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HIPK2 Regulation by MDM2 Determines Tumor Cell Response to the p53-Reactivating Drugs Nutlin-3 and RITA

Cinzia Rinaldo,[1] Andrea Prodosmo,[1] Francesca Siepi,[1] Alice Moncada,[1] Ada Sacchi,[1] Galina Selivanova,[2] and Silvia Soddu[1]

[1]Department of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Rome, Italy and [2]Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden

Abstract

In the past few years, much effort has been devoted to show the single-target specificity of nongenotoxic, p53 reactivating compounds. However, the divergent biological responses induced by the different compounds, even in the same tumor cells, demand additional mechanistic insights, whose knowledge may lead to improved drug design or selection of the most potent drug combinations. To address the molecular mechanism underlying induction of mitotic arrest versus clinically more desirable apoptosis, we took advantage of two MDM2 antagonists, Nutlin-3 and RITA, which respectively produce these two outcomes. We show that, along with p53 reactivation, the proapoptotic p53-activator HIPK2 is degraded by MDM2 in Nutlin-3–treated cells, but activated by transiently reduced MDM2 levels in RITA-treated ones. Gain- and loss-of-function experiments revealed the functional significance of MDM2-mediated HIPK2 regulation in cell decision between mitotic arrest and apoptosis in both types of p53 reactivation. These data indicate that strategies of p53 reactivation by MDM2 inhibition should also take into consideration MDM2 targets other than p53, such as the apoptosis activator HIPK2. [Cancer Res 2009;69(15):6241–8]

Introduction

Somatic missense mutations in the *TP53* gene are present in ~50% of human cancers (1). However, the observation that p53 function is lost in most wild-type p53 (wtp53)-carrying cancers, due to amplification or overexpression of its negative regulator MDM2, has rendered the strategy of releasing p53 from MDM2 an attractive therapeutic target in oncology (2). Indeed, in the past decade, induction of p53-mediated apoptosis in tumor cells by exquisitely selective MDM2 inactivation without stimulation of genotoxic damage has been one of the leading ideas to avoid unwanted side effects and limit the induction of secondary cancers resulting from genomic damage (3, 4). Several approaches have been developed and tested (5), leading to the discovery of small molecules that can restore p53 function in tumor cells by targeting MDM2 through different mechanisms, such as inhibition of MDM2-mediated ubiquitylation of p53, of p53-MDM2 interaction, or of MDM2 E3 ubiquitin ligase activity (6). Although each strategy promoted p53 reactivation in tumor cells carrying wtp53, as predicted, the biological responses were found to be significantly different. With the exception of hematologic malignancies, inherently more prone to apoptosis, cells from solid tumors undergo mitotic arrest or apoptosis depending on the p53-reactivating compound used (7, 8), suggesting that in addition to p53 release from MDM2, other mechanisms might be engaged.

Recently, it has been shown that MDM2 is not only a p53 inhibitor but contributes to modulate the p53-mediated biological outcome (i.e., mitotic arrest versus apoptosis) to diverse triggering signals (9). In particular, we have found that the cytostatic response to mild, presumably reparable DNA damage implies an active MDM2-mediated inhibition of the p53 apoptotic pathway. This event is determined by the MDM2-mediated degradation of the p53 proapoptotic activator HIPK2. In contrast, cell response to lethal damage is associated with low levels of MDM2 expression and HIPK2 activation (10). HIPK2 is a multifunctional kinase that by virtue of protein/protein interaction with a still growing list of targets and phosphorylation of specific serine/threonine residues, regulates gene transcription and the response to DNA damage (11, 12). HIPK2 is one of the kinases that phosphorylates p53 at Serine 46 (p53Ser46; refs. 13, 14), a posttranslational modification triggered by severe, presumably irreparable damage that would lead to apoptosis (15). In addition, HIPK2 can induce apoptosis in a p53-independent manner by promoting a phosphorylation-dependent proteasomal degradation of the antiapoptotic transcriptional corepressor CtBP (16).

Based on the findings described above, we asked whether the MDM2-mediated regulation of HIPK2 might be involved in cell decision between mitotic arrest and apoptosis induced by different MDM2 antagonists. To address this question, we selected two compounds, which have been extensively characterized both in tumor cell culture and *in vivo* tumor xenografts: Nutlin-3, which mainly induces mitotic arrest in solid tumor–derived cells, and Reactivation of p53 and Induction of Tumor-cell Apoptosis (RITA), which preferentially promotes apoptosis in the same tumor cells (7, 8). Nutlin-3 is a *cis*-imidazoline analogue that binds MDM2 in the p53 pocket and inhibits p53 degradation (17) without causing major conformational changes in the MDM2 molecule and preserving its E3-ligase activity (18). RITA is a furanic compound, identified in a cell-based screen, which binds with high affinity to the p53 NH_2-terminal domain. This binding induces a conformational change in p53 that reduces p53-MDM2 interaction and p53 ubiquitylation, leading to p53 accumulation and induction of the p53-dependent apoptotic pathway (8). Here, we show that the biological responses induced by these small compounds, above and beyond their specific ability of releasing p53 from MDM2, include effects on others MDM2 targets, such as the apoptosis activator HIPK2.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Silvia Soddu, Department of Experimental Oncology, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Phone: 39-065266-2492; Fax: 39-065266-2505; E-mail: soddu@ifo.it.

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Materials and Methods

Cell lines and drug treatments. U2OS, RKO, MCF-7, HCT116, H1299, SJSA-1, LNCaP, and SHEP cells were maintained in DMEM or RPMI supplemented with 10% fetal bovine serum. Primary human embryo kidney cells were kindly provided by S. Bacchetti (Regina Elena Cancer Institute, Rome, Italy; ref. 10). For compound treatment, cells were incubated with 10 μmol/L MG132 (Biomol), 50 μmol/L z-VAD-fmk (Bachem), 10 μmol/L Nutlin-3 (Cayman), or 1 μmol/L RITA, unless differently indicated. TUNEL (Upstate), cell viability, proliferation, colony formation, and cell cycle profiles were determined as described (10, 19).

Expression vectors and transfections. The expression vectors were described by Rinaldo and colleagues (10) and the interfering vectors were described by Cecchinelli and colleagues (19). The siRNA for MDM2 depletion and their control were purchased by Dharmacon (20). Plasmids were transfected by lipofectamine-plus reagent (Invitrogen) and RNA oligonucleotides by RNAiMAX reagent (Invitrogen).

Western blotting. Total cell extracts (TCE) were prepared in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8), 300 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 1 mmol/L EDTA] supplemented with protease-inhibitor mix (Roche). TCEs were resolved on precast NuPAGE 4% to 12% gels, transferred onto nitrocellulose membranes (Bio-Rad), and analyzed with the following antibodies: rabbit anti-HIPK2 (kindly provided by M.L. Schmitz, Institute of Biochemistry, Giessen, Germany); rabbit anti-p53 (FL-393), anti-p21 (C-19), and anti-SP1 (sc-59; Santa Cruz Biotechnology); sheep anti-p53 (Ab-7, Calbiochem); rabbit anti–phospho-p53Ser46 and anti-cleaved PARP (Cell Signaling Technology); mouse anti-MDM2 monoclonals (MoAb) 2A10 (Ab-2 Calbiochem) and Ab-1 (Oncogene Research Products); anti–α-tubulin MoAb (Immunologic Sciences), anti-actin MoAb (Sigma); anti-CtBP MoAb (BD Transduction Laboratories); horseradish peroxidase–conjugated goat anti-mouse, anti-rabbit, and anti-sheep (Cappel).

Real-time 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 primers used were described (19).

Results

HIPK2 is differentially expressed in response to Nutlin-3 and RITA. To evaluate whether the cell decision between mitotic arrest and apoptosis induced by different MDM2 antagonists involves

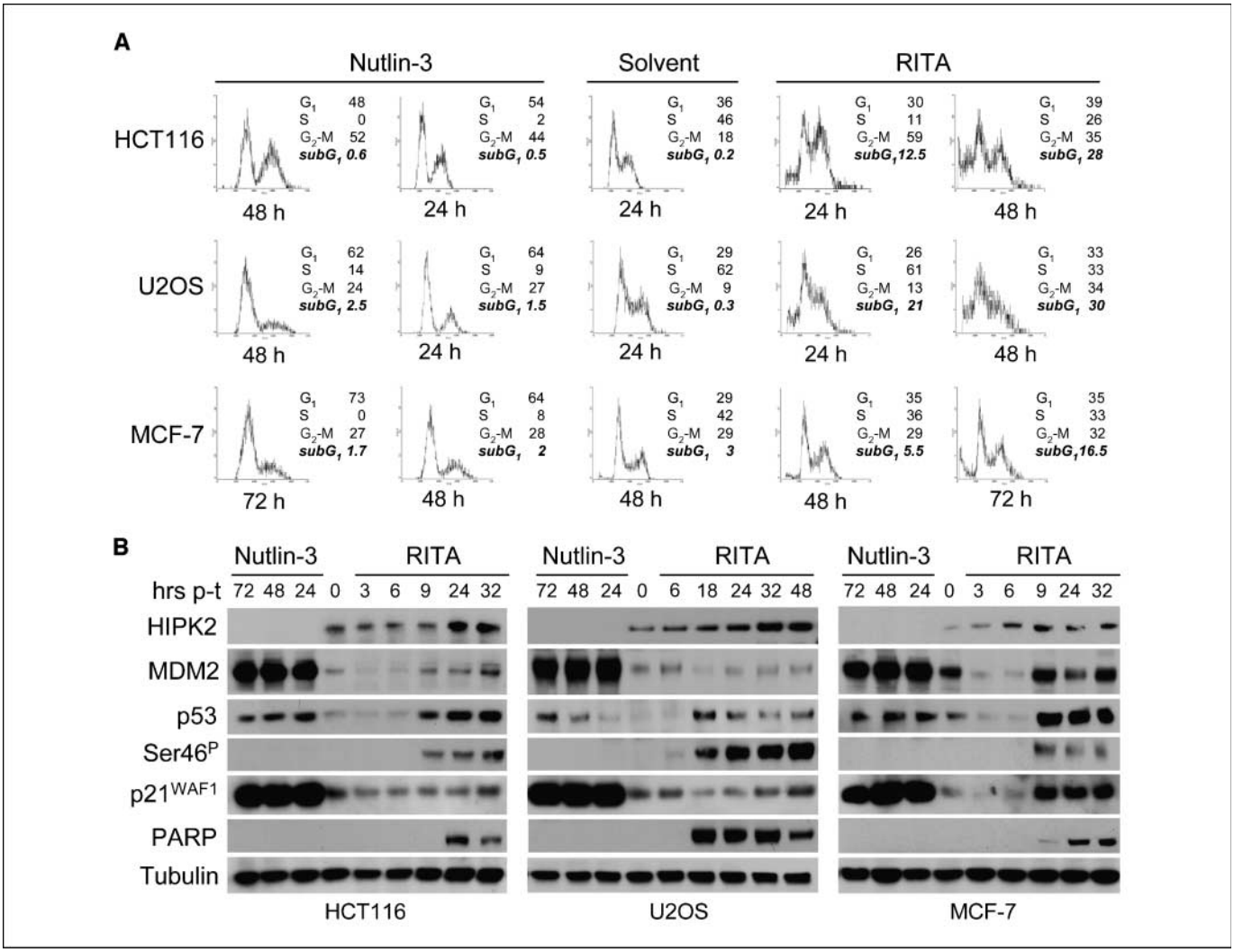

Figure 1. Biological and biochemical effects of Nutlin-3 and RITA. The indicated cells were treated with Nutlin-3 or RITA or their solvents (DMSO and PBS, respectively). *A,* cell cycle profiles were analyzed at the indicated times after treatments. The percentages of cells in the different phases of the cell cycle have been calculated excluding the sub-G_1 picks, whose amount has been independently evaluated relative to the total populations *B,* TCEs were prepared at the indicated hours after treatment (*hrs p-t*) and analyzed by WB for the indicated proteins. $Ser46^P$ indicates the reaction obtained with anti-phospho–specific p53Ser46 antibody. Tubulin expression shows equal loading of samples.

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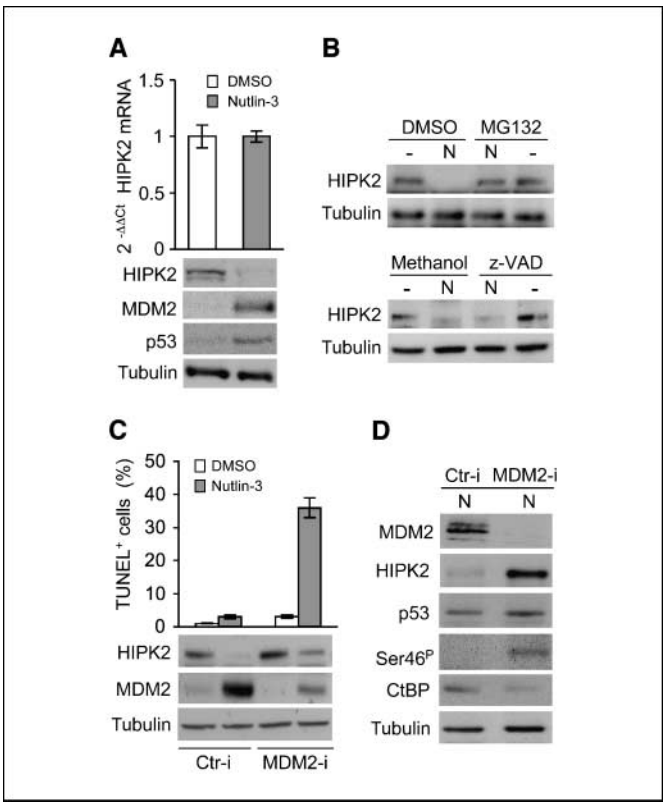

Figure 2. Nutlin-3 represses HIPK2 expression by inducing its MDM2-mediated degradation. *A,* U2OS cells were treated with Nutlin-3 or its solvent (DMSO), and RNAs and TCEs were prepared 48 h after treatment. The HIPK2 mRNA levels were analyzed by Real-time PCR (*top*). TCEs were analyzed by WB for the expression of the indicated proteins (*bottom*). *B,* U2OS cells were treated with Nutlin-3 (*N*) in the presence or absence of MG132 or its solvent (DMSO) or z-VAD-fmk or its solvent (*Methanol*). TCEs were analyzed for HIPK2 expression by WB. *C,* U2OS cells were transiently transfected with MDM2-specific siRNA (*MDM2-i*) or Ctr-i and treated with Nutlin-3 or DMSO 24 h after transfection. The percentages of apoptotic cells were measured by TUNEL assay 48 h after treatment. *Columns,* average of three independent experiments; *bars,* SD. TCEs were prepared 24 h after treatment and analyzed by WB. *D,* U2OS cells were depleted for MDM2 as in *C* and 24 h after transfection were treated with Nutlin-3. TCEs were obtained 24 h after treatment and analyzed by WB for the indicated proteins.

HIPK2, as a MDM2 target other than p53, we selected a set of wtp53-carrying cell lines derived from solid tumors. We did not include in our study hematopoietic tumors to avoid pitfalls due to high intrinsic sensitivity to apoptosis of hematopoietic cells (21). Consistent with previous reports (7, 8), cells from the same lines were cell cycle arrested but alive upon Nutlin-3 treatment, while undergoing cell death upon RITA (Supplementary Fig. S1*A*; Fig. 1*A*). Next, the effects of Nutlin-3 and RITA on the expression of different known or putative targets were analyzed by Western blot (WB; Supplementary Fig. S1*B*; Fig. 1*B*). As expected, the cleavage of PARP was present only in the apoptotic, RITA-treated cells; the cell cycle inhibitor $p21^{WAF1}$ was strongly induced only in the arrested, Nutlin-3–treated cells, whereas its amount was reduced, at least transiently, in the RITA-treated cells, as recently reported (22). The amount of p53 protein was increased by both compounds, although to a different degree, depending on the cell line; in contrast, the amount of MDM2 was strongly increased by Nutlin-3, as described (17), but transiently repressed by RITA, although with different kinetics among the cells. Interestingly, an inverse correlation with MDM2 expression was observed for HIPK2, whose expression was strongly repressed by Nutlin-3 while increased by RITA, revealing a differential regulation of this apoptosis activator by the two compounds. This differential regulation of HIPK2 is p53-dependent because it was not detected in p53-null cells (Supplementary Fig. S1*C*). When normal cells, such as primary human embryo kidney cells, were subjected to similar treatments, no effects were induced by RITA, whereas a mild inhibition of cell proliferation associated with MDM2 induction and HIPK2 reduction were observed upon Nutlin-3 treatment (Supplementary Fig. S2).

HIPK2 depletion by Nutlin-3 is caused by MDM2 up-regulation and contributes to the mitotic arrest. To identify the mechanism/s of HIPK2 modulation by Nutlin-3, we analyzed HIPK2 protein and mRNA levels. Because decreased of HIPK2 protein expression was associated with no mRNA modifications (Fig. 2*A*), we assessed protein degradation by adding Nutlin-3 in the presence or absence of the proteasome inhibitor MG132. The pan-caspase inhibitor z-VAD was included in the experiment because HIPK2 can be activated by caspase cleavage (23). HIPK2 reduction was rescued by MG132, whereas no effect was observed with z-VAD (Fig. 2*B*), suggesting that Nutlin-3 represses HIPK2 expression by proteasome-mediated degradation.

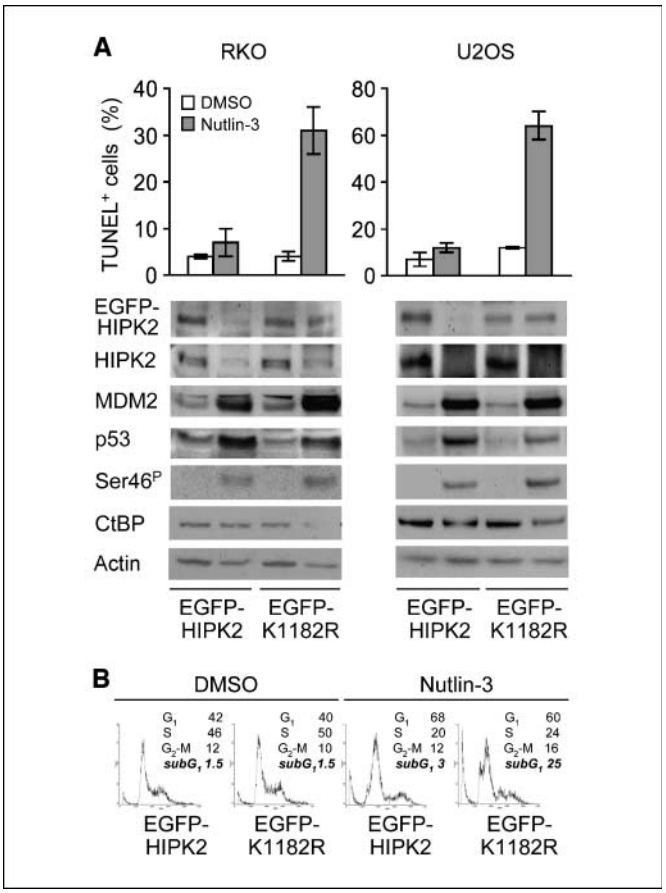

Figure 3. The mitotic arrest induced by Nutlin-3 depends on MDM2-mediated degradation of HIPK2. *A,* RKO and U2OS cells were transiently transfected with vectors expressing the indicated tagged-proteins and treated with Nutlin-3. The percentages of apoptotic cells were measured by TUNEL assay 48 h after treatment. *Columns,* average of three independent experiments; *bars,* SD. TCEs were prepared 24 h after treatment and analyzed by WB for the indicated proteins. Actin expression shows equal loading of samples. *B,* cell cycle profiles of RKO cells treated as in *A* were analyzed 48 h after treatment as described in Fig. 1*A*.

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In response to mild/reparable genotoxic damage, MDM2-mediated degradation of HIPK2 is a critical step for induction of mitotic arrest and protection from apoptosis (10). Although Nutlin-3 activates p53 without evidence of genotoxic stress (17), the associated strong expression of the MDM2 protein that preserves its E3-ligase activity (18) might be directly responsible for the degradation of HIPK2, thus promoting the mitotic arrest induced by Nutlin-3. To test this hypothesis, cells were treated with Nutlin-3 upon depletion of MDM2 by RNAi (MDM2-i). A significantly milder reduction of HIPK2 expression and a strong induction of apoptosis were observed in MDM2-i cells compared with control interfered (Ctr-i) cells (Fig. 2*C*). These events were associated with induction of p53Ser46 phosphorylation (p53Ser46^{P}) and repression of CtBP expression (Fig. 2*D*), two markers of HIPK2-induced apoptosis (13, 16), supporting the hypothesis that down-regulation of HIPK2 upon Nutlin-3 treatment is MDM2-dependent and the mitotic arrest induced by this compound depends on MDM2-mediated HIPK2 degradation.

MDM2 promotes the degradation of HIPK2 through its ubiquitylation at lysine residue 1182, whose substitution with an arginine produced a HIPK2-K1182R mutant resistant to MDM2-mediated degradation (10). Thus, we tested the effect of Nutlin-3 on EGFP-tagged wild-type HIPK2 (EGFP-HIPK2) or the degradation-resistant HIPK2-K1182R mutant (EGFP-K1182R) ectopically expressed in cells at low level. The low level of expression was required to avoid massive apoptosis by the HIPK2 expression on its own (10). As shown in Fig. 3*A*, Nutlin-3 promoted the degradation of the endogenous HIPK2 as well as of the EGFP-HIPK2, but did not reduce the expression of the MDM2-resistant EGFP-K1182R mutant. In addition, a strong induction of apoptosis with p53Ser46^{P} and CtBP repression was induced by Nutlin-3 upon expression of the HIPK2-K1182R mutant (Fig. 3*A* and *B*).

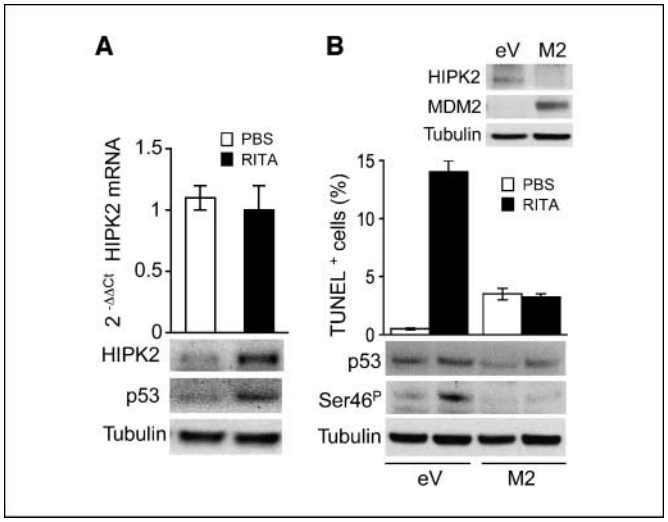

Figure 4. RITA increases HIPK2 expression by MDM2 down-regulation. *A,* U2OS cells were treated with RITA and RNAs and TCEs were prepared 36 h after treatment. The HIPK2 mRNA levels were analyzed by Real-time PCR (*top*). TCEs were analyzed by WB for expression of the indicated proteins (*bottom*). *B,* U2OS cells were transiently transfected with pCMV-MDM2 vector encoding for MDM2 (*M2*) or with the empty control vector (*eV*). After 24 h from transfection, cells were treated with RITA and harvested 24 h after treatment for TUNEL assay and WB analysis of the indicated proteins. Values of TUNEL assay correspond to the average of three independent experiments ± SD. *Insert,* WB for MDM2 overexpression and HIPK2 protein levels, 48 h after transfection in the presence of RITA.

Taken together, these results indicate that the depletion of HIPK2 induced by Nutlin-3 shifts the balance toward induction of mitotic arrest in solid tumor–derived cells.

HIPK2 induction by RITA is caused by MDM2 down-regulation and contributes to the apoptotic response. Next, we investigated the mechanism of HIPK2 induction by RITA by analyzing HIPK2 mRNA and protein levels. Increased HIPK2 protein expression was associated with no change in mRNA level (Fig. 4*A*), supporting a nontranscriptional type of regulation.

We had previously observed that MDM2 depletion by RNAi is able to increase HIPK2 expression even in the absence of genotoxic damage (Fig. 2*C*; ref. 10), suggesting that MDM2 might contribute to the maintenance of the steady-state level of HIPK2. Because our original time course analyses performed upon RITA treatment showed that HIPK2 induction is preceded by a decrease in MDM2 expression (Fig. 1*B*), we asked whether a causal role might exist between the transient MDM2 down-regulation and the induction of HIPK2 expression. Thus, we evaluated the effect of MDM2 overexpression on HIPK2 levels and apoptosis-induction in RITA-treated cells. Forced expression of MDM2 impaired HIPK2 expression as well as p53Ser46^{P} and induction of apoptosis (Fig. 4*B*), suggesting that the RITA-induced apoptosis might depend on the MDM2-mediated activation of HIPK2. However, MDM2 overexpression may protect cells from apoptosis by other mechanisms, including, of course, direct inactivation of p53 (24). Although the concomitant RITA treatment should neutralize this effect, the contribution of MDM2 is difficult to be determined upon these conditions. Therefore, we directly assessed the role of HIPK2 in the apoptotic response to RITA. Cells were depleted for HIPK2 expression by transient or stable transfection of different HIPK2-specific RNA-interfering sequences. Although partial, the HIPK2 depletion was sufficient to inhibit RITA-induced cell death, long-term colony formation, and p53Ser46P (Fig. 5*A*–*D*). Altogether, these data show that HIPK2 is an indirect target of RITA and this compound induces apoptosis in solid tumors because it can activate HIPK2.

Combined treatment with Nutlin-3 and RITA confirmed the crucial role of HIPK2 in apoptosis induction. Drug combination are currently regarded as a most efficient strategy in cancer therapy. Indeed, combination of Nutlin-3 with conventional anticancer drugs, such as doxorubicin and cisplatin, were shown to improve their therapeutic effect (18, 25). In principle, it might be expected that combination of different MDM2 antagonists would improve their therapeutic efficacy. However, given the opposite effects exerted by Nutlin-3 and RITA on HIPK2, the combination of the two drugs would not necessarily increase their therapeutic efficacy. To get clues on this issue, we first treated cells with Nutlin-3 and added RITA when HIPK2 was already repressed by the Nutlin-3 pretreatment. As shown in Fig. 6*A* an additive effect was observed on p53 expression. However, RITA treatment could only partially down-regulate MDM2 and p21^{WAF1} expression, whose absolute levels remained significantly higher than in untreated cells; HIPK2 expression was not detectably recovered and p53Ser46^{P} was reduced, as shown by the similar intensity of the p53Ser46^{P} bands, although the amount of total p53 was higher in the Nutlin-3⇒RITA–treated cells than in the RITA alone. Eventually, a reduced amount of apoptosis, relative to RITA treatment alone was observed upon Nutlin-3⇒RITA combination. Next, we treated cells with RITA and added Nutlin-3 after HIPK2 induction by the RITA pretreatment. An additive effect on p53 expression was present also upon this combination. However, Nutlin-3 treatment could only

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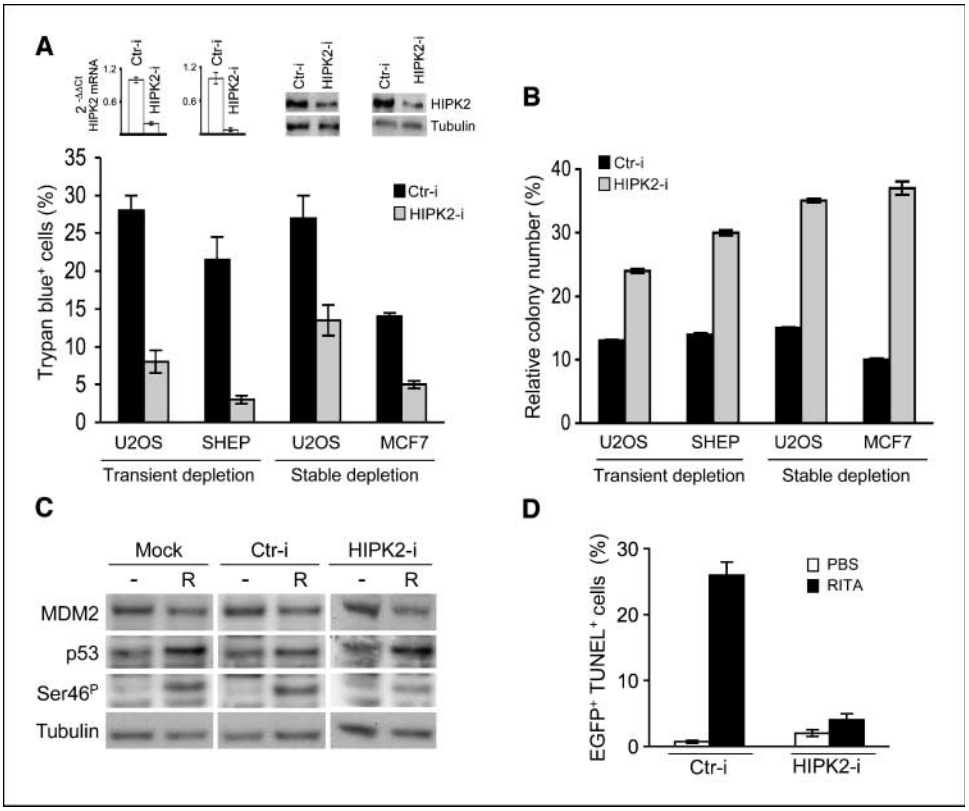

Figure 5. HIPK2 depletion inhibits RITA-induced apoptosis. *A,* the indicated cells were transiently or stably depleted for HIPK2 by RNAi (*HIPK2-i*) or Ctr-i. HIPK2 depletion was verified by quantitative Real-time PCR (*columns,* mean of two independent experiments; *bars,* SD) or by WB analysis (*top*). Interfered cells were treated with RITA, 24 h after transient transfection or 24 h after plating, in the case of stable transfection. Cell viability was assessed by trypan blue exclusion 24 h after treatment. The histogram reports the RITA-induced trypan blue positivity measured in the HIPK2-i or in the Ctr-i populations. The trypan blue positivity was calculated relative to that of solvent-treated cells. The indicative data of one of three different stable clonal populations are reported. *B,* the indicated cells were treated as in *A* and colony assays were performed 8 h after treatment. The relative colony numbers reported in the histogram have been calculated as follow:

$$\text{Relative colony number (\%)} = \frac{\text{N}^\circ \text{ of colonies in RITA-treated cells}}{\text{N}^\circ \text{ of colonies in solvent-treated cells}} \times 100$$

Columns, average of three independent experiments; *bars,* SD. *C,* TCEs from the same transiently HIPK2-depleted U2OS cells shown in *A* were obtained 24 h after treatment and analyzed by WB. All TCEs were run on the same gel; *vertical lines,* noncontiguous samples. *D,* SHEP cells were transiently depleted of HIPK2 in the presence of an auto-fluorescent marker (i.e., cotransfection with an EGFP-expressing vector at 1:10 molar ratio) and treated with RITA 24 h after transfection. TUNEL and EGFP double positive cells were counted 24 h after treatment. *Columns,* means of three independent experiments; *bars,* SD.

partially increase MDM2 and $p21^{WAF1}$ expression and partially inhibit HIPK2. These events were associated with increased amount of $p53Ser46^{P}$ and apoptotic rate (Fig. 6*B*), further supporting the model in which the MDM2-dependent regulation of HIPK2, determined by the two different MDM2 antagonists, switches the cell response induced by their p53 reactivation.

Discussion

Restoration of wtp53 function in human tumors by non-genotoxic target-specific drugs is currently regarded as a promising strategy to improve cancer therapy (26, 27). Despite the significant advances achieved in the past decade, two main problems remain unresolved: induction of permanent tumor suppressive effects (i.e., apoptosis or replicative senescence of tumor cells rather than transient mitotic arrest) and a sufficient therapeutic window (i.e., toxicity for tumors but not for normal tissues). In the present study, we addressed the molecular mechanism underlying induction of apoptosis versus growth arrest using as a tool two p53 reactivating compounds that target p53/MDM2 interaction, but produce different biological effects. According to the published data, MDM2 antagonists Nutlin-3 and RITA possess an opposing ability of inducing mitotic arrest and apoptosis in the same solid tumor–derived cells, providing an excellent tool to experimentally approach the problem. We have recently shown that the p53 proapoptotic activator HIPK2 is a critical target of the p53-MDM2 pathway, contributing to a cell decision between mitotic arrest and apoptosis upon genotoxic damage (10). Based on these findings, we asked whether HIPK2 might be involved in the different biological responses induced by Nutlin-3 and RITA, respectively. Here, we provide evidence for a strong correlation and a causal link between Nutlin-3–induced mitotic arrest and HIPK2 down-regulation as well as between RITA-induced apoptosis and HIPK2 induction. In both cases, these opposing effects on HIPK2 were mediated by MDM2, which is strongly up-regulated by Nutlin-3, as already well characterized by others (7, 18, 28), whereas transiently down-regulated by RITA. Altogether, our data show that the divergent tumor cell response to p53-reactivation is due, at least in part, to the MDM2-mediated regulation of HIPK2 (Fig. 6*C*).

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By targeting the physical interaction of p53 and MDM2, Nutlin-3 was previously shown to perform various biochemical functions: (*a*) by stabilizing p53, it elevates the cellular level of the p53 transcription target MDM2 (17); (*b*) by binding MDM2, it prevents the MDM2 association with other factors, such as the hypoxia-inducible factor 1α (29) or E2F1 (25, 30); (*c*) by preserving the E3 ubiquitin ligase activity of MDM2, it facilitates the degradation of the p53 inhibitor/regulator, MDM4 (18). The observation that, despite all these functions, Nutlin-3 induces mitotic arrest in most cancer cells with wtp53 but fails to induce effective apoptosis in many of them (7) has suggested that the cell cycle arrest function of p53 is well-preserved in human cancer but the apoptotic function may suffer from abnormalities in the downstream p53 signals (7, 17). This hypothesis led toward the identification of the cancer phenotypes susceptible to Nutlin-3–induced apoptosis. For example, MDM2 amplification or MDM4 overexpression were shown to sensitize a few tumor cells to Nutlin-3–induced apoptosis (31–33), but their role has been not confirmed in other cells (7, 30, 34). Based on the strong MDM2-mediated inhibition of HIPK2 expression we observed in all the tumor cells we treated with Nutlin-3, we propose that Nutlin-3 is intrinsically a cell-cycle arresting molecule in solid tumor–derived cells and that apoptosis is the atypical response of particularly sensitive cells. This hypothesis is supported by the results we obtained with the Nutlin-3 and RITA combination in successive administrations. Nutlin-3 pretreatment inhibited HIPK2 expression and reduced RITA-induced apoptosis, whereas the reverse administration maintained HIPK2 expression and increased p53Ser46^{P} and RITA-induced apoptosis. These results show that the downstream p53 signals required for apoptosis are present and inducible in these cells when HIPK2 is not suppressed. Furthermore, by analyzing SJSA-1 and LNCaP cells, two cell lines that were shown to be sensitive to Nutlin-3–induced apoptosis but resistant to RITA (Supplementary Fig. S3*A*; refs. 7, 17), we detected defects in HIPK2 expression or localization (Supplementary Fig. S3*B* and *C*). These defects can explain, on one side, the resistance of these cells to RITA-induced apoptosis and, on the other side, the intrinsic sensitivity to Nutlin-3 induced apoptosis through mechanisms that go beyond the simple p53 reactivation, such as the recently identified aberrant activation of E2F1 (30).

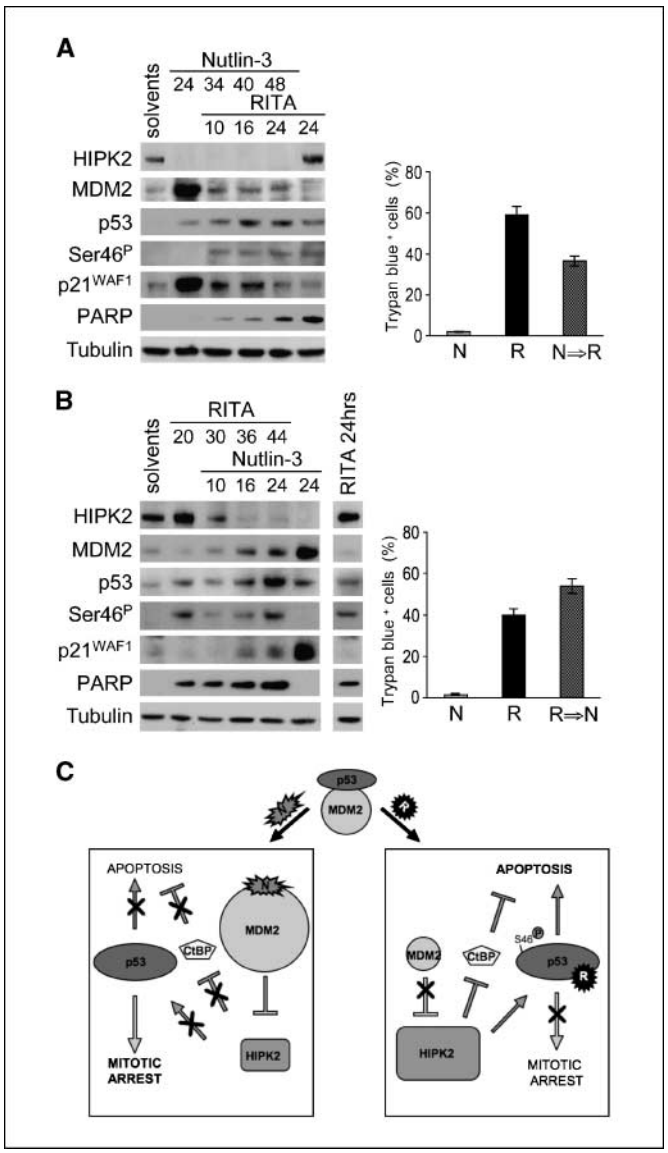

Figure 6. Different orders of administration of Nutlin-3 and RITA result in different biological outcomes. *A,* U2OS cells were treated with Nutlin-3, and after 24 h, RITA was added. At the indicated times, TCEs were analyzed by WB for the indicated proteins (*left*), whereas cell viability was measured by trypan blue exclusion 24 h after treatment (*right*). *Columns,* average of three independent experiments; *bars,* SD. *B,* U2OS cells were treated with RITA, and after 20 h, Nutlin-3 was added. At the indicated times, TCEs were analyzed by WB for the indicated proteins. All TCEs were run on the same gel; *vertical line,* noncontiguous samples. Viability of the same cells was measured 24 h after treatment; *columns,* average of three independent experiments; *bars,* SD (*right*). The increased amount of cell death measured upon RITA treatments (compare right panels in *A* and *B, central columns*) is probably due to an increase solubility of RITA when DMSO is already present in the medium. *C,* model summarizing the role of MDM2-mediated regulation of HIPK2 in response to p53 reactivation by Nutlin-3 and RITA. *Left,* binding of Nutlin-3 to MDM2 displaces it from p53, resulting in up-regulation of p53 and MDM2. Increased levels of MDM2 by degrading HIPK2, inhibits its activation of p53-dependent and p53-independent apoptotic pathways, resulting in mitotic arrest. *Right,* binding of RITA to p53 displaces it from MDM2 resulting in up-regulation of p53 and down-regulation of MDM2. Reduced levels of MDM2, by inducing HIPK2, trigger its p53-dependent and p53-independent apoptotic pathways, resulting in cell death.

By binding p53, RITA was previously shown to (*a*) block the p53-MDM2 physical interaction and promote p53 accumulation; (*b*) inhibit the interaction of p53 with other negative regulators, such as iASSP and Parc; and (*c*) activate the transcription of proapoptotic p53 target genes (8). Here, we show that RITA activates HIPK2, which triggers apoptosis by phosphorylating p53 at Ser46 and degrading CtBP. Interestingly, p53Ser46^{P} was shown to change the p53 affinity for different promoters with a shift from cell cycle arrest–related genes to apoptosis-related ones, favoring apoptosis (15, 19, 35). This modification of the transcriptional program is regulated by the prolyl-isomerase Pin1, which promotes p53 dissociation from iASPP (36, 37). Taken together, these data provide a mechanistic explanation to the original observation of RITA-induced inhibition of p53-iASSP interaction.

What remain unclear is the mechanism through which RITA transiently reduces the level of MDM2 that contribute to HIPK2 induction. We observed that RITA can repress MDM2 driven by a heterologous promoter as well as the endogenous MDM2 indicating, at least in part, a nontranscriptional type of regulation (data not shown). This effect is in apparent contrast with the mild induction of MDM2 expression recently observed by Enge and colleagues (22) that, however, analyzed only a few time points. We could reproducibly detect the transient down-regulation of MDM2 by RITA only by analyzing low-density plated cells and numerous time points. Indeed, as shown in Fig. 1*B*, different kinetics of

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MDM2 reduction were observed in the three analyzed cell lines. In addition, the temporariness of this event is consistent with the fact that, in spite of down-regulation, MDM2 is required to promote the degradation of other targets, such as p21 and hnRNPK (Fig. 1*B*; ref. 22). Of note, these last two targets, in contrast to HIPK2, are not degraded by the huge amount of MDM2 reached upon Nutlin-3 (Fig. 1*B*; ref. 22), suggesting a different target specificity of MDM2 upon RITA and Nutlin-3 treatments. Whether this different target specificity depends on the levels of MDM2, as it was shown for the monoubiquitination versus polyubiquitination of p53 (38), or whether other factors are involved, needs to be clarified. Interestingly, by a computational "blind docking" approach, it has been found that RITA should bind not only p53 but also MDM2 (39). Whether this latter binding could be experimentally proved and whether it is responsible for the different target specificity of MDM2 has to be assessed.

The complex activities and diverse indirect effects of p53-reactivating compounds point out that the direct comparison of the effect of MDM2 antagonists with MDM2 knockout mouse models might be inappropriate, as recently acknowledged by van Amerongen and Berns (40). Indeed, the strong up-regulation of MDM2 and the following degradation of HIPK2 induced by Nutlin-3 treatment, reported here, cannot be compared, for example, with the knockout of the *mdm2* gene in mice followed by inducible expression of wtp53, which produced severe tissue damage (41). In the absence of MDM2, HIPK2 should be strongly induced, which will result in significant apoptosis induction in normal tissues. Furthermore, MDM2 down-regulation by RITA is far from a complete disappearance of the protein and is also transient, which also makes the comparison with MDM2 knockout model inadequate.

Another consideration that can be derived from our observations concerns the possibility that drugs such as the inhibitors of MDM2 ubiquitin ligase activity (i.e., HLI98) might be therapeutically interesting because of their off-target activities (42). Besides the fact that HLI98 should not provoke the MDM2-dependent, ubiquitin-mediated degradation of HIPK2, the observation that it can also inhibit the activity of another RING finger E3, Siah1, recently shown to be a strong inhibitor of HIPK2 stability (43), opens the possibility of a further, direct activation of HIPK2 by inhibition of this E3. It would be interesting to evaluate whether such events contribute to the apoptotic response induced by this compound (42).

In summary, our data indicate that the biological responses induced by the MDM2 antagonists Nutlin-3 and RITA, originally thought to depend on their specific ability of releasing p53 from MDM2, rely also on the indirect effects they exert on HIPK2. This may have important implications for the improvement of drug design or the selection of most potent drug combinations in anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cinzia Rinaldo, Andrea Prodosmo, Francesca Siepi, et al.

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