ORIGINAL ARTICLE

HIPK2 phosphorylates Δ Np63 α and promotes its degradation in response to DNA damage

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Homeodomain-interacting protein kinase 2 (HIPK2) is an emerging player in cell response to genotoxic agents that senses damage intensity and contributes to the cell's choice between cell cycle arrest and apoptosis. Phosphorylation of p53 at S46, an apoptosis-specific p53 posttranslational modification, is the most characterized HIPK2 function in response to lethal doses of ultraviolet (UV), ionizing radiation or different anticancer drugs, such as cisplatin, roscovitine and doxorubicin (DOX). Indeed, like p53, HIPK2 has been shown to contribute to the effectiveness of these treatments. Interestingly, p53-independent mechanisms of HIPK2-induced apoptosis were described for UV and tumor growth factor- β treatments; however, it is unknown whether these mechanisms are relevant for the responses to anticancer drugs. Because of the importance of the so-called 'p53-independent apoptosis and drug response' in human cancer chemotherapy, we asked whether p53-independent factor(s) might be involved in HIPK2-mediated chemosensitivity. Here, we show that HIPK2 depletion by RNA interference induces resistance to different anticancer drugs even in p53-null cells, suggesting the involvement of HIPK2 targets other than p53 in response to chemotherapy. In particular, we found that HIPK2 phosphorylates and promotes proteasomal degradation of Δ Np63 α , a prosurvival Δ N isoform of the p53 family member, p63. Indeed, effective cell response to different genotoxic agents was shown to require phosphorylation-induced proteasomal degradation of Δ Np63 α . In DOX-treated cells, we show that HIPK2 depletion interferes with Δ Np63 α degradation, and expression of a HIPK2-resistant Δ Np63 α - Δ 390 mutant induces chemoresistance. We identify T397 as the Δ Np63 α residue phosphorylated by HIPK2, and show that the non-phosphorylatable Δ Np63 α -T397A mutant is not degraded in the face of either HIPK2 overexpression or DOX treatment. These results indicate Δ Np63 α as a novel target of HIPK2 in response to genotoxic drugs.

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Introduction

Homeodomain-interacting protein kinase 2 (HIPK2) is an evolutionarily conserved Serine/Threonine kinase, originally identified as co-repressor for homeodomain transcription factors (Kim *et al.*, 1998). HIPK2 interacts and phosphorylates at specific S/T residues, a still enlarging body of targets, involved in the regulation of gene transcription during development and in cell response to several types of stress (Calzado *et al.*, 2007; Rinaldo *et al.*, 2007b; Calzado *et al.*, 2009). In the latter condition, HIPK2 is activated by different genotoxic stimuli, including ultraviolet (UV) (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002), ionizing irradiation (Dauth *et al.*, 2007) and anticancer drugs such as cisplatin (CDDP) (Di Stefano *et al.*, 2004), doxorubicin (DOX) (Rinaldo *et al.*, 2007a) and roscovitine (Weierska-Gadek *et al.*, 2007). Depletion of HIPK2 expression by specific antisense oligonucleotides or interfering RNAs induces strong resistance to the apoptosis caused by these agents, supporting a functional role by the kinase in response to these stresses.

A few mechanisms of HIPK2 inactivation in human cancers have been identified, such as HIPK2-forced cytoplasmic relocation in breast carcinomas and in leukemogenesis (Pierantoni *et al.*, 2007; Wee *et al.*, 2008), HIPK2 mutations in acute myeloid leukemia (Li *et al.*, 2007) and loss of HIPK2 protein expression and allele-specific loss-of-heterozygosity in thyroid cancers (Lavra *et al.*, 2011). At least in the case of breast cancer biopsies, HIPK2 inactivation was significantly associated with overexpression of the prosurvival $\alpha_6\beta_4$ integrin (Bon *et al.*, 2009) and with tumor resistance to spontaneous apoptosis (Pierantoni *et al.*, 2007).

The tumor suppressor p53, a key mediator of DNA damage response (DDR) and a critical player in the maintenance of genomic stability, has been the first identified HIPK2 target involved in DDR. HIPK2 was

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shown to bind p53 and differentially regulate its localization, phosphorylation, acetylation and transcriptional activity, depending on the extent of the damage. In particular, upon severe, presumably irreparable DNA damage induced by lethal doses of UV, CDDP or DOX, HIPK2 is strongly upregulated and phosphorylates human p53 at S46 (Puca *et al.*, 2010), an apoptosis-specific p53 posttranslational modification (Oda *et al.*, 2000) or its mouse ortholog at S58 (Cecchinelli *et al.*, 2006b). In contrast, in less severe, presumably reparable DNA damage conditions induced by sub-lethal doses of UV or DOX, HIPK2 is ubiquitinated by MDM2 in a p53 dependent manner and targeted to proteasomal degradation (Rinaldo *et al.*, 2007a). These findings identify HIPK2 as a critical target for the p53/MDM2 pathway in the cell decision between cell cycle arrest and apoptosis during DDR, and further support the hypothesis that HIPK2 contributes to tumor cell responsiveness to anticancer treatments.

Besides p53 phosphorylation, HIPK2 promotes apoptosis by modulating other factors, directly or indirectly related to p53-mediated apoptosis, such as the β -catenin regulator Axin, the p53 family member p73, the p53 inhibitor MDM2, the acetyl transferases p300/CPB and PCaF (Rinaldo *et al.*, 2007b; Li *et al.*, 2009; Puca *et al.*, 2010), and, more recently, PML (Gresko *et al.*, 2009) and the methyl-CpG-binding protein 2 (Bracaglia *et al.*, 2009). In addition, HIPK2 depletion in human tumor cells was shown to induce p53 misfolding, yielding a 'mutant-like' p53 conformation and resistance to DOX and CDDP (Puca *et al.*, 2008).

Interestingly, p53-independent functions of HIPK2 were also identified in UV and tumor growth factor- β -induced apoptosis. In p53-deficient cells, UV-activated HIPK2 phosphorylates the transcriptional co-repressor CtBP at S422 and targets it for proteasomal degradation (Zhang *et al.*, 2003). Again, in p53-deficient cells, HIPK2 participates in the tumor growth factor- β -induced apoptosis, leading to activation of the stress-stimulated kinase, c-Jun NH(2)-Terminal Kinase (JNK) (Hofmann *et al.*, 2003). Although these events are not confined to p53-deficient cells and the experiments have been performed only upon induction of apoptosis by UV irradiation or tumor growth factor- β -treatment, these results suggest that HIPK2 might contribute to tumor chemosensitivity, even in the absence of wild type (wt) p53. Because of the relevance for human cancer treatments of p53-independent components in apoptosis and drug response (Leong *et al.*, 2007; Vilgelm *et al.*, 2008), we asked whether p53-independent factor(s) might be involved in the HIPK2-mediated response to chemotherapy.

In this study, we have shown that HIPK2 is stabilized upon treatment with different drugs independently from the *TP53* gene status, and that, HIPK2 depletion by RNA interference induces chemoresistance even in p53-defective cells. Looking for p53-independent HIPK2 targets in these conditions, we found that the prosurvival factor Δ Np63 α is directly phosphorylated by HIPK2 at T397, and this kinase activity is required for the Δ Np63 α degradation in response to anticancer therapy.

Results

HIPK2 contributes to tumor cell chemosensitivity through p53-dependent and -independent mechanisms

To analyze the role of HIPK2 in response to different anticancer drugs, we first performed time course analyses of HIPK2 expression in wtp53-carrying RKO cells upon treatment with previously defined, apoptotic doses of etoposide, DOX or bleomycin, whereas UV irradiation was used as positive control. As shown in Figure 1a, a time-dependent increase of HIPK2 levels was observed with each drug. Then, we verify whether HIPK2 has a causal role in the cell response to these different drugs by employing two stable polyclonal populations of HIPK2-interfered RKO cells (HIPK2i-1 and HIPK2i-2), obtained by transfection of two different HIPK2-specific interfering sequences (see Materials and methods; Figure 1b, insert panel). Colony

Figure 1 HIPK2 is involved in tumor cell response to antineoplastic drugs. RKO cells were treated with etoposide (EPEG), DOX or bleomycin (BLM) at the indicated doses. UV radiation at 50 J/m² was used as positive control for HIPK2 protein stabilization. (a) TCEs were prepared at the indicated times after treatment and analyzed by western blot (WB) for HIPK2 protein expression. Tubulin expression shows equal loading of samples. (b) RKO cells were stably depleted for HIPK2 by two different shRNAs. HIPK2 depletion was verified by WB analysis (small insert). Interfered cells (HIPK2i-1 and HIPK2i-2) and control cells (Ctr-i) were treated with the indicated drugs as in (a) and plated for colony forming assay 24 h post-treatment. Columns are average of three independent experiments and bars indicate standard deviation. Student's *t*-test: ***P < 0.0001.

formation assays showed that, compared with control-interfered (Ctr-i) cells, both RKO-HIPK2i populations have an increased resistance to all tested drugs, further supporting the rising idea that HIPK2 is involved in the cell response to a wide range of stresses.

As HIPK2 was shown to induce apoptosis in p53-dependent and -independent manners, we tested the same drugs on p53-null cells in the presence or absence of HIPK2 depletion. As shown in Figure 2a, HIPK2 upregulation was induced in p53-null H1299 cells, and

the resistance of these cells to etoposide and bleomycin was strongly increased in two stable HIPK2i populations obtained as for RKO cells (Figure 2b), indicating the involvement of p53-independent mechanism(s). We were not able to measure any colony forming capacity of our H1299 cells upon DOX treatment, even at doses as low as 20 nM (data not shown); thus, to test the HIPK2/p53 dependency to this drug, we employed the HCT116 isogenic cell model consisting of wtp53-carrying parental cells and their p53-null derivatives (Bunz *et al.*, 1998). To avoid selection differences between the two populations promoted by stable transfection, HIPK2 depletion in the p53-proficient and -defective HCT116 cells was induced upon transient transfection of specific Stealth RNA duplex (see Materials and methods) and quantified by real-time reverse transcription PCR (Figure 2c). As expected, in the Ctr-depleted cells (universal negative control), reduction in DOX-induced apoptosis was observed in the p53-null population, compared with the p53-proficient one (Figures 2d and e, compare the black columns). Still, a further reduction in DOX-induced apoptosis was observed in both populations upon HIPK2 depletion (Figures 2d and e), further supporting the existence of HIPK2-mediated, p53-independent mechanism(s) of chemoresponse.

HIPK2 contributes to Δ Np63 α degradation in response to DOX, independently of the TP53 gene status

It has been shown that HIPK2 interacts with the other members of the p53 family, p73 and p63; however, the functional role(s) of these interactions are still mainly unknown (Kim *et al.*, 2002). In the past few years, several studies have revealed that apoptotic doses of UV irradiation or genotoxic drugs promote a phosphorylation-induced, proteasome-mediated degradation of the prosurvival isoform Δ Np63 α and a subsequent modification of its transcriptional activity (Liefer *et al.*, 2000; Papoutsaki *et al.*, 2005; Westfall *et al.*, 2005; Zangen *et al.*, 2005; Muller *et al.*, 2006). Thus, we asked whether Δ Np63 α , that was already shown to be bound by HIPK2 (Kim *et al.*, 2002), might be one of the HIPK2 targets in DDR. To begin assessing this idea, we first confirmed the binding between the two proteins (Supplementary Figure 1a). Next, we tested HIPK2 expression in the presence or absence of apoptotic doses of DOX in three different cell lines, MCF-10A, HaCat and FaDu, known to express detectable levels of endogenous Δ Np63 α (Ciardiello *et al.*, 1990; Li *et al.*, 2008), as we also confirmed by isoform-specific RT-PCR analysis (Du *et al.*, 2010; data not shown). RKO were used as Ctr from the previous experiments (Figure 1). As shown in Figure 3a, HIPK2 expression increased upon DOX treatment, independently of the TP53 gene status, that is, wt in the MCF-10A and mutated in HaCat and FaDu cells. As expected, DOX treatment stabilized p53 only in the wtp53-carrying cells, whereas it strongly reduced Δ Np63 α expression (Figures 3b–d) in all cells, supporting its independency from p53, as previously reported (Fomenkov *et al.*, 2004). To evaluate whether the DOX-induced repression

Figure 2 HIPK2 mediates tumor cell chemosensitivity in p53 null cells. (a) H1299 cells were treated with UV or the indicated drugs at the indicated doses. TCEs were prepared 12 h after treatment and analyzed by western blot (WB) for HIPK2 expression. (b) H1299 cells were stably depleted for HIPK2 by two different shRNAs. HIPK2 depletion was verified by WB (small insert). Interfered cells (HIPK2i-1 and HIPK2i-2) and Ctr-i cells were treated with the indicated drugs and colony-forming assay was performed 24 h post-treatment. Columns are average of three independent experiments and bars indicate standard deviation. Student's *t*-test: ***P < 0.0001. (c–e) p53 proficient (p53+/+) and defective (p53-/-) HCT116 cells were transiently depleted for HIPK2. After 48 h from transfection, (c) HIPK2 depletion was verified by real-time reverse transcription PCR and drug sensitivity was assessed by treatment with 2 μ M DOX and subsequent cell death evaluation (d) by Trypan blue exclusion test and (e) by TUNEL assay 24 h post-treatment. Student's *t*-test: ***P < 0.0001, **P < 0.001, *P < 0.01.

Figure 3 HIPK2 contributes to Δ Np63 α degradation upon DOX treatment. **(a)** The indicated cells were treated with 2 μ M DOX or maintained in the absence of drug. TCEs were prepared 12h post-treatment and analyzed by western blot (WB) for the indicated proteins. Actin expression shows equal loading of samples. **(b–d)** The indicated cells were treated with DOX. TCEs were prepared at the indicated times post-treatment and analyzed by WB for Δ Np63 α and p53 expression. **(e, f)** MCF-10A and FaDu cells were transiently depleted for HIPK2; 36h after transfection, cells were treated with DOX or maintained in the absence of drug. TCEs were prepared at the indicated times post-treatment and analyzed for HIPK2 and Δ Np63 α expression. The upregulation of HIPK2 is transient due to its super-activation induced by caspase cleavage of the C-terminal portion, which can be induced even in the absence of wtp53 upon apoptotic doses of drugs (Gresko *et al.*, 2006). Density values were calculated as ratio of HIPK2 or Δ Np63 α and actin densitometry units. **(g)** Real-time reverse transcription PCR was performed for the indicated genes. The histogram reports mRNA relative value in HIPK2-i and in universal negative control (UNC) populations. mRNA levels of DOX-treated cells were calculated relative to that of untreated (Ctrl) ones. Data are mean of three independent experiments. Student's *t*-test: **P < 0.001, *P < 0.01. **(h)** FaDu cells were transfected with an expression vector coding for EGFP-HIPK2 (0.5, 1 or 2 μ g). TCEs were prepared 36h post-transfection and analyzed by WB. Density values were calculated as the ratio of Δ Np63 α and actin densitometry units. **(i)** FaDu cells were transfected with an expression vector coding for EGFP or EGFP-HIPK2 (1 and 2 μ g) and treated with 10 μ M MG132 (or its solvent, DMSO) 20h post-transfection. TCEs were prepared 10h after MG132-treatment and endogenous Δ Np63 α protein levels were analyzed by WB. **(j)** FaDu cells were transfected with an expression vector coding for Flag-HIPK2 or Flag alone and treated with cycloheximide (CHX; 25 μ g/ml). TCEs were prepared at the indicated times post-treatment and analyzed by WB for Δ Np63 α expression. Density values were calculated as ratio of Δ Np63 α and actin densitometry units.

of Δ Np63 α is mediated by HIPK2, transient depletion of the kinase was induced by three different Stealth RNA duplexes in cells of each of the three Δ Np63 α -expressing lines. A strong, though not complete, inhibition of Δ Np63 α repression was observed in all cells (Figures 3e and f and data not shown), indicating that HIPK2 contributes to the DOX-induced repression

of Δ Np63 α . This partial rescue of Δ Np63 α expression induced by HIPK2 depletion was also associated with a reduced inhibition of a few Δ Np63 α transcription target genes, already shown to be modulated by CDDP-treatment (Huang *et al.*, 2010). After assessing the modulation of *c-JUN*, *CDH1*, *CARM* and *EGFR* in CDDP- and DOX-treated FaDu cells by real-time

reverse transcription PCR (Supplementary Figure 1b), we measured their expression in Ctr and HIPK2-depleted cells, observing a partial recovery in the latter ones (Figure 3g). To evaluate more directly whether HIPK2 reduces Δ Np63 α protein expression by degradation, as described for genotoxic treatments, increasing amount of EGFP-tagged HIPK2 was transfected in FaDu cells in the absence or presence of the proteasomal inhibitor MG132. Western blot analyses of total cell extracts (TCEs) showed a reduction of the endogenous Δ Np63 α protein levels (Figures 3h and i) that was rescued by the addition of MG132 (Figure 3i), indicating that HIPK2 can promote Δ Np63 α degradation. In agreement, a reduced half-life of the endogenous Δ Np63 α protein was observed upon HIPK2 overexpression and cyclohexamide treatment (Figure 3j).

The C-terminal region of Δ Np63 α is required for its HIPK2-induced degradation

HIPK2 was previously shown to bind p63 in its C-terminal region (Kim *et al.*, 2002); thus, we tested whether a C-terminal deletion mutant of Δ Np63 α , the Δ 390 that encodes for a protein deleted from amino-acid 390 to the stop codon of the human Δ Np63 α (5c) and is not phosphorylated upon UV (Papoutsaki *et al.*, 2005), was still degraded by HIPK2 overexpression or DOX treatment. Western blot analyses of FaDu cells co-transfected with EGFP-HIPK2 and Myc-tagged

Δ Np63 α or Δ 390 showed that only the full-length Δ Np63 α expression is repressed (Figure 4a). Similar results were obtained upon DOX treatment of FaDu, HaCat and H1299 cells transfected with Myc- Δ Np63 α or Myc- Δ 390-deletion mutant (Figure 4b). Taken together, these results indicate that the C-terminal region of Δ Np63 α is required for its DOX-induced, HIPK2-mediated degradation.

As Δ Np63 α degradation is required for effective cellular response to genotoxic drugs (Liefer *et al.*, 2000; Huang *et al.*, 2008), we asked whether overexpression of the Δ 390-deletion mutant might induce resistance to DOX. Colony formation assays were performed on FaDu cells upon transfection with Δ Np63 α or Δ 390 and DOX treatment. As expected, both proteins increased the colony formation efficiency compared with the Ctr vector-transfected cells, but Δ 390 was more efficient than wt- Δ Np63 α (Figure 4c), supporting the concept that the C-terminal region of Δ Np63 α is required for cell sensitivity to DOX.

HIPK2 phosphorylates Δ Np63 α at T397 and this kinase activity is required for Δ Np63 α degradation

HIPK2 regulates several of its targets through the S/T kinase activity (Rinaldo *et al.*, 2007b). Thus, we first asked whether HIPK2 directly phosphorylates p63 in an *in vitro* kinase assay. Comparable amounts of eukaryotic GST-HIPK2 (eGST-HIPK2) and of its kinase-dead K221R mutant (eGST-K221R) were produced in

Figure 4 Role of the C-terminal region of Δ Np63 α in HIPK2 mediated degradation. (a) FaDu cells were co-transfected with an expression vector coding for EGFP or EGFP-HIPK2, together with a vector coding for the fusion protein Myc- Δ Np63 α or its deletion mutant Myc- Δ 390. TCEs were prepared 24 h post-transfection and western blot (WB) analysis of exogenous Δ Np63 α protein was performed using an α -Myc Ab. (b) The indicated cells were transfected with an expression vector coding for Myc- Δ Np63 α or Myc- Δ 390; 16 h post-transfection, cells were treated with DOX for the indicated times and TCEs were analyzed by WB. (c) FaDu cells were transfected with the indicated expression vectors and, 16 h post-transfection, treated with 1 μ M DOX. Colony formation assay was set up 24 h after DOX treatment. Representative colonies are shown. Colony numbers are reported as mean of three independent experiments $\pm$ s.d. Student's *t*-test: *** $P < 0.0001$.

H1299 cells, purified by glutathione-sepharose beads, and incubated with purified recombinant p63 protein (kindly provided by Prof. Mantovani) in the presence of [γ^{32} P]-ATP, as we previously reported for p53 (Cecchinelli *et al.*, 2006b). As shown in Figure 5a, wt-HIPK2, but not the kinase-dead mutant, was able to phosphorylate itself and p63, indicating that HIPK2 phosphorylates p63 *in vitro*.

Next, we assessed whether the HIPK2 kinase activity is required for Δ Np63 α degradation. FaDu cells were transfected with EGFP-HIPK2, EGFP-K221R kinase-dead mutant or EGFP alone, and TCEs analyzed by western blot for the endogenous Δ Np63 α protein levels. Also in this case, only wt-HIPK2 was able to reduce Δ Np63 α expression (Figure 5b), suggesting that the kinase activity of HIPK2 is required for Δ Np63 α degradation.

A strong functional link between Δ Np63 α phosphorylation and its subsequent degradation has been observed upon treatment with different genotoxic agents (Papoutsaki *et al.*, 2005; Westfall *et al.*, 2005; Huang *et al.*, 2008; Galli *et al.*, 2010). In addition, upon CDDP treatment, three specific phosphorylation sites (that is, S375, T397 and S466) have been identified in the C-terminal region of Δ Np63 α by mass spectrometry (Huang *et al.*, 2008; Figure 5c, underscored sites). Interestingly, one of these sites, the T397, belongs to the recognition motif usually phosphorylated by HIPK2, that is, S or T, followed or preceded by P. Thus, although there are 10 such motifs in the C-terminal region of Δ Np63 α (Figure 5c, big bold residues), we first tested whether HIPK2 phosphorylates Δ Np63 α at T397. *In vitro* kinase assay was performed by incubating comparable amounts of bacterially-produced proteins (wt-GST- Δ Np63 α or its non-phosphorylatable form at T397, the GST-T397A mutant; Figure 5d), with commercially available active-HIPK2 (see Materials and methods), in the presence of [γ^{32} P]-ATP. A strong phosphorylation signal was observed only with the wt-GST- Δ Np63 α protein (Figure 5e), indicating that HIPK2 phosphorylates Δ Np63 α at the T397 site.

Inhibition of Δ Np63 α phosphorylation at T397 upon CDDP treatment was shown to interfere with Δ Np63 α degradation (Huang *et al.*, 2008). Thus, to confirm that the DOX-induced, HIPK2-mediated degradation of Δ Np63 α depends on T397 phosphorylation, cells were co-transfected with EGFP-HIPK2 and wt-Myc- Δ Np63 α , or the non-phosphorylatable Myc-T397A mutant. As shown in Figures 5f and g, the T397A mutant was resistant to HIPK2-induced degradation and as a longer half-life than the wt- Δ Np63 α , as measured by cyclohexamide treatment. Comparable results were obtained in FaDu cells upon transfection with the same Myc- Δ Np63 α or Myc-T397A expressing vectors and treated with DOX. Indeed, we observed that the T397A mutant (i) is more resistant to DOX-induced Δ Np63 α repression (Figure 5h, left panel), (ii) inhibits cell death (Figure 5h, right panel) and (iii) is less ubiquitylated than the wt form (Figure 5i), indicating that in cell response to DOX, HIPK2 contributes to Δ Np63 α degradation by its phosphorylation at T397.

Discussion

In this study, we investigated whether the p53 pro-apoptotic activator HIPK2 contributes to the cellular response to chemotherapy through molecular mechanism(s) other than those mediated by p53. We show that the DOX-induced degradation of the prosurvival factor Δ Np63 α is induced by HIPK2 in a p53-independent manner. In particular, HIPK2 depletion by RNA interference was sufficient to inhibit drug-induced Δ Np63 α degradation even in mutant p53-carrying cells. Furthermore, we found that HIPK2 phosphorylates Δ Np63 α at T397, and this specific posttranslational modification strongly contributes to both HIPK2- and DOX-induced degradation of Δ Np63 α and susceptibility to DOX. In addition, we showed that a C-terminal deletion mutant of Δ Np63 α missing the T397 site, the Δ 390 mutant, strongly increase cell resistance to DOX.

Δ Np63 α is one of the N-terminal-defective isoforms encoded by the *TP63* gene. Transcription of *TP63* can be initiated at two alternative transcription start sites, resulting in the transcription-activating (TA) p63 and Δ Np63 isoforms that contain or lack, respectively, a TA domain (Yang *et al.*, 1998). The Δ Np63 isoforms possess dominant-inhibitory functions over the TA isoforms of the p53 family (TAp63, TAp73 and p53) that usually result in prosurvival and pro-proliferation activities (Parsa *et al.*, 1999; Barbieri *et al.*, 2005; Wu *et al.*, 2005; Rocco *et al.*, 2006). Overexpression of Δ Np63 has been frequently observed in squamous cell carcinoma and in some other epithelial tumors (Hibi *et al.*, 2000; Senoo *et al.*, 2001; Moll and Slade, 2004; Lin *et al.*, 2006) and, beyond its dominant negative effect over the pro-apoptotic and growth-arresting functions of the TA isoforms, Δ Np63 was found to contribute to tumor progression by favoring angiogenesis and chemoresistance (Wu *et al.*, 2005; Zangen *et al.*, 2005; Lanza *et al.*, 2006; Muller *et al.*, 2006; Rocco *et al.*, 2006) and by modulating cell adhesion processes (Carroll *et al.*, 2006; Yang *et al.*, 2006). Regardless of the HIPK2-mediated regulation of Δ Np63 α in response to DOX, we observed that HIPK2 depletion results in an increased expression of Δ Np63 α even in non-stressing conditions (Figure 3e and data not shown), suggesting that the tumor-associated HIPK2 inactivation might contribute to Δ Np63 α overexpression. We previously found a similar behavior for the prosurvival factor galectin-3, whose expression is strongly increased in several human cancers, at least in part, because of the lack of a HIPK2-dependent, p53-mediated repression of Galectin-3 transcription (Cecchinelli *et al.*, 2006a; Lavra *et al.*, submitted). In this regard, it will be interesting to evaluate Δ Np63 α expression in those human tumors in which HIPK2 has been found inactive. To this aim, we have started to analyze, by immunohistochemistry for HIPK2 and p63 staining, 20 thyroid cancers and six benign thyroid hyperplasia. As shown in Table 1, a strong inverse correlation between the two proteins was observed, supporting the idea that the tumor-associated reduction of HIPK2 expression is compatible with the induction of p63 expression.

Increasing evidence indicates that regulation of protein stability is a key mechanism to control p63 isoforms' actions through different E3-ubiquitin ligases (Muller *et al.*, 2006). Thus far, we found that neither Itch nor MDM2 are responsible for the HIPK2-induced degradation of Δ Np63 α (Supplementary Figures 1c

and d). In response to several genotoxic agents, ubiquitylation and proteasomal degradation were shown to be prevented by phosphorylation on the TA isoforms (Rossi *et al.*, 2006; Li *et al.*, 2008; MacPartlin *et al.*, 2008), whereas promoted also, by phosphorylation on the Δ N isoforms (Liefer *et al.*, 2000; Westfall *et al.*, 2005;

Zangen *et al.*, 2005). Though Y phosphorylation was overall shown to be associated to TA stability (Gonfloni *et al.*, 2009; Wang *et al.*, 2010) and S/T phosphorylation to Δ N degradation (Westfall *et al.*, 2005; Huang *et al.*, 2008; Galli *et al.*, 2010), the molecular pathways underlying these divergent events and their balance have just begun to be described. The HIPK2 activity on Δ Np63 α we are reporting here is consistent with the general idea that the cellular amount of this p63 isoform has to be reduced to enable an effective DDR. A phosphorylation-dependent prodegradation function of HIPK2 has already been reported for CtBP, c-Myb and ZBTB4 (Zhang *et al.*, 2003; Kanei-Ishii *et al.*, 2004; Yamada *et al.*, 2009), suggesting that it might be a common mechanism to destabilize prosurvival factors. Puzzling, the HIPK2 kinase activity is required for the apoptosis-specific activation of p53. How the same kinase drives molecules, even from the same family, such as p53 and Δ Np63 α to so divergent fates is presently unidentified. We know that p53 is phosphorylated by HIPK2 in its N-terminus and this homologous region in Δ Np63 α is absent. On the opposite site, HIPK2 phosphorylates Δ Np63 α at the C-terminus in the SAM domain, a region absent in p53 (Irwin and Kaelin, 2001). On the basis of these data, one might speculate that HIPK2 phosphorylation at the N- or C-terminus promotes activation or degradation, respectively. However, too little information is available thus far to make a model. It will be interesting to verify, for example, whether the described HIPK2 cooperation activity with TAp73 depends on its HIPK2-induced phosphorylation at the N-terminus, or whether p53 can be the only member of the family activated by direct HIPK2-induced phosphorylation because, at variance from the p63 and p73 isoforms, it is not phosphorylated at Y residues.

HIPK2 phosphorylates Δ Np63 α at T397, one of the three residues previously identified by mass spectrometry, as phosphorylation sites targeted in response to CDDP treatment (that is, S385, T397, S466; Huang

et al., 2008). On the basis of the kinase recognition motifs, the authors proposed that the three sites are phosphorylated by ATM, CDK2 and p70s6 K, respectively. Although specific RNA interference for ATM and p70s6 K significantly reduced phosphorylation of their relative sites, CDK2 depletion or inactivation only marginally reduced CDDP-induced Δ Np63 α degrada-

Table 1 Immunohistochemistry of HIPK2 and p63 in thyroid

	HIPK2	p63
<i>Follicular hyperplasia</i>		
Case # 1	+	Neg
Case # 2	+	Neg
Case # 3	+	Neg
Case # 4	+	Neg
Case # 5	+	Neg
Case # 6	+	Neg
<i>Follicular thyroid carcinoma</i>		
Case # 1	Neg	Neg
Case # 2	Neg	Neg
Case # 3	Neg	Neg
Case # 4	Neg	+
Case # 5	Neg	+
Case # 6	+/-	Neg
Case # 7	Neg	+
Case # 8	+	Neg
Case # 9	Neg	Neg
Case # 10	+	Neg
Case # 11	Neg	Neg
Case # 12	Neg	+
<i>Papillary thyroid carcinoma</i>		
Case # 1	Neg	Neg
Case # 2	Neg	+
Case # 3	Neg	+
Case # 4	+/-	+/-
Case # 5	Neg	Neg
Case # 6	Neg	+
Case # 7	Neg	+
Case # 8	Neg	+/-

Abbreviation: HIPK2, homeodomain-interacting protein kinase 2.

Figure 5 Phosphorylation of Δ Np63 α at T397 by HIPK2 is required for Δ Np63 α degradation. (a) eGST-HIPK2 and eGST-K221R proteins were prepared by GST pulldown from H1299 cells and incubated with a purified p63 protein in the presence of [γ ³²P]-ATP. Kinase reaction products were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and analyzed by autoradiography (upper panel). Western blot (WB) analysis of GST proteins and p63 was performed after gel rehydration with the indicated antibodies. (b) FaDu cells were transfected with EGFP-HIPK2 (2.5 μ g), EGFP-K221R (2, 3 and 5 μ g) or EGFP (2, 3 and 5 μ g). TCEs were prepared 36 h after transfection and analyzed by WB; * indicate unspecific bands. (c) Schematic representation of Δ Np63 α protein. The C-terminal aminoacidic sequence is shown in detail; underscored letters: phosphorylation sites identified by Huang *et al.* (2008); bold letters: HIPK2 recognition motifs. (d) WB analysis of bacterially produced GST, GST- Δ Np63 α and GST-T397A proteins. (e) Comparable amounts of GST proteins were incubated with active-HIPK2 in the presence of [γ ³²P]-ATP, and the kinase reaction products were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and analyzed by autoradiography. (f) HaCat cells were co-transfected with expression vectors coding for the indicated proteins. TCEs were prepared at the indicated times and analyzed by WB for expression of the exogenous proteins. (g) U2OS cells were transfected with and without HIPK2, together with expression vectors coding for Myc- Δ Np63 α or Myc-T397A, and treated with cycloheximide (CHX; 25 μ g/ml). TCEs were prepared at the indicated times post-treatment and analyzed by WB for Δ Np63 α expression. Density values were calculated as ratio of Δ Np63 α and actin densitometry units. (h) FaDu cells were transfected with Myc- Δ Np63 α - or Myc-T397A-expressing vectors. DOX was added 16 h post-transfection and TCEs were prepared 48 h after treatment and analyzed by WB. The histogram reports the DOX-induced trypan blue positivity measured in the Myc- Δ Np63 α - or in the Myc-T397A- transfected cells. The trypan blue positivity was calculated relative to that of mock-treated transfected cells. Student's *t*-test **P < 0.001. (i) FaDu cells were transfected with a vector expressing HA-ubiquitin, in combination with Myc- Δ Np63 α or Myc-T397A. DOX was added 16 h post-transfection and 5 μ M MG132 was added 36 h post-DOX treatment. TCEs were analyzed 48 h after DOX treatment by WB for the indicated proteins (lower panels) or immunoprecipitated with anti-Myc Ab. Immunocomplexes were analyzed by WB with anti-Myc and anti-HA antibodies. Ubiquitylated high molecular weight conjugates of Δ Np63 α were indicated as (Ub)n- Δ Np63 α .

tion, suggesting that other kinases recognizing the same motif might be also involved. Interestingly, Huang and collaborators (2008) elegantly showed by Flp-In technology in HNSCC 029 cells, the requirement of each of these sites in the CDDP-induced degradation of Δ Np63 α . Our data are consistent with these results, as we found that the T397A mutant is completely resistant to DOX- and HIPK2-induced degradation, indicating that at least this phosphorylation site is involved in cell response to both CDDP and DOX. In addition, the authors showed a time-dependent hierarchy in the phosphorylation sites, with S385, an ATM target, being the first to be phosphorylated upon CDDP and being required for the subsequent phosphorylation at the two other sites. This observation is also very intriguing in view of our results, because ATM is also involved in HIPK2 activation (Dauth *et al.*, 2007; Winter *et al.*, 2008), as well as in p53 phosphorylation at S15, a posttranslational modification that precede S46 (Oda *et al.*, 2000). Taken together, these observations suggest that in DDR, the ATM sensor, among different functions, activates HIPK2 and prepares p53 and its antagonist Δ Np63 α , to the subsequent HIPK2-induced phosphorylation that drives these molecules to their respective fates.

The HIPK2 destabilizing activity on Δ Np63 α we described upon treatment with different anticancer drugs, independently from the TP53 gene status, further support the concept that HIPK2 contribute to DDR in p53-dependent and -independent manners. However, this observation does not exclude the possibility that both pathways are engaged simultaneously in cells that carry wtp53 and express Δ Np63 α , such as the MCF10A

cells we employed here. In this type of cells, drug-induced activation of HIPK2 would (i) activate p53 by S46 phosphorylation, (ii) promote the degradation of Δ Np63 α by T397 phosphorylation and (iii) further improve p53 activity by removing the dominant inhibitory effect by Δ Np63 α (Figure 6). These data indicate that HIPK2 has a double commitment, working as activator for proapoptotic factors and inhibitor for antiapoptotic one. On the opposite site, these considerations would allow to suppose that tumor-associated inhibition of HIPK2 activity might strongly contribute to chemoresistance in addition to other better-characterized events, such as p53 mutation/inactivation or Δ Np63 overexpression. HIPK2-related data from human tumor samples are still insufficient to be significant. We hope that the enlarging body of experimental evidence supporting key roles for HIPK2 in tumorigenicity and drug response would encourage the development of more extensive translational studies.

Materials and methods

Cells culture conditions and treatments

RKO, FaDu, HaCat, HCT116 p53+/+ and p53-/- (Bunz *et al.*, 1998), U2OS and p53-null mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum. Immortalized human mammary MCF-10A cells (Ciardiello *et al.*, 1990) were cultured in mammary epithelium basal medium, supplemented with MEGM SingleQuots (Clonetics, Lonza Milano S.r.l., Milano, Italy). For drug treatment, subconfluent cells were incubated in the presence of DOX (Sigma, St Louis, MO, USA), bleomycin (Aventis Pharma, Varese, Italy), Etoposide (Bristol-Myers Squibb, Princeton, NJ, USA) or MG132 (Calbiochem, Gibbstown, NJ, USA), at the indicated concentrations.

Expression vectors and transfection

The following plasmids were employed: pCDNA3-Myc- Δ Np63 α , pCDNA3-Myc-T397A and pCDNA3-Myc- Δ 390 (Papoutsaki *et al.*, 2005; Di Costanzo *et al.*, 2009); pEGFP-HIPK2 and pEGFP-K221R (Cecchinelli *et al.*, 2006b); pEGFP-C2 (Stratagene, Santa Clara, CA, USA). Plasmids pGex- Δ Np63 α and pGex- Δ Np63 α (T397A) were obtained by PCR amplification from pCDNA3-Myc- Δ Np63 α and pCDNA3-Myc-T397A using specific primers: Δ Np63 BamH-upper: 5'-CGATATG-GATCCTTGACCTGGAAAA CAATGCCC-3'; Δ Np63 Xho1-lower: 5'-CGTATACTCGAGTCATTCTCCTTCCTC TTTGATACG-3' and cloned into a BamH1/Xho1 digested pGex6p-2rbs vector. The expression vectors were transfected by using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions.

Western blot analyses

TCEs were prepared in RIPA buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1% Nonidet P40, 1 mM EDTA), supplemented with protease-inhibitor mix (Roche, Indianapolis, IN, USA). TCEs were resolved on precast NuPAGE 4–12% gels (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and analyzed with the following antibodies: rabbit anti-HIPK2 (kindly provided by M.L. Schmitz), rabbit anti-p53 (FL-393) and mouse anti-p63

Figure 6 Model summarizing HIPK2 activity on p53 and Δ Np63 α in response to antineoplastic drugs.

(4A4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Myc (Upstate, Millipore, Bilerica, MA, USA), rabbit anti-GST (kindly provided by Dr M. Fanciulli), mouse anti- α -tubulin (Immunological Sciences, Rome, Italy), mouse anti-HA-peroxidase and mouse anti-actin (Sigma), HRP-conjugated goat anti-mouse and anti-rabbit (Cappel, West Chester, PA, USA). Immunoreactivity was determined using the ECL-chemiluminescence reaction (Amersham Corp., Arlington Heights, IL, USA), following the manufacturer's instructions.

Colony forming assay

Cells were treated with drugs at the indicated doses for 24 h, then plated at low density in 60 mm petridishes and grown for a week in the absence of drugs. Surviving colonies were fixed and stained with crystal violet (0.5% in methanol; Sigma) and air-dried.

RNA interference

Stable interference by shRNA was performed by transfection of pRetroSuper and two different pRetroSuper-HIPK2 vectors carrying HIPK2-1376 or HIPK2-789 sequences (Cecchinelli *et al.*, 2006a). After 24 h from transfection, stable polyclonal populations of Ctr and HIPK2-depleted cells (HIPK2i-1 and HIPK2i-2 carrying HIPK2-1376 and HIPK2-789 sequences, respectively) were obtained by selection with 2 μ g/ml puromycin. Transient interference by siRNA was obtained by HIPK2i Stealth RNAi sequences (a mix of three different sequences, transduced alone or in combination with comparable results) and universal negative control Stealth RNAi Negative, Medium GC Duplexes (Invitrogen). Cells were transduced by using the RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions.

RNA extraction and real-time reverse transcription PCR

Total RNA was extracted with Trizol (Invitrogen), reverse transcribed and amplified by using the High Capacity cDNA Reverse Transcription Kit and SYBR Green DNA Master mix (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 7500 system SDS software. The following primers were employed: upper human/mouse HIPK2 5'-AGGAAGAGTAAGCAGCACCAG-3' lower human/mouse HIPK2 5'-TGCTGATGGTGATGACACTGA-3' upper human c-JUN 5'-GCATGAGGAACCGCATCGCTGCCTCCA AGT-3' lower human c-JUN 5'-GCGACCAAGTCCTTCC CACTCGTGCACACT-3' upper human CARM1 5'-CAGGG CTGGTCCACGGCCTG-3' lower human CARM1 5'-CCTG AGAGCGTGTCCCCTGC-3' upper human CDH1 5'-CGG GAATGCAGTTGAGGATC-3' lower human CDH1 5'-AG GATGGTGTAAAGCGATGG-3' upper human EGFR 5'-CCCAGTACCTGCTCAACTGGT-3' lower human EGFR 5'-TGCCAGGTTCGCGGTG-3' upper human GAPDH 5'-

TCCCTGAGCTGAACGGGAAG-3' lower human GAPDH 5'-GGAGGAGTGGGTGTCGCTGT-3'. Each target amplification was performed in duplicate.

In vitro kinase assay

Recombinant eGST-HIPK2 and eGST-K221R were produced in H1299 cells by infection with the vaccinia virus vTF7-3 (kindly provided by Dr B Moss), followed by transfection with 2 μ g of pcDNA3-eGST-HIPK2 or pcDNA3-eGST-K221R plasmids using Lipofectamine (Invitrogen) (Cecchinelli *et al.*, 2006b). TCEs were prepared 24 h post-transfection by incubation for 30 min at 4 °C in lysis buffer (50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 150 mM KCl, 1 mM dithiothreitol, 1% Nonidet P-40). After centrifugation, eGST-fusion proteins were purified from supernatant by overnight incubation with glutathione-sepharose beads (Sigma) at 4 °C and used as enzymatic source. Alternatively, the active-HIPK2 fragment was purchased from Upstate and employed at the concentration of 50 ng/reaction. Recombinant GST- Δ Np63 α and GST-T397A fusion proteins were produced in BL21 bacteria and purified on glutathione-sepharose resin (Sigma). For kinase assay, recombinant proteins were washed and incubated for 30 min at 30 °C in kinase buffer (20 mM Hepes (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂) in the presence of 185 KBq [γ -³²P]-ATP. The phosphorylated substrates were resolved on precast NuPAGE 4-12% gels and analyzed by autoradiography.

Abbreviations

CDDP, cisplatin; Ctr, control; DDR, DNA damage response; DOX, doxorubicin; eGST, eukaryotic-Glutathione S Transferase; HIPK2, homeodomain-interacting protein kinase 2; TCE, total cell extract.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)