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Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction
via activation of ATM and inhibition of HdmX

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ABSTRACT

Inactivation of the p53 tumour suppressor, either by mutation or by over-expression of its inhibitors Hdm2 and HdmX is the most frequent event in cancer. Reactivation of p53 by targeting Hdm2 and HdmX is therefore a promising strategy for therapy. However, Hdm2 inhibitors do not prevent inhibition of p53 by HdmX, which impedes p53-mediated apoptosis. Here we show that p53 reactivation by the small molecule RITA leads to efficient HdmX degradation in tumour cell lines of different origin and in xenograft tumours *in vivo*. Notably, HdmX degradation occurs selectively in cancer cells, but not in non-transformed cells. We identified the inhibition of the Wip1 phosphatase as the major mechanism important for full engagement of p53 activity accomplished by restoration of the ATM kinase-signaling cascade which leads to HdmX degradation. In contrast to previously reported transactivation of its own inhibitor Wip1 by p53, we observed p53-dependent repression of Wip1 expression, which disrupts the negative feedback loop conferred by Wip1. Our study reveals that the depletion of both HdmX and Wip1 potentiates cell death due to sustained activation of p53. Thus, RITA is an example of a p53-reactivating drug that not only blocks Hdm2, but also inhibits two important negative regulators of p53 - HdmX and Wip1, leading to efficient elimination of tumour cells.

INTRODUCTION

By regulating cell cycle control, DNA repair and apoptosis, p53 serves as a major gatekeeper of tumour suppression (1). Upon oncogene activation, p53 is released from its negative regulators, Hdm2 and HdmX, becomes activated and induces transcription of its target genes (2, 3). Hdm2 and HdmX are structurally alike and both inhibit the transcriptional activity of p53 by binding to it. In addition, Hdm2 possesses an intrinsic E3 ubiquitin ligase activity which targets p53 for proteasomal degradation (4). Upon certain types of DNA damage Hdm2 autoubiquitinates itself (5) as well as ubiquitinating HdmX, leading to the degradation of both proteins (2, 3, 6-8), thus allowing continuous p53 activation.

The function of p53 is impaired in the majority, if not all human cancers, either by mutation, or by functional deregulation of its inhibitors. Both Hdm2 and HdmX are overexpressed in many tumours (9, 10). Major efforts aimed at p53 reactivation have been focused on targeting the Hdm2/p53 interaction (11-13), the enzymatic activity of Hdm2 (14, 15), or restoring mutant p53 function (16). Several lines of evidence highlight the importance of developing inhibitors towards HdmX, since downregulation of HdmX by means of siRNA in cancer cell lines induces p53, suppresses colony outgrowth (17) and reduces tumour xenograft growth (18). Importantly, HdmX forms heterodimers with Hdm2, which enhances the E3 ligase activity of Hdm2; therefore HdmX depletion leads to elevated p53 levels (19). A number of studies have demonstrated that the p53-reactivating compound nutlin3a has a 30-fold lower efficiency in disrupting the p53/HdmX interaction compared to the p53/Hdm2 complex (20, 21). Furthermore,

activation of p53 after nutlin3a treatment is significantly enhanced upon downregulation of HdmX by siRNA in a number of human tumour cell lines (20, 21), which is in line with the notion that downregulation of HdmX is necessary for complete p53 activation (22, 23). There have been reports of short peptides inhibiting the interaction between HdmX and p53 (24, 25), however, the clinical application of peptides is not optimal due to their proteolytic degradation and the insufficient cellular uptake. Recently the chemical inhibitor of HdmX/p53 binding SJ-172550 was found, which displays additive effect in cancer cells when combined with an Hdm2 inhibitor (26).

Wild-type p53 induced phosphatase 1 (Wip1), encoded by the *PPM1D* gene, is a serine/threonine phosphatase that exhibits oncogenic features. It is overexpressed in a vast number of tumours (27) and is important for the survival of tumour stem cells (28). As its name indicates, Wip1 is a direct transcriptional target of p53 (29), which inhibits p53, creating a negative feedback loop. Wip1 counteracts p53 activation following DNA damage on several levels, including reversion of the activating p53 phosphorylation at Ser15 (30) and stabilization of p53's negative regulators Hdm2 and HdmX by dephosphorylating sites that trigger their proteasomal degradation (31). Importantly, the two major DNA-damage sensors ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are targeted by Wip1, blocking DNA-damage signalling (32). This interference with the DNA damage response confers resistance to standard treatments in tumours overexpressing Wip1 and therefore makes Wip1 an attractive target for anti-cancer therapies (33, 34).

Here we report that p53 activated by RITA, a chemical inhibitor of the HDM2/p53 interaction, efficiently counteracts the potent oncogenes Wip1 and HdmX by reducing their levels in cancer cells of different origin. This disables the p53/Wip1 negative feedback loop and prevents inhibition of p53 by HdmX thus facilitating robust apoptosis induction by p53.

MATERIALS AND METHODS

Cell lines and plasmids

The colon carcinoma cell-line HCT116 and its p53-null derivative, HCT116 *TP53*^{-/-}, were a gift from B. Vogelstein, John Hopkins University, USA. The Wip1 shRNA encoding plasmid as well as the Flag-Wip1 encoding construct were a gift from René H. Medema, Utrecht, The Netherlands.

In vitro assays

Western blotting was performed according to standard procedures. The proteins were detected using the following antibodies: anti-p53 DO-1 (Santa Cruz Biotechnologies, Santa Cruz/CA), anti-Hdm2 2A10 supernatant (a gift from M. Perry, NIH, USA), anti-HdmX (Bethyl laboratories), anti-Wip1 (Bethyl laboratories, Montgomery/TX) and anti- β -actin AC-15 (Sigma, Stockholm/Sweden). The p53-inhibitor, pifithrin- α (PFT- α), a gift from A. Gudkov, Roswell Park Cancer Institute, USA, was used at 10 μ M for 3h prior to addition of RITA. Protein degradation was inhibited by treatment of cells with 30 μ M of the proteasomal inhibitor MG132 (Sigma, Stockholm/Sweden). For inhibition of ATM signaling cells were pre-treated with 10 μ M KU55933 (Tocris Bioscience, Bristol/UK) for 2h. HdmX half-life after RITA treatment was detected after blocking protein synthesis using cycloheximide (chx) (Sigma, Stockholm/Sweden). Cells were pre-treated with 1 μ M RITA for 3h, incubated with 30 μ g/ml chx, and harvested 0, 2, 4 and, 6h after chx treatment. For densitometrical analysis the programme Image J was used.

Animal experiments

The Northern Stockholm Animal Ethical Committee approved all animal studies, and animal care was in accordance with Karolinska Institute guidelines. Animal experiments were performed as described in (35).

Affimetrix Microarrays

MCF-7 cells were treated with DMSO, 1 μ M RITA or 10 μ M nutlin3a for the indicated time. Raw data from the hgu219 chips were normalized using the robust multiarray average procedure, implemented in the R package affy (36). Per gene hypothesis testing was performed using Benjamini & Hochberg method implemented in R package LIMMA using linear models for microarray data (37).

Quantitative real-time PCR

Cells were treated with 1 μ M RITA, 10 μ M nutlin3a (Merck, Darmstadt/Germany) or 100 μ M 5-FU (Sigma, Stockholm/Sweden) for 8h. mRNA was isolated and reverse transcribed to cDNA according to manufacturer's instructions (5 Prime, Hamburg/Germany; Invitrogen, Stockholm/Sweden). For quantitative real-time PCR (qPCR) the following concentrations were used: 150nM primers; 10ng cDNA; 7.5 μ L 2x master mix (BioRad, Sundbyberg/Sweden); water to a total of 15 μ L; Primers used: GAPDH forward 5'-TCA-TTT-CCT-GGT-ATG-ACA-ACG-3'; reverse 5'-ATG-TGG-GCC-ATG-AGG T-3'; *PPM1D* forward 5'-ATA-CCT-GAA-CCT-GAC-TGA-C-3';

reverse 5`-CTC-CTC-CAG-TGA-CTT-GAC-3`; data were analyzed using the BioRadiQ5 system software.

Growth suppression assays

For fluorescence-activated cell sorting (FACS) analysis of sub-G1 (apoptotic) fraction, cells were seeded in 6-well plates and treated with RITA. After 48h of incubation, cells were harvested by trypsinization, fixed with 70% ethanol, treated with RNaseA (0.25mg/ml), and stained with propidium iodide (0.02mg/ml).

For long-term viability assay, 200 cells per well were seeded into six-well plates and treated with 1 μ M RITA or nutlin3a for 24h, the medium was changed and cells were allowed to grow for 7 days, unless indicated otherwise. The colonies were then stained with crystal violet.

RESULTS

RITA-activated p53 induces depletion of HdmX protein in tumour cells

Comparison of the biological effects of two inhibitors of the p53/Hdm2 interaction, nutlin3a and RITA showed that in HCT116 and MCF7 cells RITA induces apoptosis whereas nutlin3a triggers mainly growth arrest (35, 38). Several studies have demonstrated that HdmX impedes the induction of apoptosis upon inhibition of Hdm2 by nutlin3a in MCF7 cells. Therefore we tested whether nutlin3a and RITA differentially affect HdmX. Indeed, we found that HdmX is potently downregulated after RITA treatment, but not after nutlin3a treatment (Fig 1A and 5C). Kinetic analysis demonstrated that RITA treatment reduced HdmX protein levels in MCF7 and HCT116 cells in a time-dependent manner (Fig 1B). In addition, we observed oscillation of Hdm2 levels (Fig 1B and 3B), which is in line with our previous study (39) and several reports which have demonstrated that Hdm2 oscillates upon p53 activation by DNA damage due to opposing processes: transcriptional activation of its expression by p53 and enhanced proteasomal degradation (40).

Next we investigated whether the downregulation of HdmX was p53-dependent. We compared the effect of RITA in the wild-type p53 expressing HCT116 cells with that in the isogenic HCT116 p53-null derivative, HCT116 *TP53*^{-/-}. In contrast to p53-positive cells, HdmX was not affected by RITA in p53-null cells (Fig 1B). Concordantly, HdmX was not depleted by RITA treatment in two other p53-null cell lines, H1299 and Saos-2 (Fig 1C), suggesting that the effect of RITA on HdmX is p53-dependent. This conclusion was further corroborated employing the previously described p53 inhibitor pifithrin- α .

(PFT- α) (41), which abolished HdmX downregulation in HCT116 and MCF-7 cells (Fig 1D). We extended our studies using a set of wild type p53-expressing human tumour cell lines of different origin. The observed decline of HdmX levels upon RITA treatment was unambiguous in a majority of the cell lines tested (Fig 2A).

To investigate the importance of our finding in a more physiological setting, we examined the level of HdmX upon RITA treatment in human xenografts grown in SCID mice. Notably, we detected a decline of HdmX in p53-positive HCT116, but not in the isogenic p53-null HCT116 *TP53*^{-/-} tumour xenografts (Fig 1E).

Since HdmX is an important negative regulator that keeps p53 activity low in the absence of stress in untransformed cells, depletion of HdmX might lead to toxicity in normal tissues. Therefore, we tested whether the decline of HdmX upon RITA treatment occurs in untransformed cells. As we have previously reported the p53 protein level is not elevated by RITA in untransformed cells (12, 35). In line with the absence of p53 induction, we did not detect significant changes in HdmX levels in several untransformed cell lines, including normal human diploid fibroblasts (NHDF) and mammary epithelial cells, 184A1 and MCF10A (Fig 1F and 1G). Thus, downregulation of HdmX requires complete activation of p53 by RITA, which occurs only in oncogene-expressing cells (12, 35). Taken together, these results demonstrate that RITA induces p53-dependent downregulation of HdmX exclusively in tumour cells.

Downregulation of HdmX correlates with RITA-induced apoptosis

The levels of HdmX after RITA treatment were examined in a panel of human tumour cell lines of different origin, including breast, lung, and colon carcinoma, as well as lung fibrosarcoma and osteosarcoma. In the majority of the cell lines we observed a decline of HdmX (as well as Wip1, which will be discussed later) after 8h of RITA treatment, while the osteosarcoma SJSA and the colon carcinoma RKO were exceptions (Fig 2A and 2B). To exclude the possibility that the downregulation of HdmX is delayed in these cells, we investigated HdmX kinetics. As shown in Fig. 2B, the HdmX level did not decrease even after 24h of treatment.

Next we tested whether there is a correlation between the downregulation of HdmX and sensitivity to RITA. In line with previously published data, we observed that the majority of wild type p53-expressing human tumour cell lines underwent apoptosis after RITA treatment, as evidenced by the induction sub-G1 fraction (Fig 2C) (38). However, we did not detect a significant induction of apoptosis by RITA treatment in SJSA and RKO lines (Fig 2C). Thus, downregulation of HdmX correlated with the induction of apoptosis by RITA in human tumour cell lines.

The results presented above suggest that HdmX downregulation contributes to the efficient apoptosis induction by RITA. To test this further, we assessed whether depletion of HdmX by shRNA enhanced the biological effect of RITA. In line with Lineares *et al* (19), HdmX depletion led to an elevated p53 level. This results in stronger p53 induction upon RITA treatment (Fig 2D). Moreover, HdmX depletion significantly enhanced

apoptosis induction by p53 upon treatment with RITA (Fig 2E). We also observed augmented growth suppression in a long-term growth suppression assay (Fig 2F and 6C). Taken together, these results clearly show that the potent depletion of HdmX contributes to the robust apoptosis induction and thereby aids the anti-tumour effects conferred by RITA-activated p53.

The decrease of HdmX protein levels occurs through a proteasome-dependent mechanism independently of Hdm2

As HdmX is a long-lived protein (see Fig 3A), the reduction of its level observed already 4h after treatment with RITA suggests that enhanced protein degradation might be involved in HdmX depletion. To test this notion, we examined whether RITA treatment leads to decreased HdmX protein stability. As shown in Fig 3A, the half-life of HdmX was significantly shortened upon RITA treatment, indicating that RITA triggered active degradation of HdmX. Next, we studied whether inhibition of the proteasome by MG132 could stabilize HdmX, and indeed the protein levels were rescued (Fig 3B).

Hdm2 is the most prominent negative regulator of HdmX. It is an E3 ligase that, upon certain signals, forms a heterodimer with HdmX through binding via the RING domain, leading to its ubiquitination and subsequent degradation by the proteasome (2, 3, 6-8). To address whether Hdm2 is involved in the decrease of HdmX levels after RITA treatment, we depleted Hdm2 by means of siRNA. As expected, protein levels of HdmX and p53 were elevated upon the Hdm2 knockdown. RITA-induced downregulation of HdmX levels was, however, not compromised in the absence of Hdm2 (Fig 3C). Similarly,

inhibition of Hdm2 by overexpression of dominant negative Hdm2 mutants did not rescue the level of HdmX (data not shown). Thus, we conclude that RITA-induced downregulation of HdmX occurs in a proteasome- dependent manner, but is not mediated by Hdm2.

ATM is essential for HdmX downregulation by RITA

Several studies have demonstrated that DNA damage signalling induces ubiquitination and consequent degradation of HdmX (2, 3). RITA has been shown to induce p53-dependent DNA damage signalling, including activation of ATM (42). As ATM directly phosphorylates and thereby destabilizes HdmX, we tested whether ATM is involved in the HdmX downregulation. Upon ATM depletion by means of siRNA, p53 was induced by RITA to the same extent as in control cells, suggesting that DNA damage signalling is downstream of p53 induction and is not required for p53 activation by RITA (Fig 4A). However, HdmX degradation was substantially inhibited (Fig 4A). Further, pre-treatment with the selective ATM inhibitor KU55933 partially prevented the degradation of HdmX (Fig 4B), suggesting its involvement in HdmX degradation. Since the rescue was incomplete, we cannot rule out the possibility that another kinase, such as ATR, could be involved as well (43).

To confirm the involvement of ATM we investigated its phosphorylation upon RITA treatment. ATM phosphorylation was increased, as demonstrated by Western blot and immunocytochemistry (Fig 4C and 4D), supporting the notion that ATM was activated. In line with ATM activation, HdmX phosphorylation at S367 and S403 was increased (Fig 4E). To further address the importance of this phosphorylation for RITA-induced

HdmX degradation, we assessed whether mutation of serine residues can rescue HdmX from degradation induced by RITA. As shown in Fig. 4F, in contrast to wild type HdmX (Fig 3A), the half-life of non-phosphorylatable triple mutant HdmX-TM carrying S342A, S367A and S403A substitutions did not decrease after RITA treatment (Fig 4F). These data demonstrate that signalling via ATM is essential for RITA-induced depletion of HdmX.

p53-dependent downregulation of both mRNA and protein levels of Wip1

Activation of ATM in cancer cells is frequently neutralised by the p53-activated Wip1 (*PPM1D*) (30), which inhibits both ATM and p53 by removing activating phosphorylation marks (31, 32). Microarray analysis of changes in gene expression induced by RITA in MCF7 cells revealed a potent downregulation of *PPM1D* (Fig 5A). In contrast, treatment with the p53 activator nutlin3a, led to an induction of *PPM1D* expression (Fig 5A). Quantitative RT-PCR confirmed the repression of *PPM1D* expression by RITA and induction of *PPM1D* mRNA by nutlin3a (Fig 5B). In addition, treatment with the p53 activator 5-FU also resulted in *PPM1D* induction (Fig 5B). Consistent with these results, the level of Wip1 protein was induced by nutlin3a and 5FU (Fig 5C). In line with mRNA decrease upon RITA treatment, Wip1 protein was drastically downregulated by RITA (Fig 5C and 5D).

We found a strong correlation between the decrease of HdmX and Wip1 and induction of apoptosis in a panel of cancer cell lines (Fig 2A and 2C). Importantly, neither HdmX nor Wip1 were downregulated after RITA treatment in RKO and SJS cells, (Fig 5E and Fig

2A). Similarly to the downregulation of HdmX, the effect on Wip1 was p53-dependent, since it was not observed in p53-null cells (Fig 5F).

Wip1 depletion results in HdmX downregulation and aids apoptosis induction by p53

The important role of Wip1 for maintaining a high level of HdmX was underscored by substantially decreased HdmX expression upon Wip1 knockdown by means of shRNA (Fig 6A). The HdmX level was further decreased upon RITA treatment, along with Wip1, most probably due to the incomplete depletion of Wip1 by shRNA (Fig 6A).

Our results presented above (Fig 3B) showed clear proteasome dependency of RITA-induced HdmX downregulation. Thus we addressed the question, whether HdmX decrease observed upon Wip1 depletion shows the same characteristics. Using parental HCT116 cells as reference, we estimated that HCT116-Wip1 shRNA cells exhibit 65% of HdmX protein level. Blocking of the proteasome with MG132 up to 8h gradually restored HdmX level to 95% (Fig 6B). These data support the notion that Wip1 is important for maintaining the stability of the HdmX protein in cancer cells. Furthermore, our results suggest that Wip1 inhibition is important for the p53-mediated biological response in cancer cells. As shown in Fig 6C, downregulation of Wip1 by means of shRNA facilitated the growth suppression effect of nutlin3a and RITA in MCF7 cells, assessed by a long-term growth suppression assay. Since Wip1 decrease seems to be crucial for HdmX degradation, we expressed ectopic Wip1 in U2OS cells. We found that Wip1 overexpression prevents HdmX degradation after RITA treatment (Fig 6D). Further, it

partially rescued from RITA-induced cell death in U2OS cells (Fig 6E) and to a lesser extent also in HCT116 and MCF7 cells (data not shown).

Taken together, our data show that Wip1 is efficiently downregulated in a p53-dependent manner after RITA treatment. Depletion of Wip1 plays a crucial role in the decrease of HdmX protein levels and contributes to the efficient growth suppression by pharmacologically activated p53.

DISCUSSION

Diverse ranges of alterations, which inactivate the p53 tumour suppressor function in cancers with non-mutated *TP53* gene, converge on two p53 inhibitors, Hdm2 and HdmX. Since HdmX and Hdm2 regulate p53 independently of each other and act synergistically, the inhibition of both Hdm2 and HdmX is required for full p53 activation (22). This makes both proteins important targets for cancer therapy. Several small molecules inhibiting Hdm2 have been identified, however, these do not affect HdmX, impeding their efficiency, in particular in tumours with overexpressed HdmX (20). An inhibitor of HdmX/p53 binding SJ-172550 has been described recently (26); it does not kill cancer cells on its own, but has an additive effect when combined with Hdm2 inhibitors. However, simultaneous targeting of Hdm2 and HdmX by a single molecule has not yet been reported.

In the present study we found that in addition to preventing the p53/Hdm2 interaction, the small molecule RITA (12) induces a potent destabilization of HdmX protein in cancer cells *in vitro* and *in vivo*. Downregulation of HdmX was p53-dependent, since it did not occur in p53-null cells.

Our results suggest that active ATM is required for downregulation of HdmX by RITA. Several studies have demonstrated that DNA damage signalling regulates HdmX and Hdm2 protein stability (3, 44). The checkpoint kinase ATM and its downstream kinase Chk2 induce phosphorylation of Hdm2 (5, 45) and HdmX (2, 3), leading to degradation of Hdm2 and HdmX and activation of p53. Our data showing partial rescue of HdmX

level upon RITA treatment by both ATM knockdown and the ATM inhibitor KU55933 supports the involvement of ATM in HdmX downregulation. This notion was further strengthened by our finding that an HdmX mutant that cannot be phosphorylated by ATM was rescued from RITA-induced degradation.

p53 induces DNA damage signalling upon activation by RITA (42). We have previously shown the induction of p53 phosphorylation at Ser15 upon RITA treatment (46). Notably, it appears that the induction of p53 level upon RITA treatment is not dependent on DNA damage signalling, since ATM inhibition by siRNA and KU55933 did not prevent p53 accumulation. These data suggest that the induction of DNA damage signalling occurs downstream of p53.

Further elucidation of the factors involved in DNA damage signalling upon RITA treatment allowed us to identify the involvement of the Wip1 phosphatase in HdmX depletion. It has been reported that Wip1 can directly reverse the ATM-mediated phosphorylation of HdmX, which leads to its stabilization (31). We observed a substantial downregulation of Wip1 on protein and mRNA levels upon p53 activation by RITA. Further, our results showed that inhibition of Wip1 plays a causal role in the decrease of HdmX, suggesting that the low level of Wip1 helps to unleash HdmX degradation following p53 activation by RITA.

Based on our results we suggest a model (Fig 6D) in which inhibition of Wip1 by pharmacologically activated p53 creates a positive feedback loop uncoupling p53 from its

negative regulators Hdm2 and HdmX. RITA-activated p53 represses the expression its own inhibitor Wip1, which confers robust activation of p53 by acting on several levels. First, it restores active ATM, which phosphorylates p53, thereby activating it. Second, ATM-mediated phosphorylation of HdmX induces its degradation, negating HdmX-mediated inhibition of p53. Further, in the absence of Wip1 the activating phosphorylation of p53 and inhibitory phosphorylation of HdmX by ATM cannot be removed, which amplifies the signal, triggering full-scale p53 activation.

Normal cell homeostasis requires keeping p53 activity at bay in the absence of stress. This is achieved by a combination of two negative feedback loops, including the one between p53 and Hdm2/HdmX, and the negative feedback between p53, ATM and Chk2 mediated by the phosphatase Wip1. Both these feedback loops are corrupted in cancers, as evidenced by overexpression and amplification of Hdm2, HdmX and Wip1 in numerous cancers that do not carry *TP53* mutations. We demonstrate that pharmacological reactivation of p53 by RITA is able to counteract this imbalance and restore an appropriate p53 response, ultimately leading to apoptosis in tumour cells.

As inhibition of Wip1, Hdm2 and HdmX by a single small molecule is unprecedented, RITA might open a novel therapeutic approach for tumours that are dependent on the overexpression of these factors. In line with this notion, high HdmX levels have been shown to correlate with RITA sensitivity in a panel of breast cancer lines (47). Simultaneous inhibition of HdmX, Hdm2 and Wip1 might be an essential component for anti-cancer therapies targeting p53. This capability of p53 might allow us to manage with the daunting challenge of anti-cancer therapy - diverse genetic abnormalities in individual

cancers. It is worth noting that in accordance with the absence of p53 activation we did not observe inhibition of HdmX in non-transformed cells by RITA, suggesting that the possibility of toxic effects of RITA in normal cells due to HdmX inhibition is low.

Our finding that Wip1, which has been shown to be transcriptionally activated by p53, was repressed upon RITA treatment in a p53-dependent manner is very intriguing. It implies that the p53-mediated negative feedback loops insuring survival of cells following stress signalling could be reversed under certain conditions. We have previously shown that RITA-induced p53 activation can block the pause-and-repair survival pathway mediated by induction of p21 (38). In this study we demonstrated that the p53/Wip1 negative feedback loop that negates p53 activation could be converted to a positive feedback loop that potentiates p53 activity. This opens a possibility to irreversibly shift the survival-death balance and to produce a robust apoptotic outcome, leading to elimination of tumour cells.

Notably, treatment with the p53 activator nutlin3a did not lead to downregulation of HdmX or Wip1 levels in HCT116 and MCF7 cells. In order to address the mechanism underlying the differential regulation of Wip1 by RITA and nutlin3a, i.e., repression versus induction, respectively, we analyzed the contribution of several p53 cofactors that are differentially regulated by RITA and nutlin3a, including hnRNP K (38) and HIPK2 (39). However, our data did not support the involvement of these factors (data not shown). An interesting example of a transcription factor, which can both activate and repress the same genes upon binding to their promoters is MyoD. Depending on its

interaction with HDAC1 or LSD1 at the promoter of its target genes, MyoD can repress or activate genes, respectively (48, 49).

A similar mechanism could be engaged in the switch between p53-mediated activation and repression of its target gene Wip1. Since there are more than fifty known p53 partner proteins, which can modulate p53-mediated regulation of gene expression, high throughput proteomic approach is required to identify such a cofactor(s).

We showed that the downregulation of HdmX and Wip1 contribute to the induction of apoptosis by p53. As has been reported previously, nutlin3a induces growth arrest in cell lines with high HdmX expression (38, 50). Upon downregulation of HdmX by shRNA or by combinational treatment with DNA damaging drugs that induce HdmX degradation, nutlin3a induces apoptosis (20, 21, 50, 51), suggesting that neutralization of the inhibitory effect of HdmX is crucial for the induction of apoptosis by p53. This notion is corroborated by our results showing that shRNA-mediated depletion of HdmX induced p53 level and enhanced apoptosis upon RITA treatment. Further, depletion of Wip1 facilitated growth suppression by nutlin3a, supporting the notion that simultaneous targeting of both Wip1 (this study) and HdmX (20, 21, 50, 51) is required for a full-scale induction of apoptosis upon inhibition of Hdm2.

Two cell lines in which RITA treatment did not induce apoptosis, namely, the colon carcinoma cell line RKO and the osteosarcoma cell line SJSA, showed neither Wip1 and HdmX downregulation, nor Hdm2 oscillation. Interestingly, the activity of the p53 kinase

HIPK2, which is important for the pro-apoptotic function of p53, was found to be defective in SJSA and RKO cells (39). However, depletion of HIPK2 by shRNA in MCF7 cells did not prevent the effect of RITA on these factors (data not shown). It is plausible that certain undefined defects in the p53 pathway may account for the difference in response to RITA in these cell lines. Further studies will help to elucidate these issues.

In conclusion, our study provide several lines of evidence showing that in addition to blocking the inhibitory effects of Hdm2 on p53, activation of p53 by RITA simultaneously abrogates the two oncogenes, Wip1 and HdmX, creating a positive feedback loop which potentiates apoptosis induction by p53. These findings open an approach for the development of anticancer drug(s) with a broader spectrum than drugs that merely target Hdm2.

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Figure 1

HdmX protein levels are downregulated in a p53-dependent manner upon RITA treatment as assessed by Western blotting. (A) HdmX protein levels were decreased in HCT116 and MCF7 cells 8 hours after treatment with 1 μ M RITA, but unchanged upon 10 μ M nutlin3a or in control DMSO-treated cells. (B) The p53-positive cell lines HCT116 and MCF7 showed a continuous decline in HdmX protein levels over time, while HdmX was unaffected in the p53-null cells HCT116 *TP53*^{-/-} after treatment with 1 μ M RITA for the indicated time spans (C) Accordingly, the p53-null cell lines H1299 and Saos-2 did not respond to RITA by HdmX downregulation. (D) Pretreatment with the p53 inhibitor pifithrin- α for 3h prevented the decline of HdmX protein in HCT116 and MCF-7 cells. (E) HCT116, HCT116 *TP53*^{-/-} cells were injected into mice and the arising tumours were treated intra-tumourally with RITA (1mg/kg). In line with cell culture results, HdmX protein was downregulated in the p53-positive, but not in the p53-null cells. (F) HdmX protein was not downregulated in the untransformed cell lines NHDF, MCF-10A and 184A1 upon 8h of 1 μ M RITA treatment. (G) Also in a longer time-course HdmX protein levels were unchanged upon 1 μ M RITA treatment in the untransformed NHDF cells.

Figure 2

Downregulation of HdmX contributes to RITA-induced apoptosis (A) A panel of different cell lines was treated with RITA for 8h, and HdmX as well as Wip1 was downregulated in all of them except RKO and SJSA, as detected by immunoblotting. (B) Assessment of the kinetics of HDMX levels by Western blotting in RKO and SJSA cells did not detect downregulation of HdmX protein after treatment with 1 μ M RITA. (C)

Induction of apoptosis in different cell lines by 48h of RITA treatment correlated with HdmX downregulation. The cells were stained with PI and the sub-G1 fraction representing apoptotic cells with fragmented DNA was quantified by FACS analysis (mean \pm SEM; n=3). RKO and SJSA were the only cell lines that did not show a substantial increase of the sub-G1 fraction. (D) Depletion of HdmX by shRNA results in lower levels of HdmX upon RITA treatment. HCT116 and HCT116 shHdmX cells were treated with DMSO or RITA for the indicated time points and detected by immunoblotting. (E) Knockdown of HdmX by means of siRNA increased the efficiency of RITA to induce sub-G1 cells as assessed by FACS as in (C) in HCT116, HCT116 *TP53*^{-/-} and HCT116 siHdmX. (F) HdmX depletion facilitates the killing of tumor cells by RITA, as detected using long-term viability assay. Cells were seeded at low density and exposed to 1 μ M RITA for 24h, the medium was changed and cells were grown for 7 days. The colonies were then stained with crystal violet.

Figure 3

The reduction of HdmX levels is mediated by the proteasome and is Hdm2-independent. Protein levels were assessed by Western blotting. (A) HdmX protein half-life is drastically decreased upon RITA treatment in MCF-7 cells. Cells were treated with DMSO or RITA, followed by a cycloheximide chase. (B) HdmX degradation is proteasome-dependent, since it is rescued by pretreatment with the proteasome inhibitor MG132 for 3h before RITA treatment of HCT116 and MCF7 cells. (C) HdmX degradation induced by RITA was not rescued by an siRNA mediated knockdown of Hdm2 in HCT116 cells, as assessed by Western blotting.

Figure 4

The downregulation of HdmX is ATM-dependent. Protein levels were assessed by immunoblotting (A) HdmX degradation was prevented by siRNA-mediated ATM depletion prior to RITA treatment of HCT116 and MCF-7 cells. (B) The ATM inhibitor KU55933 partially prevented HdmX downregulation by RITA in HCT116 cells. (C) RITA treatment induces phosphorylation of ATM and its downstream target KAP-1 in HCT and MCF7 cells. (D) RITA induced ATM phosphorylation was confirmed in HCT *TP53*^{+/+} cells by immunofluorescence. (E) HA-HdmX or the non-phosphorylatable mutant HA-HdmX-TM was expressed in MCF7 cells. After RITA treatment for 8h, an immunoprecipitation was performed using anti-HA or nonspecific control antibodies. RITA induces phosphorylation of HdmX at S367 and S403 of the wild-type protein while no phosphorylation was detected at the mutant. (F) HdmX-TM which cannot be phosphorylated by ATM is protected from degradation induced by RITA treatment. HdmX levels were assessed in MCF-7 cells stably expressing a non-phosphorylatable triple mutant of HdmX pretreated with RITA for 3h, followed by cycloheximide treatment for the indicated time spans.

Figure 5

Wip-1 is downregulated by RITA-reactivated p53 on mRNA and protein level. (A) Microarray analysis of MCF7 cells revealed the downregulation of *PPM1D* mRNA upon RITA treatment and upregulation upon nutlin3a treatment. MCF-7 cells were treated with 1 μ M RITA or 10 μ M nutlin3a, or DMSO as a control, for the indicated times, mRNA was

collected and resulting cDNA hybridized to Affimetrix microarrays. The microarray data were analysed using the computer language R. (B) Downregulation of *PPM1D* mRNA upon RITA and upregulation upon nutlin3a and 5-FU was detected by qPCR. HCT116 and MCF-7 cells were treated with RITA, nutlin3a or 5-FU for 8h and mRNA levels of *PPM1D* (Wip1) were assessed by qPCR (mean \pm SEM; n=3). (C) The protein level of Wip1 (*PPM1D*) was downregulated in MCF-7 cells treated with RITA, but upregulated by nutlin3a or 5-FU, as assessed by Western blot after 8h of treatment. (D) Wip1 was not downregulated in the two cell lines RKO and SJSA, as shown by Western blotting. The cells were treated with RITA or DMSO as a control for the indicated time spans. (E) The downregulation of Wip1 is p53-dependent. HCT116 cells and their isogenic p53-null derivate HCT116*TP53*^{-/-} were treated with DMSO or RITA for 8h. Wip1 level was decreased only in the p53-positive cells.

Figure 6

Wip1 depletion contributes to HdmX downregulation and p53-induced apoptosis. (A) Wip1 depletion results in low HdmX level, which was further decreased upon treatment with RITA, as assessed by Western blotting. Wip1 was stably knocked down by lentiviral-expressed Wip1 shRNA. shRNA against GFP was used as a control. (B) Wip1 depletion leads to the destabilization of HdmX, as evidenced by the restoration of low HdmX levels in *PPM1D* knockdown cells by exposure to the proteasome inhibitor MG132. Parental HCT116 cells were used as a reference. (C) Wip1 depletion sensitizes MCF7 cells to nutlin3a and as well as to RITA as tested by a growth suppression assay. Cells were seeded at low density, treated with 10 μ M nutlin3a, 1 μ M RITA or DMSO for

48h and stained with crystal violet. Also HdmX depletion sensitizes to RITA in MCF7 cells. (D, E) Overexpression of FLAG-Wip1 prevented downregulation of HdmX upon RITA treatment and partially rescued from RITA-induced apoptosis in U2OS cells. 48h after transfection with Flag-Wip-1 encoding or empty vector cells were treated with RITA for 8h to detect protein levels (D) or for 48h to assess cell survival (E). (F) Model of p53-dependent downregulation of HdmX. Left panel, p53 activation is blunted by a combination of two negative feedback loops, including the negative feedback between p53 and Hdm2/HdmX, and the negative feedback between p53 and ATM mediated by the phosphatase Wip1. Upon RITA treatment, repression of Wip1 disrupts both negative feedback loops, ultimately leading to ATM activation and HdmX downregulation and thus activating p53 by three different ways, namely (1) reversing the repression by Wip1, (2) abolishing the inhibition by HdmX and (3) inducing activation by ATM.

Figure 1

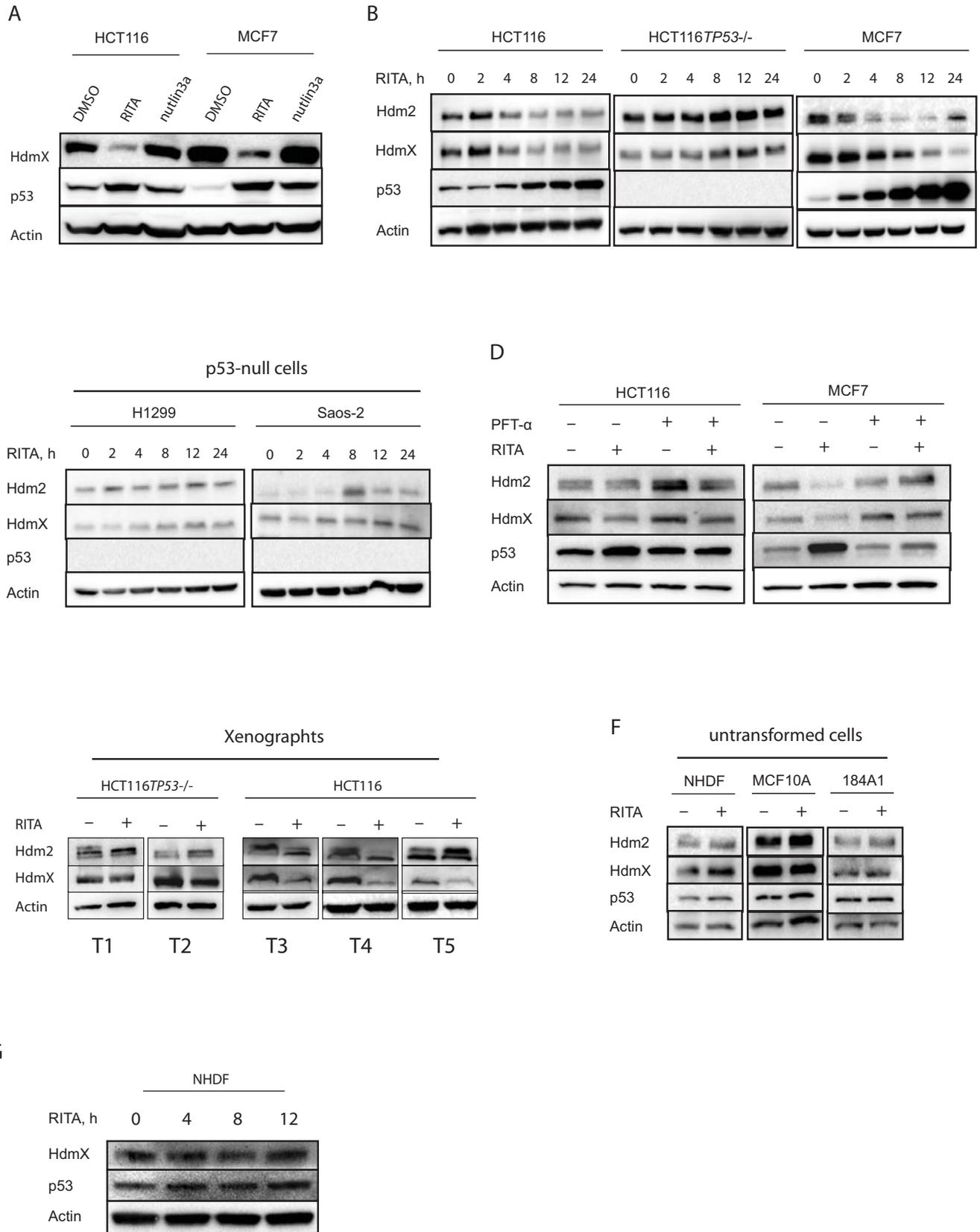


Figure 2

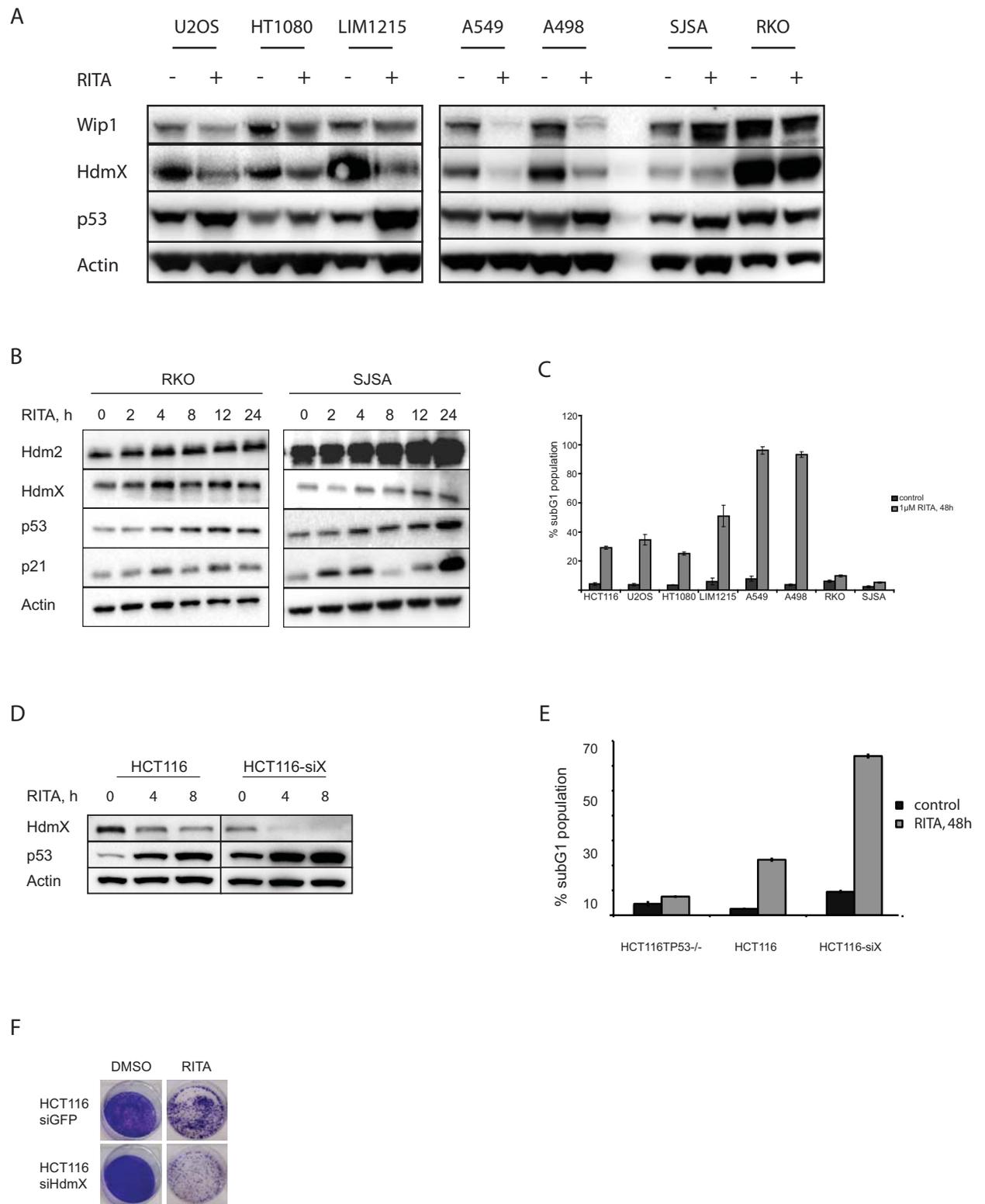
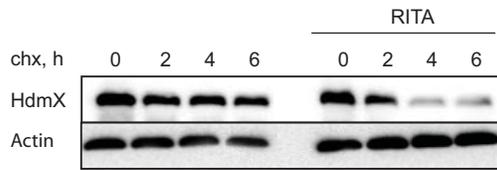
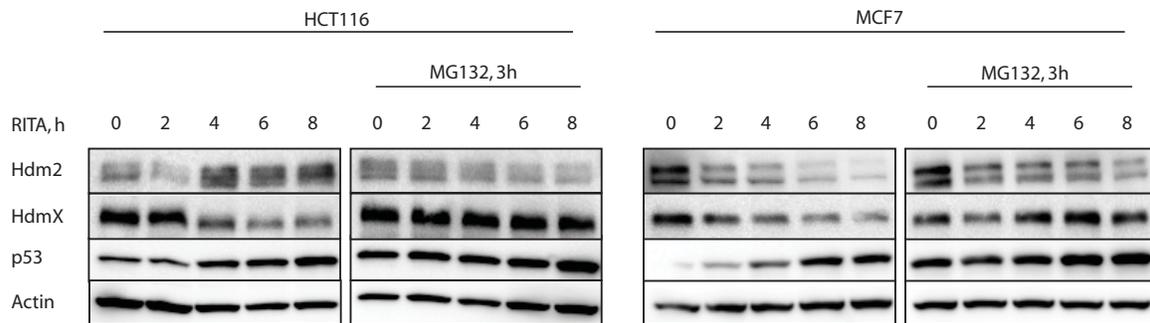


Figure 3

A



B



C

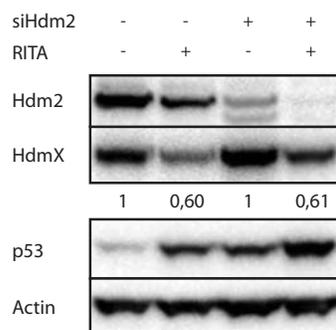


Figure 4

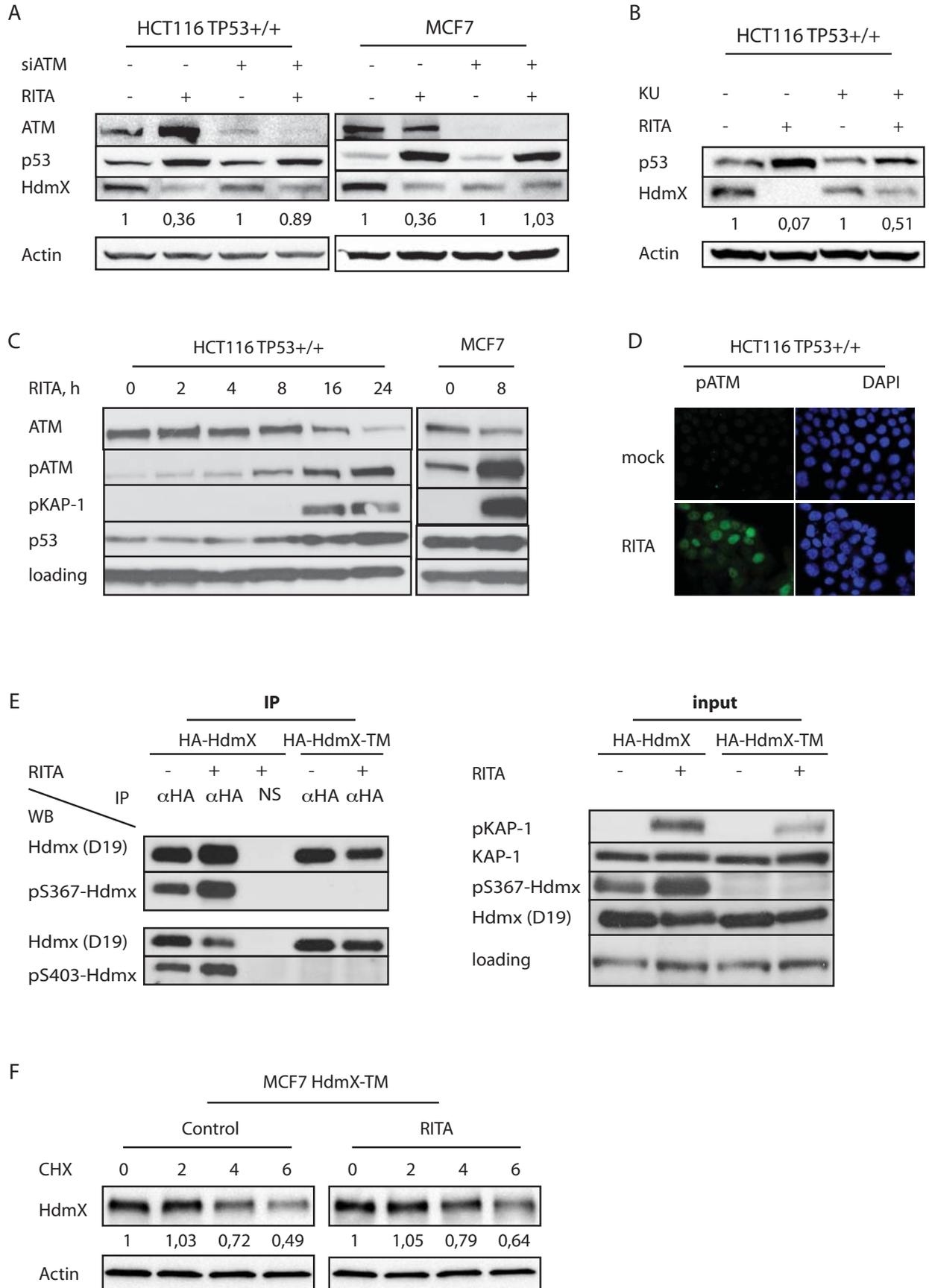
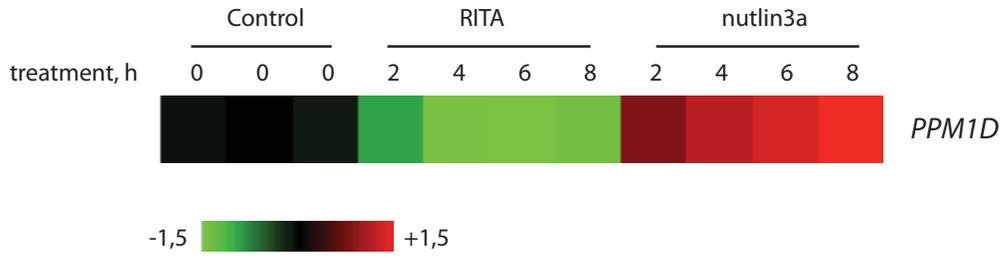
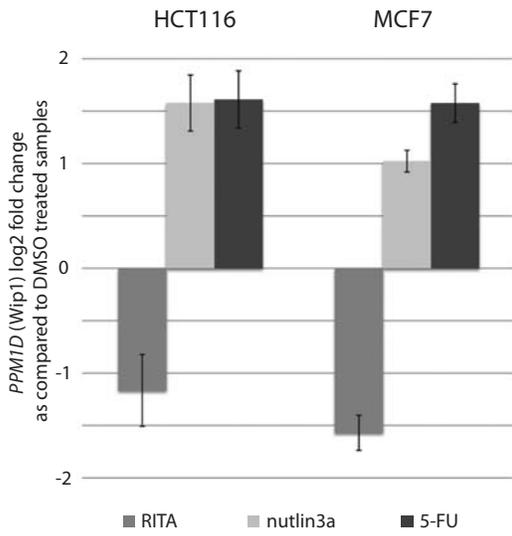


Figure 5

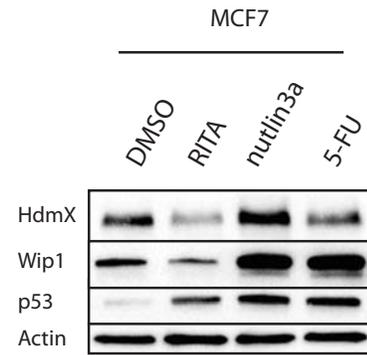
A



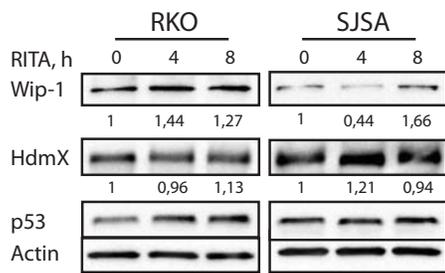
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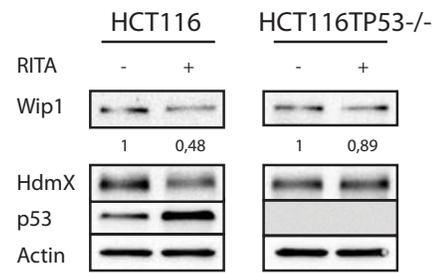


Figure 6

