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HIPK2 knock-down compromises tumor cell efficiency to repair damaged DNA

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Abstract

Homeodomain Interacting Protein Kinase-2 (HIPK2) is a protein with many functions and a modulator of p53 oncosuppressor functions. *TP53* is the "guardian of the genome" thus, is the most critical tumor suppressor gene product that inhibits malignant transformation. P53R2 gene is directly induced by p53 in response to DNA damage and is involved in the p53 checkpoint for repairing damaged DNA to block genome instability. Here we wanted to explore the involvement of HIPK2 in damaged-DNA repair by regulating p53-induced p53R2 gene. We show that, induction of p53R2 expression, p53 recruitment onto p53R2 promoter, and its transcriptional activation was strongly impaired by HIPK2 knock-down, in response to drug. The failure of p53-induced p53R2 activation markedly compromised damaged-DNA repair efficiency. Finally, overexpression of exogenous p53 overcame the inability of endogenous p53 to activate p53R2-luc promoter in HIPK2 depleted cells. These data suggest that HIPK2 is involved in damaged-DNA repair taking part in restraining tumor progression, at least in part depending on p53 regulation.

Keywords: p53; p53R2; DNA repair; Host-reactivation; RNA interference; Tumor progression

HIPK2 is a serine–threonine kinase mostly localized in subnuclear dot-like structures [1]. HIPK2 regulates transcription, apoptosis, cell growth, and development through its interaction with a variety of functional proteins including, not only transcriptional regulators and chromatin modifiers, but also cytoplasmic signal transducers, transmembrane proteins, and the E2 component of SUMO ligase [2]. HIPK2 is a positive regulator of p53 oncosuppressor functions. Thus, in response to severe DNA damage, such as UV irradiation or drugs treatment, HIPK2 interacts with and specifically phosphorylates p53 oncosuppressor protein at residue Ser46 with subsequent transcriptional activation of pro-apoptotic target genes [3–5]. We also identified a novel mechanism for HIPK2 regulation of p53 oncosuppressor functions after non-severe DNA damage, independently from Ser46 phosphorylation. We found that HIPK2 mediates PCAF-dependent p53Lys320 acetylation, selectively inducing $p21^{Waf1}$ gene related to growth arrest response [6]. The biological importance of these findings has been evidenced by inactivation of HIPK2 function. Silencing of endogenous HIPK2 impairs p53Ser46-mediated apoptotic response after severe DNA damage and strongly reduces p53 binding to $p21^{Waf1}$ promoter after non-apoptotic DNA damage [5,6]. Either way, loss of HIPK2 function strongly harms p53 functions.

The *TP53* tumor suppressor gene plays an important role in preventing cancer development and most tumor cells show either mutations in the *TP53* gene or defects in the pathways responsible for the activation of p53 protein [7,8]. Activation of p53 induces its oncosuppressor functions including cell cycle arrest, apoptosis, DNA repair, and replicative senescence, that are thought to prevent the replication of abnormal cells and either allow their

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repair or target their elimination [9]. Thus, defects in proper DNA repair have been suggested to lead to genomic instability, resulting in cancer [10,11]. In this regard, absence of functionally active p53 has been shown to result in spontaneous and induced tumor susceptibility in mice and men [12–14]. How p53 activation and stabilization occur following stress is still not completely understood. In cancers harboring wtp53 its tumor suppressor activity may be circumvented by genetic alterations including overexpression of MDM2 that promotes the degradation of p53 inhibiting its transcription activity [15–17]. Alternatively, p53 activity may be compromised by other mechanisms such as deregulation of regulatory proteins [8,18]. Either way, loss of p53 function becomes a key element for tumor development and progression. Therefore, agents that are able to regulate p53 functions might be considered as an approach auxiliary to halt tumor progression. These considerations led us to examine whether HIPK2 was involved in damaged-DNA repair through p53 activity to identify potential markers of tumor progression.

Materials and methods

Cell cultures and treatments. Human colon carcinoma RKO, RKO-pSuper, RKO-HIPK2-interfered (HIPK2i) [5], RKO-p53-interfered (p53i), H1299-pSuper (p53 null), and H1299-HIPK2i (all kindly provided by S. Soddu), and human lung carcinoma A549 cell lines were cultured in RPMI-1640 (GIBCO-BRL, Life Technology, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL) plus glutamine and antibiotics in humidified atmosphere with 5% CO_2 at 37 °C.

For DNA damage, subconfluent cells were irradiated with UV light at 10 J/m^2 and treated with adriamycin (ADR) diluted into the medium to a final concentration of 0.2 μg/ml.

Western blot, antibodies, and plasmids. Cells were washed with ice-cold phosphate-buffered saline (PBS), collected by trypsinization, rinsed with PBS, and lysed for 20 min on ice in lysis buffer (50 mM Tris–HCl, pH 7.5; 1 mM EDTA; 150 mM NaCl; 0.5% sodium deoxycolate; 1% SDS) plus protease inhibitors. Equal amount of proteins were mixed with Laemmli sample buffer, resolved in 12% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Western blot analysis was performed with an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) in accordance with the manufacturer's instructions.

The following antibodies were used: anti-p53 (DO1) mouse monoclonal, anti-p53R2 goat polyclonal, anti-p21^{Waf1} rabbit polyclonal (all from Santa Cruz Biotechnology), anti-p53 sheep polyclonal (Ab7; Oncogene Science), and anti-tubulin mouse monoclonal (Immunological Sciences). Immunoreactivity was detected by ECL chemiluminescence reaction kit (Amersham).

The expression vectors used were: pSUPER and pSUPER-HIPK2 [5], wild-type-p53 [3], pGL3-luc, and p53R2-luc (kindly provided by K. Sabapathy, University of Singapore).

Transactivation assay. Cells were plated in 35 mm dishes and transfected with the luciferase reporter driven by the p53-dependent promoter p53R2, using Lipofectamine Plus (Invitrogen) method according to manufacturer's instructions. Twenty-four hours later cells were treated with ADR or irradiated with UV and incubated for additional 24 h before analyzing luciferase activity. Transfection efficiencies were normalized with the use of a co-transfected β-galactosidase construct (β-gal) and luciferase activity was assayed as previously described [3].

Host reactivity assay. pGL3-luciferase plasmid was damaged by UV (700 J/m^2) and co-transfected into pSuper and HIPK2i cells along with β-gal expression vector, using Lipofectamine Plus (Invitrogen) method according to manufacturer's instructions. Cells were cultured for 24 and 48 h to allow repair of damaged DNA and the luciferase activity was determined thereafter. Values were normalized to the β-gal internal standard and were expressed relative to the luciferase activity obtained for the undamaged PGL3-luc plasmid in each cell line (fixing the untreated values as 100%). High luciferase activity corresponds to efficient DNA repair and vice versa.

Student's *t*-test was used for statistical significance of the differences between cell lines in repairing damaged DNA.

siRNA interference and Reverse Transcriptase-PCR (RT-PCR). For siRNA transfection, RKO cells were plated at semiconfluence in 35 mm dishes the day before transfection. si-Control and siRNA-p53R2 (Dharmacon) were transfected overnight using Lifectamine Plus method (Invitrogen) according to the manufacturer's protocol and 48 h later cells were transfected with UV-damaged PGL3 plasmid as above for the host-reactivation assay. RNA was isolated 48 h after siRNA transfection by using the RNeasy mini kit (Qiagen S.P.A., Milano, Italy) and cDNA and PCR were performed essentially as previously described [5]. Briefly, 2 μg of total RNA were reverse transcribed using the MuLV reverse transcriptase and the reverse transcribed material was used for PCRs with the AmpliTaq DNA Polymerase (Gene Amp RNA PCR kit, Perkin-Elmer, Roche Molecular System, Brachburg, NJ, USA). The p53R2 transcript was amplified with primers: Forward 5′-AGGACTGTTCGTGTTCA GCTC-3′ and Reverse 5′-GTCCACCTCCTGAGAAAACTC-3′. The HIPK2 and GAPDH primers were described elsewhere [5]. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Chromatin immunoprecipitation (ChIP) analysis. Cells were treated with ADR (0.2 μg/ml) for 12 h. DNA and protein complexes were cross-linked in living cells for ChIP analysis as described previously [6]. Cell lysates were incubated with specific anti-p53 antibody (Ab7) and no specific IgG and DNA bound to immunoprecipitates amplified with promoter-specific primer for *p53R2*. 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*^{Waf1} and *p53R2*. The primer sequences for *p53R2* promoter are: Forward 5′-caaagaaacggaagtggtgg-3′ and Reverse 5′-agtttttggggacacagg-3′. *GAPDH* was used as control for p53 DNA-binding specificity. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Results

HIPK2 knock-down impairs p53-dependent p53R2 transcription

Among the p53 target genes responsible of p53 tumor suppressor activities, p53R2 induces ribonucleotide reductase activity providing nucleotides during the repair process, and therefore inducing repair of damaged DNA after exposure to various genotoxic agents [19]. Reduced p53-dependent transcription of p53R2 gene in response to DNA damage leads to deregulation of DNA repair machinery and likely increases genomic instability [19]. To study the impact of HIPK2 in DNA repair, we took advantage of the role that HIPK2 plays in regulating p53 functions and first explored the involvement of HIPK2 in regulating p53-dependent p53R2 expression. We performed immunoblot analysis of RKO-pSuper control and HIPK2 interfered (HIPK2i) cells (Fig. 1A) that were exposed to ADR (0.2 μg/ml) treatment (Fig. 1B) and UV (10 J/m^2) irradiation

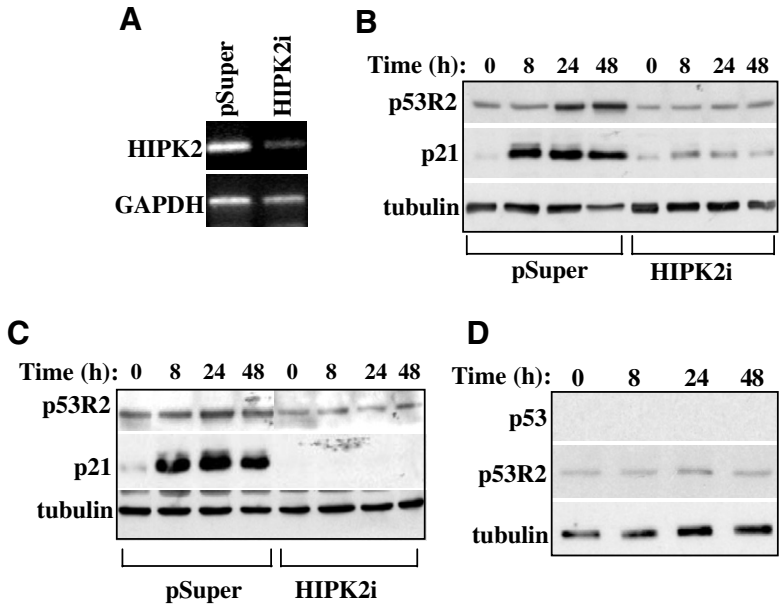

Fig. 1. HIPK2 depletion inhibits p53R2 induction. (A) RT-PCR analysis of RKO cells stably transfected with pSuper and pSUPER-HIPK2 expression vectors. Equal amounts of RNA were reverse-transcribed and analyzed by PCR for expression of HIPK2 gene. GAPDH was amplified as control. RKO-pSuper and -HIPK2i cell lines were treated with ADR (0.2 μg/ml) (B) and UV (10 J/m^2) (C) and chased at the indicated times. (D) RKO-p53i cells were treated with ADR (0.2 μg/ml) and chased at the indicated times. Equal amounts of total cell extracts were separated on denaturing 12% SDS–PAGE and analyzed by immunoblotting with the indicated antibodies. Anti-tubulin was used as protein loading control. The data are representative of three independent experiments.

(Fig. 1C), doses suitable for induction of p53R2, as reported [19], but not for induction of apoptosis (not shown). We observed induction of p52R2 expression in pSuper cells in response to both damages while the induction was inhibited by HIPK2 depletion (Fig. 1B and C). In agreement with what we have previously shown for non-severe DNA-damage by cisplatin [6], p21^{Waf1} was also induced following both treatments, only in pSuper cells compared to HIPK2i cells (Fig. 1B and C). To ascertain that p53R2 induction was p53-dependent, we analyzed RKO cells depleted of p53, by RNA interference. We found that after ADR treatment, p53R2 was not induced (Fig. 1D), in accordance with what was previously shown [19]. These findings confirm that HIPK2 plays a role in regulating p53 transcription activity after non-apoptotic DNA damage.

We next tested the involvement of HIPK2 in p53-induced p53R2 transactivation. As shown in Fig. 2, the p53R2 reporter activity was induced by both ADR treatment (Fig. 2A) and UV irradiation (Fig. 2B) only in pSuper cells, while it was significantly inhibited by HIPK2 knock-down. Similar results were obtained in A549 cells transiently transfected with pSUPER-HIPK2 interfering vectors (Fig. 2C). As expected, RKO-p53i cells failed to activate p53R2-luc reporter (not shown). To evaluate the *in vivo* recruitment of p53 onto *p53R2* promoter, chromatin immunoprecipitation assay (ChIP) was performed on ADR treated cells and immunoprecipitated DNA was analyzed by PCR using promoter specific primers for *p53R2*, *p21*^{Waf1}, and *GAPDH* genes under conditions of linear amplification. We found that p53 was bound *in vivo* to *p53R2* promoter in response to ADR treatment only in pSuper cells (Fig. 2D) compared to HIPK2i cells (Fig. 2E). Additionally, p53 was no longer bound to *p21*^{Waf1} promoter in HIPK2i cells, compared to pSuper cells (Fig. 2). *GAPDH* promoter was used as control for specificity of p53 binding. These results show that HIPK2 is involved in p53 recruitment onto *p53R2* promoter and its transcriptional activation.

p53-dependent p53R2 DNA repair is impaired by HIPK2 depletion

As we found that HIPK2 was necessary for efficient induction of p53 target p53R2, we next evaluated whether the DNA repair capacity was compromised in cells depleted of HIPK2 function. To this aim we used the host-cell reactivation assay, a method of investigating the DNA-repair capacity of cells by quantifying the function of repaired exogenous DNA that has been damaged before introducing it into cells [20]. PGL3-luciferase reporter plasmid was exposed to UV irradiation (700 J/m^2) to induce damage and subsequently transfected into pSuper and HIPK2i cells. Untreated pGL3-luc vector was transfected alone as positive control. Expression of luciferase activity, which corresponds to the DNA-repair rate, was monitored. The damaged reporter, unless repaired, does not display any transcriptional activity. As shown in Fig. 3A, pSuper cells consistently demonstrated increasing levels of luciferase activity 48 h after transfection similar in extent to what found with untreated plasmid. Conversely, HIPK2i cells showed a lesser level of luciferase activity 24 h after transfection, compared to pSuper control cells, and did not

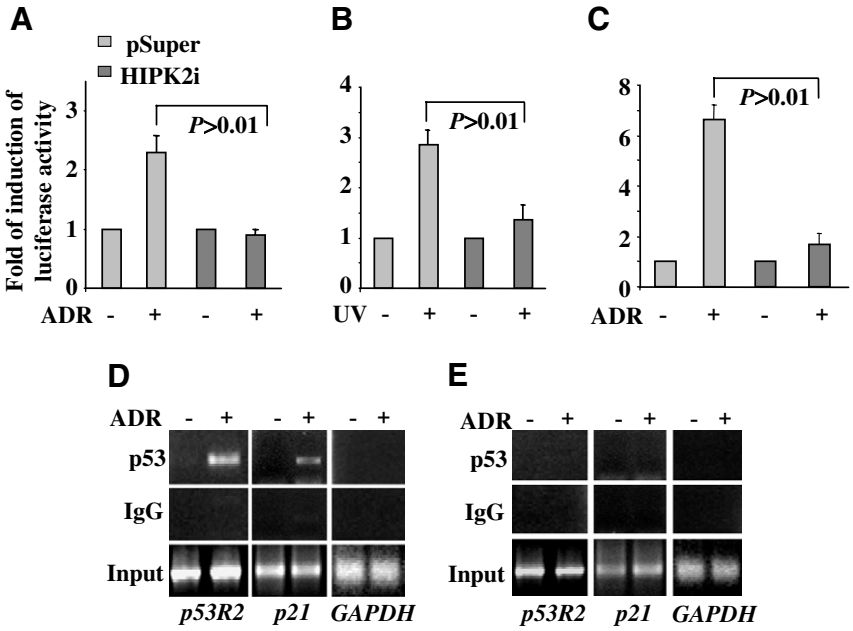

Fig. 2. HIPK2 is required for p53R2 transcription. RKO-pSuper and -HIPK2i cells were co-transfected with p53R2-luc reporter and β-gal plasmids, using the Lipofectamine Plus method. Twenty-four hours later, cells were treated with ADR (0.2 μg/ml) (A) and UV (10 J/m^2) (B) for 24 h. Luciferase activity was determined following normalization to β-gal activity. (C) A549 lung cancer cells were transiently transfected with pSUPER and pSUPER-HIPK2 vectors and 36 h later, co-transfected with β-gal and p53R2-luc reporter and treated with ADR as above for luciferase activity. All transfections were performed in duplicate. Data are represented as fold of induction with respect of untreated control cells and standard deviation of four independent experiments performed in duplicate is reported. *P*-values are indicated. *In vivo* recruitment of p53 onto promoters. RKO-pSuper (D) and -HIPK2i (E) cells were left untreated or treated with ADR (0.2 μg/ml) for 12 h and subjected to chromatin immunoprecipitation (ChIP) using specific polyclonal anti-p53 (Ab7) antibody and no specific IgG as control. Immunoprecipitates from each sample were analyzed by PCR using specific primers for *p53R2* and *p21*^{Waf1} promoters. *GAPDH* promoter was used as control for specificity of p53 binding. A sample representing linear amplification (1:10) of the total chromatin (Input) was included as control for PCR promoter amplification.

promote reactivation of UV-damaged luciferase reporter after 48 h of transfection (Fig. 3A). These data suggest that abrogation of HIPK2 function may render tumor cells less efficient in repairing damaged DNA. To evidence the involvement of endogenous p53 in reactivating damaged DNA, we transfected RKO-p53i cells with pGL3-luciferase reporter plasmid damaged with UV, as above. As shown in Fig. 3A, p53i cells did not promote reactivation of UV-damaged luciferase reporter compared to pSuper control cells. Next we knocked down *p53R2* gene by siRNA and showed good reduction of p53R2 expression (Fig. 3B, inset). We transfected si-Control and si-p53R2 cells with pGL3-luciferase reporter plasmid damaged with UV, as above. In agreement with our hypothesis, p53R2-interfered cells failed to reactivate damaged DNA, compared to si-Control cells (Fig. 3B). These differences were reproducible and consistent in several experiments. Taken together these data suggest that HIPK2 plays a role in DNA repair at least in part through p53-dependent p53R2 gene.

Exogenous p53 overexpression overcomes endogenous p53 inability to induce p53R2

We finally evaluated whether exogenous p53 could overcome the endogenous p53 inability, in HIPK2 depleted cells, to induce p53R2 gene. To this aim, RKO-pSuper and HIPK2i cells were co-transfected with reporter plasmids encoding for p53R2-luc and wtp53 or treated with non-apoptotic dose of ADR. As shown in Fig. 4, pSuper (upper panel) and HIPK2i (lower panel) cells showed induction of p53R2-luc activity following wtp53 co-expression, while ADR treatment induced p53R2-luc activity only in pSuper cells, as shown previously. These data show that, overexpression of exogenous wtp53 can induce transcription of p53R2-luc promoter, likely overcoming the mechanisms of p53 inhibition triggered by HIPK2 knockdown.

Discussion

When activated, the p53 protein turns on genes that can halt cell division until the DNA is repaired, or induce apoptosis when the damage is irreversible [21]. Thus, p53 can help prevent the accumulation of potentially cancer-causing mutations and also put brakes on abnormal cell growth. The results presented here show that HIPK2 is involved in regulating damaged-DNA repair at least in part through p53. It has been described that inactivation of p53 directly interferes with the transcription of p53R2 in response to DNA damage and that faulty regulation of p53R2 might enhance misincorporation of dNTPs and dysregulation of DNA repair machinery and thereby increase

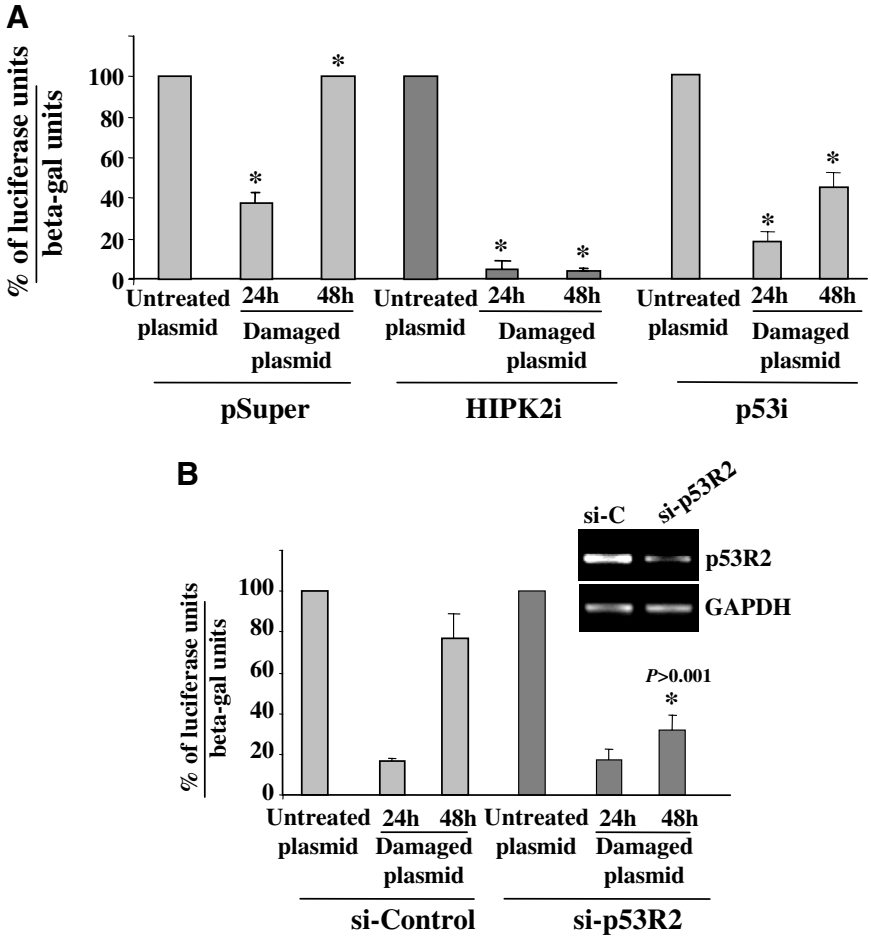

Fig. 3. Host-reactivation assay showing that damaged-DNA repair is impaired by HIPK2 depletion. (A) pGL3-luciferase plasmid was either left untreated or damaged with UV irradiation (700 J/m^2) before co-transfecting it in pSuper and HIPK2i cells along with β-gal expression vector. The relative luciferase units were determined 24 and 48 h after transfection. (B) RKO-p53i cells were transfected with pGL3-luciferase plasmid for host-reactivation assay, as above. All experiments were performed in duplicate al least three times, independently. Data are represented as ratio of luciferase activity over β-gal units. *P* value is indicated (*$P < 0.001$). (C) RKO cells were transiently transfected with si-Control and siRNA-p53R2 for 48 h (inset, RT-PCR for p53R2 mRNA levels). pGL3-luciferase plasmid was either left untreated or damaged with UV irradiation (700 J/m^2) before co-transfecting it along with β-gal expression vector in RKO cells, transiently interfered for p53R2 function. The relative luciferase units were determined 24 and 48 h after transfection. All experiments were performed in duplicate al least three times, independently. Data are represented as ratio of luciferase activity over β-gal units. *P* value is indicated (*$P > 0.001$).

the frequency of mutations [19]. Hence, the existence of a functional p53 is mandatory for the selection of antitumor response. Loss of p53 function, mostly conferred by its transactivation activity, can be obtained either by mutations or by deregulation of regulatory proteins [8,18]. Either way, p53 malfunction would contribute to tumor progression because it increases the rate of genetic mutations. About half of all cancers express normal p53 protein and many of these tumors show defects in the ability to respond to p53 [8]. In cancers carrying wtp53 its tumor suppressor activity may be circumvented by genetic alterations including overexpression of MDM2 that promotes the degradation of p53 inhibiting its transcriptional activity [15–17]. However, identification of alternative mechanisms by which p53 function is impaired may provide further insights into the molecular aetiology of human cancers harboring wild-type p53 and the consequent cancer therapy.

One of the most recent regulators of p53 is the kinase HIPK2, involved in p53-dependent growth arrest and apoptosis. In this report, we show that HIPK2 knockdown strongly abolished damaged-DNA repair, at least in part through impairment of p53-dependent induction of p53R2 gene, suggesting the existence of a switch, raised by the absence of HIPK2, that might increase genomic instability and thereby favor tumor progression. HIPK2 is an important regulator of p53 functions through it direct phosphorylation at Ser46 after severe DNA damage [3–5] or PCAF-mediated p53Lys320 acetylation in response to non-severe DNA damage [6]. Hence, one could hypothesize that altered HIPK2-mediated p53 acetylation, rather than phosphorylation, might be involved in induction of p53R2 gene. Thus, recruitment of both general and specialized transcriptional co-regulators, histone modifications and the subsequent alterations in chromatin structure seem

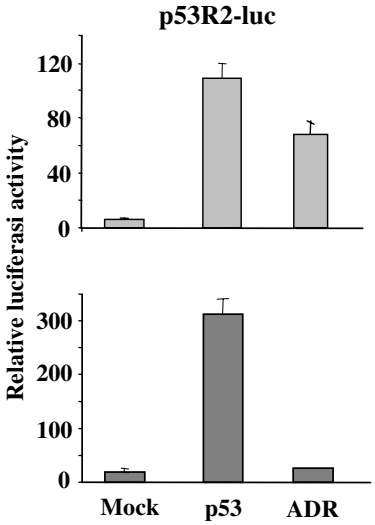

Fig. 4. Exogenous p53 overexpression induces p53R2-luc activity. RKO-pSuper (upper panel) and HIPK2i (lower panel) cells were co-transfected with p53R2-luc plasmid and wtp53 expression vectors or treated with ADR 0.2 μM. Luciferase activity was determined following normalization to β-gal activity. Data are represented as relative luciferase activity with respect to β-gal units. Standard deviation of three independent experiments performed in duplicate is reported.

to be one of the major outcomes of p53 binding to the consensus recognition element and activation of transcription [22]. Intriguingly though, abrogation of HIPK2 function impaired the cell capacity to repair damaged DNA in a stronger manner compared to p53 interference (Fig. 3), suggesting the existence of additional mechanisms and interacting proteins regulated by HIPK2 other than p53. This is reasonable because HIPK2 relies in a crossroad for multiple p53-dependent and -independent biological functions, including transcription regulation, apoptosis, growth-arrest, and development, thanks to its interaction with multiple proteins involved in chromatin and protein posttranslational modifications [2]. It seems plausible that lack of HIPK2 will have effects on cell growth and survival that would not be mimicked in cells where p53 function has been lost directly. At present, reduced HIPK2 mRNA expression has been found in breast, thyroid, and colon cancer tumors, often associated with increased malignancy [23,24]. Thus, is becoming evident that HIPK2 plays a key role in restraining tumor progression, however, much more work needs to be done in order to fully elucidate p53-dependent and -independent functions of HIPK2.

Despite the potent antitumor properties of p53, it is not clear whether its loss of function simply facilitates the genetic changes that contribute to tumor development, or whether tumor growth is dependent on keeping the p53 pathway turned off permanently [25]. Interestingly, we found that, overexpression of exogenous p53 was able to induce transcription of p53-dependent p53R2-luc promoter, likely overcoming the inhibitory effect generated by HIPK2 depletion. These findings suggest that p53 oncosuppressor pathways were not misplaced following HIPK2 depletion and open a way to reconstitute p53 activities, through, for instance, gene therapy to halt tumor growth, in agreement with recent publications [26–29]. These findings also highlight the need for further studies to better understand the role for HIPK2 in regulating p53 activity.

In conclusion, we have shown that HIPK2 knock-down strongly reduced damaged-DNA repair. This has potential implications in tumor prognosis since the reduced DNA repair activity would promote genomic instability and tumor progression. In this regard, HIPK2 appears as an interesting prognostic marker to be evaluated in tumors.

Acknowledgments

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