPK2 and PCAF colocalize in the nuclear compartment. To this aim, we transfected pSuper control and HIPK2-interfered cells with PCAF-Flag expression vector. At 24 h after transfection, cells were treated with cisplatin (1.75 μg/ml for 12 h) and analysed by indirect immunofluorescence. Interestingly, PCAF showed changed distribution in HIPK2-interfered cells, compared to the pSuper control counterparts, with an increased cytoplasmic pattern (Figure 8a, Flag panel), suggesting that HIPK2 is involved in the subcellular distribution of PCAF. Next, to test the involvement of HIPK2 catalytic activity in regulating PCAF nuclear localization, 293 cells were cotransfected with PCAF-Flag along with HIPK2-GFP and K221R-GFP (kinase-dead mutant) expression vectors and

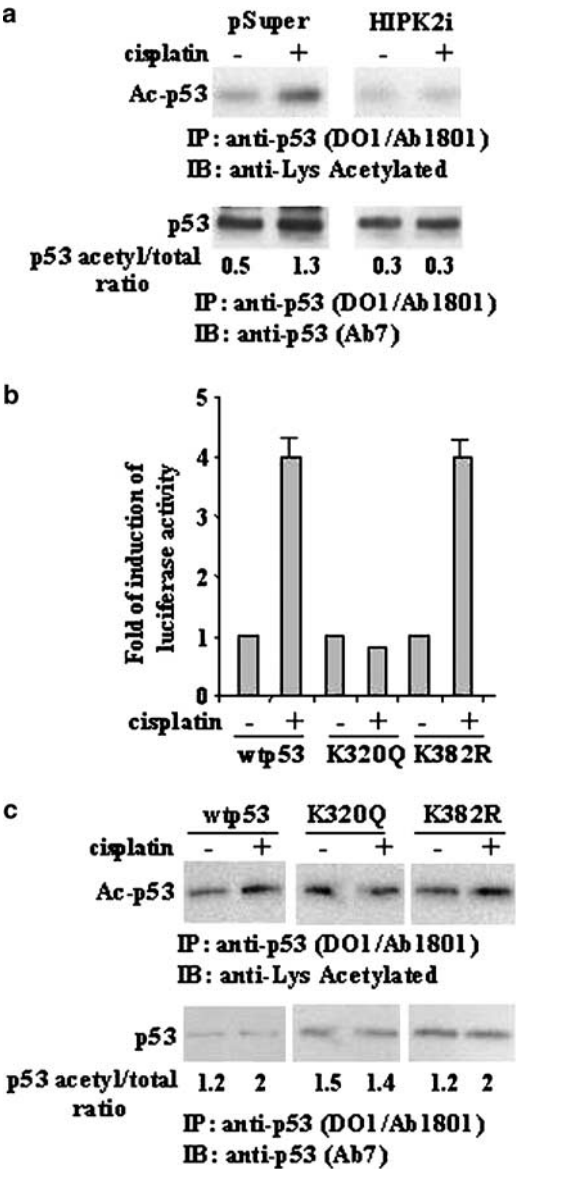

Figure 5 HIPK2 is required for p53 acetylation. (**a**) Cisplatin-induced p53-acetylation is abrogated in HIPK2-depleted cells. RKO-pSuper and HIPK2-interfered (HIPK2i) cells were treated with cisplatin (1.75 μg/ml for 24 h). Equal amounts of total cell extracts were subjected to immunoprecipitation with anti-p53 antibodies (a mix of DO1 and Ab1801) and Western blot analysis with anti-lysine acetylated antibody (upper panel). The same filter was reprobed with polyclonal anti-p53 antibody (Ab7) (lower panel). (**b**) Cisplatin treatment does not potentiate the transcriptional activity of nonacetylatable p53K320Q mutant. H1299 cells were cotransfected with p21-luc reporter and the indicated p53 mutants (50 ng), in the presence or absence of cisplatin (1.75 μg/ml). Cells were harvested 24 h after drug treatment, lysed, and assayed for luciferase activity following normalization to β-gal activity. Results are shown as fold of induction with respect of untreated control cells of three independent experiments performed in duplicate $\pm$s.d. (**c**) H1299 cells were transfected with wtp53 and the indicated mutants (1 μg) and 24 h later treated with cisplatin (1.75 μg/ml for 18 h). Equal amounts of total cell extracts were subjected to immunoprecipitation with anti-p53 antibodies (a mix of DO1 and Ab1801) and Western blot analysis with anti-lysine acetylated antibody (upper panel). The same filter was reprobed with polyclonal anti-p53 antibody (Ab7) (lower panel)

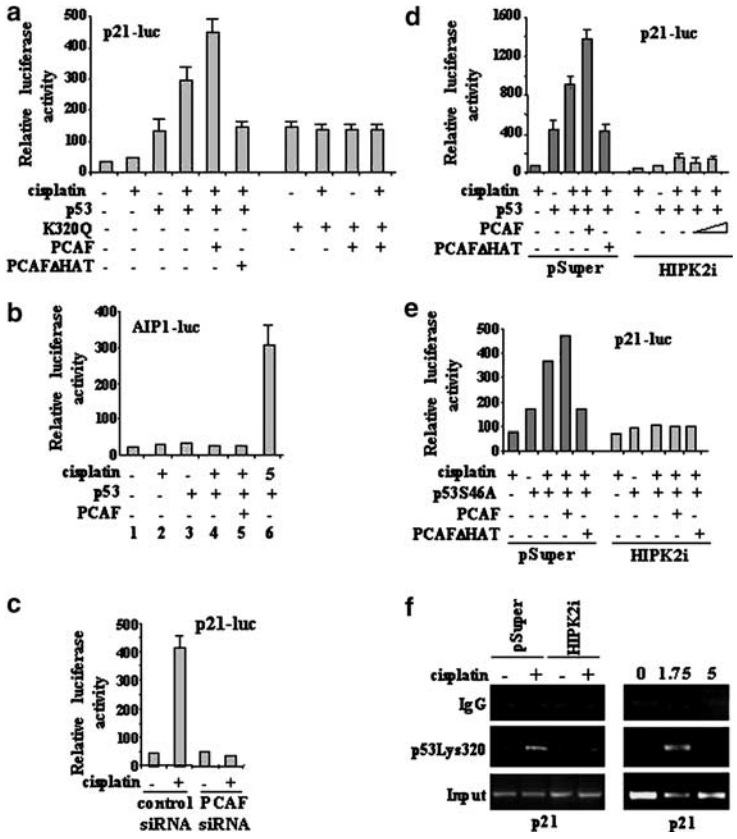

Figure 6 p53-transcriptional activity by PCAF. (**a**) H1299 cells were cotransfected with p21-luc reporter, and expression vectors encoding for p53 (50 ng), K320Q (50 ng), PCAF (250 and 500 ng), and PCAFΔHAT (500 ng) proteins, treated with cisplatin (1.75 μg/ml) for 24 h and assayed for luciferase activity. Results, normalized to β-gal activity, are representative of three independent experiments performed in duplicate $\pm$s.d. (**b**) H1299 cells were cotransfected with p53AIP1-luc reporter vector and the selected expression vectors, treated with the cytostatic (1.75 μg/ml, lanes 2, 4, 5), and the cytotoxic doses of cisplatin (5 μg/ml, lane 6), and assayed for luciferase activity. (**c**) Selective degradation of PCAF by specific siRNA prevents *p21* promoter activation in response to cisplatin. RKO cells were first transfected with 0.5 μg of either PCAF siRNA or control siRNA and then with p21-luc reporter in the presence or absence of cisplatin (1.75 μg/ml for 24 h) and assayed for luciferase activity. (**d**) HIPK2 is required for the cisplatin-induced PCAF-mediated p53-transcriptional activity. H1299 cells were first transfected with pSuper control and HIPK2-interfering vectors and then with the p21-luc reporter along with selected plasmids in the presence of cisplatin (1.75 μg/ml for 24 h) and assayed for luciferase activity. (**e**) Same experiment as in (**d**) using p53Ser46A mutant. (**f**) RKO pSuper and HIPK2-interfered cells were left untreated or treated with cisplatin (1.75 μg/ml for 12 h, left panels) and subjected to ChIP using polyclonal anti-p53Lys320 antibody and no specific IgGs as control. RKO cells were left untreated or treated with the apoptotic dose of cisplatin (5 μg/ml for 12 h, right panels) and subjected to ChIP as above. Immunoprecipitates from each sample were analysed by PCR using specific primers for the *p21* promoter

analysed by indirect immunofluorescence. We found that HIPK2 was mostly in the nuclear compartments as dots (D'Orazi *et al*., 2002; Hofmann *et al*., 2002) (Figure 8b, upper panels, GFP panel) and that the almost exclusively nuclear staining of PCAF (Flag panel) showed a strong overlapping with HIPK2 (Merge panel). Similarly, also the K221R mutant showed overlapping nuclear staining with PCAF (Figure 8b, lower panels) suggestig that HIPK2 but not its catalytic activity is important for PCAF nuclear localization.

Finally, we performed ChIP analysis in pSuper and HIPK2-interfered cells transfected with PCAF-Flag expression vector and treated with cisplatin. The immunoprecipitated DNA using anti-Flag and anti-acetylated Histone H4 antibodies was analysed by PCR using primers for *p21* promoter. Consistent with the data previously shown, PCAF was preferentially recruited onto the *p21* promoter in pSuper control cells, compared to the HIPK2-interfered cells (Figure 7c). PCAF-transfected pSuper control cells also showed higher levels of acetylated histone H4, in response to cisplatin, compared to the HIPK2-interfered counterparts (Figure 7c).

Taken together, these data indicate that HIPK2 plays a role in p53 activation upon nonapoptotic DNA damage favoring PCAF nuclear localization and contributing to PCAF-mediated p53Lys320 acetylation to selectively transactivate *p21* promoter.

Discussion

To investigate the involvement of HIPK2 in regulating p53 functions, we analysed the p53 response to different degrees of DNA damage using the chemotherapeutic

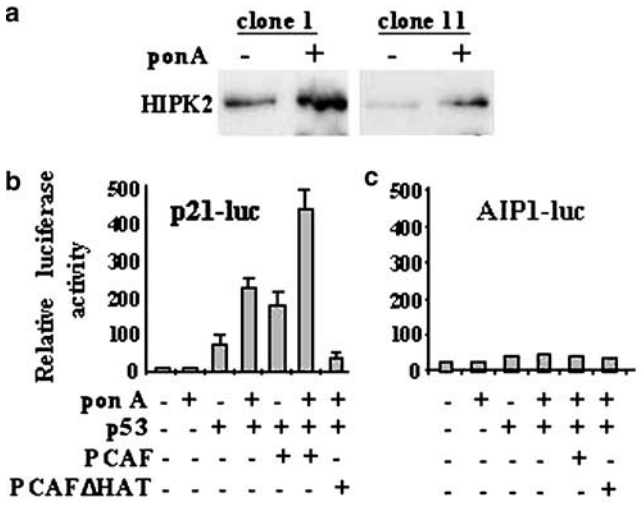

Figure 7 HIPK2-inducible expression selectively affects p53 activity. (**a**) Immunoblot analysis of ponasterone A-induced HIPK2 in H1299 cells. Total cell extracts from two selected clones incubated in the presence or absence of ponasterone A (2.5 μM for 8 h) were immunoblotted with specific anti-HIPK2 antibody. (**b**, **c**) HIPK2-induced expression selectively drives p53 transcriptional activity. H1299-HIPK2-inducible cells were cotransfected with p21- (**b**) and p53AIP1-luc (**c**) reporters and expression vectors encoding for p53 (50 ng), PCAF (500 ng), and PCAFΔHAT (500 ng) proteins. At 24 h after transfection, cells were induced with ponasterone A (2.5 μM for 8 h) and assayed for luciferase activity

drug cisplatin. Here we show that in nonapoptotic conditions, HIPK2 contributes to p53 activation, independently from Ser46 phosphorylation, by directing the PCAF-mediated p53Lys320 acetylation and selective transcription of p21 cell cycle inhibitor.

In response to nonapoptotic DNA damage, we detected the selective p53 binding to *p21* promoter, *in vivo*, while the proapoptotic *p53AIP1* target gene promoter was bound by p53 only in apoptotic conditions. We found that HIPK2-depletion impairs p53 transcriptional activity and that cisplatin-induced p53 acetylation, mediated by PCAF, is important for specifying the p53 transcriptional potential. Indeed, the finding that p53 is no longer acetylated nor transcriptionally active in HIPK2-depleted cells strongly suggests that additional HIPK2-dependent pathways, other than p53Ser46 phosphorylation known to activate p53 apoptotic function in the presence of severe DNA damage (D'Orazi *et al*., 2002; Hofmann *et al*., 2002; Di Stefano *et al*., 2004a, b), are required to activate p53 in response to nonapoptotic, genotoxic damage. In the latter condition, we found that: (1) only the p53K320Q mutant is unable to increase the transcriptional activity of the *p21* promoter; (2) the levels of p53 acetylated at Lys320 increase onto the *p21* promoter *in vivo*; (3) the HAT-PCAF able to acetylate p53 at Lys320 *in vitro,* cooperates with p53 and HIPK2 in the activation of the *p21* promoter; (4) depletion of PCAF by siRNA abrogates the drug-induced transcriptional activity of the *p21* promoter; and (5) HIPK2 is involved in maintaining PCAF nuclear localization. Altogether, these results strongly suggest that HIPK2 regulates p53-induced *p21* transcription by promoting PCAF-mediated p53 acetylation at Lys320. The molecular mechanisms by which HIPK2 regulates this post-translational modification are still unclear and need to be elucidated. One hypothesis is that the HIPK2 catalytic activity we detected following nonapoptotic DNA damage (Figure 1e) could directly or indirectly modify PCAF to promote p53-acetylation. We could not find direct phosphorylation of PCAF by HIPK2 by either using the commercially available anti-phospho-serine-threonine antibodies or performing kinase assay using HIPK2 immunocomplexes and GST-PCAF as substrate (data not shown). On the other hand, we have found that HIPK2 as well as its kinase-dead mutant are important in maintaining the nuclear localization of PCAF, suggesting that HIPK2 catalytic activity might be involved in other modifications of PCAF such as acetylation, as also suggested for regulation of other proteins (i.e., p53) (D'Orazi *et al*., 2002; Hofmann *et al*., 2002). In HIPK2-depletd cells, PCAF overexpressed is shown as diffuse, nuclear, and cytoplasmic staining, suggesting its impairment to function as HAT on nuclear proteins.

PCAF is a coactivator that enhances the activity of numerous other activators and proteins involved in transcription including MyoD (Sartorelli *et al*., 1999), E2F1 (Martinez-Balbas *et al*., 2000; Pediconi *et al*., 2003), and the architectural protein HMGA-1 (Munshi *et al*., 2001). PCAF can function alone or together with p300/CBP coactivators depending on the target promoters. Interestingly, the HAT activity of p300/CBP and PCAF is often differentially used in transcription activation. It has been shown that PCAF acetylates p53 *in vitro* at Lys320, a residue distinct from Lys382, one of those acetylated by p300, and thereby increases p53's ability to bind to its cognate DNA site (Liu *et al*., 1999). We found that PCAF cooperates with p53 in specifically increasing the transcription of the *p21* promoter. Interestingly, the lack of p53 acetylation in HIPK2-depleted cells might account for the impaired p53 activity since PCAF-mediated acetylation was shown to recruit transcriptional coactivators to p53-dependent promoters (Barlev *et al*., 2001). The difference in p53 post-translational modifications, in response to different degrees of genotoxic damage, represents an important step in the elucidation of the signaling pathways that regulate p53. However, the significance of particular post-translational modifications in mediating p53 cellular activities is still unclear and controversial. In particular, the role of p53 acetylation in the regulation of the *p21* gene has not been completely clarified (Prives and Manley, 2001), although, as previously shown, PCAF and CBP have clearly distinct specificities for p53 acetylation *in vitro*. A possible explanation for distinct acetylation by different enzymes is that it allows p53 to respond to different activating signals (Liu *et al*., 1999).

Another novel finding from our studies is that the role of p53Lys320 acetylation, in regulating the transcription of p53 target genes, appears to be promoter-specific

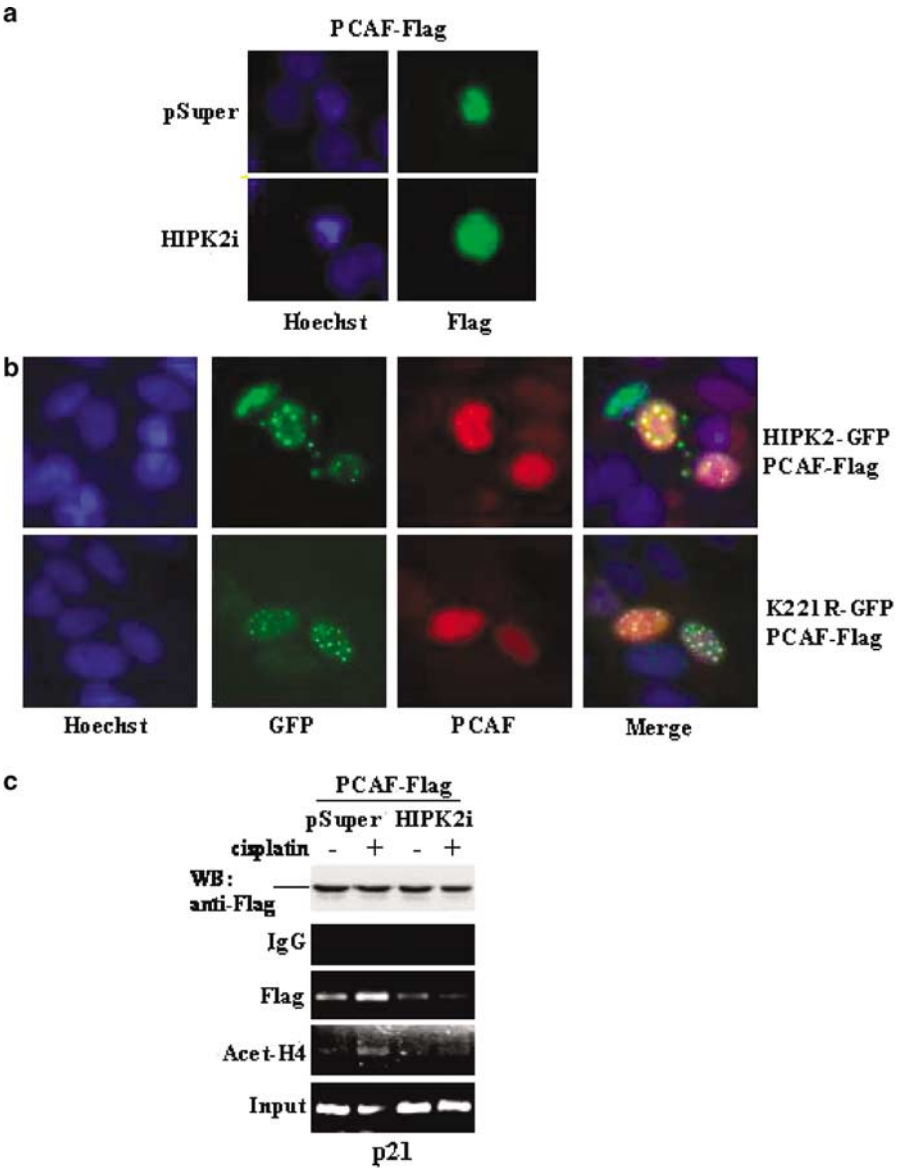

Figure 8 Analysis of HIPK2 and PCAF localization. (**a**) RKO-pSuper and HIPK2-interfered cells were transiently transfected with PCAF-Flag expression vector and analysed by indirect immunofluorescence for distribution of PCAF (green). Nuclear DNA was stained with Hoechst. (**b**) 293 cells were plated in 35 mm Petri dishes and transfected with HIPK2-GFP (3 μg), K221R-GFP (3 μg), and PCAF-Flag (2 μg) expression vectors. HIPK2- and K221R-GFP were detected by the intrinsic fluorescence of GFP. Indirect immunofluorescence was used to detect colocalization of HIPK2 (green) with PCAF (red). The merge panel shows overlapping localization (yellow) between HIPK2 proteins and PCAF. Nuclear DNA was stained with Hoechst. (**c**) RKO pSuper and HIPK2-interfered (HIPK2i) cells were transiently transfected with PCAF-Flag expression vector. At 24 h after transfection, cells were treated with cisplatin (1.75 μg/ml for 12 h) and subjected to ChIP using monoclonal anti-Flag antibody, polyclonal anti-acetyl-histone H4 antiserum, and no specific IgGs. Immunoprecipitates from each sample were analysed by PCR using specific primers for the *p21* promoter

towards the *p21* promoter. The possibility that conformational changes, imposed by mutations or post-translational modifications, may alter the affinity of the p53 protein to different target promoters is supported by several observations (Friedlander *et al*., 1996; Ryan and Vousden, 1998; Saller *et al*., 1999). Moreover, in agreement with this hypothesis, it was recently shown that DNA-damage-induced acetylation improve the apoptotic function of the p53-family member p73 by enhancing its ability to activate selectively the transcription of proapoptotic target genes (Costanzo *et al*., 2002; Pediconi *et al*., 2003).

Our data strongly support the idea that different p53 post-translational modifications, such as those occurring in response to different degrees of DNA damage, contribute to the p53 selection between growth-arrest or apoptosis target genes. Furthermore, our results point towards a novel function of HIPK2 as 'directional

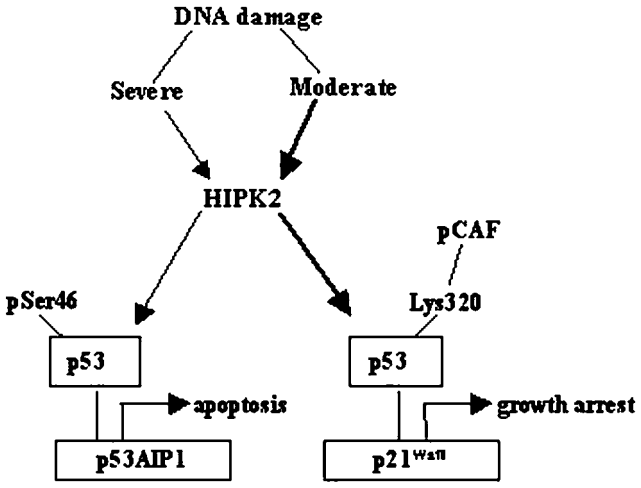

Figure 9 A proposed model for the selective activation of the p53-target genes in response to different degrees of genotoxic damage. In response to genotoxic damage, severe DNA damage activates HIPK2 that directly phosphorylates p53 at Ser46 and induces proapoptotic target genes whereas, nonapoptotic DNA damage activates HIPK2 that contributes to p53 acetylation at Ly320, mediated by PCAF, and induces the growth arrest-related p21 target gene

modulator' of the p53 oncosuppressor functions. Accordingly, we propose a model (Figure 9) for the selection of different p53-dependent regulatory pathways. When DNA damage occurs, the DNA damage sensing machinery estimates the level of damage. Next p53 is post-translationally modified and activated. The cellular response occurs according to the DNA damage level by tightly regulated p53 post-translational modifications. In our model, HIPK2 mediates, at least in part, p53 activation acting both after severe DNA damage by directly phosphorylating Ser46 and after nonapoptotic DNA damage by contributing to its PCAF-mediated acetylation. These HIPK2-mediated modifications contribute to the selective activation of p53 target promoters.

In conclusion, we have provided evidence suggesting a novel function for HIPK2 in *in vivo* activation of p53 and its involvement in regulating p53 oncosuppressor functions (growth arrest *versus* apoptosis) by mediating its acetylation that appears as a fundamental mechanism in regulating p53 transcriptional activity.

Materials and methods

Cell culture, reagents, transfection, and luciferase assays

293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Life Technology, Grand Island, NY, USA), RKO colon cancer (wtp53), the modified RKO-pSuper, and RKO-HIPK2 interfered (Di Stefano *et al*., 2004a), H1299 lung adenocarcinoma (p53 null), H1299-pVgRXR cells (Wang *et al*., 1998) (kindly provided by G Blandino, Regina Elena Cancer Institute, Rome, Italy), and 2008 ovarian cancer cells (wtp53) (kindly provided by S Howell, UCSD Cancer Center, La Jolla, CA, USA), were cultured in RPMI-1640 (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL).

Cisplatin was purchased from TEVA Pharma-Italia. To induce HIPK2 expression in the H1299-pIND-HIPK2 cells, ponasterone A, a synthetic analog of ecdysone (Alexis Biochemicals, San Diego, CA, USA), was added to the medium to a final concentration of 2.5 μM.

Transient transfection assays were performed using the BES (for 293, H1299, and 2008 cells) or the Lipofectamine Plus reagent (Invitrogen) (for RKO cells) methods, as described earlier (D'Orazi *et al*., 2002). The amount of plasmid DNA was equalized in each sample by supplementing with empty plasmid.

Luciferase activity was assayed as previously described (Manni *et al*., 2001). Cells were transfected with the luciferase reporter gene driven by the p53-dependent promoters and transfection efficiencies were normalized with the use of a cotransfected β-galactosidase vector. RKO cells were stably transfected with the p21- and AIP1-luc reporters along with the pBabe-puro vector (1 : 10 molar ratio). At 24 h after transfection, puromicin (2 μg/ml) was added to the medium for selection.

Antibodies and plasmids

The antibodies used were: anti-Bax, anti-p21, anti-p53 (Ab1801, DO1) (Santa Cruz Biotechnology); anti-acetylated-lysines, anti-phospho-p53Ser46, and anti-phospho-p53Ser15 (Cell Signaling Technology); anti-acetyl-p53 (Lys320), anti-acetyl-histone H4 (Upstate Biotechnologies); anti-p53 antibody (Ab7) (Oncogene Science); anti-PARP (BD PharMingen); anti-Flag (M5), anti-tubulin (SIGMA, BIO-Sciences); and anti-HIPK2 (kindly provided by ML Schmitz, University of Bern, Switzerland).

The expression vectors used in this study were: human wild-type CMVp53; human CMVp53K320Q (lysine 320 mutated to glutamine) and CMVp53K381/382R (lysines 381 and 382 mutated to arginine), obtained from the CMVp53 vector with the QuickChange site-directed mutagenesis kit (Stratagene); pSuper and pSuper-HIPK2 interfering vectors (Di Stefano *et al*., 2004a); human PCAF-Flag and the HAT-defective PCAF-Flag (a kind gift of, respectively, M Fanciulli, Regina Elena Cancer Institute Rome, Italy and M Levrero, 'Andrea Cesalpino' Foundation, Rome, Italy); HIPK2-GFP and the kinase-dead K221R-GFP (D'Orazi *et al*., 2002); AIP1-luc (Oda *et al*., 2000a) (kindly provided by H Arakawa, National Cancer Center, Tokyo, Japan) and p21^{Waf1}-luc (el Deiry *et al*., 1993) (kindly provided by B Vogelstein, Johns Hopkins University, School of Medicine, Baltimore, MD, USA). For the construction of the HIPK2-inducible vector, pIND-HIPK2 was prepared by cloning the murine HIPK2 cDNA into the *Eco*R1/*Xho*1 insertion sites of the pIND plasmid (Invitrogen).

Western blotting, immunoprecipitations, and kinase assays

Immunoblotting, immunoprecipitation, and kinase assays were performed essentially as described (D'Orazi *et al*., 2002). Immunoprecipitations were carried out by incubating 300–2000 μg of total protein extracts with, respectively, anti-p53 (a mix of DO1 and Ab1801) and anti-HIPK2 antibodies preadsorbed to protein G-agarose (Pierce). Immunocomplexes were collected by centrifugation, separated by 9–10% SDS–PAGE and blotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). For Western blotting, membranes were incubated with the indicated antibodies. Immunoreactivity was detected with the ECL chemoluminescence reaction kit

(Amersham Corp., Arlington Heights, IL, USA). For kinase assay, HIPK2-immunocomplexes from RKO cells treated for 12 h with cisplatin (1.75 and 5 μg/ml) were incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 200 μM sodium orthovanadate) in the presence of 5 μCi [γ-^{32}P]-ATP, 50 mM unlabeled ATP, and 5 μg of myelin basic protein (MBP) (SIGMA) as substrate, for 30 min at 30°C. Reaction products were resolved by 10% SDS–PAGE and [^{32}P]-labeled proteins were detected by autoradiography.

ChIP assay

DNA and protein complexes were cross-linked in living cells by the addition of formahaldeyde (Merck, Inc.) directly to cell culture medium to 1% final concentration. Cross-linking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine, pH 2.5 (final concentration of 0.125 M) for 5 min at room temperature. Cells were scraped off the plates and resuspended in hypotonic buffer (5 mM piperazine *N*,*N*-bis zethone sulfonic acid pH 8, 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors). Nuclei were centrifugated, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, 0.5% deoxycholic acid, and a mix of protease inhibitors), and sonicated for 10 pulses of 20 s at 80% power to generate 500–2000-bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). The cell lysates were precleared by incubation at 4°C with 15 μl of protein-G agarose beads (Pierce) preadsorbed with sonicated single-stranded DNA and bovine serum albumin (SIGMA). The cleared lysates were incubated with the indicated antibodies on a rotating platform for 12–16 h at 4°C. Antibody–protein–DNA complexes were bound to 30 μl of protein-G agarose beads preadsorbed with sonicated single-stranded DNA and bovine serum albumin, for 3 h at 4°C. After centrifugation, the beads were extensively washed, and the antigen was eluted with 1% SDS, 50 mM sodium carbonate at 37°C for 30 min with vigorous shaking. DNA–protein crosslinks were reversed by heating at 65°C for 12–16 h. DNA was phenol-extracted, ethanol-precipitated, and analysed by subsequent PCR. In each experiment, the linearity of the signal was insured by amplification of increasing amounts of template DNA. Generally, DNA representing 0.005–0.01% of the total chromatin sample (Input) or 1–10% of the immunoprecipitates was amplified using promoter-specific primers for *p21*, *p53AIP1*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *thymidine kinase* (*Tk*) genes. Primer sequences are available upon request. Immunoprecipitation with no specific immunoglobulins (IgGs, Santa Cruz) was performed as negative control.

Reverse transcriptase–PCR (RT–PCR)

RNA was isolated by using the RNeasy mini kit (Qiagen S.P.A., Milano, Italy) following the manufacturer's instructions. In all, 5 μg of total RNA was reverse transcribed using the MuLV reverse transcriptase and the reverse transcribed material was used in PCR reactions with the AmpliTaq DNA Polymerase (Gene Amp RNA PCR kit, Perkin Elmer, Roche Molecular System, Brachburg, NJ, USA). The p21 transcript was amplified with primers F1 5′-CCT CTT CGG CCC GGT GGA-3′ and R1 5′-CCG TTT TCG ACC CTG AGA G-3′. The HIPK2 and GAPDH primers were described elsewhere (Di Stefano *et al*., 2004a). PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Acknowledgements
We thank all the people cited in the text for their generous gifts of reagents. We are particularly grateful to M Levrero, A Damalas, M Fanciulli, and G Blandino for constant availability, scientific support, and critical reading of the manuscript. We also thank G Fontemaggi, A Gurtner, and T Bruno for their expert technical assistance. This work was supported by grants from AIRC, MIUR-Cofin, MURST-Fondi Ateneo, and FIRB-MIUR.

References

Appella E and Anderson CW. (2000). *Pathol. Biol. (Paris)*, **48,** 227–245.
Appella E and Anderson CW. (2001). *Eur. J. Biochem.*, **268,** 2764–2772.
Ashcroft M, Taya Y and Vousden KH. (2000). *Mol. Cell. Biol.*, **20,** 3224–3233.
Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y. (1998). *Science*, **281,** 1674–1677.
Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD and Berger SL. (2001). *Mol Cell.*, **8,** 1243–1254.
Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E and Fornace AJ. (1999). *EMBO J.*, **18,** 6845–6854.
Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB and Siliciano JD. (1998). *Science*, **281,** 1677–1679.
Chehab NH, Malikzay A, Appel M and Halazonetis TD. (2000). *Genes Dev.*, **14,** 278–288.
Costanzo A, Merlo P, Pediconi N, Fulco M, Sartorelli V, Cole PA, Fontemaggi G, Fanciulli M, Schiltz L, Blandino G, Balsano C and Levrero M. (2002). *Mol. Cell*, **9,** 175–186.
Di Stefano V, Rinaldo C, Sacchi A, Soddu S and D'Orazi G. (2004a). *Exp. Cell Res.*, **293,** 311–320.
Di Stefano V, Blandino G, Sacchi A, Soddu S and D'Orazi G. (2004b). *Oncogene*, **23,** 5185–5192.
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). *Nat. Cell Biol.*, **4,** 11–19.
el Deiry W, Tokino T, Velculescu VE, Levy DB, Parson R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75,** 817–825.
Espinosa JM and Emerson BM. (2001). *Cell*, **8,** 57–69.
Friedlander P, Haupt Y, Prives C and Oren M. (1996). *Mol. Cell. Biol.*, **16,** 4961–4971.
Giaccia AJ and Kastan LB. (1998). *Genes Dev.*, **12,** 2973–2983.
Gottlieb T and Oren M. (1996). *Biochim. Biophys. Acta*, **1287,** 77–102.
Gu W and Roeder RG. (1997). *Cell*, **90,** 595–606.
Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ and Mak TW. (2000). *Science*, **287,** 1824–1827.
Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, Droge W, Will H and Schmitz ML. (2002). *Nat. Cell Biol.*, **4,** 1–10.

Ko LJ, Shieh SY, Chen X, Jayaraman L, Tamai K, Taya Y, Prives C and Pan ZQ. (1997). *Mol. Cell. Biol.*, **17,** 7220–7229.

Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E and Anderson CW. (1992). *Mol. Cell. Biol.*, **12,** 5041–5049.

Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD and Berger SL. (1999). *Mol. Cell. Biol.*, **19,** 1202–1209.

Luo J, Li M, Tang Y, Laszkowska M, Roeder RG and Gu W. (2003). *Proc. Natl. Acad. Sci. USA*, **101,** 2259–2264.

Luo J, Su F, Chen D, Shiloh A and Gu W. (2000). *Nature*, **408,** 377–381.

Manni I, Mazzaro G, Gurtner A, Mantovani R, Haugwitz U, Krause K, Engeland K, Sacchi A, Soddu S and Piaggio G. (2001). *J. Biol. Chem.*, **276,** 5570–5576.

Martinez-Balbas MA, Bauer UM, Nielsen SJ, Brehm A and Kouzarides T. (2000). *EMBO J.*, **19,** 662–671.

Miyashita T and Reed JC. (1995). *Cell*, **80,** 293–299.

Moller A, Sirma H, Hofmann TG, Rueffer S, Klimczak E, Droge W and Schmitz ML. (2003). *Cancer Res.*, **63,** 4310–4314.

Munshi N, Agalioti T, Lomvardas S, Merita M, Chen G and Thanos D. (2001). *Science*, **293,** 1133–1136.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y and Taya Y. (2000a). *Cell*, **102,** 849–862.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N. (2000b). *Science*, **288,** 1053–1058.

Pediconi N, Ianari A, Costanzo A, Belloni L, Gallo R, Cimino L, Porcellini A, Screpanti I, Balsano C, Alesse E, Gulino A and Levrero M. (2003). *Nat. Cell Biol.*, **5,** 552–558.

Prives C and Hall PA. (1999). *J. Pathol.*, **187,** 112–126.

Prives C and Manley JL. (2001). *Cell*, **107,** 815–818.

Ryan KM and Vousden KH. (1998). *Mol. Cell. Biol.*, **18,** 3692–3698.

Saito S, Yamaguchi H, Higashimoto Y, Chao C, Xu Y, Roenace AJ, Appella E and Anderson CW. (2003). *J. Biol. Chem.*, **39,** 37536–37544.

Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW and Appella E. (1998). *Genes Dev.*, **12,** 2831–2841.

Saller E, Tom E, Brunori M, Otter M, Estreicher A, Mack DH and Iggo R. (1999). *EMBO J.*, **18,** 4424–4437.

Sartorelli V, Puri PL, Hamamori Y, Ogryzko V, Chung G, Nakatani Y, Wang JY and Kedes L. (1999). *Mol. Cell*, **4,** 725–734.

Shieh SY, Ahn J, Tamai K, Taya Y and Prives C. (2000). *Genes Dev.*, **14,** 289–300.

Shieh S-Y, Ikeda M, Taya Y and Prives C. (1997). *Cell*, **9,** 325–334.

Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C and Abraham RT. (1999). *Genes Dev.*, **13,** 152–157.

Vogt Sionov R and Haupt Y. (1999). *Oncogene*, **18,** 6145–6157.

Wang Y, Blandino G, Oren M and Givol D. (1998). *Oncogene*, **177,** 1923–1930.