HIPK2 phosphorylates ΔNp63α and promotes its degradation in response to DNA damage

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Running title: HIPK2/ΔNp63α axis in DNA damage response

Abstract

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 UV, ionizing radiation, or different anticancer drugs, such as cisplatin, roscovitine, and doxorubicin. 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 TGF-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 the response to chemotherapy. In particular, we found that HIPK2 phosphorylates and promotes proteasomal degradation of ΔNp63α, a prosurvival ΔN isoform of the p53 family member, p63. Indeed, effective cell response to different genotoxic agents was shown to require phosphorylation-induced proteasomal degradation of ΔNp63α. In doxorubicin treated cells, we show that HIPK2 depletion interferes with ΔNp63α degradation and expression of a HIPK2-resistant ΔNp63α-Δ390 mutant induces chemoresistance. We identify T397 as the ΔNp63α residue phosphorylated by HIPK2 and show that the non-phosphorylatable ΔNp63α-T397A mutant is not degraded in the face of either HIPK2 overexpression or doxorubicin treatment. These results indicate ΔNp63α as a novel target of HIPK2 in the response to genotoxic drugs.

Keywords: HIPK2/chemotherapy/ΔNp63/phosphorylation/degradation

Introduction

HIPK2 (Homeodomain-Interacting Protein Kinase 2) is an evolutionarily conserved S/T kinase originally identified as co-repressors 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 (reviewed by Rinaldo *et al*., 2007b; Calzado *et al*., 2007; Calzado *et al*., 2009). In the latter condition, HIPK2 is activated by different genotoxic stimuli, including 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 (Wesierska-Gadek *et al*., 2007). Depletion of HIPK2 expression by specific anti-sense 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 relocalization 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-heterozigosity in thyroid cancers (Lavra *et al*., submitted). 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 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 (reviewed by Puca *et al*., 2010), an apoptosis-specific p53 posttranslational modification (Oda *et al*., 2000), or its mouse ortholog at S58 (Cecchinelli *et al.*, 2006a). In contrast, in less severe, presumably reparable DNA damage conditions induced by sub-lethal doses of UV or DOX, HIPK2 is ubiquitylated 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 (reviewed by 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 TGF-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 TGF-β-induced apoptosis leading to activation of the stress-stimulated 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 TGF-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 ΔNp63α is directly phosphorylated by HIPK2 at T397 and this kinase activity is required for the ΔNp63α 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 DOX, Bleomycin (BLM), or Etoposide (EPEG), while 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 a stable polyclonal population of HIPK2 interfered (HIPK2i) RKO cells (Fig. 1b, insert panel). Colony formation assays showed that, compared to control-interfered (Ctr-i) cells, RKO-HIPK2i cells 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.

Since 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 EPEG and BLM was strongly increased in the stable HIPK2i population (Fig. 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 20nM (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 verified by Real-time RT-PCR (Fig. 2c). As expected, in the control-depleted cells (UNC), reduction in DOX-induced apoptosis was observed in the p53-null population compared to the p53-proficient one (Fig. 2d, compare the black columns). Still, a further reduction in DOX-induced apoptosis was observed in both populations upon HIPK2 depletion (Fig. 2d) further supporting the existence of HIPK2-mediated, p53-independent mechanism(s) of chemoresponse.

HIPK2 contributes to ΔNp63α 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, ΔNp63α (Liefer et al., 2000; Papoutsaki et al., 2005; Westfall *et al*., 2005; Zangen *et al*., 2005; Muller *et al*., 2006). Thus, we asked whether ΔNp63α, 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 tested HIPK2 expression in the presence or absence of apoptotic doses of DOX in three different cell lines with detectable levels of endogenous ΔNp63α (*i.e.*, MCF-10A, HaCat, and FaDu cells) (Ciardiello *et al*., 1990; Li *et al*., 2008) while RKO were used as control from the previous experiments (Fig. 1). As shown in Figure 3a, HIPK2 expression increased upon DOX treatment, independently of the *TP53* gene status, that is wild type in the MCF-10A and mutated in HaCat and FaDu cells. As expected, DOX treatment stabilized p53 only in the wtp53-carrying cells (Fig. 3a) while strongly reduced ΔNp63α expression (Fig. 3b, 3c, and 3d) in all cells, supporting its independency from p53, as previously reported (Fomenkov *et al.*, 2004).

To evaluate whether the DOX-induced repression of ΔNp63α is mediated by HIPK2, transient depletion of the kinase was induced by Stealth RNA duplex in cells of each of the three ΔNp63α-expressing lines. A strong, though not complete inhibition of ΔNp63α repression was observed in all cells (Fig. 3e and data not shown), indicating that HIPK2 contributes to the DOX-induced degradation of ΔNp63α. To further support this aspect and verify whether HIPK2, as described for genotoxic treatment, reduces ΔNp63α protein expression by proteasomal degradation, increasing amount of EGFP-tagged HIPK2 was transfected in FaDu cells in the absence or presence of the proteasomal inhibitor MG132. Western blot (WB) analyses of total cell extracts (TCEs) showed a dose-response reduction of the endogenous ΔNp63α protein levels (Fig. 3f) that was rescued by the addition of MG132 (Fig. 3g), indicating that HIPK2 can promote ΔNp63α degradation.

The C-terminal region of ΔNp63α 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 ΔNp63α, the Δ390 that encodes for a protein deleted from amino-acid 390 to the stop codon of the human ΔNp63α (see below, Fig. 5c) and is not phosphorylated upon UV (Papoutsaki *et al*., 2005), was still degraded by HIPK2 overexpression or DOX treatment. WB analyses of FaDu cells co-transfected with EGFP-HIPK2 and Myc-tagged ΔNp63α or Δ390 showed that only the full-length ΔNp63α expression is repressed (Fig. 4a). Similar results were obtained upon DOX treatment of FaDu, HaCat, and H1299 cells transfected with Myc-ΔNp63α or Myc-Δ390-deletion mutant (Fig. 4b). Taken together, these results indicate that the C-terminal region of ΔNp63α is required for its DOX-induced, HIPK2-mediated degradation.

Since ΔNp63α degradation is required for effective cellular response to genotoxic drugs (Liefer *et al*., 2000; Huang *et al*., 2008), we asked whether overexpression of the Δ390-deletion mutant might induce resistance to DOX. Colony formation assays were performed on FaDu cells upon transfection with ΔNp63α or Δ390 and DOX treatment. As expected, both proteins increased the colony formation efficiency compared to the control vector-transfected cells, but Δ390 was more efficient than wt-ΔNp63α (Fig. 4c), supporting the concept that the C-terminal region of ΔNp63α is required for cell sensitivity to DOX.

HIPK2 phosphorylates ΔNp63α at T397 and this kinase activity is required for ΔNp63α degradation

HIPK2 regulates several of its targets through the S/T kinase activity (reviewed by 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 (KD) K221R mutant (eGST-K221R) were produced in 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*., 2006a). As shown in Figure 5a, wt-HIPK2 but not the KD 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 ΔNp63α degradation. FaDu cells were transfected with EGFP-HIPK2, EGFP-K221R KD mutant, or EGFP alone, and TCEs analyzed by WB for the endogenous ΔNp63α protein levels. Also in this case, only wt-HIPK2 was able to reduce ΔNp63α expression (Fig. 5b), suggesting that the kinase activity of HIPK2 is required for ΔNp63α degradation.

A strong functional link between ΔNp63α 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 (*i.e.*, S375, T397, and S466) have been identified in the C-terminal region of ΔNp63α by mass spectrometry (Huang *et al*., 2008) (Fig. 5c, underscored sites). Interestingly, one of these sites, the T397, belongs to the recognition motif usually phosphorylated by HIPK2, *i.e.*, S or T followed or preceded by P. Thus, although there are 10 such motifs in the C-terminal region of ΔNp63α (Fig. 5c, big-bold residues), we first tested whether HIPK2 phosphorylates ΔNp63α at T397. In vitro kinase assay was performed by incubating comparable amounts of bacterially produced proteins (wt-GST-ΔNp63α or its non-phosphorylatable form at T397, the GST-T397A mutant) (Fig. 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-ΔNp63α protein (Fig. 5e) indicating that HIPK2 phosphorylates ΔNp63α at the T397 site.

Inhibition of ΔNp63α phosphorylation at T397 upon CDDP treatment was shown to interfere with ΔNp63α degradation (Huang *et al*., 2008). Thus, to confirm that the DOX-induced, HIPK2-mediated degradation of ΔNp63α depends on T397 phosphorylation, HaCat cells were cotransfected with EGFP-HIPK2 and wt-Myc-ΔNp63α or the non-phosphorylatable Myc-T397A mutant. As shown in Figure 5f, the T397A mutant was completely resistant to HIPK2-induced degradation. Similar results were obtained in FaDu cells upon transfection with the same Myc-ΔNp63α or Myc-T397A expressing vectors and treated with DOX (Fig. 5g), indicating that in cell response to this drug, HIPK2 contributes to ΔNp63α degradation by its specific phosphorylation at T397.

Discussion

In this study, we investigated whether the p53 proapoptotic 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 ΔNp63α is induced by HIPK2 in a p53-independent manner. In particular, HIPK2 depletion by RNA interference was sufficient to inhibit drug-induced ΔNp63α degradation even in mutant p53-carrying cells. Furthermore, we found that HIPK2 phosphorylates ΔNp63α at T397 and this specific posttranslational modification strongly contributes to both HIPK2- and DOX-induced degradation of ΔNp63α. In addition, we showed that a C-terminal deletion mutant of ΔNp63α missing the T397 site, the Δ390 mutant, strongly increase cell resistance to DOX.

ΔNp63α 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 TAp63 and ΔNp63 isoforms, that contain or lack, respectively, a transcription-activating

(TA) domain (Yang *et al*., 1998). The ΔNp63 isoforms possess dominant-inhibitory functions over the TA isoforms of the p53 family (TAp63, TAp73, and p53) that usually result in prosurvival and proproliferation activities (Parsa *et al*., 1999; Barbieri *et al*., 2005; Wu *et al*., 2005; Rocco *et al*., 2006). Overexpression of ΔNp63 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 proapoptotic and growth arresting functions of the TA isoforms, ΔNp63 was found to contribute to tumor progression by favoring angiogenesis and chemoresistance (Zangen *et al*., 2005; Wu *et al*., 2005; Rocco *et al*., 2006; Lanza *et al*., 2006; Muller *et al*., 2006) and by modulating cell adhesion processes (Yang *et al*., 2006; Carroll *et al*., 2006). Regardless of the HIPK2-mediated regulation of ΔNp63α in response to DOX, we observed that HIPK2 depletion results in an increased expression of ΔNp63α even in non-stressing conditions (Fig. 3e and data not shown) suggesting that the tumor-associated HIPK2 inactivation might contribute to ΔNp63α 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*., 2006b; Lavra *et al*., submitted). In this regard, it will be interesting to evaluate ΔNp63α expression in those human tumors in which HIPK2 has been found inactive.

Increasing evidence indicates that regulation of protein stability is a key mechanism to control p63 isoforms' actions. In particular, 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), while promoted also by phosphorylation on the ΔN 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 ΔN 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 ΔNp63α 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 c-Myb and CtBP (Kanei-Ishii *et al*., 2004; Zhang *et al*., 2003) 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 ΔNp63α to so divergent fates is presently unidentified. We know that p53 is phosphorylated by HIPK2 in its N-terminus and this homologous region in ΔNp63α is absent. On the opposite site, HIPK2 phosphorylates ΔNp63α at the C-terminus in the SAM domain, a region absent in p53 (Irwin and Kaelin, 2001). Based on 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 ΔNp63α at T397, one of the three residues previously identified by mass spectrometry as phosphorylation sites targeted in response to CDDP treatment (*i.e.*, S385, T397, S466) (Huang *et al*., 2008). Based on the kinase recognition motifs, the authors proposed that the three sites are phosphorylated by ATM, CDK2, and p70s6K, respectively. While specific RNA interference for ATM and p70s6K significantly reduced phosphorylation

of their relative sites, CDK2 depletion or inactivation only marginally reduced CDDP-induced ΔNp63α degradation, suggesting that other kinases recognizing the same motif, might be also involved. Interestingly, Huang and collaborators elegantly showed by Flp-In technology in HNSCC 029 cells, the requirement of each of these sites in the CDDP-induced degradation of ΔNp63α. Our data are consistent with these results since 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 ΔNp63α to the subsequent HIPK2-induced phosphorylation that drives these molecules to their respective fates.

The HIPK2 destabilizing activity on ΔNp63α 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 carries wtp53 and express ΔNp63α, 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 ΔNp63α by T397 phosphorylation, and *iii*) further improve p53 activity by removing the dominant inhibitory effect by ΔNp63α (Fig. 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 ΔNp63 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.

Conflict of interest

The authors declare no conflict of interest.

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Materials and Methods

Cells culture conditions and treatments

RKO, FaDu, HaCat, HCT116 p53+/+ and p53-/- (Bunz *et al*., 1998) cells were cultured in DMEM 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., Italy). For drug treatment, sub-confluent cells were incubated in the presence of DOX

(Sigma), BLM (Aventis Pharma), EPCT (Bristol-Myers Squibb), or MG132 (Calbiochem), at the indicated concentrations.

Expression vectors and transfection

The following plasmids were employed: pCDNA3-Myc-ΔNp63α, pCDNA3-Myc-T397A and pCDNA3-Myc-Δ390 (Papoutsaki *et al*., 2005; Di Costanzo *et al*., 2009); pEGFP-HIPK2 and pEGFP-K221R (Checchinelli *et al*., 2006b); pEGFP-C2 (Stratagene). Plasmids pGex-ΔNp63α and pGex-ΔNp63α(T397A) were obtained by PCR amplification from pCDNA3-Myc-ΔNp63α and pCDNA3-Myc-T397A using specific primers: ΔNp63 BamH-upper: 5'-CGATATGGATCCTTGTACCTGGAAAA CAATGCCC-3'; ΔNp63 Xho1-lower: 5'-CGTATACTCGAGTCATTCTCCTTCCTCTTTG ATACG-3' and cloned into a BamH1/Xho1 digested pGex6p-2rbs vector. The expression vectors were transfected by using Lipofectamine Plus reagent (Invitrogen), according to manufacturer's instructions.

Western blot analyses

TCEs were prepared in RIPA buffer [50mM Tris-HCl pH 8, 300mM NaCl, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40, 1 mM EDTA], supplemented with protease-inhibitor mix (Roche). TCEs were resolved on precast NuPAGE 4-12% gels (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad) and analyzed with the following antibodies (Abs): rabbit anti-HIPK2 (kindly provided by M.L. Schmitz); rabbit anti-p53 (FL-393) and mouse anti-p63 (4A4) (Santa Cruz Biotechnology); mouse anti-Myc (Upstate); rabbit anti-GST (kindly provided by Dr. M. Fanciulli); mouse anti-α-tubulin (Immunological Sciences); mouse anti-actin (Sigma); HRP-conjugated goat anti-mouse and anti-rabbit (Cappel). 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 hours, then plated at low density in 60 mm Petri dishes and grown for a week in the absence of drugs. Surviving colonies were fixed and stained with Cristal Violet (0.5% in methanol) (Sigma) and air-dried.

RNA interference

Stable interference by shRNA was performed by transfection of pRetroSuper and pRetroSuper-HIPK2 vectors (Cecchinelli *et al*., 2006b). After 24 hours from transfection, stable polyclonal populations of control and HIPK2-depleted cells were obtained by selection with 2μg/ml puromycin. Transient interference by siRNA was obtained by HIPK2i Stealth RNAi sequences (a mix of 3 different sequences) and universal negative control (UNC) 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 RT-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) 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 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 2μg of pcDNA3-eGST-HIPK2 or pcDNA3-eGST-K221R plasmids using Lipofectamine (Invitrogen) (Cecchinelli *et al*., 2006a). TCEs were prepared 24 hours post-transfection by incubation for 30 min at 4°C in lysis buffer [50mM Tris-HCl (pH 7.4), 300mM NaCl, 150mM KCl, 1mM DTT, 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 50ng/reaction. Recombinant GST-ΔNp63α 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 [20mM Hepes pH 7.4, 50mM NaCl, 10mM $MgCl_2$, 10mM $MnCl_2$] in the presence of 185 KBq [γ^{32}P]-ATP. The phosphorylated substrates were resolved on precast NuPAGE 4-12% gels and analyzed by autoradiography.

Abbreviations

BLM, bleomycin; CDDP, cisplatin; Ctr, control; DDR, DNA damage response; DOX, doxorubicin; EPEG, etoposide; eGST, eukaryotic-GST; HIPK2, Homeodomain-Interacting Protein Kinase 2; KD, kinase-dead; TCE, total cell extract; WB, Western blot.

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Figure Legends

Figure 1 HIPK2 is involved in tumor cell response to antineoplastic drugs. RKO cells were treated with DOX, EPEG, or BLM at the indicated doses. UV radiation at 50 J/m^2 was used as positive control for HIPK2 protein stabilization. (**a**) TCEs were prepared at the indicated times after treatment (Hrs) and analyzed by WB for HIPK2 protein expression. Tubulin expression shows equal loading of samples. (**b**) RKO cells were stably depleted for HIPK2 by shRNA. HIPK2 depletion was verified by WB analysis (small insert). Interfered cells (HIKP2-i) and control cells (Ctr-i) were treated with the indicated drugs and plated for colony forming assay 24 hours post-treatment. Columns are average of three independent experiments and bars indicate standard deviation.

Figure 2 HIPK2 mediates tumor cell chemosensitivity in p53 null cells. (**a**) H1299 cells were treated with the indicated drugs at the indicated doses. TCEs were prepared 12 hours after treatment and analyzed by WB for HIPK2 expression. (**b**) H1299 cells were stably depleted for HIPK2 by shRNA. HIPK2 depletion was verified by WB (small insert). HIPK2-i and Ctr-i cells were treated with the indicated drugs and colony forming assay was performed 24 hours after treatment. Columns are average of three independent experiments and bars indicate standard deviation. (**c** and **d**) p53 proficient (p53+/+) and defective (p53-/-) HCT116 cells were transiently depleted for HIPK2. After 48 hours from transfection, (**c**) HIPK2 depletion was verified by Real-time PCR and (**d**) drug sensitivity was assessed by treatment with 2μM DOX and subsequent cell death evaluation by Trypan blue exclusion test 24 hours post-treatment.

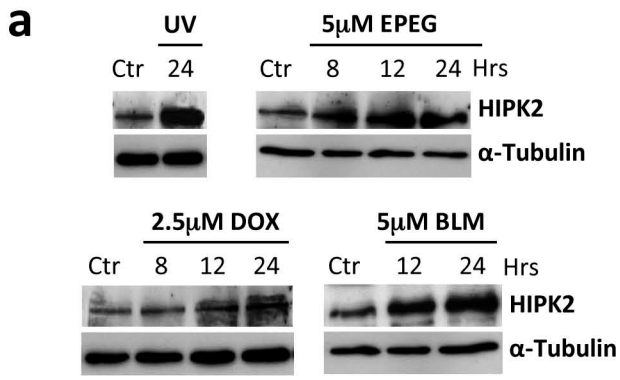
Figure 3 HIPK2 contributes to ΔNp63α degradation upon DOX treatment. (**a**) The indicated cells were treated with 2μM DOX or maintained in the absence of drug. TCEs were prepared 12 hours post-treatment and analyzed by WB for the indicated proteins. Actin expression shows equal loading of samples. (**b**, **c**, and **d**) The indicated cells were treated with DOX. TCEs were prepared at the indicated times post-treatment and analyzed by WB for ΔNp63α expression. (**e**) FaDu cells were transiently depleted for HIPK2; 36 hours 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 ΔNp63α expression. (**f**) FaDu cells were transfected with an expression vector coding for EGFP-HIPK2 (0.5, 1 or 2 μg). TCEs were prepared 36 hours post-transfection and analyzed by WB. (**g**) FaDu cells were transfected as in (**f**) (1 and 2 μg) and treated with 10μM MG132 (or its solvent, DMSO) 20 hours post-transfection. TCEs were prepared 10 hours after MG132-treatment and endogenous ΔNp63α protein levels were analyzed by WB.

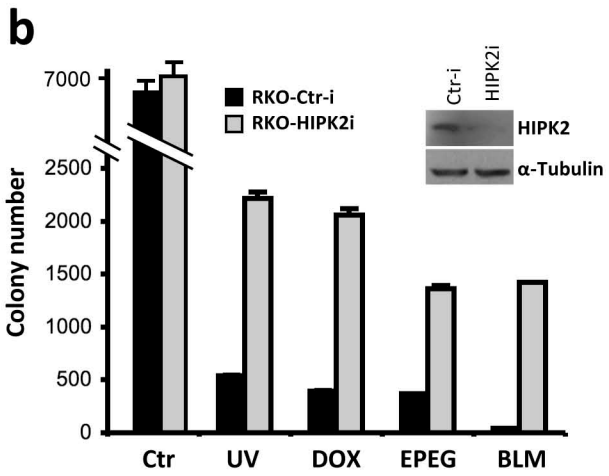
Figure 4 Role of the C-terminal region of ΔNp63α in HIPK2 mediated degradation. (**a**) FaDu cells were co-transfected with an expression vector coding for EGFP or EGFP-HIPK2 together with a vector coding for the fusion protein Myc-ΔNp63α or its deletion mutant Myc-Δ390. TCEs were prepared 24 hours post-transfection and WB analysis of exogenous ΔNp63α protein was performed using an α-Myc Ab. (**b**) The indicated cells were transfected with an expression vector coding for Myc-ΔNp63α or Myc-Δ390; 16 hours post-transfection cell 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 hours post-transfection treated with 1μM DOX. Colony formation assay was set up 24 hours after DOX treatment.

Figure 5 Phosphorylation of ΔNp63α at T397 by HIPK2 is required for ΔNp63α degradation. (**a**) eGST-HIPK2 and eGST-K221R proteins were prepared by GST pull down from H1299 cells and incubated with a purified p63 protein in the presence of [γ^{32}P]-ATP. Kinase reaction products were resolved by SDS-PAGE and analyzed by autoradiography (upper panel). WB analysis of GST proteins and p63 was performed after gel rehydration with the indicated Abs. (**b**) FaDu cells were transfected with EGFP-HIPK2 (2.5 μg), EGFP-K221R (2, 3, and 5 μg), or EGFP (2, 3, and 5 μg). TCEs were prepared 36 hours after transfection and analyzed by WB; ∗ indicate unspecific bands. (**c**) Schematic representation of ΔNp63α 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-ΔNp63α, and GST-T397A proteins. (**e**) Comparable amounts of GST proteins were incubated with active-HIPK2 in the presence of [γ^{32}P]-ATP and the kinase reaction products were resolved by SDS-PAGE 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**) FaDu cells were transfected with Myc-ΔNp63α and Myc-T397A expressing vectors. DOX was added 16 hours after transfection and TCEs were prepared 48 hours after treatment and analyzed by WB.

Figure 6 Model summarizing HIPK2 activity on p53 and ΔNp63α in response to antineoplastic drugs.

Lazzari *et al.*, Figure 1





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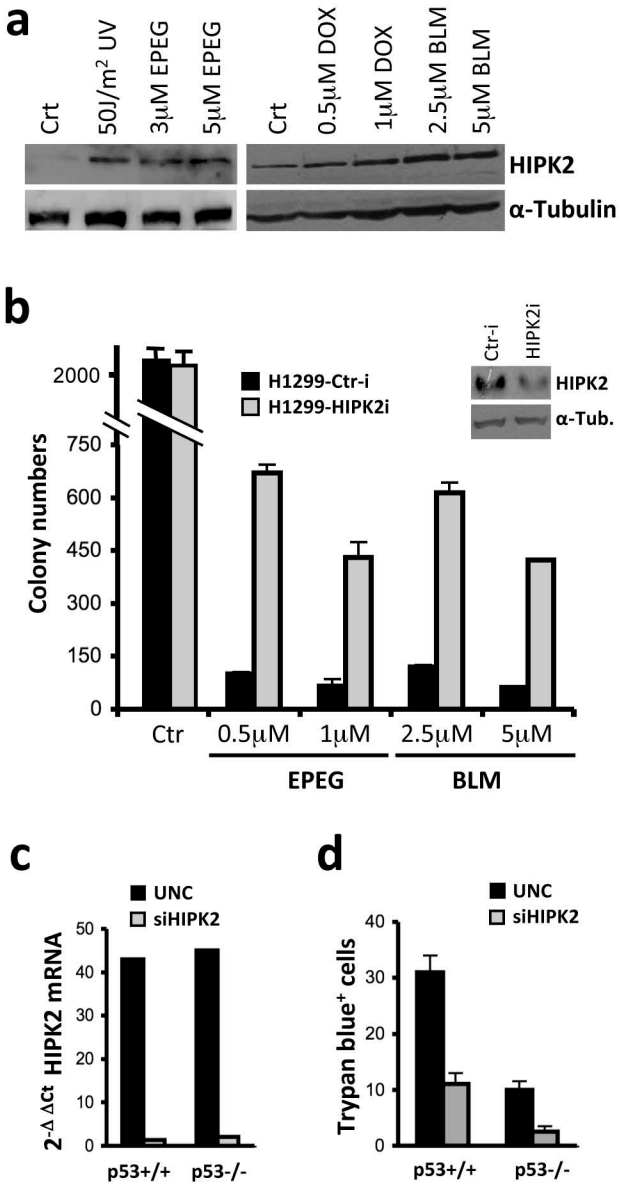

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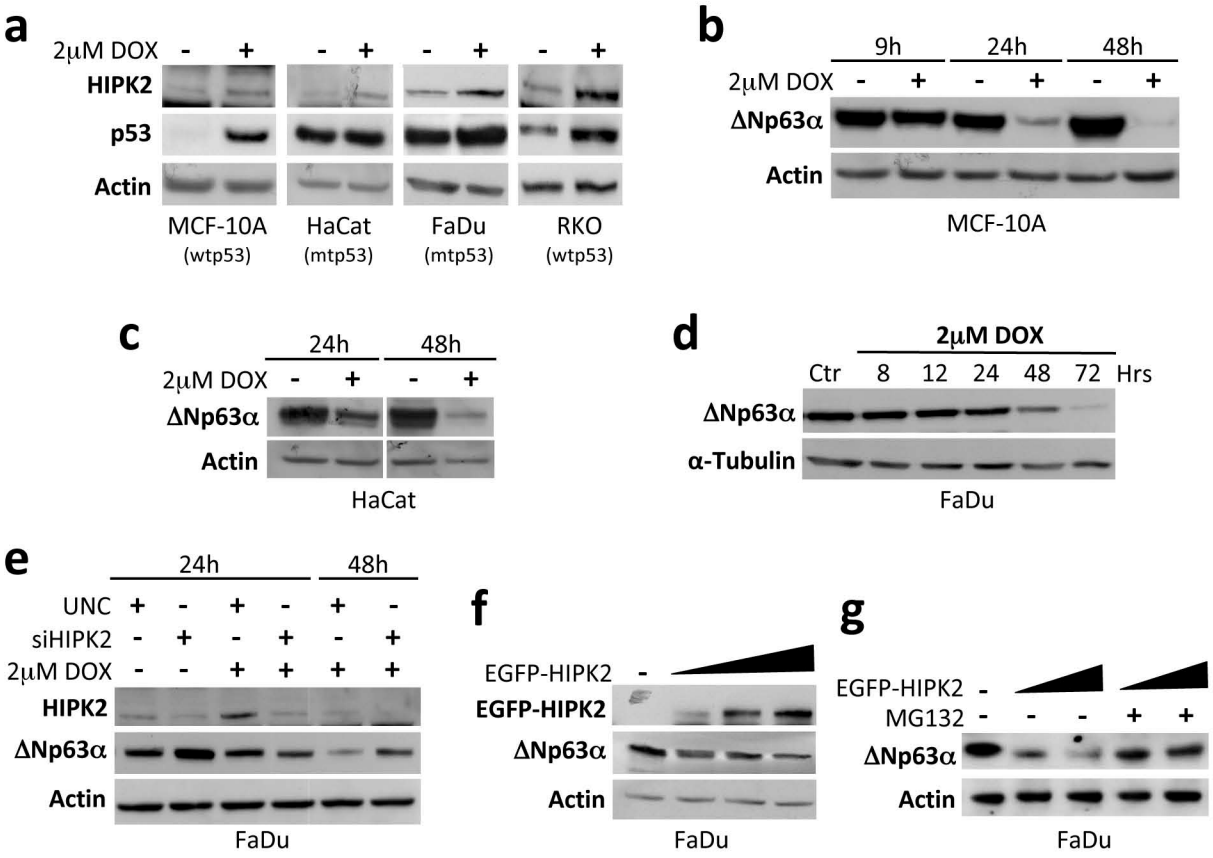

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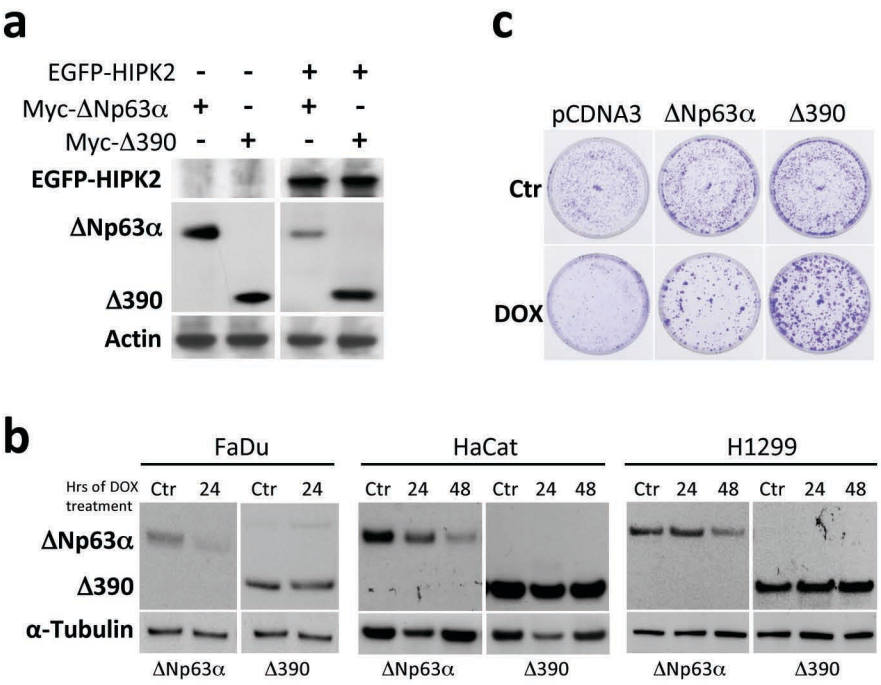

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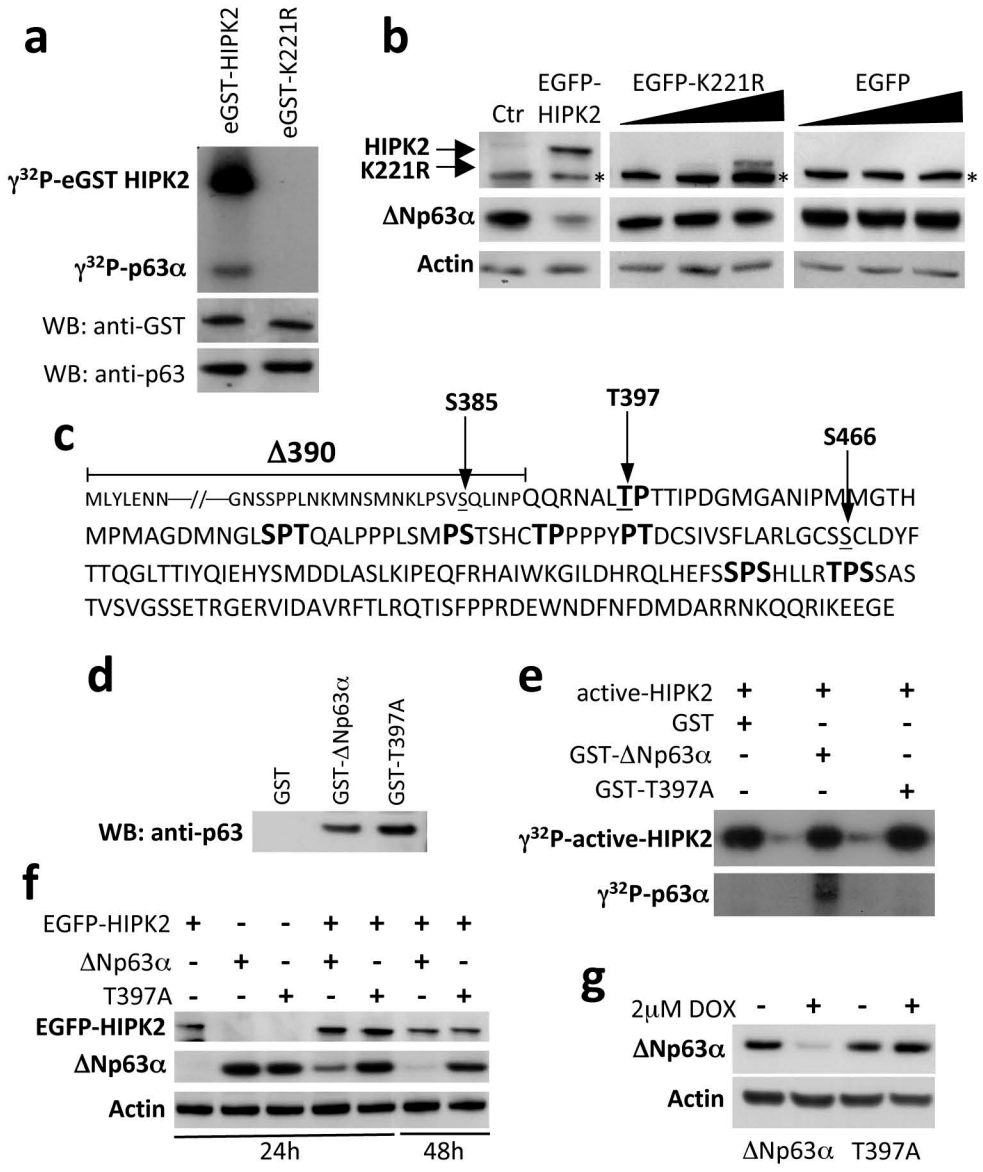
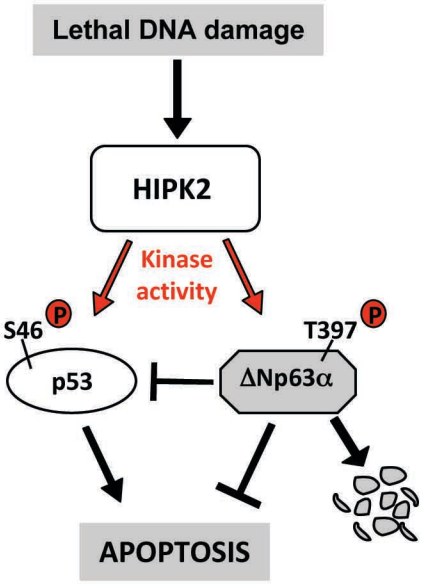
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