Article

MDM2-Regulated Degradation of HIPK2 Prevents p53Ser46 Phosphorylation and DNA Damage-Induced Apoptosis

Cinzia Rinaldo,[1] Andrea Prodosmo,[1] Francesca Mancini,[1,2] Stefano Iacovelli,[1,3] Ada Sacchi,[1]
Fabiola Moretti,[1,2] and Silvia Soddu[1,*]
[1] Department of Experimental Oncology, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy
[2] Institute of Neurobiology and Molecular Medicine, National Research Council, 00137 Rome, Italy
[3] Present address: San Raffaele Scientific Biomedical Park of Rome, Via di Castel Romano 100, 00128 Rome, Italy.
*Correspondence: soddu@ifo.it
DOI 10.1016/j.molcel.2007.02.008

SUMMARY

In response to DNA damage, p53 induces either cell-cycle arrest or apoptosis by differential transcription of several target genes and through transcription-independent apoptotic functions. p53 phosphorylation at Ser46 by HIPK2 is one determinant of the outcome because it takes place only upon severe, nonrepairable DNA damage that irreversibly drives cells to apoptosis. Here, we show that p53 represses its proapoptotic activator HIPK2 via MDM2-mediated degradation, whereas a degradation-resistant HIPK2 mutant has increased apoptotic activity. Upon cytostatic, nonsevere DNA damage, inhibition of HIPK2 degradation is sufficient to induce p53Ser46 phosphorylation and apoptosis, converting growth-arresting stimuli to apoptotic ones. These findings establish HIPK2 as an MDM2 target and support a model in which, upon nonsevere DNA damage, p53 represses its own phosphorylation at Ser46 due to HIPK2 degradation, supporting the notion that the cell-cycle-arresting functions of p53 include active inhibition of the apoptotic ones.

INTRODUCTION

In response to DNA damage, eukaryotic cells activate checkpoint pathways to arrest the cell cycle and facilitate DNA repair or induce apoptosis and eliminate damaged cells (Bartek and Lukas, 2001). The tumor suppressor p53 is a key mediator of the DNA damage response and plays critical role in the transcriptional regulation of a large number of cell-cycle arrest- and apoptosis-related genes as well as in the activation of transcription-independent apoptosis (Oren, 2003; Vousden, and Lu, 2002; Moll et al., 2005). To exert these functions, the latent p53 protein constitutively expressed in an inactive state is stabilized and activated (Lavin and Gueven, 2006). These

events are thought to depend largely on posttranslational modifications and protein-protein interactions with an increasing array of cofactors that regulate the p53 response at a multiplicity of levels (Appella and Anderson, 2001; Yee and Vousden, 2005).

The best characterized p53-related feedback loop is that of the p53 inhibitor *mouse double minute 2* (MDM2) (Prives, 1998; Harris and Levine, 2005). MDM2, as well as its human homolog HDM2 (here, collectively called MDM2), is one of the main targets and a negative regulator of p53. MDM2 is an E3-ubiquitin (Ub) ligase that binds and inactivates p53 by both repressing its transcriptional activity and promoting ubiquitylation and proteasomal degradation of p53 and several other proteins, including itself (Honda et al., 1997; Haupt et al., 1997; Kubbutat et al., 1997; Iwakuma and Lozano, 2003). MDM2 is responsible for the maintenance of the basal, inactive state of p53 under normal conditions (Marine et al., 2006). In response to DNA damage, the MDM2 negative regulation of p53 is released, leading to stabilization of transcriptionally competent p53 (Perry, 2004). These events are independent from the intensity of the damage because they take place upon both sublethal and lethal types of damage. Thus, they do not directly account for the divergence in the functional outcomes (e.g., cell-cycle arrest versus apoptosis). In contrast, among the posttranslational modifications, phosphorylation of human p53 at Ser46 (Bulavin et al., 1999; Oda et al., 2000), or of its mouse homolog Ser58 (Cecchinelli et al., 2006b), was identified as a specific modification involved in apoptotic regulation. It was proposed that severe, nonrepairable DNA damage, which would lead to cell suicide rather than cell-cycle arrest, promotes phosphorylation at Ser46 (Oda et al., 2000). In particular, phosphorylation at this site can promote changes in p53 affinity for different promoters with a shift from cell-cycle arrest-related genes to apoptosis-related ones (Oda et al., 2000; Mayo et al., 2005; Cecchinelli et al., 2006a). Based on these findings, p53Ser46 phosphorylation is considered a "point of no return" because it would irreversibly drive cells toward apoptosis (Oda et al., 2000). This model is further supported by the recent observation that, like polymorphism at codon 72, the only other known

polymorphism in p53 protein, codon 47, is functionally relevant. Substitution at this codon of the wild-type proline residue, which belongs to the phosphorylation consensus of Ser46, with a serine, significantly decreases Ser46 phosphorylation and p53's ability to induce apoptosis (Li et al., 2005). In addition, the resistance of some tumor cells to *TP53* gene transfer-induced apoptosis was shown to depend on a defect in p53Ser46 phosphorylation and was overcome by transduction of a mutant p53 that mimics Ser46 phosphorylation (Ohtani et al., 2004; Rodicker and Putzer, 2003; Ichwan et al., 2005).

*H*omeodomain-*i*nteracting *p*rotein *k*inase *2* (HIPK2) is a member of a nuclear Ser/Thr kinase family originally identified as corepressor for homeodomain transcription factors (Kim et al., 1998). Recently, we and others have shown that HIPK2 interacts with the carboxyl terminus of p53 through its speckle retention signal. HIPK2 and p53 colocalize with PML-3 into the nuclear bodies and cooperate in the activation of p53-dependent transcription and induction of apoptosis (D'Orazi et al., 2002, Hofmann et al., 2002). Upon severe DNA damage by UV irradiation or antineoplastic treatments such as doxorubicin (Adriamycin, ADR) and cisplatin, HIPK2 specifically phosphorylates human p53 at Ser46 (D'Orazi et al., 2002, Hofmann et al., 2002; Moller et al., 2003; Di Stefano et al., 2004b) and mouse p53 at Ser58 (Cecchinelli et al., 2006b) and this kinase activity is required for the induction of apoptosis. Furthermore, as little as a 50% reduction in the endogenous HIPK2 levels in tumor cells can robustly reduce p53Ser46 phosphorylation and apoptosis upon cisplatin treatment or UV irradiation (Di Stefano et al., 2004b; Cecchinelli et al., 2006a), strongly supporting a relevant role for HIPK2 in cell response to DNA damage. HIPK2 was shown to promote apoptosis by targeting factors other than p53, such as the CtBP transcriptional corepressor (Zhang et al., 2003), and to modulate the activity of other proteins directly or indirectly related to apoptosis, such as the p53 family members p73 and p63 (Kim et al., 2002), the p53 inhibitor MDM2 (Wang et al., 2001; Di Stefano et al., 2004a), and the β-catenin regulator Axin (Rui et al., 2004), further linking HIPK2 to apoptosis regulation.

During studies of HIPK2 activation in response to DNA damage, we noticed that, although a lethal damage is associated with the previously characterized increment of HIPK2 expression and p53Ser46 phosphorylation, a sublethal, cytostatic damage strongly represses HIPK2 expression. Further exploration of this phenomenon revealed that HIPK2 is a target for MDM2-mediated, Ub-dependent degradation and that HIPK2 degradation takes place only in growth-arresting conditions when MDM2 is efficiently induced by p53. Inhibition of this pathway by MDM2 depletion is sufficient to promote HIPK2 induction and p53Ser46 phosphorylation and to convert a cell-cycle-arresting stimulus by a sublethal dose of ADR into a lethal effect. Our findings suggest that the cytostatic response to DNA damage may imply an active p53-induced, MDM2-mediated inhibition of the p53 apoptotic pathways, at least in part, through suppression of the

apoptosis-specific p53 posttranslational modification at Ser46.

RESULTS

HIPK2 Is Differentially Expressed in Cells Exposed to Cytostatic or Lethal Doses of DNA Damaging Agents

To analyze the role of HIPK2 in DNA damage response, we tested the sensitivity of human HCT116 colon cancer cells to ADR by a dose-response curve (Figure 1A). The 0.5 μM dose, compatible with a 99% survival at 48 hr posttreatment, and the 3 μM dose that killed ∼50% of the cells at the same time were selected for further analyses. The cell-cycle profiles confirmed cell death at the higher dose, whereas they showed cell-cycle arrest at the lower dose (Figure 1B, upper panels). The long-term survival and the reversibility of the cell-cycle arrest were tested by colony-formation assay. As shown in the lower panels of Figure 1B, the cells treated for 24 hr with 0.5 μM ADR were able to form colonies when replated in the absence of drug, whereas those treated with 3 μM ADR did not recover from the damage. We analyzed the expression of HIPK2 and p53 and the phosphorylation of p53Ser46 by western blotting (WB). As expected, p53 was induced by either cytostatic or lethal doses of drug, whereas p53Ser46 phosphorylation was detected only in cells treated with the lethal dose. HIPK2 expression was increased by the lethal dose of ADR, but surprisingly, it was strongly repressed upon cytostatic treatment (Figure 1C, left and middle panels, and Figure S1A in the Supplemental Data available with this article online). This repression was detectable in wild-type p53 (*WTp53*)-carrying HCT116 cells, but not in their *p53*-null derivatives (Bunz et al., 1998) (Figure 1C, right panel), suggesting that p53 is involved in HIPK2 repression in these experimental conditions. Repression of HIPK2 was also observed in WTp53-carrying RKO and MCF-7 cells in response to cytostatic doses of ADR or UV irradiation (Figure S1B).

HIPK2 Is Regulated at the Posttranslational Level

To test directly whether p53 promotes HIPK2 repression, p53-null H1299 cells were infected with a p53 recombinant adenovirus (Adp53) at multiplicities compatible with cell survival. Repression of HIPK2 protein was induced by p53 expression (Figure S1C). To evaluate whether HIPK2 is a transcriptional target of p53, a time-course analysis of HIPK2 protein and mRNA levels was performed on pair samples after Adp53 infection. Although the protein levels decreased with time, the RNA was not reduced (Figure 1D), supporting nontranscriptional regulation. In agreement with this assumption, an exogenous EGFP-tagged HIPK2 driven by a heterologous promoter was repressed similarly to the endogenous HIPK2 when H1299 cells were cotransfected with EGFP-HIPK2 and p53 (Figure S1D). A test for protein degradation showed that HIPK2 levels could be rescued by cell treatment with the proteasome inhibitor MG132. As shown in Figure 1E, when Adp53-infected H1299 cells were treated
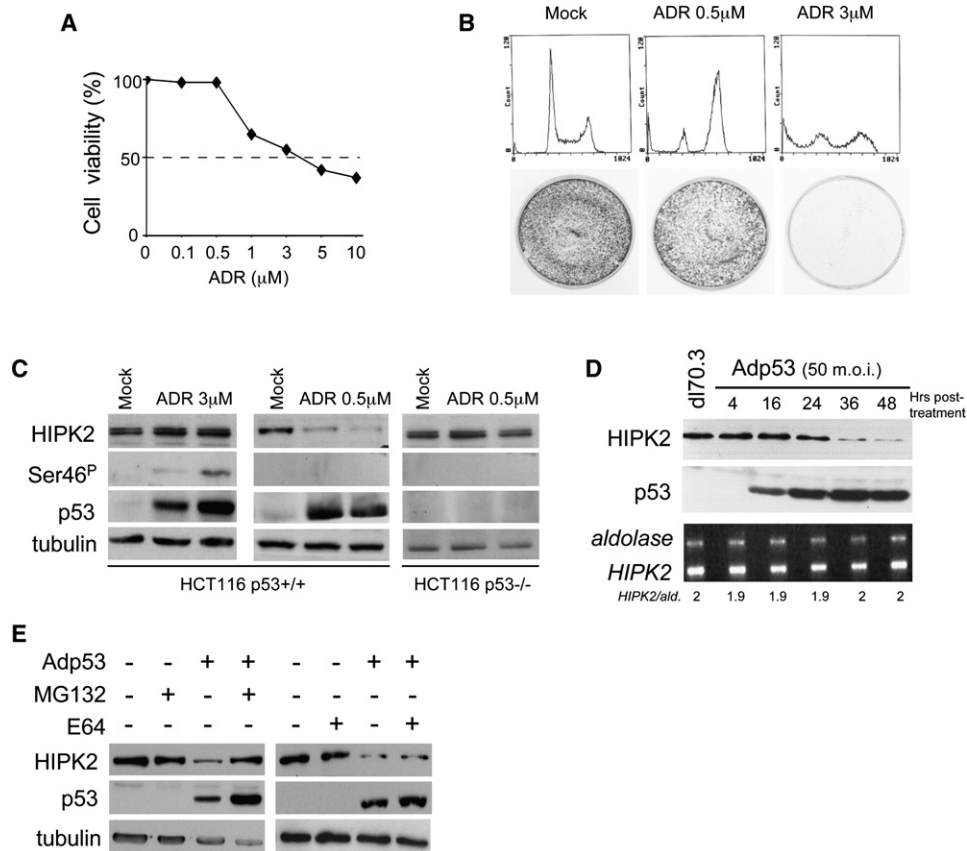
Figure 1. HIPK2 Expression Is Different in Cells Exposed to Cytostatic or Lethal Doses of ADR

(A) Percentage of cell viability of *WTp53*-carrying HCT116 cells treated with the indicated doses of ADR and measured 48 hr posttreatment by trypan blue-exclusion test.

(B) Cell-cycle analysis of HCT116 cells treated with the indicated doses of ADR performed 48 hr posttreatment by flow cytometry (upper panels). Colony-formation assay performed 24 hr after treatment of HCT116 cells with the indicated doses of ADR (lower panels).

(C) HCT116 cells and their *p53*^{−/−} derivatives were treated with lethal (3 μM) or cytostatic (0.5 μM) doses of ADR. The immunoblot kinetics (i.e., 4 and 8 hr for the lethal dose, 24 and 32 hr for the cytostatic one) of the indicated proteins is shown. Ser46[P] indicates the reaction obtained with the anti-phosphospecific p53Ser46 Ab. Tubulin expression shows equal loading of samples.

(D) H1299 cells were infected with dl70.3 or Adp53 at 50 multiplicity of infection (m.o.i.). RNA and protein extracts were obtained at the indicated times. WB for HIPK2 and p53 proteins (upper panel) and semiquantitative reverse transcription-PCR assays (Cecchinelli et al., 2006a) for *Hipk2* and the housekeeping gene *aldolase* (lower panel) are reported. The PCR products were quantified by densitometry. Density values were calculated as the ratio of *Hipk2* and *aldolase* densitometry values.

(E) H1299 cells infected as in (D) and treated with MG132 (10 μM), E64 (50 μM), or their relative solvent (− symbols) for an additional 16 hr. TCEs were analyzed by WB.

with MG132, but not with the calpain inhibitor E64, HIPK2 was not repressed by p53, even though p53 expression was increased by the MG132 treatment, as expected (Honda et al., 1997, Haupt et al., 1997; Kubbutat et al., 1997). These results suggest that, as for p53, HIPK2 levels are controlled by proteasome-mediated degradation.

HIPK2 Is a Target of MDM2-Mediated Degradation

To test whether MDM2 is involved in p53-induced HIPK2 degradation, mouse embryo fibroblasts (MEFs) from *Mdm2/p53* double-knockout (dKO) mice (Montes de Oca Luna et al., 1995) were transiently transfected with expression vectors carrying EGFP, EGFP-HIPK2, MDM2, or p53 in different combinations. WB showed that reduction of

EGFP-HIPK2 was induced by MDM2 coexpression even in the absence of p53, whereas p53 alone did not promote HIPK2 degradation in this cell context (Figure 2A, left panels), suggesting that MDM2 can be a p53 effector of this biological process. No modification in the levels of EGFP was observed in any tested conditions (Figure 2A, right panels). Matching results were obtained by counting the number of EGFP-positive, fluorescent dKO MEFs upon a similar pattern of transfections. A strong reduction in the percentage of fluorescent cells was observed only upon coexpression of EGFP-HIPK2 chimera and MDM2 protein, whereas no modification was detected in the EGFP-expressing cells (Figure 2B). HIPK2 degradation by MDM2 was further supported by the reduced HIPK2 half-life
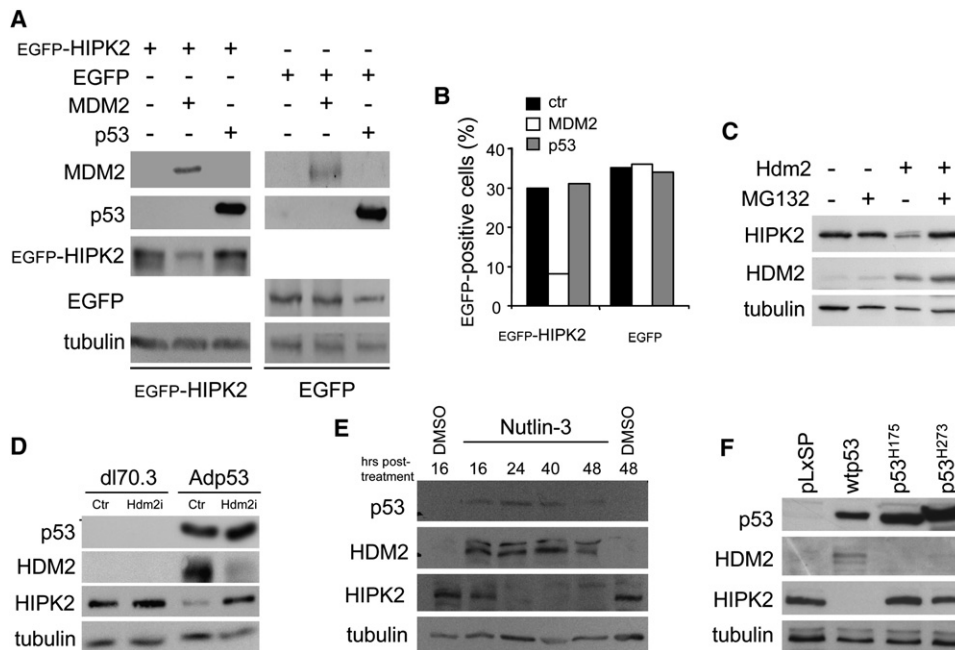
Figure 2. MDM2 Mediates p53-Dependent Degradation of HIPK2

(A) dKO MEFs were transfected with the indicated combinations of expression vectors. TCEs were obtained 24 hr posttransfection and analyzed for the indicated proteins. EGFP alone and EGFP-HIPK2 chimera are compared.

(B) dKO MEFs were transfected as in (A), and the percentages of EGFP-positive, fluorescent cells were counted by using a fluorescent microscope. Ctr indicates cells transfected with the relative control vector.

(C) H1299 cells were transfected with the indicated expression vectors and 40 hr later treated with MG132 (10 μM) or its solvent DMSO for an additional 8 hr. TCEs were analyzed by WB for the indicated proteins.

(D) H1299 cells were transfected with *HDM2*-specific siRNA (HDM2i) or with control siRNA (Ctr), infected with dl70.3 or Adp53 at 50 m.o.i., and analyzed by WB.

(E) *WTp53*-carrying RKO cells were treated with Nutlin-3 (10 μM) or its solvent (DMSO). Immunoblot kinetics of the indicated proteins is shown.

(F) H1299 cells were transduced with the indicated proteins. TCEs were obtained 40 hr posttransfection and analyzed by WB for the indicated proteins.

induced by HDM2 overexpression (Figure S2A) and by a test for proteasomal degradation showing that, in HDM2 overexpressing cells, HIPK2 levels are rescued by MG132 treatment (Figure 2C).

Next, we confirmed the MDM2 requirement in HIPK2 degradation by RNA interference or Nutlin-3 treatment. In the first case, *p53*-null H1299 cells were transiently transfected with siRNA to *HDM2* or control sequences as described (Bres et al., 2003). In these conditions, Adp53 infection promoted degradation of endogenous HIPK2 protein only in the control-interfered cells, whereas it had no effect in the HDM2-interfered cells (Figure 2D). In the second case, a time-course analysis of *WTp53*-carrying RKO cells treated with Nutlin-3, an antagonist of the HDM2-p53 interaction (Vassilev et al., 2004), showed that the HDM2 upregulation induced by this compound was associated with a strong repression of HIPK2 (Figure 2E).

MDM2 is a transcription target of p53, thus, we evaluated whether transcription-defective mutants of p53 (Kern et al., 1992), such as the tumor-derived p53H175 and p53H273 mutants, are defective for HIPK2 repression. WB showed reduction of HIPK2 in H1299 cells induced to express endogenous HDM2 protein through

WTp53 overexpression. In contrast, HIPK2 was not reduced in the p53H175- and p53H273-expressing cells where HDM2 was not induced (Figure 2F). Together, these results reveal that MDM2 can be a p53 effector for HIPK2 degradation.

MDM2 Interacts with HIPK2 In Vitro and In Vivo

Given the dependence of HIPK2 degradation on MDM2, we explored the potential interaction between the two proteins by immunoprecipitation. We found that endogenous HDM2 coimmunoprecipitates with endogenous HIPK2 upon ADR treatment, before HIPK2 starts to be degraded, whereas no interaction was detectable in unstressed cells (Figure 3A). To verify whether this interaction is direct, in vitro-translated human and mouse proteins were employed. MDM2 and HIPK2 proteins were found to interact also in these conditions (Figure S2B). To determine the MDM2 region involved in the interaction, different HDM2 deletion mutants were coexpressed with HIPK2 and a coimmunoprecipitation assay was performed with anti-HIPK2 antibody (Ab). As shown in Figure 3B, only the HDM2$^{\Delta442-491}$ was not bound to HIPK2, indicating that the C terminus of MDM2, which includes the RING domain, is involved in the interaction.
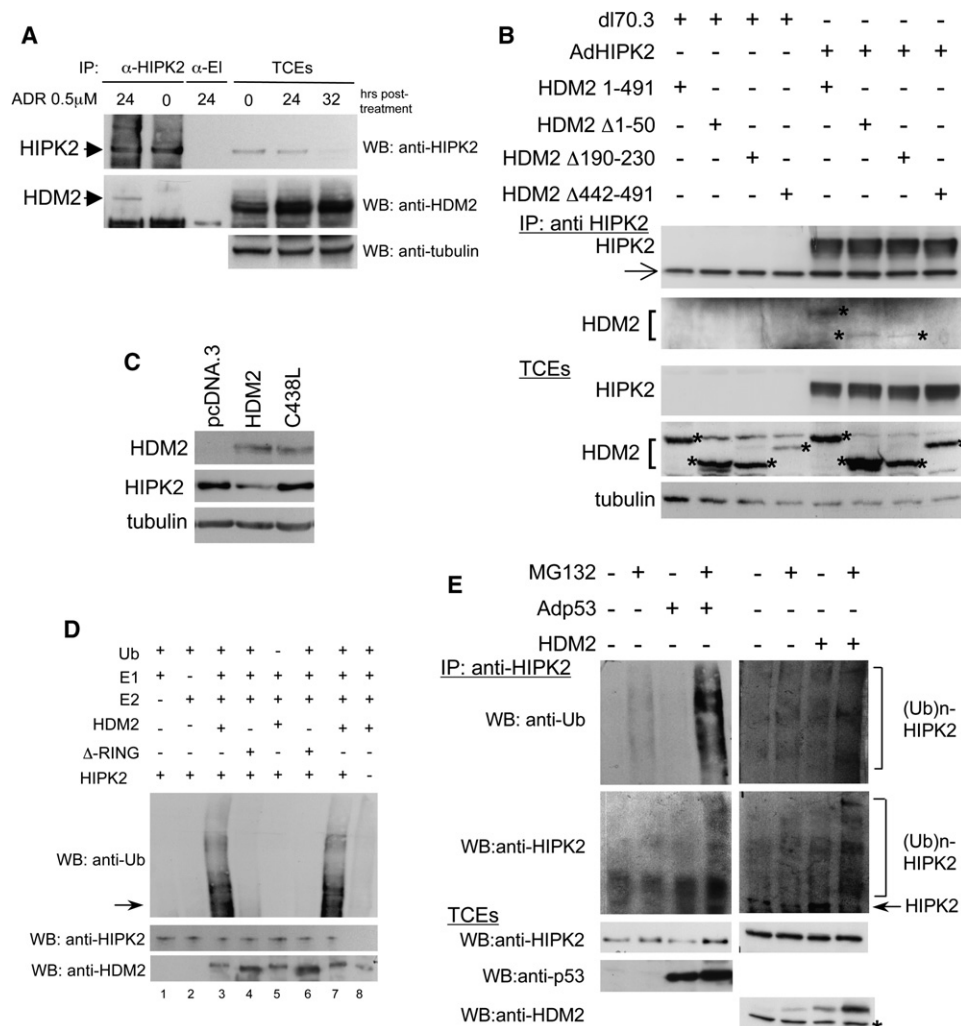
Figure 3. MDM2 Binds and Ubiquitylates HIPK2

(A) RKO cells were treated with ADR for the indicated times. TCEs were directly analyzed by WB or immunoprecipitated with the indicated Abs before WB. Anti-elastase (α-El) Ab was used as a negative control of immunoprecipitation (IP).

(B) dKO MEFs were infected and transfected with the indicated vectors. TCEs were either immunoprecipitated with anti-HIPK2 Ab and subsequently analyzed by WB or directly analyzed by WB. The arrow indicates a nonspecific band. The asterisks indicate the specific bands of HDM2 full-length (1-491) and deletion mutants.

(C) H1299 cells were transfected with the indicated expression vectors. TCEs were obtained 40 hr posttransfection and analyzed by WB for the indicated proteins.

(D) In vitro ubiquitylation reactions were performed in the presence of recombinant E1, E2, and Ub with addition of the immunocomplexes obtained by dKO MEFs transduced with HIPK2, HDM2, or its Δ-RING deletion mutant as source of substrate or of WT and defective E3 activity. Reactions were performed for 2 hr at 30°C (lanes 1–5 and 8) or for 30 min at 37°C (lanes 6 and 7). Reaction products were subdivided in two aliquots and resolved by NuPAGE (3%–8%) followed by WB with anti-Ub Ab (upper panel) and by SDS-PAGE (7%) followed by WB with anti-HIPK2 and anti-HDM2 Abs (lower panels). The arrow indicates the position of the hypothetically unmodified HIPK2 protein.

(E) H1299 cells were infected with Adp53 or transfected with HDM2 and treated with or without MG132. TCEs were analyzed directly by WB for the indicated proteins (lower panels) or immunoprecipitated with anti-HIPK2 Ab. Immunocomplexes were analyzed by WB with anti-Ub or anti HIPK2 Abs, as indicated. Ubiquitylated high molecular-weight conjugates of HIPK2 were indicated as (Ub)n-HIPK2. The arrow indicates the unmodified HIPK2 protein. The asterisk indicates a nonspecific band.

MDM2 Catalyzes Ubiquitylation of HIPK2

Protein degradation by the proteasome is frequently modulated by polyubiquitylation (Welchman et al., 2005). Because MDM2 is an E3-Ub ligase, we tested whether the C438L mutant of HDM2 that lacks this activity (Fang et al., 2000) degrades HIPK2. As shown in Figure 3C, this mutant did not reduce HIPK2 expression in H1299 cells, suggesting that the E3 ligase activity is required for the MDM2-mediated degradation of HIPK2. Thus, we analyzed directly whether MDM2 can catalyze ubiquitylation of HIPK2 in vitro. HIPK2, WT HDM2, or its Δ-RING deletion mutant, which has no E3 ligase activity, was

immunoprecipitated from total cell extracts (TCEs) of transduced dKO MEFs and employed for in vitro ubiquitylation assay. As shown in Figure 3D, slowly migrating bands recognized by anti-Ub Abs were detectable only in the presence of WT HDM2 while the Δ-RING deletion mutant was unable to modify HIPK2, indicating that HDM2 can catalyze HIPK2 ubiquitylation in vitro.

Next, we investigated whether the HIPK2 degradation after MDM2 or p53 overexpression is linked to an increase in HIPK2 ubiquitylation in vivo. HIPK2 was immunoprecipitated from TCEs of H1299 cells transduced with HDM2 or p53 in the presence or absence of MG132 and analyzed by WB with anti-Ub and anti-HIPK2 Abs. Similar patterns of slowly migrating bands were detectable in MG132-treated cells upon p53 or MDM2 expression (Figure 3E), supporting the presence of HIPK2-Ub conjugates in vivo upon MDM2 activation. Overall, these results indicate that HIPK2 is a target of MDM2 ubiquitylation.

HIPK2 Lysine Residue 1182 Is Critical for the MDM2-Mediated Degradation of HIPK2

HIPK2 has 47 lysine residues along its sequence that are possible sites for Ub conjugation (Welchman et al., 2005) (Figure 4A). To define the HIPK2 region responsible for its p53-induced, MDM2-mediated degradation, we first tested the N- and C terminus halves of the HIPK2 protein in an in vivo degradation assay in Adp53-infected H1299 cells. Although the N-terminal portion of HIPK2 possesses 31 of the 47 total lysines, this region was completely resistant to degradation, whereas the C-terminal was still degraded (Figure 4A). Because the C-terminal portion of HIPK2 contains a PEST sequence, which is frequently associated with protein degradation, with 11 of the 15 residual lysines in and around this region, we constructed a series of HIPK2 deletion mutants within this area (Figures 4A and 4B). However, the only mutants resistant to p53-induced degradation were those lacking the entire C terminus, suggesting that the PEST region and the lysine residues between the kinase- and homeodomain-interacting domain are not per se relevant for HIPK2 degradation. Because the only remaining lysine was at residue 1182, we substituted it with an arginine in EGFP-HIPK2 (EGFP-HIPK2^{K1182R}) and FLAG-HIPK2 (FLAG-HIPK2^{K1182R}) proteins. A strong reduction of degradation was observed when this mutant was coexpressed with p53 (Figure 4C) or HDM2 proteins (Figure 4D). This event did not depend on modifications of the MDM2-HIPK2 interaction, because WT HIPK2 and the HIPK2^{K1182R} mutant can still be coimmunoprecipitated with HDM2 (Figure 4E).

The Degradation-Resistant HIPK2^{K1182R} Mutant Has Increased Apoptotic Activity

HIPK2 binds and phosphorylates p53 through the PEST region and the kinase domain, respectively (D'Orazi et al., 2002; Hofmann et al., 2002; Rui et al., 2004). Although the K1182R substitution is outside these regions, we tested whether the HIPK2^{K1182R} maintained p53 binding and phosphorylation activities by coimmunoprecipitation

and in vitro kinase assays. As shown in Figure 5A, FLAG-HIPK2^{K1182R} was as efficient as the WT HIPK2 in either assay. In addition, similarly to the WT HIPK2 (D'Orazi et al., 2002; Hofmann et al., 2002), the HIPK2^{K1182R} mutant colocalizes in the nuclear speckles with p53 (Figure 5B).

HIPK2 is a strong inhibitor of colony formation and a potent inducer of apoptosis in WTp53-carrying cells (D'Orazi et al., 2002; Hofmann et al., 2002; Rui et al., 2004). Thus, we tested the biological activity of the degradation-resistant HIPK2^{K1182R} mutant in RKO cells by comparison with WT HIPK2 and the kinase-dead HIPK2^{K221R} mutant that has no apoptotic activity. Puromycin selection showed that the HIPK2^{K1182R} mutant is more effective than WT HIPK2 in inhibiting colony formation (Figure 5C). Comparable results were obtained in MEFs from WT mice (data not shown). To evaluate whether this increased biological activity was mainly due to increased expression levels of the HIPK2^{K1182R} mutant, RKO cells were induced to express similar levels of EGFP-HIPK2 and EGFP-HIPK2^{K1182R} proteins by transfection of different amounts of expression vectors. EGFP-HIPK2^{K1182R} induced 8-fold more apoptosis than WT EGFP-HIPK2 protein (Figure 5D), showing a stronger apoptotic activity of the HIPK2^{K1182R} mutant compared to the WT protein. Although, at this point, we cannot rule out whether this effect is only due to protein stability, or if it also involves some other aspects of HIPK2 pro-apoptotic activity, overall these results support the idea that p53-induced, MDM2-mediated degradation of HIPK2 might have antiapoptotic functions.

HIPK2 Degradation Prevents p53Ser46 Phosphorylation and DNA Damage-Induced Apoptosis

To investigate whether MDM2 regulates HIPK2 function in vivo, we employed early passage, primary human embryonic kidney cells (HEK) to avoid accumulation of genetic alterations in their response to DNA damage. After assessing the cell sensitivity to ADR (Figure 6A), we performed time-course analyses of p53 and p53-related markers of DNA damage response upon treatment with cytostatic (0.5 μM) or lethal (5 μM) doses of ADR. Consistent with many reports, the cytostatic dose of ADR induced increased expression of p53 and of its targets p21^{Waf1} and HDM2, whereas the lethal dose increased p53 expression, but not p21^{Waf1} and HDM2 expression (Perry et al., 1993; Ashcroft et al., 2000). In addition, p53Ser46 was phosphorylated only upon lethal dose of ADR (Figure 6B). In agreement with the results obtained with tumor cell lines (Figure 1C), HIPK2 expression was induced by the lethal dose of ADR and repressed by the cytotoxic one, confirming the expected correlation with p53Ser46 phosphorylation and revealing an inverse correlation between HIPK2 and HDM2 levels (Figure 6B) at the two different doses. To evaluate whether this inverse correlation has a causal role, we asked whether modulation of HDM2 expression modifies the cell response to cytostatic and/or lethal doses of ADR. HEK cells were transfected with an *HDM2* expression vector and treated with the lethal dose of ADR. As
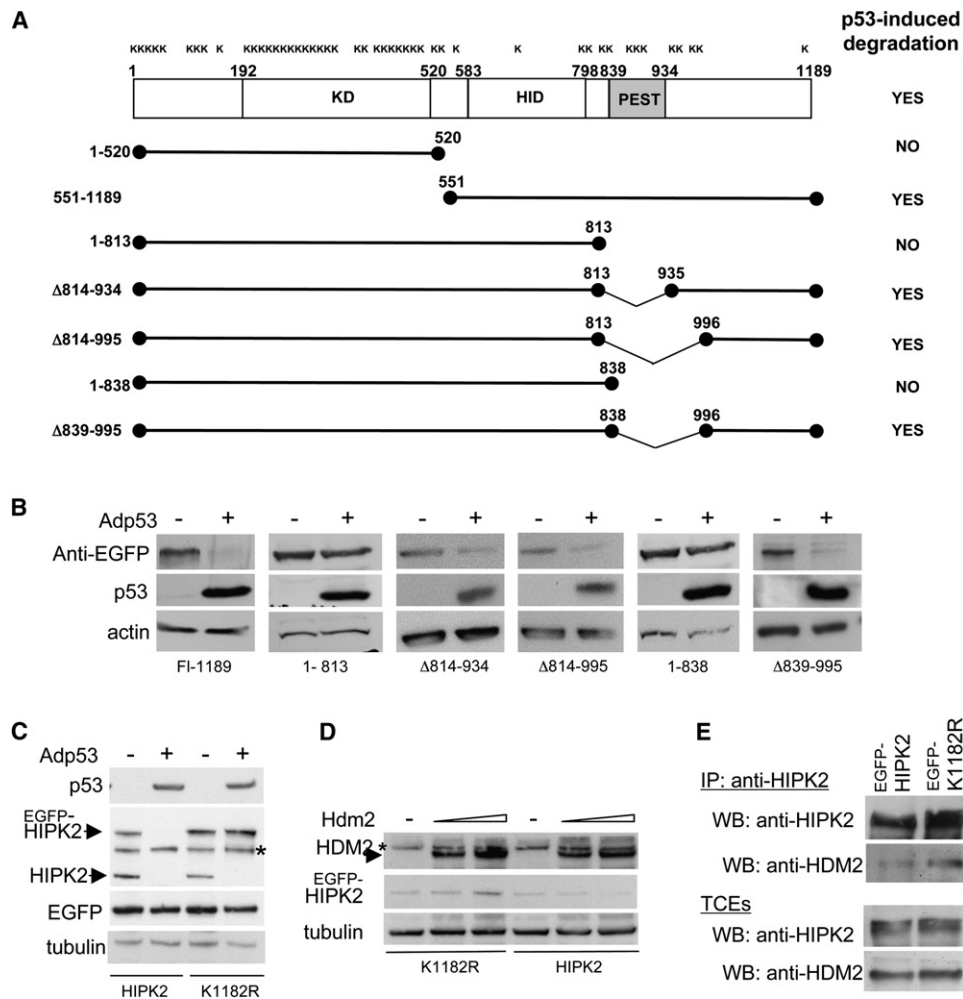
Figure 4. Mapping of the HIPK2 Degradation-Responsible Site

(A) Schematic representation of full-length (FL-1189) HIPK2 protein with the upper "K" indicating the position of the total lysine residues in its amino acid sequence. KD, kinase domain; HID, Homeobox-interacting domain; and PEST, in silico identified PEST-containing region. The HIPK2 deletion mutants we constructed starting from the EGFP-HIPK2 WT-carrying vector are schematically reported below the FL-1189 protein. On the right of each schematic protein is summarized its sensitivity to p53-induced degradation.

(B) H1299 cells were infected with dl70.3 or Adp53 at 50 m.o.i., transiently transfected with the indicated EGFP-HIPK2 expressing vectors, and analyzed by WB. Actin was used as loading control.

(C) H1299 cells were infected as in (B) and transfected with the chimera EGFP-HIPK2 or EGFP-K1182R point mutant together with EGFP alone as internal control. Expression levels of exogenous (EGFP-HIPK2) and endogenous (HIPK2) proteins, p53, EGFP, and tubulin were detected by WB. The asterisk indicates a nonspecific band.

(D) dKO MEF cells were transfected with two different concentrations of HDM2 in combination with EGFP-HIPK2 or EGFP-K1182R expression vectors and analyzed by WB for the indicated proteins. HIPK2 was reacted with anti-GFP Ab.

(E) dKOs were transfected with HDM2 in combination with EGFP-HIPK2 or EGFP-K1182R expression vectors, and TCEs were prepared 24 hr post-transfection, immunoprecipitated with anti-HIPK2 Ab, and resolved by SDS-PAGE followed by WB with anti-HIPK2 and anti-HDM2 Abs (upper panels). TCEs were analyzed directly by WB for the transfected proteins (lower panels).

shown in Figure 6C, HDM2 overexpression reduced the basal level of HIPK2 and significantly impaired HIPK2 induction, p53Ser46 phosphorylation, and cell death upon treatment with the lethal dose of ADR. Similar results were obtained with an HDM2 point mutant that does not bind p53 but maintains the capacity of binding and degrading HIPK2 (Figure S3). Mirroring results were obtained with HDM2-depleted HEK cells. Indeed, HDM2 depletion

by siRNA increased the basal level of HIPK2 in mock-treated cells and impaired HIPK2 repression upon treatment with the cytostatic dose of ADR. This partial rescue of HIPK2 expression was sufficient to promote p53Ser46 phosphorylation and cell death in the presence of a suble-thal dose of drug (Figure 6D), converting a cytostatic signal into an apoptotic one. These results are consistent with several observations by us and others showing that
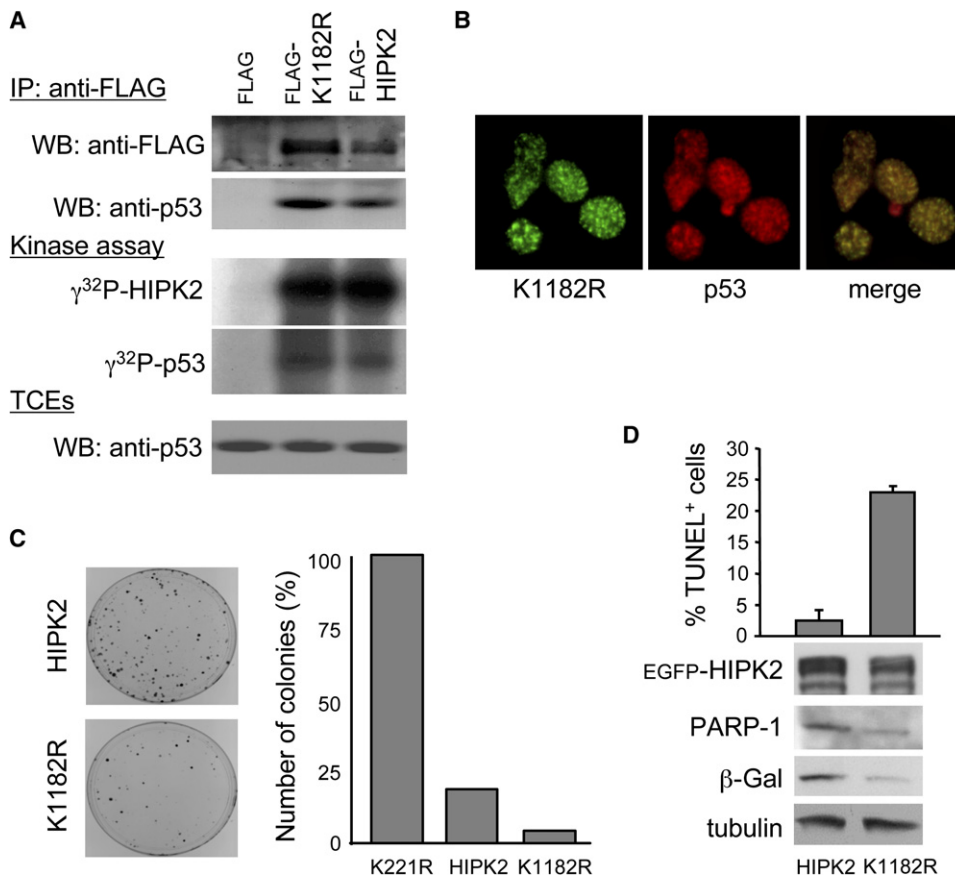
Figure 5. Biological Activity of HIPK2-K1182R Mutant

(A) HEK293 cells were transiently transfected with the indicated FLAG-HIPK2 vectors. TCEs were immunoprecipitated with anti-FLAG Ab and processed as follows: SDS-PAGE and WB with anti-FLAG and anti-p53 Abs, or incubation in the presence of γ^{32}P-ATP for kinase assay, SDS-PAGE, and autoradiography. TCEs were also analyzed by WB with anti-p53 Abs.

(B) H1299 cells were transfected with EGFP-K1182R and p53. HIPK2-K1182R is visible by its intrinsic green fluorescence. p53 was detected by indirect immunofluorescence staining with anti-p53 Ab followed by TRITC-conjugated Ab.

(C) Colony-formation assay was performed upon transfection of RKO cells with low doses (0.3 μg per 60 mm dish) of the indicated expression vectors. Representative HIPK2 and K1182R plates are shown. The percentage of the number of colonies counted in each plate is reported in the histogram. The percentages were calculated relative to the number of colonies obtained by transfection of the kinase-dead HIPK2 mutant (K221R) that does not inhibit colony formation (D'Orazi et al., 2002, Hofmann et al., 2002).

(D) Apoptotic cell death (percentage of TUNEL-positive cells) in RKO cells transfected with 2 μg of EGFP-HIPK2 or 0.5 μg of EGFP-K1182R expression vectors in combination with the pCMV-β-Gal vector at 1:20 molar ratio (upper panel). Results are mean of three different experiment ± standard deviation. For one representative experiment out of the three performed, the expression of the indicated proteins assessed by WB is reported (lower panel). Reduction of the full-length form of PARP-1 is shown. β-Gal expression was used as an internal control for transfection efficiency.

HIPK2 overexpression or depletion induces or represses, respectively, p53Ser46 phosphorylation and cell death in the presence or absence of DNA damage (D'Orazi et al., 2002; Di Stefano et al., 2004b; data not shown). Taken together, these results indicate that MDM2 may inhibit HIPK2 function and p53 proapoptotic activation in vivo.

DISCUSSION

p53, which plays a major role in cell response to DNA damage and other types of stress, is strictly regulated by several posttranslational modifications and a complex networks of positive and negative feedback loops, most of which act through MDM2. In this study, we show that MDM2 promotes ubiquitylation and degradation of the p53 proapoptotic activator HIPK2, whereas a degradation-resistant HIPK2 mutant has increased apoptotic activity. Expression or activation of a transcriptionally competent p53 induces MDM2 and subsequent repression of HIPK2, whereas MDM2 depletion causes accumulation of HIPK2. During cell response to DNA damage, these effects are associated with a modulation of p53Ser46 phosphorylation, a target of HIPK2 kinase activity, and the following induction of apoptosis. As sketched in Figure 7, a series of our results support a model in which the MDM2-dependent degradation of HIPK2 may
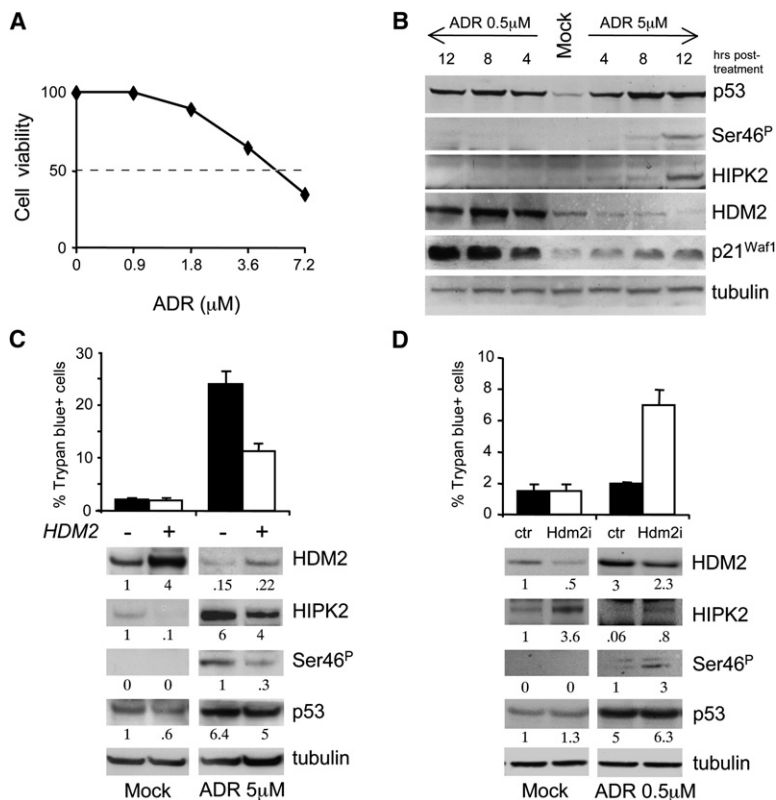
Figure 6. Modulation of Cell Response to Cytostatic or Apoptotic Doses of ADR by Altering HDM2 Expression

(A) Percentage of cell viability of HEK cells treated with different doses of ADR measured 24 hr posttreatment by trypan blue-exclusion test.

(B) Immunoblot kinetics for the indicated proteins in HEK cells treated with cytostatic (0.5 μM) or lethal (5 μM) doses of ADR.

(C) HEK cells were transfected with *HDM2*-carrying or the empty vector (− symbol in the *HDM2* row) and 36 hr posttransfection were maintained as mock or treated with lethal dose of ADR (5 μM). The percentage of cell death (trypan blue+ cells) was calculated 24 hr posttreatment (upper panel). The results reported in the histogram are means of two independent experiments performed in duplicate ± standard deviation. For one representative experiment out of two performed, the expression of the indicated proteins assessed by WB is reported (lower panel). Numbers below each row of WB indicate the densitometric values normalized with the relative tubulin value.

(D) HEK cells were transfected with HDM2-specific siRNA (Hdm2i) or control siRNA (ctr) and 24 hr posttransfection were maintained as mock or treated with cytostatic dose of ADR (0.5 μM). The percentage of cell death and the WB analyses were performed as in (C).

contribute to cell-cycle arrest by interfering with the activation of the p53-mediated apoptotic pathway.

As shown by many previous studies (reviewed by Vogelstein et al. [2000], Vousden and Lu [2002], and Oren [2003]), p53 is able to sense the intensity of the damage and modulate the following biological responses that can range from transient growth arrest to permanent replicative senescence or apoptosis. p53 is known to reach these different goals by differential activation of a large number of target genes and by mitochondrial functions.

However, the mechanisms responsible for the selection of the targets are still largely unknown. p53Ser46 phosphorylation has been proposed as a damage-intensity sensor because it occurs only upon severe DNA damage and irreversibly drives the cells to apoptosis by promoting changes in p53 affinity for different promoters with a shift from cell-cycle arrest-related genes to apoptosis-related ones. Given the involvement of HIPK2 in p53Ser46 phosphorylation and the subsequent induction of apoptosis, the MDM2-mediated degradation of HIPK2 might be
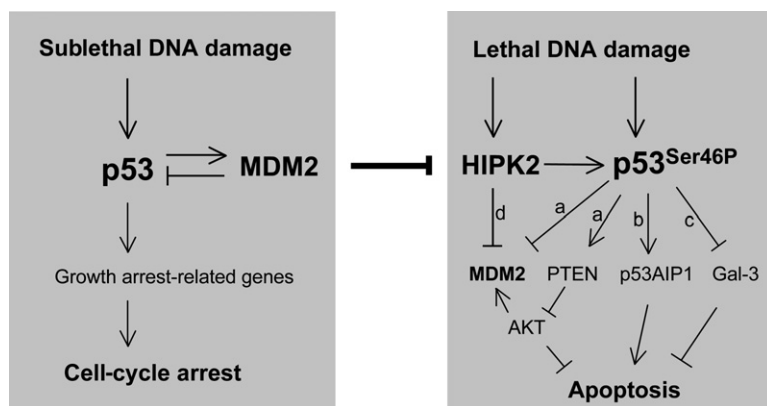

Figure 7. Model Summarizing the Role of HIPK2 Regulation in Cell Response to DNA Damage

In the left panel, it is envisaged that sublethal DNA damage activates the classical p53-MDM2 feedback loop and the transcription of growth arrest-related genes that will arrest the cell cycle. In the right panel, there are the apoptotic pathways shown to be regulated by p53 phosphorylated at Ser46 (a, Mayo et al. [2005]; b, Oda et al. [2000]; and c, Cecchinelli et al. [2006a]). In addition, the inactivation role that HIPK2 has directly on MDM2 is shown (d: Wang et al., 2001; Di Stefano et al., 2004a). The events outlined in the two panels are linked by the p53-induced, MDM2-mediated degradation of HIPK2 we are reporting here, which would actively block activation of the apoptotic pathways when the cell-cycle arrest is required.

interpreted as a negative feedback loop by which p53 represses one of its own proapoptotic activators. However, p53Ser46 phosphorylation is considered a "point of no return," seemingly contradicting the existence of a negative loop in this pathway. The active inhibition of HIPK2 expression upon sublethal damage and the possibility of transforming this type of damage into an apoptotic one by rescuing HIPK2 expression and p53Ser46 phosphorylation through MDM2 depletion (Figure 6) is consistent with the "point of no return" hypothesis and strongly suggests that the MDM2-mediated degradation of HIPK2 is not a negative feedback loop to recover from the apoptotic pathway activated by p53Ser46 phosphorylation but rather a preemptive inhibition of the p53 proapoptotic activation when cell-cycle arrest is required. These results provide functional relevance to observations reported by a few groups (reviewed by Oren [2003]) and support the existence, together with the well-documented role in apoptosis, of antiapoptotic effects of p53.

Recently, Gresko and coworkers showed that a p53-induced, caspase-mediated cleavage of the C-terminal region of HIPK2 is required for full activation of its apoptotic function (Gresko et al., 2006). They showed that a caspase-resistant HIPK2 mutant is less active in induction of apoptosis, whereas a C-terminal HIPK2 deletion mutant that mimics the caspase-cleaved protein has increased apoptotic activity. Interestingly, the C-terminal portion of HIPK2 that is removed from the full-length protein by caspase activity includes the lysine residue 1182 we are describing here, strongly supporting the model of a positive feedback loop that reinforces HIPK2 apoptotic function by elimination of a degradation site. Indeed, the ΔC-HIPK2 deletion mutants we employed, although efficiently bound by MDM2, are significantly more stable than the full-length protein (Figure 4B and data not shown).

MDM2 acts as a survival factor in many cell types and is overexpressed in a subset of human tumors where it is thought to contribute to the development of resistance to radio and chemotherapy (Iwakuma and Lozano, 2003). In response to UV irradiation, regulation of the p53-MDM2 pathway was shown to be significantly different between cells that will undergo transient cell-cycle arrest or apoptosis (Perry et al., 1993; Ashcroft et al., 2000; Perry, 2004). In the first case, one can observe the classical feedback loop behavior of p53-MDM2 regulation that leads to p53 activation followed by MDM2 upregulation that limits the p53 growth arresting activity (Figure 7, left panel). In contrast, cells undergoing apoptosis lack accumulation of MDM2 and consequently increase p53 expression that would now allow transcription of apoptosis-related targets to whose promoter binding sites p53 has, on average, lower affinity (Vousden, and Lu, 2002). Based on our results, this model could be enriched by the fact that accumulation of MDM2 would also degrade HIPK2 and block the apoptosis-specific p53Ser46 phosphorylation. In contrast, low levels of MDM2 would release HIPK2 degradation and allow p53Ser46 phosphorylation

that would further repress MDM2 transcription (Mayo et al., 2005) and neutralize the MDM2 inhibitory activity on p53, including p53 ubiquitylation and degradation, p53 cytoplasmic relocalization, and p53 transcriptional inactivation (Di Stefano et al., 2004a) (Figure 7).

In summary, our data demonstrate that HIPK2 is a target for MDM2-mediated degradation and strongly suggest that the cell-cycle-arresting functions of p53 include the suppression of the apoptotic ones. This may have important implications in tumor resistance to therapy and for the development of overcoming strategies.

EXPERIMENTAL PROCEDURES

Cell-Culture Conditions, Transfection, Adenoviral Infection, and RNA Interference

Human H1299, RKO, MCF-7, HEK293, HCT116 (both p53$^{+/+}$ and p53$^{-/-}$, kindly provided by B. Vogelstein) cells, and mouse fibroblasts from WT or *Mdm2/p53* dKO mice (kindly provided by G. Lozano) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Primary HEK cells (kindly provided by S. Bacchetti) were maintained in α-MEM supplemented with 10% FBS and used between the first 12 doublings. siRNAs were transfected by oligofectamine reagent (Invitrogen) as suggested by the manufacturer. HDM2-specific and control siRNA sequences were as described (Bres et al., 2003). The recombinant adenoviruses dl70.3, Adp53 (kindly provided by S. Bacchetti and F. Graham), and AdHIPK2 (Cecchinelli et al., 2006b) were amplified, titrated, and employed as described (Cecchinelli et al., 2006b). For DNA damage, subconfluent cells were UV irradiated at different doses or treated with ADR (Pharmacia Italia). MG132 and E64 (Calbiochem) and Nutlin-3 (Cayman) were added to subconfluent cells at the indicated doses. Cell viability, colony-formation assay, cell-cycle profiles, and TUNEL assay were performed as described (D'Orazi et al., 2002; Cecchinelli et al., 2006a).

Immunoprecipitation, Western Blotting, Immunofluorescence, and Kinase Assay

In vitro-translated proteins were produced by TNT-coupled wheat germ extract system (Promega). TCEs were prepared in RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, and 1 mM EDTA) for immunoprecipitations and in nondenaturing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton, and 5 mM EDTA) for coimmunoprecipitations. Immunocomplexes were resolved by SDS-PAGE, and WBs were performed with the following Abs: rabbit anti-HIPK2 (kindly provided by L. Schmitz); rabbit anti-p53 (FL-393) (Santa Cruz Biotechnology); sheep anti-p53 (Ab-7, Calbiochem); rabbit anti-phospho-p53Ser46 (Cell Signaling Technology); anti-MDM2 monoclonal (MoAb) (2A10, kindly provided by A. Levine; Ab1, Oncogene Research Products); anti-Ub MoAb (FK2, Affiniti Research Product); anti-GFP and anti-PARP MoAb (clone 42, BD PharMingen); anti-FLAG MoAb (M5 for WB and M2 for IP and IF, Sigma); anti-α-tubulin MoAb (Immunological Sciences), anti-actin MoAb (Sigma); anti-β-Gal MoAb (Roche) and rabbit anti-p21 (C-19, Santa Cruz Biotechnology); and HRP-conjugated goat anti-mouse, anti-rabbit, and anti-sheep (Cappel).

Kinase assays and immunofluorescences were performed as reported (D'Orazi et al., 2002).

Expression Vectors

To construct the pEGFP-HIPK2 deletion mutants, DNA fragments of the murine *Hipk2* gene were amplified from pBKS clone 46 (D'Orazi et al., 2002) by PCR in the presence of specific primers. HIPK2-K1182R, HDM2-C438L, and HDM2-D68A mutants were constructed by the QuikChange site-directed mutagenesis kit (Stratagene). pCMV-Hdm2 was kindly provided by K. Vousden, pCMV-Hdm2ΔRing

by M. Oren, and pCMV-Hdm2Δ190-230 by V. Calabro'. All the other vectors employed are described elsewhere (D'Orazi et al., 2002; Cecchinelli et al., 2006a).

In Vitro and In Vivo Ubiquitylation Assays

For in vitro ubiquitylation assay, dKO MEFs were transiently induced to express high levels of HIPK2 by infection with AdHIPK2 adenovirus, or WT HDM2 or its Δ-RING deletion mutant by plasmid transfection. TCEs were immunoprecipitated with anti-HIPK2 or anti-HDM2 Abs, respectively. Immunocomplexes were incubated in 35 μl ubiquitylation buffer (25 mM Tris-HCl [pH 7.5], 60 mM NaCl, 1 mM DTT, 4 mM ATP, and 9 mM MgCl2) supplemented with 200 ng of recombinant E1, 200 ng of E2 (UbcH5b), and 15 μg of Ub (all from Sigma) for 2 hr at 30°C or for 30 min at 37°C. Proteins were resolved by Tris-Acetate Gel (Invitrogen) followed by WB. For in vivo ubiquitylation assay, TCEs were obtained with RIPA buffer and either analyzed directly by WB or immunoprecipitated with anti-HIPK2 Ab, resolved by SDS-PAGE (5%), or NuPAGE (3%–8%) followed by WB.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/25/5/739/DC1/.

REFERENCES

Appella, E., and Anderson, C.W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. Eur. J. Biochem. 268, 2764–2772.

Ashcroft, M., Taya, Y., and Vousden, K.H. (2000). Stress signals utilize multiple pathways to stabilize p53. Mol. Cell. Biol. 20, 3224–3233.

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

Bres, V., Kiernan, R.E., Linares, L.K., Chable-Bessia, C., Plechakova, O., Treand, C., Emiliani, S., Peloponese, J.M., Jeang, K.T., Coux, O., et al. (2003). A non-proteolytic role for ubiquitin in Tat-mediated transactivation of the HIV-1 promoter. Nat. Cell Biol. 5, 754–761.

Bunz, F., Dutriax, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282, 1497–1501.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J., Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 18, 6845–6854.

Cecchinelli, B., Lavra, L., Rinaldo, C., Iacovelli, S., Gurtner, A., Gasbarri, A., Ulivieri, A., Del Prete, F., Trovato, M., Piaggio, G., et al. (2006a). Repression of the anti-apoptotic molecule galectin-3 by HIPK2-activated p53 is required for p53-induced apoptosis. Mol. Cell. Biol. 26, 4746–4757.

Cecchinelli, B., Porrello, A., Lazzari, C., Gradi, A., Bossi, G., D'Angelo, M., Sacchi, A., and Soddu, S. (2006b). Ser58 of mouse p53 is the homologue of human Ser46 and is phosphorylated by HIPK2 in apoptosis. Cell Death Differ. 11, 1994–1997.

Di Stefano, V., Blandino, G., Sacchi, A., Soddu, S., and D'Orazi, G. (2004a). HIPK2 neutralizes MDM2 inhibition rescuing p53 transcriptional activity and apoptotic function. Oncogene 23, 5185–5192.

Di Stefano, V., Rinaldo, C., Sacchi, A., Soddu, S., and D'Orazi, G. (2004b). Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. Exp. Cell Res. 293, 311–320.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., et al. (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser46 and mediates apoptosis. Nat. Cell Biol. 4, 11–19.

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.

Gresko, E., Roscic, A., Ritterhoff, S., Vichalkovski, A., Del Sal, G., and Schmitz, M.L. (2006). Autoregulatory control of the p53 response by caspase-mediated processing of HIPK2. EMBO J. 25, 1883–1894.

Harris, S.L., and Levine, A.J. (2005). The p53 pathway: positive and negative feedback loops. Oncogene 24, 2899–2908.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature 387, 296–299.

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.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. 420, 25–27.

Ichwan, S.J., Yamada, S., Sumrejkanchanakij, P., Ibrahim-Auerkari, E., Eto, K., and Ikeda, M.A. (2005). Defect in serine 46 phosphorylation of p53 contributes to acquisition of p53 resistance in oral squamous cell carcinoma cells. Oncogene 25, 1216–1224.

Iwakuma, T., and Lozano, G. (2003). MDM2 an introduction. Mol. Cancer Res. 1, 993–1000.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256, 827–830.

Kim, Y.H., Choi, C.Y., Lee, S.J., 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, 25875–25879.

Kim, E.-J., Park, J.-S., and Um, S.-J. (2002). Identification and characterization of HIPK2 interacting with p73 and modulating functions of the p53 family in vivo. J. Biol. Chem. 277, 32020–32028.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature 387, 299–303.

Lavin, M.F., and Gueven, N. (2006). The complexity of p53 stabilization and activation. Cell Death Differ. 13, 941–950.

Li, X., Dumont, P., Della Pietra, A., Shetler, C., and Murphy, M.E. (2005). The codon 47 polymorphism in p53 is functionally significant. J. Biol. Chem. 280, 24245–24251.

Marine, J.-C., Francoz, S., Maetens, M., Wahl, G., Toledo, F., and Lozano, G. (2006). Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ. 13, 927–934.

Mayo, L.D., Seo, Y.R., Jackson, M.W., Smith, M.L., Rivera Guzman, J., Korgaonkar, C.K., and Donner, D.B. (2005). Phosphorylation of human p53 at Ser46 determines promoter selection and whether apoptosis is attenuated or amplified. J. Biol. Chem. 280, 25953–25959.

Moll, U.M., Wolff, S., Speidel, D., and Deppert, W. (2005). Transcription-independent pro-apoptotic functions of p53. Curr. Opin. Cell Biol. 17, 631–636.

Moller, A., Sirma, H., Hofmann, T.G., Rueffer, S., Klimczak, E., Droge, W., Will, H., and Schmitz, M.L. (2003). PML is required for homeodomain-interacting protein kinase 2 (HIPK2)-mediated p53 phosphorylation and cell cycle arrest but is dispensable for the formation of HIPK domains. Cancer Res. 63, 4310–4314.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378, 203–206.

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-phsphorylated p53. Cell 102, 849–862.

Ohtani, S., Kagawa, S., Tango, Y., Umeoka, T., Tokunaga, N., Tsunemitsu, Y., Roth, J.A., Taya, Y., Tanaka, N., and Fujiwara, T. (2004). Quantitative analysis of p53-targeted gene expression and visualization of p53 transcriptional activity following intratumoral administration of adenoviral p53 in vivo. Mol. Cancer Ther. 3, 93–100.

Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ. 10, 431–442.

Perry, M.E. (2004). Mdm2 in the response to radiation. Mol. Cancer Res. 2, 9–19.

Perry, M.E., Piette, J., Zawadzki, J.A., Harvey, D., and Levine, A.J. (1993). The mdm-2 gene is induced in response to UV light in a p53-dependent manner. Proc. Natl. Acad. Sci. USA 90, 11623–11627.

Prives, C. (1998). Signaling to p53: breaking the MDM2-p53 circuit. Cell 95, 5–8.

Rodicker, F., and Putzer, B.M. (2003). p73 is effective in p53-null pancreatic cancer cells resistant to WT TP53 gene replacement. Cancer Res. 63, 2737–2741.

Rui, Y., Xu, Z., Lin, S., Li, Q., Rui, H., Luo, W., Zhou, H.M., Cheung, P.Y., Wu, Z., Ye, Z., et al. (2004). Axin stimulates p53 functions by activation of HIPK2 kinase through multimeric complex formation. EMBO J. 23, 4583–4594.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., et al. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 303, 844–848.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature 408, 307–310.

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

Wang, Y., Debatin, K.M., and Hug, H. (2001). HIPK2 overexpression leads to stabilization of p53 protein and increased p53 transcriptional activity by decreasing Mdm2 protein levels. BMC Mol. Biol. 2, 8–16.

Welchman, R.L., Gordon, C., and Mayer, R.J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell Biol. 6, 599–609.

Yee, K.S., and Vousden, K.H. (2005). Complicating the complexity of p53. Carcinogenesis 26, 1317–1322.

Zhang, Q., Yoshimatsu, Y., Hildebrand, J., Frisch, S.M., and Goodman, R.H. (2003). Homeodomain interacting protein kinase 2 promotes apoptosis by down-regulating the transcriptional co-repressor CtBP. Cell 115, 177–186.