Homeodomain-Interacting Protein Kinase 2 Is the Ionizing Radiation–Activated p53 Serine 46 Kinase and Is Regulated by ATM

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Abstract

Phosphorylation of p53 at Ser^{46} is important to activate the apoptotic program. The protein kinase that phosphorylates p53 Ser^{46} in response to DNA double-strand breaks is currently unknown. The identification of this kinase is of particular interest because it may contribute to the outcome of cancer therapy. Here, we report that ionizing radiation (IR) provokes homeodomain-interacting protein kinase 2 (HIPK2) accumulation, activation, and complex formation with p53. IR-induced HIPK2 up-regulation strictly correlates with p53 Ser^{46} phosphorylation. Down-regulation of HIPK2 by RNA interference specifically inhibits IR-induced phosphorylation of p53 at Ser^{46}. Moreover, we show that HIPK2 activation after IR is regulated by the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM). Cells from ataxia telangiectasia patients show defects in HIPK2 accumulation. Concordantly, IR-induced HIPK2 accumulation is blocked by pharmacologic inhibition of ATM. Furthermore, ATM down-regulation by RNA interference inhibited IR-induced HIPK2 accumulation, whereas checkpoint kinase 2 deficiency showed no effect. Taken together, our findings indicate that HIPK2 is the IR-activated p53 Ser^{46} kinase and is regulated by ATM. [Cancer Res 2007;67(5):2274–9]

Introduction

Tumor suppressor p53 is important to maintain genomic stability and to suppress carcinogenesis. Consistently, p53 is mutated or functionally inactivated in most human cancers (1). p53 gets activated after genotoxic stress and, depending on cell type, nature, and the extent of damage, activates different cellular responses including DNA repair, cellular senescence, and apoptosis (2). The activity of p53 is mainly regulated via its subcellular distribution and its protein stability, which is controlled through posttranslational modifications in particular by site-specific phosphorylation (3). In response to DNA damage, a set of protein kinases is activated that phosphorylate p53 at multiple residues, leading to its stabilization and transcriptional activation (4).

The differential phosphorylation patterns of p53 are thought to regulate the expression of different sets of target genes that determine the cellular decision between the p53 effector pathways. p53 phosphorylated at Ser^{46}, in concert with Ser^{15} and Ser^{20}, triggers the apoptotic program by activating proapoptotic target genes such as *p53DINP1* and *p53AIP1* (5, 6). Interestingly, the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) is required for p53 Ser^{46} phosphorylation, although it fails to phosphorylate p53 at Ser^{46} directly (7). These findings argue for an important role of ATM in regulating the currently unknown ionizing radiation (IR)–activated p53 Ser^{46} kinase.

DNA double-strand breaks (DSB) are highly dangerous for the cell and, if remain unrepaired, lead to cell death or to genomic instability, a driving force for carcinogenesis. Numerous proteins are involved in the mammalian DSB response and deficiencies in many of these proteins predispose to cancer. DSBs trigger a signaling pathway that results in activation of ATM, which coordinates the DNA damage response through direct phosphorylation of a series of effector proteins, including Ser^{15} of p53 (8–10). Genetic defects in ATM cause ataxia telangiectasia, a severe inherited genomic instability syndrome characterized by radiation sensitivity, defective DNA damage checkpoint signaling, neurodegeneration, premature ageing, and increased cancer susceptibility (8–10).

The serine/threonine protein kinase homeodomain-interacting protein kinase 2 (HIPK2; ref. 11) is an important regulator of growth suppression and apoptosis (12). HIPK2 directly interacts with p53 *in vitro* and *in vivo* and phosphorylates p53 Ser^{46} after UV damage, thereby activating the apoptotic program (13, 14). Although stress-activated protein kinase p38 also phosphorylates p53 Ser^{46} after UV damage, it fails to mediate IR-induced p53 Ser^{46} phosphorylation (7, 15, 16). Thus, the IR-activated p53 Ser^{46} kinase remained thus far unknown (17).

Here, we explored the role of HIPK2 in p53 Ser^{46} phosphorylation after IR. Our results identify HIPK2 as the IR-activated p53 Ser^{46} kinase and provide evidence for its regulation by the DNA damage checkpoint kinase ATM.

Materials and Methods

Cell culture and antibodies. Hep3B, HepG2, MCF7, and HT1080 cells were obtained from the American Type Culture Collection (Rockville, MD). HCT116, HCT116 p53$^{-/-}$ and HCT116 Chk2$^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein. Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 1% (w/v) penicillin/streptomycin, and 20 mmol/L HEPES buffer. Ataxia telangiectasia fibroblasts (GM02052), healthy control fibroblasts (GM03491), and WI38 fibroblasts were obtained from the Coriell Cell Repositories (Campden, NJ) and maintained in DMEM/15% FCS/1% (w/v) penicillin/streptomycin/20 mmol/L HEPES. All cells were cultured in a humidified incubator at 37°C at 5% CO_2. DMEM and supplements were purchased from Invitrogen (Karlsruhe, Germany).

The following antibodies were used: p53 (DO-1) and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz, Inc., Heidelberg, Germany), α-tubulin (Sigma, Munich, Germany), p53 phosphorylated Ser^{46} and phosphorylated Ser^{15} (Cell Signaling Technologies, Danvers, MA), p53DINP1 (Novus Biologicals, Littleton, CO), actin clone C4 (MP Biomedicals, Illkirch, France). The affinity-purified HIPK2 antibody has been previously described (13).

Drug treatments. Caffeine (Sigma) was solved in cell culture medium and sterile filtered. Wortmannin (Sigma) and LY294002 (Merck Biosciences,

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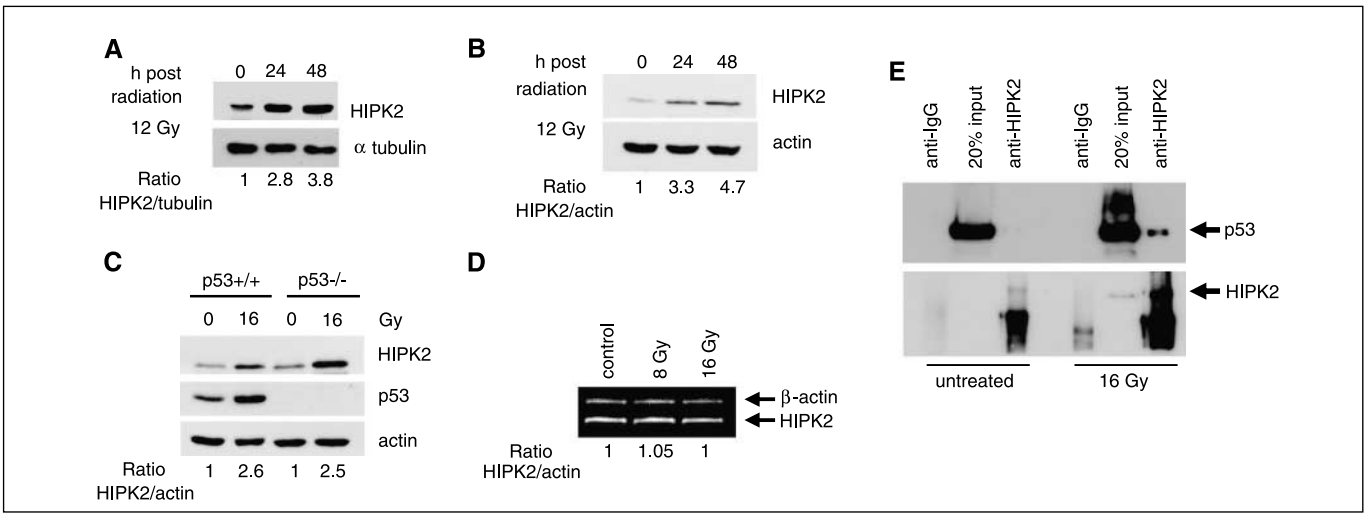

Figure 1. HIPK2 accumulates after ionizing radiation and forms a complex with p53. HepG2 (*A*) and HCT116 (*B*) cells were irradiated with 12 Gy and harvested at the given time points posttreatment, and total cell extracts were analyzed by immunoblotting. *C,* HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were irradiated as indicated, and total cell extracts were analyzed by immunoblotting. *D,* reverse transcription-PCR analysis of HIPK2 and β-actin expression in untreated and irradiated HepG2 cells. Cells were harvested 24 h posttreatment, and total RNA was used for the reverse transcription-PCR reaction. *E,* total cell lysates from HepG2 cells either left untreated or irradiated with 16 Gy were subjected to immunoprecipitation using rabbit IgG control antibodies or affinity-purified rabbit HIPK2 antibodies. The precipitated complexes were analyzed by immunoblotting with HIPK2 and p53 specific antibodies. Signals were quantified using the ImageQuant software package. Representative of three (*A–D*) or two (*E*) independent experiments.

Darmstadt, Germany) were solved in sterile DMSO. Cells were irradiated with the doses indicated; 8 h later, the inhibitory drugs were added, and cells were incubated for further 16 h before harvesting.

RNA interference. The HIPK2 (targeting nucleotides 570-589; ref. 18) and luciferase control small interfering RNA (siRNA) and the ATM-specific siRNA were previously described (19) and were synthesized by Dharmacon (Lafayette, CO) or Qiagen (Hilden, Germany). Transfections were done with 75 nmol/L double-stranded RNA using HiPerFect transfection reagent (Qiagen). Cells were irradiated 20 h post transfection or left untreated as indicated.

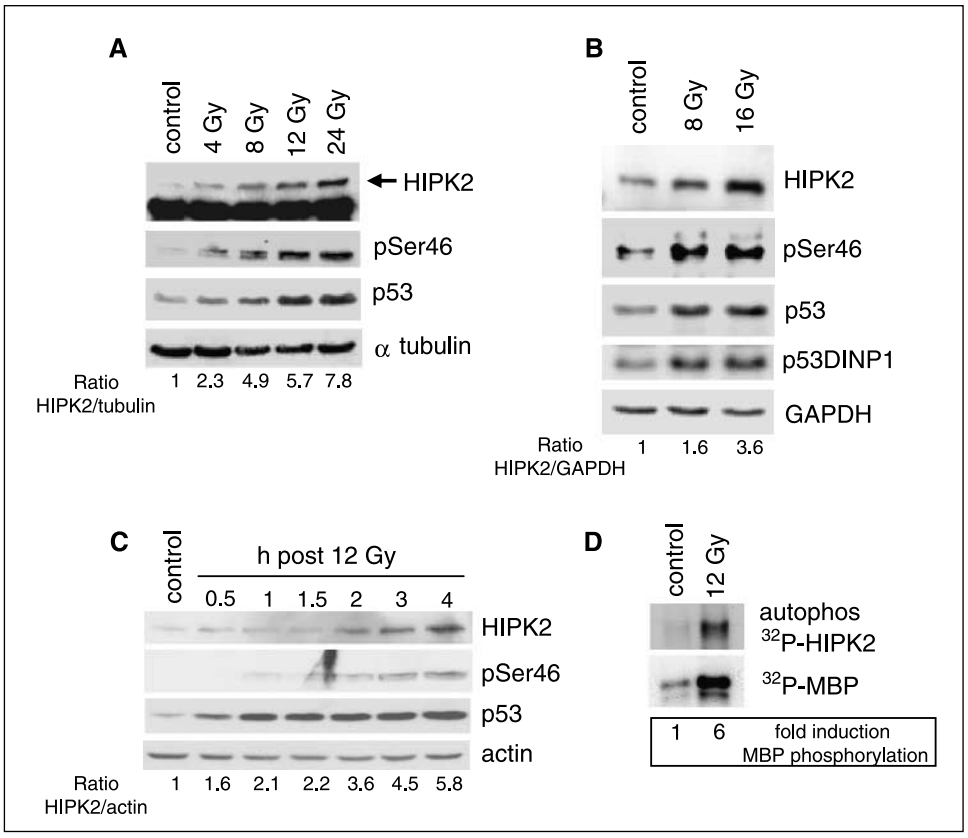

Figure 2. IR-induced HIPK2 accumulation and activation correlates with p53 Ser^{46} phosphorylation (*pSer46*). HepG2 (*A*) and WI38 (*B*) cells were irradiated with the given dose, and 24 h later, total cell lysates were prepared and analyzed by immunoblotting with the antibodies indicated. *C,* HepG2 cells were irradiated with 12 Gy, and cell lysates were prepared at the time point indicated and analyzed by immunoblotting. *D,* endogenous HIPK2 was immunoprecipitated from lysates of untreated and irradiated HepG2 cells. HIPK2 kinase activity was analyzed by immunocomplex kinase assays using MBP as a substrate. MBP phosphorylation was quantified using a phosphorimager. Representative of three independent experiments.

Immunoprecipitations, immunoblotting, and HIPK2 kinase assay. Cells were lysed in lysis buffer [25 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 10% glycerol, 1% SDS, 1% NP40, 0.5 mmol/L EDTA, 25 mmol/L NaF, protease inhibitors] and fractionated on SDS-PAGE, transferred to Hybond-P (GE Healthcare, Munich, Germany), and treated as described (13). Proteins were detected by enhanced chemiluminescence using Western Blot Dura and Femto from Pierce Biosciences (Perbio Science, Bonn, Germany). Immunoprecipitation of endogenous HIPK2 and kinase assays using myelin basic protein (MBP; Sigma) as a substrate were done as published (13). Quantification of the immunoblots and reverse transcription-PCR were done using the Image Quant (TL v2005) software package from Amersham Biosciences (GE Healthcare). Arbitrary units are shown.

IR treatment. Cells were irradiated using a Gammacell 40 or Gammacell 1000C Cesium-137 source, with the doses indicated in the figure legends. Cells were harvested at the indicated time points, and total cell lysates were prepared and further analyzed as described.

mRNA purification and reverse transcription-PCR analysis. Total RNA was isolated using the RNeasy kit (Qiagen); 2 μg total RNA was reverse transcribed using the cDNA Cycle kit (Invitrogen) according to the manufacturer's instruction. Twenty percent of the reverse transcription reaction was used as template for PCR using the following primer pairs: HIPK2 (sense, 5′-GGCCTCACATGTGCAAGTTTTC-3′; antisense, 5′-TTGGTAGGTATCAAGGAGGCTC-3′); ATM (sense, 5′-CACACTTAGCAGGTTGCAGGCCATTG-3′; antisense, 5′-GTTCCCTAAGGAGACCTACTTCCTC), β-actin (sense, 5′-CCTCGCCTTTGCCGATCC-3′; antisense, 5′-GGATCTTCATGAGGTAGTCAGTC-3′). PCR was done using β-actin primers as an internal control in each reaction using the following conditions: 1 min 95°C, 1 min 56°C, 1 min 72°C (25 cycles). PCR reactions were analyzed on 1% agarose gels.

Results

HIPK2 accumulates in response to IR. To study the role of HIPK2 in the DSB-induced DNA damage response, we treated human cancer cell lines with IR and analyzed endogenous HIPK2 protein levels by immunoblotting. Interestingly, induction of DSBs by exposure to IR resulted in elevated HIPK2 levels both in HepG2 hepatocellular carcinoma cells (Fig. 1*A*) and in HCT116 colon carcinoma cells (Fig. 1*B*). A similar HIPK2 up-regulation was found in HT1080 fibrosarcoma cells (data not shown).

To test whether HIPK2 accumulation is dependent on p53, isogenic HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with IR and HIPK2 protein levels were analyzed by immunoblotting. HIPK2 levels were comparably increased in both cell lines, irrespective of the cellular p53 status (Fig. 1*C*). Similar HIPK2 up-regulation was found in p53-deficient Hep3B hepatocellular carcinoma cells (data not shown). These results indicate that p53 is dispensable for HIPK2 accumulation. Furthermore, reverse transcription-PCR analysis of IR-treated HepG2 cells revealed no increase in HIPK2 mRNA levels (Fig. 1*D*), indicating that HIPK2 accumulation is regulated through a posttranscriptional mechanism. These data imply a role of HIPK2 in the IR-induced DNA damage response.

Complex formation of endogenous HIPK2 and p53 after IR. Previous work has established a direct interaction of HIPK2 and p53 (13, 14). To find out whether endogenous HIPK2 forms a complex with p53 in response to IR, we did immunoprecipitation assays using lysates from irradiated and untreated HepG2 cells. A small fraction of p53 (~0.5% of the input) was coimmunoprecipitated with endogenous HIPK2 from lysates of IR-treated cells but not from untreated cells (Fig. 1*E*). These data indicate complex formation of endogenous HIPK2 and p53 in response to IR.

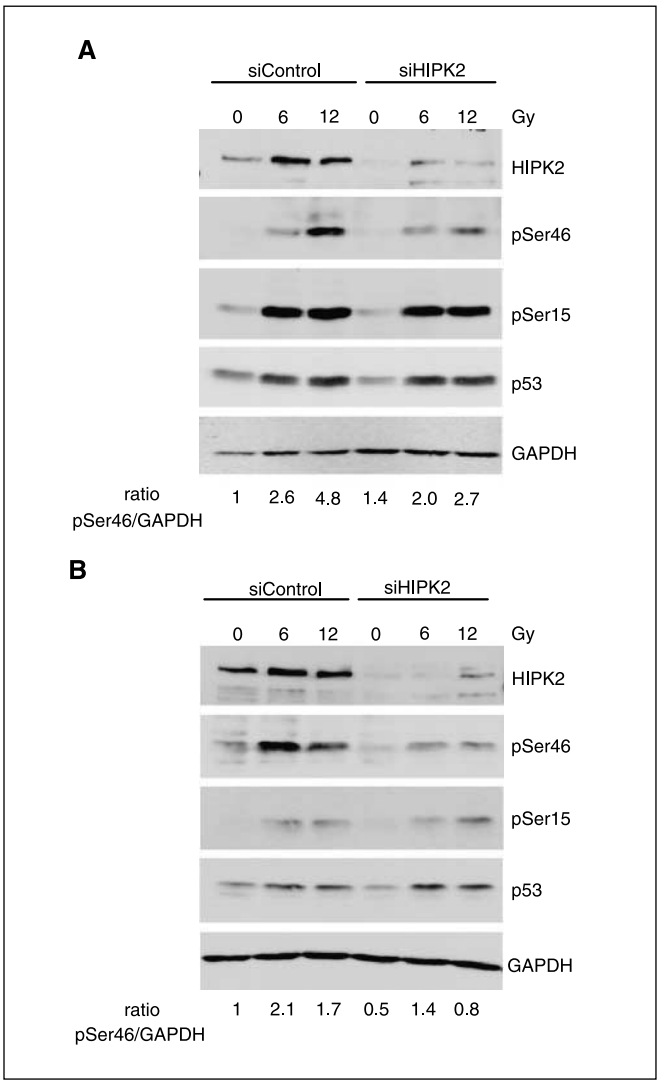

Figure 3. HIPK2 down-regulation specifically inhibits IR-induced p53 Ser^{46} phosphorylation. HepG2 (*A*) and MCF7 (*B*) cells transfected with control siRNA (*siControl*) or HIPK2-specific siRNA (*siHIPK2*) were either left untreated or irradiated with the indicated doses. Twenty-four hours later, cells were harvested; total cell lysates were analyzed by immunoblotting, and the signals were quantified using the ImageQuant software package. The phosphorylated p53 Ser^{46} signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and are given in arbitrary units. Representative of four independent experiments.

IR-induced p53 Ser^{46} phosphorylation correlates with HIPK2 accumulation. Immunoblot analysis of cell lysates from IR-treated cells revealed that HIPK2 protein levels were increased in a dose-dependent manner after IR both in HepG2 cells (Fig. 2*A*) and in primary human diploid WI-38 fibroblasts (Fig. 2*B*), suggesting a similar regulation of HIPK2 in primary cells and cancer cells. The elevated HIPK2 levels correlated with increased p53 Ser^{46} phosphorylation and up-regulation of the p53 target gene *p53DINP1* (Fig. 2*B*), a gene product specifically induced by Ser^{46} phosphorylated p53 (6).

To study the kinetics of HIPK2 up-regulation more detailed, we irradiated HepG2 cells and analyzed the cell lysates by immunoblotting. After 2 to 3 h after irradiation, HIPK2 levels were visibly increased (Fig. 2*C*). Again, HIPK2 up-regulation correlated with increased phosphorylation of p53 at Ser^{46} (Fig. 2*C*). Taken together,

these findings suggest a role of HIPK2 in IR-induced p53 Ser[46] phosphorylation.

HIPK2 is activated in response to IR. Next, we determined whether HIPK2 is activated in response to IR, which is a prerequisite to mediate p53 Ser[46] phosphorylation. To this end, we immunoprecipitated endogenous HIPK2 from lysates of untreated and IR-treated cells and measured its activity by performing *in vitro* kinase assays using MBP as a substrate as previously published (13). A strong increase in HIPK2 autophosphorylation and concomitant MBP phosphorylation was detected when HIPK2 was precipitated from lysates of IR-treated HepG2 cells (Fig. 2*D*). These findings show that IR results in HIPK2 activation, most likely through accumulation of HIPK2 rather than through an increase in its specific kinase activity.

Knockdown of HIPK2 abrogates IR-induced p53 Ser[46] phosphorylation. As HIPK2 interacts with p53 after IR and its accumulation and activation correlate with p53 Ser[46] phosphorylation, we next addressed the *in vivo* function of HIPK2 in IR-induced p53 Ser[46] phosphorylation. Therefore, we down-regulated endogenous HIPK2 by RNA interference in HepG2 and in MCF7 breast cancer cells and analyzed p53 Ser[46] phosphorylation after IR by immunoblotting. Strikingly, knockdown of HIPK2 expression reduced IR-induced p53 Ser[46] phosphorylation in both cancer cell lines (Fig. 3*A* and *B*). By contrast, IR-induced p53 phosphorylation at Ser[15], which is phosphorylated by ATM (8–10), was not affected through HIPK2 down-regulation. These results indicate a specific role of HIPK2 in p53 Ser[46] phosphorylation. Along with previous reports that showed direct interaction of HIPK2 and p53 (13, 14), these data strongly suggest a direct role of HIPK2 in IR-induced Ser[46] phosphorylation.

ATM regulates HIPK2 accumulation after IR damage. The signaling pathway governing HIPK2 activation after DNA damage is currently unknown. Because IR activates DNA damage checkpoint kinase ATM (8–10), which plays an important role in regulating IR-induced p53 Ser[46] phosphorylation (7), we tested whether ATM controls HIPK2 accumulation.

To this end, we treated irradiated HepG2 cells with the ATM/ATR inhibitor caffeine. Caffeine did only slightly affect HIPK2 steady-state levels in undamaged HepG2 cells (Fig. 4*A*). By contrast, HIPK2 accumulation in response to IR was abrogated by caffeine treatment (Fig. 4*A*). To confirm the activity of caffeine, we

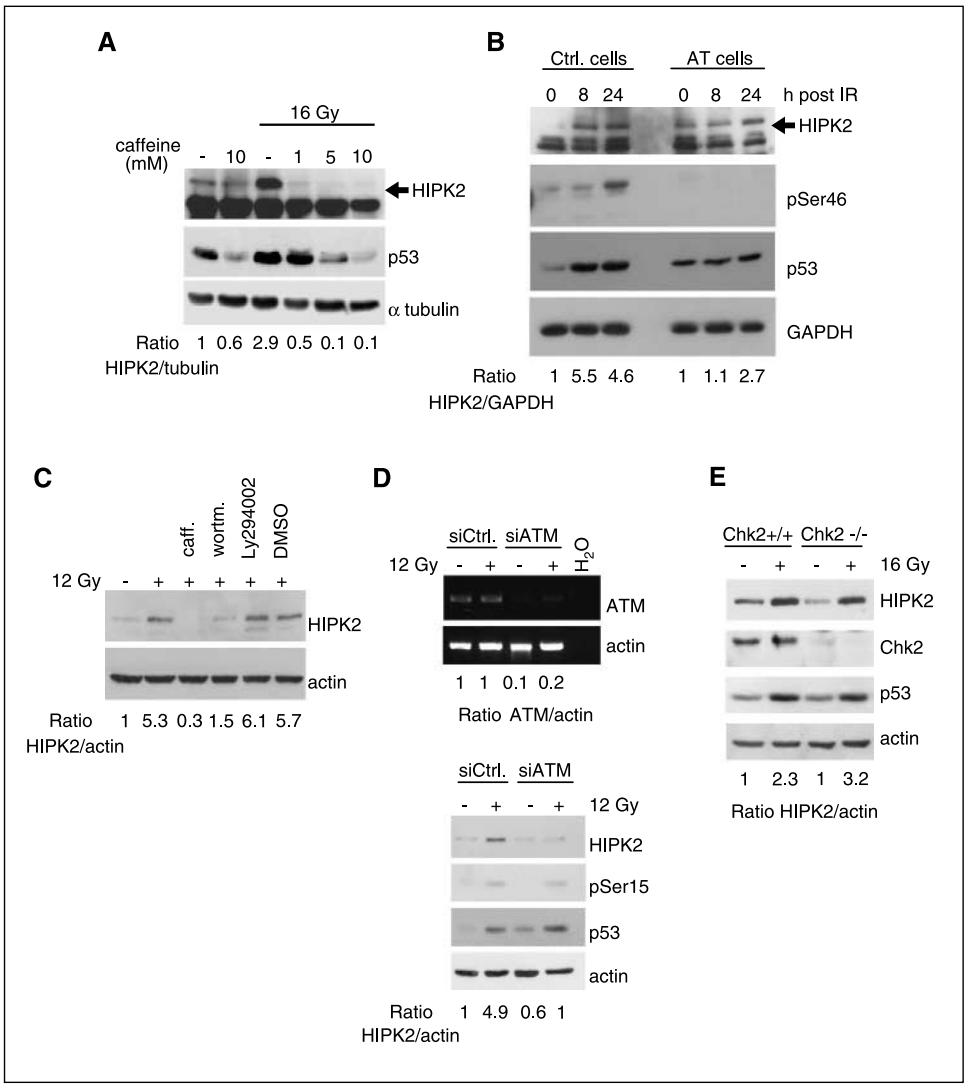

Figure 4. HIPK2 is regulated by the ATM signaling pathway. *A,* HepG2 cells were treated with caffeine and irradiated or left untreated. Twenty-four hours after irradiation, total cell lysates were prepared and analyzed by immunoblotting with the antibodies indicated. *B,* human primary diploid fibroblasts (GM03491) and ataxia telangiectasia fibroblasts (GM02052) were irradiated with 12 Gy, and total cell lysates were prepared and analyzed by immunoblotting. *C,* HepG2 cells were irradiated and treated with 5 mmol/L caffeine, 20 μmol/L wortmannin, or 50 μmol/L Ly294002, respectively. Twenty-four hours after irradiation, total cell lysates were prepared and analyzed by immunoblotting. *D, top,* HepG2 cells transfected with control siRNA (*siCtrl*) or ATM-specific siRNA (*siATM*) were either left untreated or irradiated with 12 Gy. Total RNA was analyzed by reverse transcription-PCR for ATM and actin expression. *Bottom,* total cell lysates of siRNA-treated cells were prepared 24 h after irradiation and were analyzed by immunoblotting. *E,* HCT116 Chk2$^{+/+}$ and Chk2$^{-/-}$ cells were irradiated as indicated. Twenty-four hours later, total cell lysates were prepared and analyzed by immunoblotting. Representative of three independent experiments.

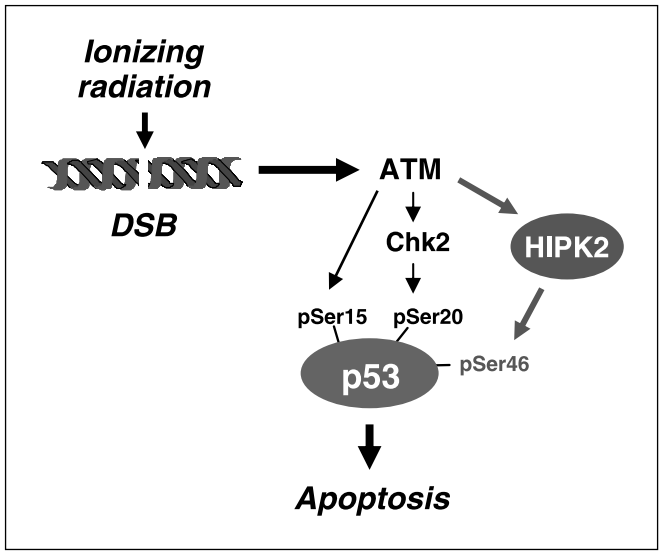

Figure 5. Model for the regulation of HIPK2 activity and p53 phosphorylations by the ATM pathway. DNA DSBs trigger activation of the DNA damage checkpoint kinase ATM. Activated ATM controls p53 phosphorylations through direct phosphorylation at Ser^{15}. In addition, ATM controls p53 Ser^{20} and Ser^{46} phosphorylation indirectly by regulating the activity of Chk2 and HIPK2, respectively. p53 phosphorylated at Ser^{15}, Ser^{20}, and Ser^{46} activates proapoptotic target genes and triggers apoptosis.

analyzed p53 protein levels, which are at least in part controlled through the ATM pathway (4). Concordant with a functional inhibition of ATM, p53 levels were decreased after IR in presence of caffeine (Fig. 4*A*).

Next, we analyzed HIPK2 levels in irradiated primary human ataxia telangiectasia fibroblasts and control fibroblasts. Although, out of currently unknown reasons, ataxia telangiectasia cells showed increased steady-state levels of HIPK2 in comparison to normal fibroblasts, IR-induced HIPK2 accumulation was reduced in ataxia telangiectasia cells (Fig. 4*B*). Consistent with a previous report (7), p53 Ser^{46} phosphorylation was absent in ataxia telangiectasia cells.

Treatment with wortmannin, an inhibitor that blocks activation of ATM and DNA-PK, also reduced HIPK2 accumulation in response to IR (Fig. 4*C*). In contrast, DNA-PK inhibitor LY294002 (20) did not inhibit HIPK2 up-regulation after IR (Fig. 4*C*), implying that IR-induced HIPK2 accumulation is regulated by ATM.

Finally, we down-regulated ATM expression in HepG2 cells by RNA interference (Fig. 4*D*) and studied HIPK2 accumulation after IR damage by immunoblotting. ATM depletion inhibited IR-induced HIPK2 accumulation (Fig. 4*D*). Unexpectedly, IR-activated p53 Ser^{15} phosphorylation was virtually unchanged upon ATM down-regulation, suggesting that other p53 Ser^{15} kinases, including ATR, DNA-PK, and hSMG-1, may compensate ATM reduction (8–10, 21). Collectively, these data show that IR-induced HIPK2 accumulation is essentially regulated by the ATM pathway.

Chk2 is dispensable for IR-induced HIPK2 activation. ATM coordinates the DNA damage response through direct phosphorylation of diverse substrate proteins, including checkpoint kinase Chk2 (9). To further dissect the HIPK2 activation pathway downstream of ATM, we used HCT116 cells that are deficient for Chk2 (22). Chk2 deficiency did not inhibit IR-induced HIPK2 accumulation (Fig. 4*E*), indicating that Chk2 is dispensable for this event.

Discussion

p53 Ser^{46} phosphorylation plays a critical role in activating the apoptotic program in response to different types of DNA damage (5). Previous work showed an essential role of HIPK2 in mediating UV-activated p53 Ser^{46} phosphorylation through direct interaction with p53 (13, 14). Interestingly, the IR-activated p53 Ser^{46} kinase remained thus far unknown.

HIPK2 is the IR-activated p53 Ser^{46} kinase. Our data here establish a clear link between HIPK2 and IR-induced p53 Ser^{46} phosphorylation. Several lines of evidence strongly suggest that HIPK2 is the IR-activated p53 Ser^{46} kinase. First, HIPK2 accumulation and activation sharply correlates with IR-induced p53 Ser^{46} phosphorylation. Second, endogenous HIPK2 and p53 form a complex in response to IR, showing specific interaction of HIPK2 with its bona fide substrate after IR. Third, knockdown of HIPK2 expression specifically inhibited p53 Ser^{46} phosphorylation in response to IR, whereas Ser^{15} phosphorylation was not compromised, suggesting that HIPK2 is dispensable for ATM activation. Collectively, these results show the importance of HIPK2 in IR-induced p53 Ser^{46} phosphorylation and indicate that HIPK2 is the IR-activated p53 Ser^{46} kinase.

Role of ATM in HIPK2 regulation. The signal transduction pathway governing HIPK2 activation in response to DNA damage remained unclear. Our experiments here provide first insight into the pathway regulating HIPK2 activation in response to DNA damage and strongly support a model that HIPK2 activation is controlled by ATM (Fig. 5). Several lines of evidence show that IR-induced HIPK2 accumulation, and by implication its activation, requires ATM. First, pharmacologic inhibition of ATM strongly inhibited IR-induced HIPK2 accumulation. Second, ataxia telangiectasia fibroblasts were defective in IR-induced HIPK2 accumulation and p53 Ser^{46} phosphorylation. Third, ATM depletion by RNA interference clearly inhibited HIPK2 accumulation in response to IR. These data provide compelling evidence for a critical role of ATM in HIPK2 regulation and are consistent with a previous report showing a requirement of ATM for p53 Ser^{46} phosphorylation after IR (7). IR-induced HIPK2 accumulation was not compromised in Chk2-deficient cells, showing that Chk2 is dispensable for HIPK2 activation.

Although, out of currently unknown reasons, HIPK2 was robustly expressed in ataxia telangiectasia cells, p53 Ser^{46} phosphorylation after IR was absent. This might be explained, at least in part, by absence of an essential HIPK2 cofactor, which is required to form an active p53 Ser^{46} kinase complex *in vivo*. Future work is required to clarify this point.

In summary, our data show that HIPK2 is the IR-activated p53 Ser^{46} kinase and provide evidence for its functional regulation through checkpoint kinase ATM. Because HIPK2 can activate the apoptotic program both in a p53-dependent and a p53-independent manner (18, 23–25), it might prove to be a promising target in cancer therapy.

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