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p53 mediates the negative regulation of MDM2 by orphan receptor TR3

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MDM2 is an oncoprotein whose transforming potential is activated by overexpression. The expression level of MDM2 is negatively regulated by orphan receptor TR3 that mainly acts as a transcriptional factor to regulate gene expression. However, the underlying mechanism is largely unclear. Here, we present the first evidence that inhibition of TR3 on MDM2 is mediated by p53. We found that TR3 directly interacts with p53 but not MDM2, and such interaction is critical for TR3 to inhibit MDM2 expression. TR3 downregulates p53 transcriptional activity by blocking its acetylation, leading to a decrease on the transcription level of MDM2. Furthermore, TR3 binding to p53 obstructs its ubiquitination and degradation induced by MDM2, resulting in the MDM2 ubiquitination and degradation. In addition, TR3 could enhance p53-mediated apoptosis induced by UV irradiation. Taken together, our findings demonstrate that p53 mediates the suppression of TR3 on MDM2 at both transcriptional and post-transcriptional level and suggest TR3 as a potential target to develop new anticancer agents that restrict MDM2-induced tumor progression.

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Introduction

The tumor suppressor protein p53 is a well-characterized transcription factor which directly activates numerous genes that are involved in many cellular functions (Vogelstein *et al*, 2000). Its function is crucial for the prevention of tumor development. For example, p53 can initiate cell cycle arrest and trigger cell death by apoptosis in response to anticancer therapy (Vousden, 2000). It has been found that p53 is mutated in about 55% of human tumors. These mutations are mainly single amino-acid substitutions in the DNA-binding domain of p53(Hollstein *et al*, 1994).

In normal cells, p53 is maintained at very low levels due to rapid degradation mediated by MDM2 (mouse double minute) protein. MDM2 is an oncoprotein whose transforming potential is activated by overexpression (Fakharzadeh *et al*, 1991). It binds to p53 and functions as an ubiquitin E3 ligase to promote p53 ubiquitination and degradation by the proteasomes, thus restraining the oncosuppressor functions of p53(Momand *et al*, 1992; Haupt *et al*, 1997; Kubbutat *et al*, 1997; Roth *et al*, 1998). Therefore, MDM2 is a negative regulator of p53, which contributes to the dysfunctions of p53. The negative regulation of MDM2 on p53 can be neutralized by partner proteins and specific protein modifications. In response to stress or DNA damage, p53 is stabilized through phosphorylation on itself by other kinases or expression of the MDM2 inhibitor ARF (Prives and Hall, 1999). HIPK2 contributes to drug-induced modulation of MDM2 activity at transcriptional (through p53Ser46 phosphorylation) and post-transcriptional (through p53-independent subcellular relocalization and proteasomal degradation) levels (Peng *et al*, 2001). Transcription regulator YY1 interacts with MDM2, stimulating MDM2–p53 complex formation and p53 ubiquitination (Sui *et al*, 2004). YY1 also directly binds to p53 transactivation domain and blocks p53–p300 binding (Gronroos *et al*, 2004).

TR3 (also known as NGFI-B or Nur77), an orphan receptor (Chang and Kokontis, 1988; Hazel *et al*, 1988), belongs to the steroid/thyroid/retinoid receptor superfamily and functions as a transcription factor that negatively or positively regulates gene expression (Kastner *et al*, 1995; Mangelsdorf and Evans, 1995). TR3 is a unique nuclear protein required not only for cell proliferation but also for apoptosis. Our previous studies revealed that TR3 can heterodimerize with retinoid X receptor (RXR) or another orphan receptor COUP-TF to regulate their transcriptional activity, which subsequently contributes to regulation of cell growth and apoptosis in response to retinoids and hormones (Wu *et al*, 1997a, b). Recently, Zhang's group found that the mitogenic effect of TR3 was associated with its presence in the nucleus, whereas its apoptotic effect was correlated with its mitochondrial localization in the same lung cancer (Kolluri *et al*, 2003). We also demonstrated that treatment of phorbol ester 12-*O*-tetradecanoyl-13-phorbol acetate (TPA) induced translocation of TR3 from the nucleus to the mitochondria where it triggered cytochrome *c* release and initiated apoptosis in gastric cancer cells (Wu *et al*, 2002; Lin *et al*, 2004). Upon stimulation of apoptosis by a variety of apoptosis-inducing agents, translocation of nuclear TR3 to the mitochondria also occurred in prostate cancer (Li *et al*, 2000), colon cancer (Wilson *et al*, 2003), lung cancer (Kolluri *et al*, 2003), and ovarian (Holmes *et al*, 2003) cells. Therefore, TR3 is indeed involved in both cellular proliferation and apoptosis, which are considered to be resulted from a disturbance in the balance between these two processes.

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The fact that TR3 functions in the stabilization of hypoxia-inducible factor-1α through decrease of MDM2 expression (Yoo *et al*, 2004) suggests that MDM2 may be a major target of TR3 function. The mechanism by which TR3 downregulates the expression of MDM2 has not been reported. Direct transcriptional regulation on the promoter of MDM2 by TR3 is unlikely, because DNA binding of TR3 is not required for this process (Yoo *et al*, 2004). Therefore, there might be some additional factors involved in the TR3–MDM2 cross-talk.

In this report, we demonstrated for the first time that p53 physically interacts with TR3 and the interaction is crucial for TR3 to inhibit MDM2. TR3 binding suppresses the transcriptional activity of p53 by blocking its acetylation, leading to the repression of MDM2 transcription. TR3 also enhanced p53-mediated apoptosis induced by irradiation of UV. In addition, TR3 binding prevents p53 from MDM2-induced degradation through the obstruction of p53 ubiquitination, which subsequently results in MDM2 ubiquitination and degradation. Taken together, our study has revealed a unique TR3–p53–MDM2 pathway in which TR3 and p53 cooperatively act to regulate MDM2 functions.

Results

p53 is required for the inhibition of MDM2 by TR3

Previous studies have shown that the expression of MDM2 is negatively regulated by TR3 (Yoo *et al*, 2004), although little is known about the underlying mechanism. We also observed that TR3 could downregulate the endogenous level of MDM2 in a dose-dependent manner in HEK293 cells (Figure 1A). However, we could not detect any physical interactions between TR3 and MDM2 (data not shown), indicating that TR3 does not directly act on MDM2.

As p53 is a well-known transcriptional activator of the MDM2 gene, we tested whether p53 could mediate the inhibition of MDM2 by TR3. Firstly, TR3 was transfected into the p53-null cells H1299. Interestingly, TR3 failed to alter the expression level of MDM2 in these cells (Figure 1B, left panel). However, when the wild-type p53 was cointroduced, TR3 restored its ability to downregulate MDM2 expression (Figure 1B, left panel). Next, we introduced TR3 into 293 cells that had been transfected with a dominant-negative p53 (p53-R175H) (Rui *et al*, 2004). Again, TR3 failed to inhibit MDM2 expression in these cells, although it could effectively reduce the level of MDM2 in 293 cells without p53-R175H (Figure 1B, right panel). We further investigated the effect of endogenous TR3 on the cellular levels of MDM2 in hepatoma cell line HepG2 that harbors wild-type p53 (Bressac *et al*, 1990). The siRNA-p53 was employed to these cells, and followed by VP-16 treatment. As shown in Figure 1C, VP-16 was able to induce the endogenous TR3 and inhibit MDM2 expression. However, the p53 siRNA drastically attenuated the inhibition of TR3 on MDM2 expression. Finally, we determined the physiological relevance of p53 in mediating TR3 inhibition on MDM2 expression in the case of UV irradiation. Indeed, UV stimulation could activate p53 in HepG2 cells, resulting in upregulation of MDM2 expression (Figure 1D), which is consistent with MDM2–p53 feedback loop (Lee *et al*, 2005). Transfection of TR3 did not impaired p53 level, but led to enhancement of inhibition on MDM2

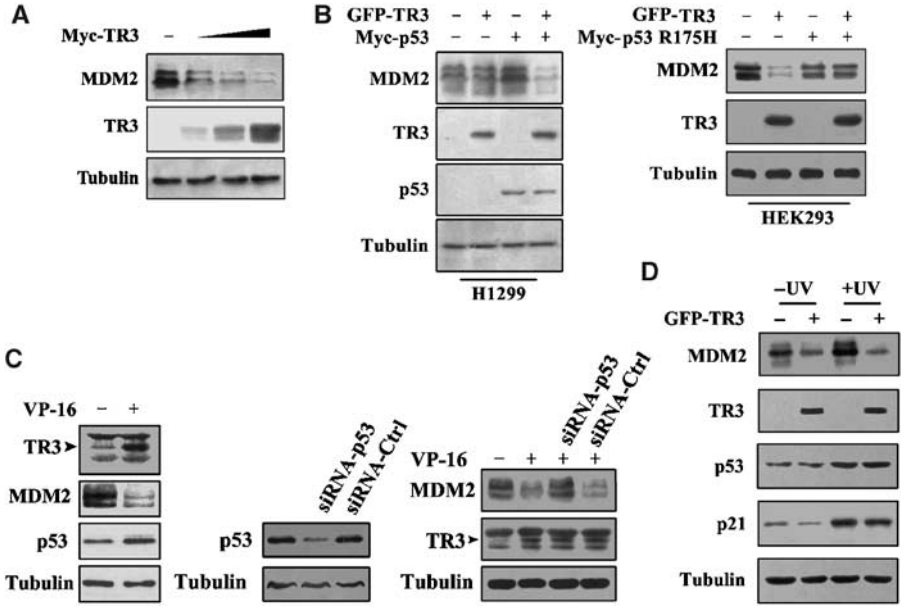

Figure 1 TR3 inhibits cellular MDM2 expression mediated by p53. (**A**) TR3 inhibited MDM2 expression in a dose-dependent manner. Increasing amounts of Myc-TR3 were transfected into 293 cells. After transfection, cell lysates were prepared and analyzed by Western blotting using anti-MDM2 antibody to indicate endogenous MDM2 level. (**B**) Inhibition of MDM2 by TR3 required p53. Flag-TR3, Myc-p53, or Myc-p53R175H (a dominant-negative form of p53) expression vectors were transfected into H1299 or 293 cells as indicated, and then monitored for their expression by Western blotting using anti-Flag or anti-Myc antibody. Anti-MDM2 antibody was used to indicate endogenous MDM2 level. (**C**) Effect of VP-16 on endogenous TR3, p53, and MDM2 expression. HepG2 cells were transfected with siRNA-p53 or its control siRNA-Ctrl. Cells were treated with VP-16 for 6 h, and lysates were then subjected to Western blot analysis using anti-TR3, -MDM2, or -p53 antibody. (**D**) Effect of UV on the expressions of TR3, p53, MDM2, and p21waf1/cip1. HepG2 cells were transfected with GFP-TR3 expression vector. After transfection, cells were irradiated with UV (50 J/m^2), and then harvested 8 h later. Cell lysates were prepared and analyzed for expressions of p53, MDM2, p21waf1/cip1, and TR3 by Western blot using their corresponding antibodies (for p53, MDM2, and p21waf1/cip1) or GFP antibody (for TR3). In each panel, Tubulin expression served as control for indicating the similar loading proteins in each lane.

(Figure 1D), the inhibition rate of MDM2 by TR3 was higher in UV irradiation group (7.2-fold) than that in non-UV irradiation group (2.3-fold), as quantified by densitometry. Taken together, these results for the first time suggest that p53 plays a critical role in mediating the inhibitory effect of TR3 on endogenous MDM2 expression.

TR3 physically interacts with p53

Given the results above, it is possible that TR3 may interact with p53. To test this possibility, 293 cells were transfected with p53 accompanied by TR3 or MDM2, and then subjected to co-immunoprecipitation (Co-IP) experiment. We found that p53 bound to TR3 as effectively as it did with MDM2 (Figure 2A). To further test the interaction between endogenous TR3 and p53, the total cell lysate of HepG2 cells was immunoprecipitated with TR3 antibody, and p53 antibody was used in Western blotting to examine the existence of p53 in the TR3 immunoprecipitates. As shown in Figure 2B, TR3 did interact with p53, and VP-16 enhanced the TR3–p53 interaction. Similar TR3–p53 interaction could also be demonstrated in GST pull-down assays, in which the wild-type p53 was found to bind to the bacterially expressed GST-TR3 fusion protein but not to GST alone (Figure 2C, left panel). In contrast, TR3 did not bind to bacterially expressed GST-MDM2, although it interacted, as expected, with bacterially expressed GST-p53 (Figure 2C, right panel), which is consistent with our Co-IP result that TR3 did not interact with MDM2. The TR3–p53 interaction could also be reproduced by the yeast two-hybrid assay. Cotransformation of TR3 with p53 into yeast induced a strong β-galactosidase (β-gal) activity as compared to transformation of TR3 or p53 alone (Figure 2D). Together, we demonstrated that it is p53 but not MDM2 that directly interacts with TR3.

Interaction with p53 is crucial for TR3 to inhibit MDM2

Next, we continued to determine the region of TR3 that interacts with p53. Different truncation mutants of TR3 were constructed as indicated in Figure 3A and coexpressed with p53 in 293 cells. Co-IP assays revealed that TR3/ND1, TR3/ND2, TR3/ND3, and TR3/CD1, but not TR3/MD1, retained the ability to interact with p53 (Figure 3A), indicating that the region of amino acid 341–521 is critical for TR3 to interact with p53.

We then found that cellular levels of MDM2 in 293 cells were reduced when the cells were transfected with TR3 truncation mutants that interacted with p53 (i.e. TR3/ND1, TR3/ND2, TR3/ND3, and TR3/CD1) (Figure 3B). In contrast, we did not observe any reduction of MDM2 expression in cells transfected with TR3/MD1, the only truncation mutant that did not interact with p53 (Figure 3B). Clearly, these results demonstrate that interaction with p53 is a prerequisite for TR3 to inhibit MDM2 expression.

To further determine the mutual interaction between p53 and TR3, different constructs of p53 as indicated (Figure 3C) were cotransfected with TR3 into 293 cells. Co-IP assays showed that the N-terminal region of amino acids (aa) 1–290 (p53/N2), but not that of aa 1–113 (p53/N1), interacted with TR3 (Figure 3C), indicating that the region spanning aa 113–290 of p53, which is the DNA-binding domain, bound to TR3. Surprisingly, when the DNA-binding domain

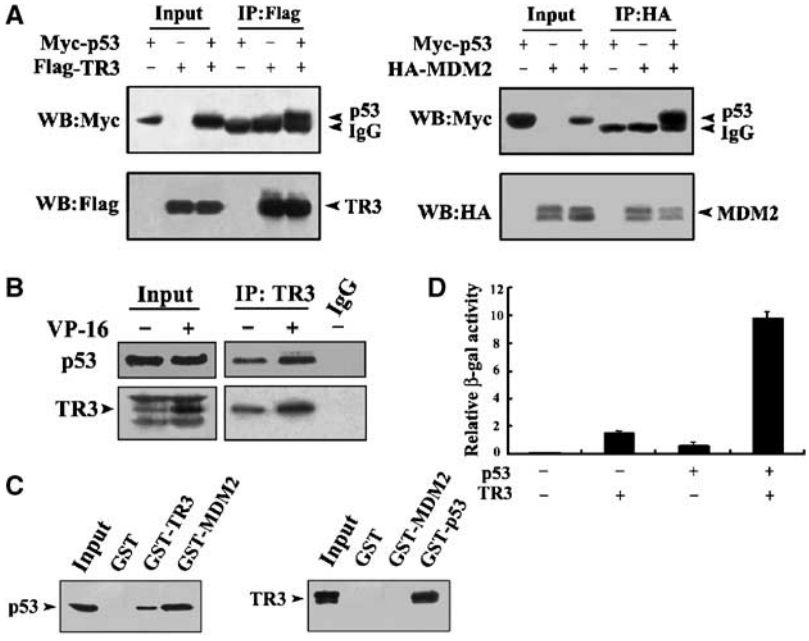

Figure 2 TR3 interacts with p53. (**A**) *In vitro* Co-IP experiment in 293 cells. Flag-TR3, Myc-p53, or HA-MDM2 were transfected into 293 cells as indicated and cell lysates were then immunoprecipitated using anti-Flag or -HA antibody. The immunoprecipitates were examined by Western blotting using anti-Myc antibody. Input represented 10% of cell lysates used in the Co-IP experiment. (**B**) *In vivo* Co-IP experiment in HepG2 cells. Cell lysates from cells treated with or without VP-16 for 6 h were incubated with anti-TR3 antibody. For Western blotting of immunoprecipitates, anti-p53 antibody was used. (**C**) GST pull-down assay for determination of TR3–p53 interaction. GST-TR3, GST-p53, GST-MDM2, or GST control protein was incubated with related protein as described in Materials and methods. Bound proteins were analyzed by Western blotting. (**D**) TR3 interacted with p53 in yeast. TR3 or p53 cDNA was cloned into the yeast expression vector as described in Materials and methods. The resulting expression vectors were introduced into yeast cells. β-Gal activity was assayed. The result represented the average ± mean from three independent experiments.

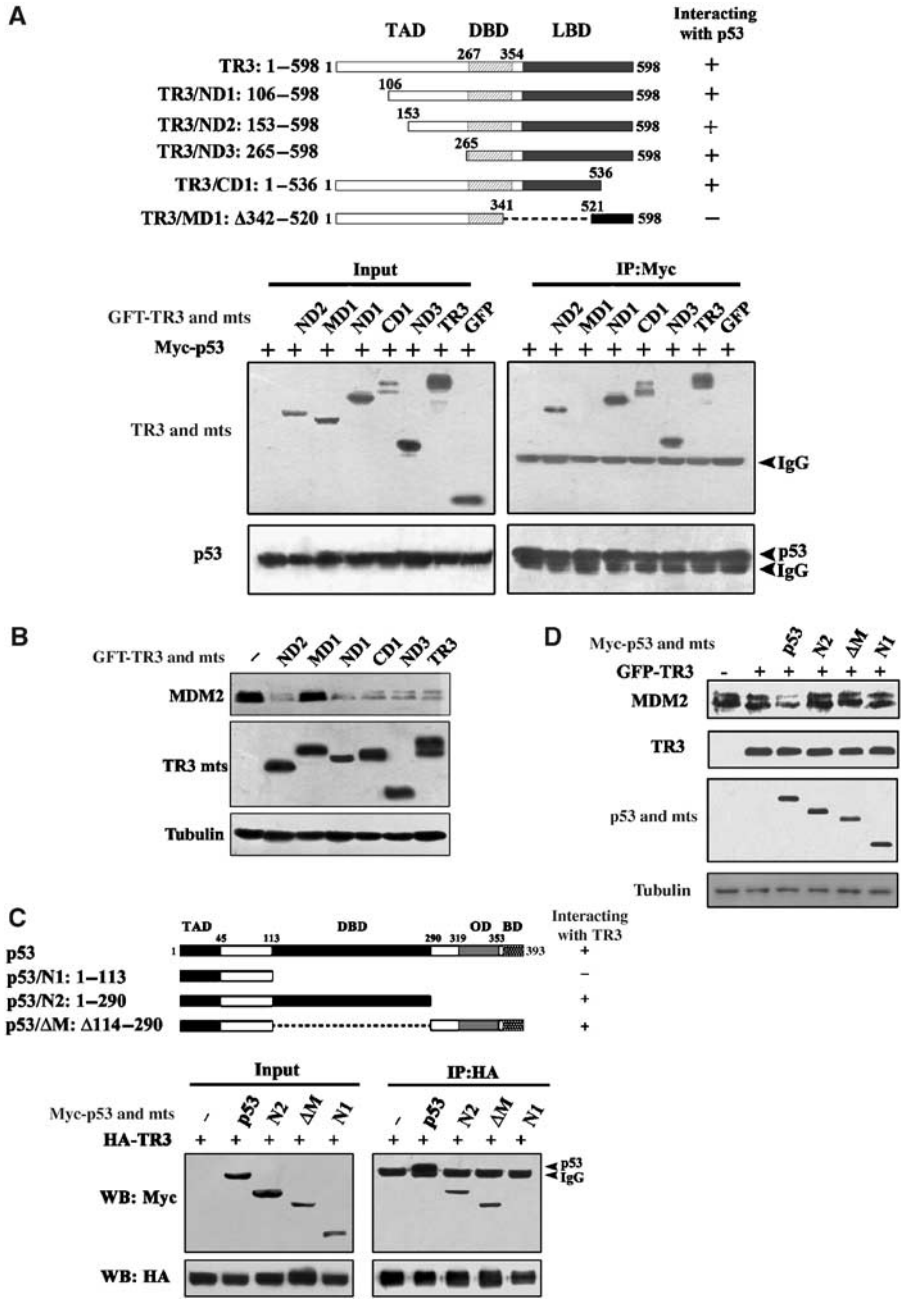

Figure 3 Interaction of TR3 with p53 is required for inhibition of MDM2. (**A**) Identification of TR3 sequence critical for p53 binding. Schematic diagrams depict different TR3 deletion constructs used in the domain mapping experiments (Top). 293 cells were transfected with Myc-p53 and GFP-TR3 truncation mutants. Cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates and cell lysates were then analyzed by Western blotting separately using anti-GFP-antibody for GFP-TR3 and its truncation mutants, and anti-Myc antibody for Myc-p53. GFP was used as a negative control. (**B**) Effect of different TR3 truncation mutants on MDM2 inhibition. GFP-TR3 and its truncation mutants were transfected into 293 cells. Endogenous MDM2 level was monitored by Western blotting by using anti-MDM2 antibody. GFP-TR3 and its mutants were revealed by anti-GFP antibody. (**C**) Interaction of TR3 with p53 and its deletion mutants. Structures of deletion mutants of p53 were shown on the top. 293 cells were transfected with HA-TR3 and different Myc-p53 deletion mutants as indicated. Cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitates and cell lysates were then analyzed by Western blotting separately using anti-Myc-antibody for Myc-p53 and its deletion mutants, and anti-HA antibody for HA-TR3. (**D**) Effect of TR3 on MDM2 inhibition mediated by different p53 deletion mutants. GFP-TR3, together with Myc-p53 or its deletion mutants as indicated, was transfected into H1299 cells. Endogenous MDM2 level was monitored by Western blotting by using anti-MDM2 antibody. GFP-TR3, p53, and its deletion mutants were revealed by anti-GFP antibody or anti-Myc antibody.

(aa 113–290) was deleted, p53 (p53/ΔM) still bound to TR3 (Figure 3C), suggesting that the C-terminal region of aa 290–393 also interacted with TR3. p53, therefore, appears to possess two domains, the DNA-binding domain and C-terminal region, for interacting with TR3. Nevertheless, neither p53/N2 nor p53/ΔM could mediate the inhibition of TR3 on MDM2 (Figure 3D), indicating that the integration of p53 is requisite for its TR3-mediating function.

Binding of TR3 represses p53 transcriptional activity

As p53 is the transcription activator of MDM2, TR3 may simply repress the transcriptional activity of p53 to exert its

inhibitory effect on MDM2. We investigated this possibility using a p53-Luceferase reporter system. When transfected into 293, U2OS, and H1299 cells, respectively (with or without coexpression of p53), TR3 significantly reduced the p53-reporter-gene activity in a dose-dependent manner (Figure 4A–C). One of the p53-interacting truncation mutants of TR3, TR3/ND1, could also repress the p53 transcriptional activity efficiently (Figure 4D–F). In contrast, the TR3/MD1 mutant that did not interact with p53 failed to do so (Figure 4D∼F). Therefore, it could be concluded that binding of TR3 to p53 resulted in inhibition of p53 transcriptional activity.

It has been reported that acetylation of p53 by p300/CBP at multiple lysine residues leads to the activation of p53 transcriptional activity (Avantaggiati *et al*, 1997; Gu *et al*, 1997; Lill *et al*, 1997). Thus, it is possible that binding of TR3 to p53 may block its acetylation by p300, leading to repression of the transcriptional activity. To test this possibility, p53 and p300 expression vector were transfected into 293 cells

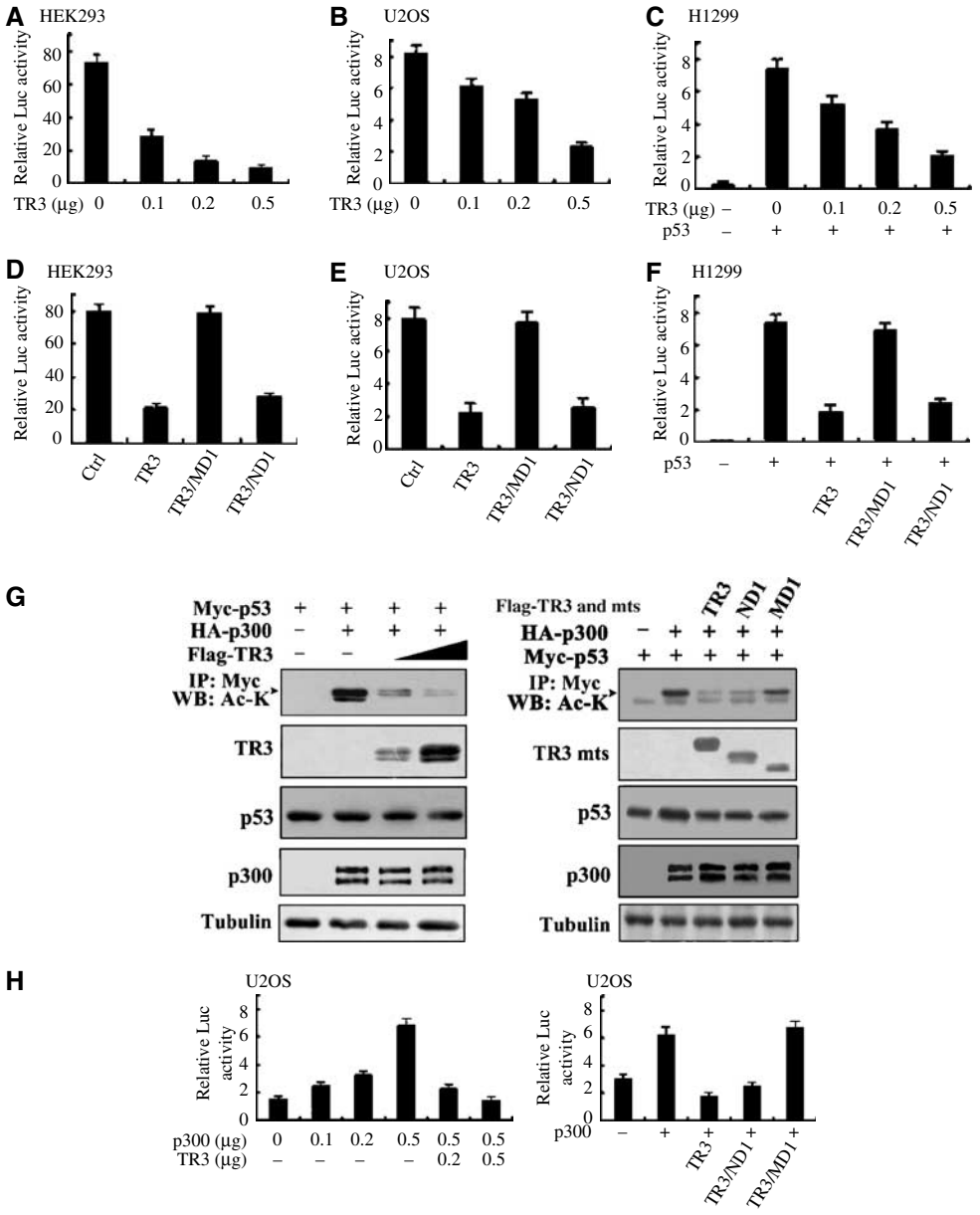

Figure 4 Regulation of p53 transcriptional activity by TR3. (**A–C**) TR3 repressed p53 transcriptional activity. p53-Luciferase reporter and β-gal gene expression vector, together with different dose of PECE-TR3 expression vector as indicated, was transfected into 293, U2OS, and H1299 cells in the absence or presence of p53 expression vector. Reporter gene activity was determined and normalized in relation to the cotransfected β-gal activity. The bars represent the average±mean from three independent experiments. (**D–F**) Effect of TR3 truncation mutants on transcriptional activity of p53. p53-Luciferase reporter and β-gal gene expression vector, together with different combinations of TR3 truncation mutants in the absence or presence of p53 expression vector, were transfected into 293, H1299, and U2OS cells as indicated. Reporter gene activity was determined and normalized as described above. (**G**) TR3 inhibited p300-induced p53 acetylation. Myc-p53, HA-p300, and different amount of Flag-TR3 were transfected into 293 cells. After transfection, cell lysates were immunoprecipitated using anti-Myc antibody for p53. Acetylated p53 was determined by anti-acetylated Lys antibody in Western blot analysis. The expressions of TR3 and p300 were immunoblotted by anti-Flag and -HA antibody, respectively. (**H**) Effect of TR3 on p300-induced p53 transcriptional activity. TR3, TR3 mutants or p300 expression vector, p53-Luciferase reporter, and β-gal gene expression vector were transfected into U2OS cells as indicated. Reporter gene activity was determined and normalized as described above.

and acetylation assays were performed. With the presence of p300, the immunoprecipitated p53 exhibited strong immunoreactivity to the anti-acetyl-lysine antibody (Figure 4G). As expected, cotransfection of TR3 greatly decreased the p300-induced acetylation level of p53 in a dose-dependent manner (Figure 4G, left panel). Similar results were also observed with the use of the TR3 truncation mutant TR3/ND1, but not TR3/MD1 (Figure 4G, right panel). We concluded that binding of TR3 to p53 inhibits p53 acetylation by p300, which might contribute to the inhibition of p53 transcriptional activity.

Next, we went on to confirm that the TR3 binding will directly inhibit p300-induced p53 transcriptional activity with the use of p53-Luciferase as a reporter in U2OS cells. The results showed that the dose-dependent enhancement of p53 transcriptional activity by p300 was significantly repressed in the presence of TR3 (Figure 4H). Again, TR3/ND1, but not TR3/MD1, could inhibit the p300-induced p53 transcriptional activity (Figure 4H), Together, these results suggest that TR3 represses the p53 transcriptional activity by binding to p53 and blocking its acetylation by p300.

TR3 downregulates MDM2 transcription via p53

We further investigated whether MDM2 gene expression was downregulated after the p53 transcriptional activity had been inhibited by TR3 through the acetylation pathway. We carried out RT–PCR and real-time PCR experiments to measure MDM2 mRNA levels. As shown in Figure 5A, transfection of 293 cells with p53 upregulated the mRNA level of MDM2 by 1.7-fold. In contrast, transfection of TR3 decreased the mRNA level of MDM2 by 9.0-fold. Again, different TR3 truncation mutants were used to examine MDM2 mRNA levels in 293 cells. The mRNA level of MDM2 was decreased by the transfection of TR3/ND1 but not TR3/MD1 (Figure 5A). In addition, when siRNA-p53 was introduced into 293 cells to inhibit endogenous p53 activity, MDM2 mRNA retained its original level even in the presence of TR3 (Figure 5B). We further used MDM2 promoter that contains a p53 response element (Rui *et al*, 2004) to test weather TR3 could modulate the transcriptional activity of MDM2 promoter in 293 cells. Luciferase assays showed that TR3 and TR3/ND1, but not TR3/MD1, also downregulated the transcriptional activity of MDM2 promoter (Figure 5C). Taken together, these results clearly demonstrated that repression of p53 transcription activity by TR3 results in the downregulation of MDM2 mRNA expression via inhibiting the transcriptional activity of MDM2 promoter, and reconfirmed that p53 indeed functions as an adaptor in the TR3–MDM2 signaling pathway.

The fact that repression of p53 transcription activity by TR3 contributes to MDM2 mRNA inhibition raises an interesting question: does this inhibition also affects expression of other p53 target genes? To answer this question, we first assessed the expression of p21waf1/cip1, one of p53 target genes. In non-UV-irradiated HepG2 cells, expression of p21waf1/cip1 could be detected, and such expression was downregulated by transfection of TR3 (Figure 1D). UV irradiation significantly induced both p53 and p21waf1/cip1 expression, whereas repression of p53-dependent p21waf1/cip1 expression by transfection of TR3 was hardly observed even in response to UV (Figure 1D). We further investigated the biological significance of TR3 in this inhibition by analy-

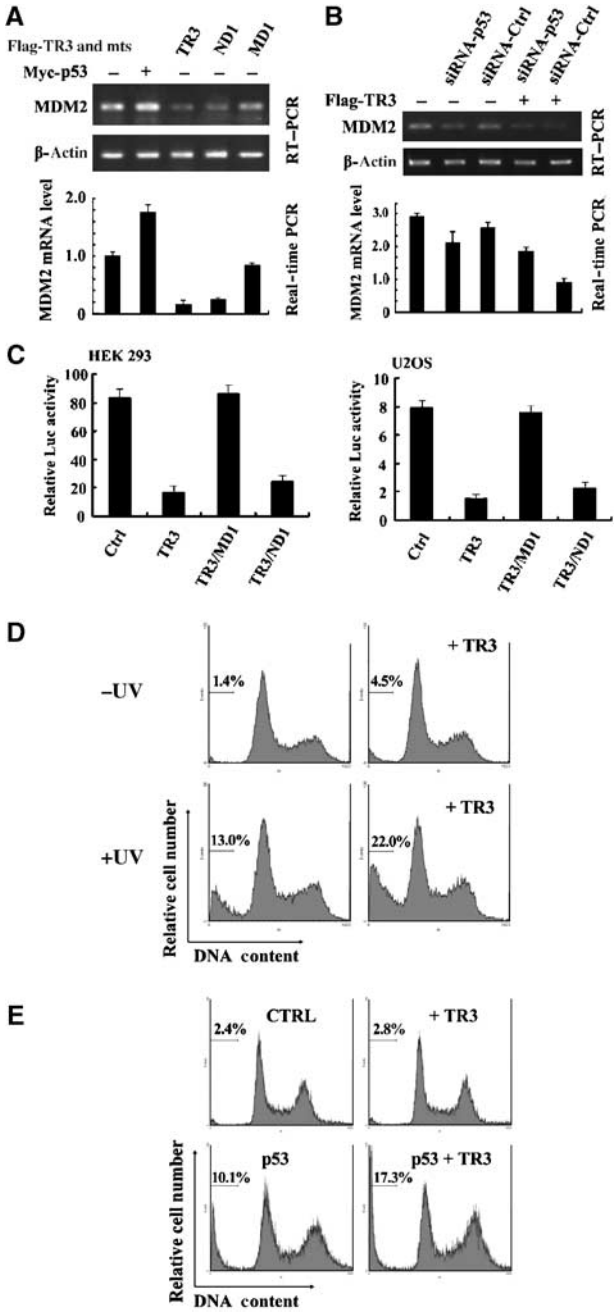

Figure 5 Effect of TR3 on p53-induced MDM2 mRNA expression. (**A, B**) TR3 regulated MDM2 mRNA expression through p53. 293 cells were transfected with different expression vectors, including Flag-TR3 and its mutants, Myc-p53, and SiRNA-p53 as indicated. After transfection, total RNA was prepared and analyzed for expression of MDM2 mRNA by RT–PCR and real-time PCR. Representatives of at least three independent experiments with similar results are shown. (**C**) TR3 decreased MDM2 promoter-Luc reporter activity. 293 and U2OS cells were transfected with different expression vectors, including PECE-TR3 and its deletion mutants as indicated. Measurement of MDM2 promoter-Luc activity and data presentation were as described in the legend to Figure 4A. (**D**) Effect of UV on regulation of apoptosis. HepG2 cells were transfected with TR3 expression vector. After transfection, cells were irradiated with UV (50 J/m^2), and then harvested 8 h later. Cells were immunostained with PI as described in Materials and methods, then analyzed by using flow cytometer. The numbers indicate the apoptotic rate. Two independent experiments were carried our. (**E**) Effect of TR3 on p53-induced apoptosis in H1299 cells. TR3, with or without p53, was transfected into cells. Apoptotic rate was determined as described in Figure 5D.

sis of cell cycle progression and apoptosis in HepG2 cells. Out of our expectation, TR3, in the absence or presence of UV irradiation, did not exert the regulatory effect on cell cycle progression (Figure 5D), whereas it did have the ability to induce apoptosis (4.5%), even could effectively enhance p53-dependent apoptosis (22.0%) induced by UV irradiation (Figure 5D). Such effect of TR3 on enhancement of apoptosis was further examined in p53-null cells H1299. As shown in Figure 5E, TR3 alone did not showed its ability to induce apoptosis in the absence of p53 (2.8%), almost the same as the control (2.4%). However, TR3 effectively enhanced p53-induced apoptosis when p53 was reintroduced (17.3%), further supporting the enhancement of TR3 on p53-dependent apoptosis. These results not only indicate a unique role of TR3 in apoptosis induction through cooperation with p53 but also suggest that inhibition of p53 transcription by TR3 may not be a sole event of its contribution to MDM2 inhibition, as in our current case.

TR3 inhibits MDM2-induced degradation of p53

It is well-known that MDM2 has an ability to degrade p53 (Zhang and Xiong, 2001). However, in our experiment, we did not observe the degradation of p53 by MDM2 in the presence of TR3 (Figure 1), suggesting another role of TR3 in stabilizing p53 protein. Just as such case, we found that the MDM2-induced degradation of p53 could be obviously inhibited by TR3 in a dose-dependent manner (Figure 6A, left panel). As MDM2 induces the degradation of p53 through the ubiquitin/proteasome pathway, we suspected that the ubiquitination of p53 was inhibited by the increased amount of TR3. This turned out to be the case. As shown in Figure 6A (right panel), MDM2 enhanced the ubiquitination level of p53, but the level was reduced when TR3 was coexpressed, regardless the presence or absence of MG132, a potential proteasomal inhibitor (Rock *et al*, 1994). Substitution of TR3 with its p53-interacting truncation mutant TR3/ND1 showed similar result, but such effect did not happen when the other mutant TR3/MD1 was used (Figure 6B), indicating that binding of TR3 protects p53 from being degraded through the MDM2-induced ubiquitin/proteasome pathway.

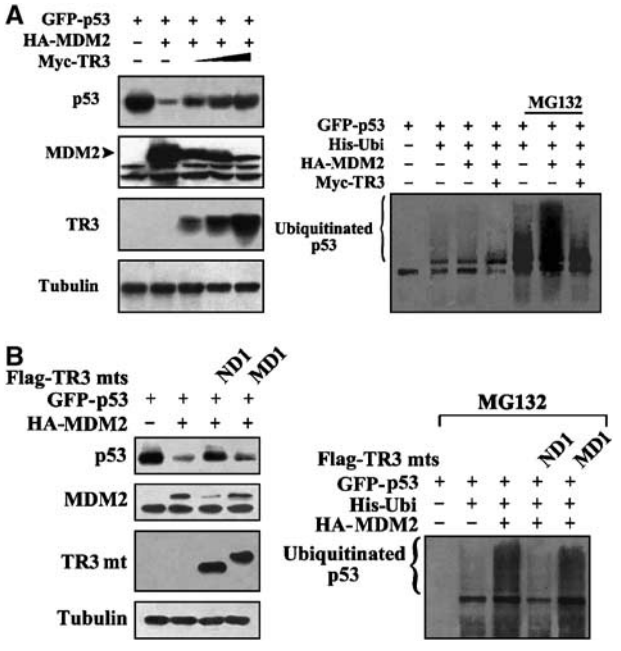

Figure 6 (**A**, **B**) TR3 protects p53 from ubiquitination and degradation by MDM2. Different expression vectors as indicated were transfected into 293 cells. The expression of p53, MDM2, and TR3 was analyzed by Western blotting. For ubiquitination assay, His-ubiquitin and different expression vectors indicated were co-expressed in 293 cells. p53 ubiquitination was monitored in immunoblots performed on nickel–agarose beads-purified proteins, and then probed with antibody to GFP to identify any p53-associated ubiquitin.

TR3 binding to p53 promotes MDM2 ubiquitination

How TR3 inhibits MDM2-induced degradation of p53? Wild-type p53 localizes in the nucleus and MDM2 triggers its nuclear export for degradation (Stommel *et al*, 1999). We also detected the nuclear export of p53 in MDM2-transfected 293 cells (Figure 7A). More than 75% of MDM2-positive cells accumulated p53 in cytoplasm. However, coexpression of TR3 blocked MDM2-induced p53 nuclear export (Figure 7A), indicating that TR3 binding detained p53 in the nucleus. Therefore, TR3 may have stronger affinity to p53 than to MDM2. We, next, transfected Myc-p53, HA-TR3, and HA-MDM2 expression vectors into 293 cells. Western blot by using anti-HA antibody against the common HA tag allowed us to compare the expression levels of TR3 and MDM2. When HA-TR3 and HA-MDM2 were expressed at similar levels, we detected significantly higher level of TR3 than MDM2 in p53 immunoprecipitates (Figure 7B). This result implies that TR3 binds to p53 with a higher affinity than MDM2 does.

To further compare the interaction affinity between p53–TR3 and p53–MDM2, we analyzed their binding constants by the method of fluorescence spectroscopy. As shown in Figure 7C, p53 protein had the same maximal emission wavelength at 310 nm as MDM2 or TR3 did, but it was unable to affect the quenching when incubated with MDM2 or TR3 protein, as its maximal fluorescence intensity is far lower than MDM2 and TR3 (Figure 7C, upper panel). When TR3 and MDM2 protein were incubated with p53 protein, they bound to p53 in a dose-dependent manner (Figure 7C, down panel). However, their binding constants were different, the value of K_a (i.e, the binding constant) for TR3–p53 was 4.3×10^7/M, while K_a for MDM2–p53 was 4.0×10^6/M, as calculated from the formula (Jiang *et al*, 2004). Clearly, the affinity of TR3–p53 is more 10-folds than that of MDM2–p53, which is consistent with the result of Figure 7B examined by Co-IP assay.

As TR3 binds to p53 with a higher affinity than MDM2, it may sequester p53 from MDM2 and MDM2 will thus release, and undergoing its degradation through ubiquitin/proteasome pathway. Indeed, we could detect ubiquitinated MDM2 in the presence of MG132 in 293 cells (Figure 7D and E, lane 4). When p53 was coexpressed, ubiquitinated MDM2 became weaker, probably due to a portion of ubiquitin being targeted to p53 (Figure 7D, lane 5), as ubiquitinated MDM2 was not weaken in the presence of TR3 (Figure 7E, lane 5). However, when TR3 was introduced, the ubiqitination level of MDM2 became much higher (Figure 7D and E, lane 6), indicating that TR3 binding to p53 promoted MDM2 self-ubiquitination. To further confirm the TR3 and p53 effects, different TR3 or p53 deletion mutants were introduced into 293 cells in the presence of both MDM2 and

ubiquitin. Consistent with the above data, TR3/ND1 that interacted with p53 could enhance MDM2 ubiquitination (Figure 7D, lane 7); in contrast, TR3/MD1 that was unable to interact with p53 failed to augment MDM2 ubiquitination (Figure 7D, lane 8). Neither p53/N2 nor p53/ΔM showed any ability to affect MDM2 ubiquitination (Figure 7E, lanes 7 and 8), which was consistent with our conclusion from Figure 3D that integrated structure of p53 is critical to mediate TR3 function. These findings suggest an important role of TR3 in the promotion of MDM2 self-ubiquitination. To further ascertain the augmentative effect of TR3 on MDM2 ubiquitination-dependent degradation, we constructed a unique MDM2 point mutant MDM2/C464A that could interact with p53, but not undergo autoubiquitination by itself (Zhao *et al*,

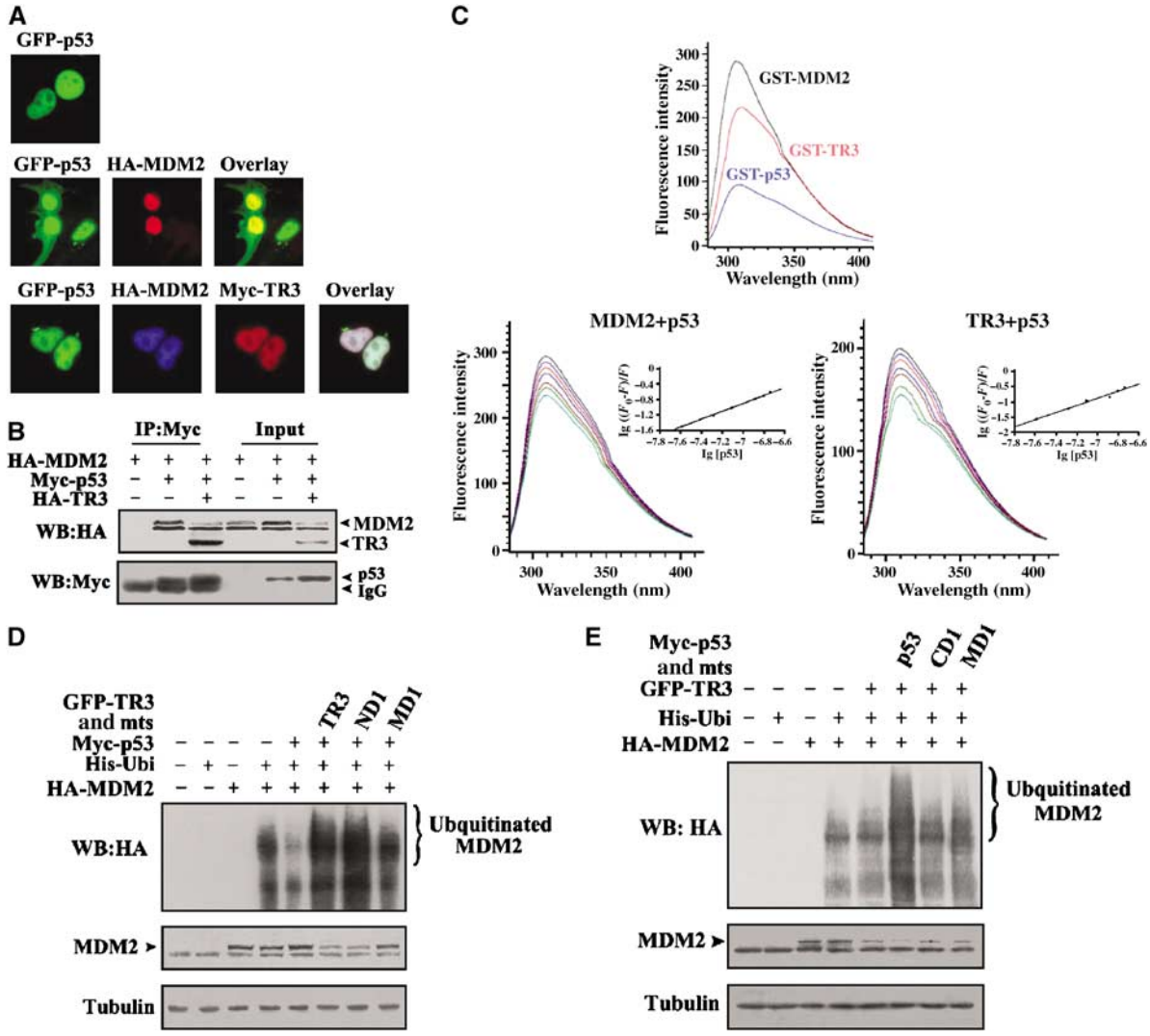

Figure 7 Interaction of TR3 with p53 promotes MDM2 self-degradation. (**A**) Subcellular localization of TR3, p53, and MDM2 in 293 cells. Myc-TR3, GFP-p53, and/or HA-MDM2 were transfected into cells as indicated. Cells were immunostained for detecting TR3 by Myc antibody followed by Texas Red-conjugated secondary antibody and for detecting MDM2 by HA antibody followed by Texas Red or Alexa flour 350-conjugated secondary antibody. Stained cells were visualized with the confocal microscope. (**B**) The relative binding affinity of MDM2 and TR3 to p53 by Co-IP assay. 293 cells were transfected with Myc-p53, HA-TR3, and HA-MDM2. Western blot against the common HA tag was used to compare the expression levels of TR3 and MDM2. Binding affinity of TR3 and MDM2 to p53 was shown by using anti-HA antibody in p53 immunoprecipites. (**C**) Fluorescence emission spectra of MDM2 and TR3. GST-p53, in a final concentration of 2 μM, was incubated with 1 μM of GST-MDM2 or GST-TR3 protein (upper panel). Either 1 μM of MDM2 or TR3 protein was incubated with different concentration of p53 (from 26 to 182 nM). After incubation for 2 min at pH 8.0, 25°C, the fluorescence intensity was detected (down panel). All spectra were recorded at $\lambda_{ex} = 275$ nm. (**D**, **E**) TR3 binding to p53 resulted in MDM2 ubiquitination. Different expression vectors, including TR3 and p53 deletion mutants as indicated, were cotransfected into 293 cells and then treated with MG132 for 3 h. MDM2 ubiquitination was monitored in immunoblots performed on nickel resin-purified proteins, and then probed with antibody against HA to identify any MDM2-associated ubiquitin. To show the expression levels of MDM2 protein, the same cell lysates were subjected to Western blotting, probing with antibody against HA. Tubulin was used as a loading control. (**F**) Interaction among TR3, p53, and MDM2 point mutant. Myc-p53, HA-MDM2/C464A, and increasing Flag-TR3 expression vectors were transfected into 293 cells as indicated, then Co-IP assay was performed as described in Materials and methods. (**G**) TR3 inhibited exogenous MDM2 expression in a dose-dependent manner. Myc-TR3 and HA-MDM2 were transfected into 293 cells. MDM2 expression level was determined by Western blotting using anti-HA antibody. (**H**) Effect of CHX on MDM2 degradation. Myc-TR3 and HA-MDM2 were transfected into 293 cells, and then treated with CHX (100 μg/ml) for the indicated times. MDM2 expression level was determined by Western blotting using anti-HA antibody. The levels of MDM2 protein were quantified by densitometry. (**I**) Effect of CHX on p53 degradation. Myc-p53 and Flag-TR3 were transfected into 293 cells, and then treated with CHX (100 μg/ml) for the indicated times. p53 expression level was determined by Western blotting using anti-Myc antibody. The levels of p53 protein were quantified by densitometry.

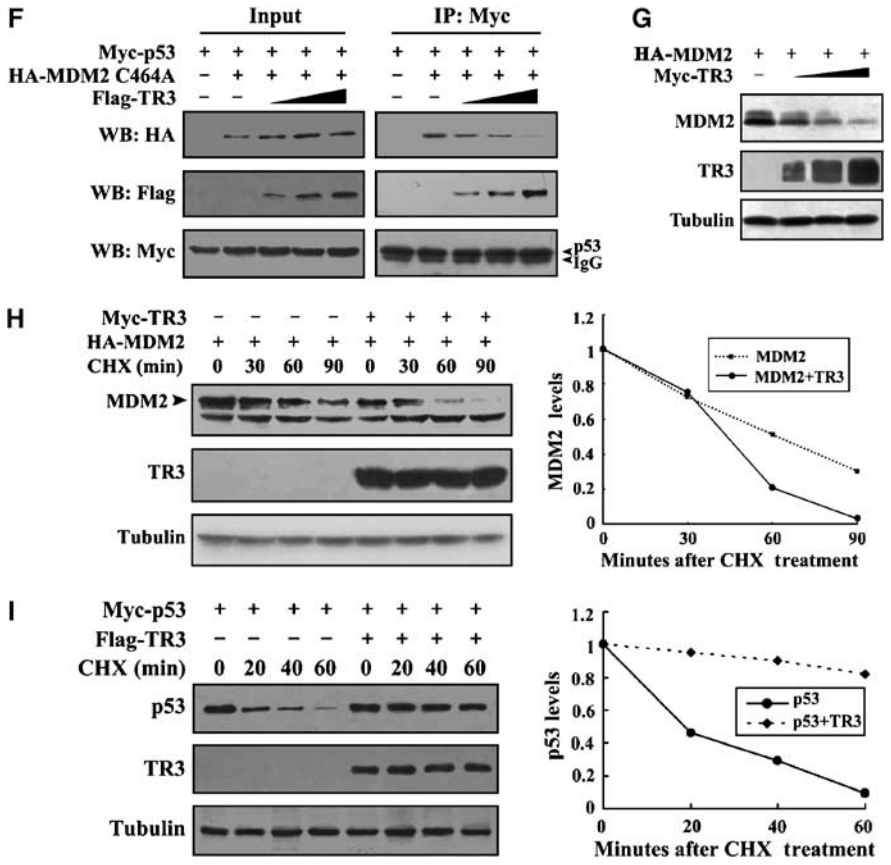

Figure 7 Continued.

2004). Co-IP assay showed that with the increasing of TR3 protein levels, TR3–p53 interaction was gradually increased, and p53–MDM2 interaction was gradually reduced in parallel (Figure 7F), further supporting that TR3 binding to p53 is stronger than MDM2. We also noted that increasing levels of TR3 did not impair the expression level of MDM2/C464A, which also supported that MDM2 degradation induced by TR3–p53 binding was through ubiquitination pathway. In addition, exogenous MDM2 expression could also be degraded by TR3 in a dose-dependent manner (Figure 7G), and such augmentative effect of TR3 on MDM2 degradation occurred earlier by 30 min in the presence of TR3 than in the absence of TR3 assessed qualitatively and quantitatively (Figure 7H). We also examined effect of TR3 on p53 stability, by parallel experiment, without transfection of MDM2. As shown in Figure 7I, p53 degraded by itself obviously after treatment of CHX for 60 min. However, transfection of TR3 strongly enhanced stabilization of p53, the half-life of p53 was prolonged obviously, as quantified by densitometry (Figure 7I). Therefore, these results further confirmed that TR3 is not able to increase p53 levels, while is capable of maintaining p53 levels. Taken together, a series of experiments above demonstrate that TR3 has a higher p53-binding affinity, which sequestrates p53 from MDM2, and then results in MDM2 autoubiquitination and degradation.

Discussion

MDM2 protein is overexpressed in a series of human tumors underscoring its involvement in the development of these human disease (Leach *et al*, 1993). Although expression of MDM2 is activated by p53 at the transcription level (Barak *et al*, 1993), the major function of MDM2 is to induce p53 proteasomal degradation (Haupt *et al*, 1997; Kubbutat *et al*, 1997). Therefore, MDM2 functions as a negative feedback regulator to maintain p53 at a low level. And thus, the destabilization of MDM2 and stabilization of p53 are of importance to provide a potential approach for cancer therapy. Recent studies indicate an important role of orphan receptor TR3 in inhibition of MDM2 protein expression (Yoo *et al*, 2004). However, the regulatory mechanism between TR3 and MDM2 cross-talk is largely unknown. In the present study, we showed a novel signal pathway in which p53 acts as an inter-link mediating TR3–MDM2 cross-talk. We demonstrated that (1) TR3 did not interact directly with MDM2, but directly interacted with p53, and its inhibition on MDM2 required the mediation of p53; (2) TR3–p53 binding led to the decrease of p53 transcriptional activity through inhibition of p53 acetylation, resulting in repression of MDM2 mRNA; (3) TR3 had a higher binding affinity to p53 than to MDM2. TR3–p53 interaction led to the separation of MDM2 from p53, which protected p53 from MDM2-induced degradation, and promoted the ubiquitination and degradation of MDM2 itself; (4) p53-dependent TR3 inhibition on MDM2 did not impair cell cycle progression while induced apoptosis, and TR3 could enhance p53-mediated apoptosis induced by UV irradiation. Therefore, our study reveals a novel model for regulatory mechanism of TR3 on MDM2 at both transcriptional and post-transcriptional levels (Figure 8).

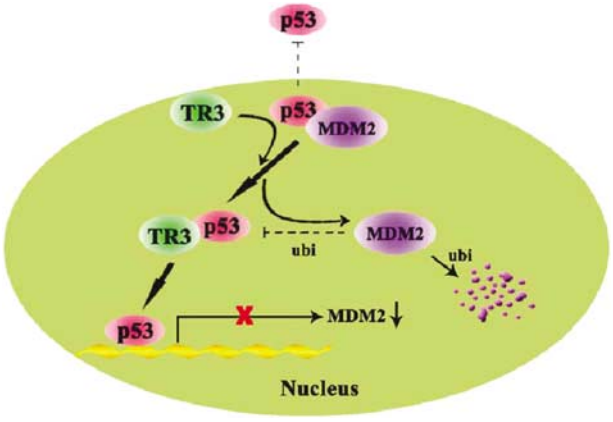

Figure 8 A proposed model for p53 to mediate the negative regulation of MDM2 by TR3.

p53 occupies a central position in signaling network through its specific transcriptional activations and post-translational modifications (Vogelstein *et al*, 2000). Although TR3 has been reported to exert its inhibition on endogenous MDM2 expression (Figure 1A) (Yoo *et al*, 2004), direct interaction or binding between TR3 and MDM2 could not be observed (Figure 2). In the cross-talk between TR3 and MDM2, p53 appears to act as an adapting protein, as it physically interacted with TR3 as well as MDM2 (Figure 2), and such interaction brought TR3 to the proximity of MDM2, facilitating TR3 to inhibit MDM2 (Figure 3). Moreover, the integration of p53 was requisite in its mediation role (Figure 3C and D). Thus, we demonstrated, for the first time, the requirement of p53 in mediating the inhibitory effect of TR3 on MDM2 expression. The role of p53 as a mediator in signal transduction has often been identified. For example, the multifunctional transcription regulator YY1 interacts with MDM2 and such interaction is competitively inhibited by ARF, which is probably mediated by p53 (Gronroos *et al*, 2004; Sui *et al*, 2004). Further study showed that as a result of downregulating p53 transcriptional activity, TR3 indirectly extended its inhibitory effect on the transcriptional activity of MDM2 promoter (Figure 5C) and expression of MDM2 gene (Figure 5A). In this process, the event of TR3–p53 binding was crucial, as the truncation mutant TR3/MD1, which is noninteractive with p53, failed to exert the above-mentioned function (Figure 5A). Regulation of p53 acetylation by TR3 seems to be an essential step. TR3 significantly repressed p300-induced p53 transcriptional activity (Figure 4H), and drastically diminished p300-induced p53 acetylation (Figure 4G). p300 can form a complex with MDM2, which facilitates MDM2-mediated p53 degradation (Grossman *et al*, 1998). Conversely, p300 was also shown to stabilize p53 after DNA damage (Yuan *et al*, 1999). These evidences gave rise to another intriguing possibility that whether TR3 could maintain p53 stability by means of competing with p300 in binding p53? Further studies with regard to these interesting issues are been carrying out.

p53 normally functions as an integrator of stress response signals by activating or repressing the transcription of genes that regulate cell cycle progression and/or apoptosis (Meek, 1999; Prives and Hall, 1999). Although Daxx, which has been shown to potentiate Fas-mediated apoptosis (Zhao *et al*, 2004), repressed the transcriptional activity of p53 toward the p21waf1/cip1 promoter, it had no effects on the induction of proapoptotic genes such as bax, PIG3, and AIP1(Gostissa *et al*, 2004). Here, we showed that TR3 failed to regulate cell cycle progression but could induce apoptosis via activation of p53 under UV irradiation, although it repressed p53-dependent transcription of the p21waf1/cip1 gene (Figures 1D, 4 and 5D). We also found that overexpression of TR3 sensitized hepatoma cells to UV-induced cell death (Figure 5D), and this process was dependent on p53 (Figure 5E). These results indicate that such an effect requires p53, and the physiological levels of p53 protein are important in setting the threshold for TR3-mediated apoptosis. The fact that activation of p53 results in elevated levels of apoptosis, which could be suppressed by MDM2 expression (Zhao *et al*, 2004), suggests that MDM2 can protect cells from p53-mediated apoptosis. In line with these observations, we also found that TR3 was able to enhance p53-dependent apoptosis induced by UV irradiation (Figure 5D), probably through the inhibition of MDM2 expression (Figure 1D). Therefore, in light of our evidence, it is fascinating to speculate that TR3 may exert a dual role through both transcriptional and post-transcriptional pathways to inhibit MDM2 expression, and p53 protein provides a linking platform for the interaction between TR3 and MDM2. Indeed, this was the unique role of TR3 in maintaining p53 protein levels by inducing MDM2 ubiquitination and degradation (Figure 7D and E). Therefore, the role of TR3 on MDM2 self-ubiquitination may be a side effect, which ensures the normalization of p53–MDM2 feedback loop. In addition, our study also points out that when p53 acts as a mediator in TR3–MDM2 pathway, although its transcriptional activity is inhibited by TR3, it could still utilize another avenue to cooperate with TR3 in inhibiting MDM2 expression. How TR3 involves in such a complex regulatory process and the exact mechanism by which the repressive function of TR3 operates specifically toward some p53-induced apoptotic genes requires further elucidation.

MDM2 functions as a ubiquitin ligase E3 to promote its self-degradation and the degradation of p53 (Fang *et al*, 2000). Thus, the MDM2–p53 feedback loop represents a cellular autoregulatory paradigm, finely tuning p53 function (Momand *et al*, 2000). TR3 also inhibited exogenous MDM2 expression (Figure 7G), is such inhibition by TR3 associated with the regulation of MDM2–p53 feedback loop? Here, we propose a mechanism for the MDM2–p53 loop regulated by TR3, that is, interaction with TR3 prevented p53 from MDM2 ubiquitination-degradation, resulting in MDM2 self-degradation. When TR3, p53, and MDM2 coexpressed in 293 cells, MDM2-induced p53 degradation was dramatically neutralized by TR3, and p53 kept its normal level even in the presence of MDM2 (Figure 6A), strongly suggesting a novel function of TR3 in maintaining p53 stability. TR3 downregulated the expression levels of MDM2 but not p53 (Figure 7H and I). Further experiment verified that this action was via the blockage of ubiquitination pathway induced by MDM2 (Figure 6A). Importantly, this effect was also closely associated with the TR3–p53 interaction, because the p53 noninteractive TR3/MD1 lost such function (Figure 6). Therefore, repression of MDM2 by TR3 may lead to stabilization of p53 and a shift in the negative MDM2 feedback loop. Recent studies point out that HIPK2 is able to rescue p53 transcriptional activity and apoptotic outcome by

overcoming MDM2-mediated proteasomal degradation (Di *et al*, 2005). HIPK2 and MDM2 form a stable complex that, in response to DNA damage, allows MDM2 detachment from phosphorylated p53Ser46. The end result of this action is HIPK2-dependent MDM2 destabilization and p53 stabilization (D'Orazi *et al*, 2002; Di *et al*, 2005). Conversely, the nuclear corepressor KAPI, as a novel MDM2-interacting protein, cooperates with MDM2 to inhibit p53 acetylation, stimulate p53 ubiquitination, and inhibit p53 transcription and apoptosis functions (Wang *et al*, 2005). In addition, the ability of MDM2 to promote p53 turnover might be tightly linked to its own instability. Deletion of the central domains or the C-terminal RING finger of MDM2 not only destroys its ability to degrade p53 but also cause stabilization of MDM2 itself (Oren, 2003). Here, we demonstrated that the TR3-induced ubiquitination and degradation of MDM2 (Figure 7D–F) is due to a higher affinity of TR3 for p53 than that between p53 and MDM2 (Figure 7B and C). In addition, we observed that p53 was located in the nucleus when transfected by itself, and MDM2 could promote p53 nuclear export. However, cotransfection of TR3 hindered p53 nuclear export even in the presence of MDM2 (Figure 7A), which might provide a mechanism for TR3 to protect p53 from MDM2 degradation. These observations revealed a connection between p53 stabilization and MDM2 destabilization regulated by TR3, not only demonstrating that inhibition of MDM2 by TR3 is important for p53 stabilization but also suggesting that TR3 stabilizing p53 protein is a unique property that is independent of other previously described functions of TR3, such as its transcriptional regulation of mitogenic activities in the nucleus (Kolluri *et al*, 2003).

In summary, our studies identified the nuclear transcription factor TR3 as a novel p53-interaction protein. TR3, via p53, inhibited MDM2 mRNA and protein expression, finally sequestrated MDM2 from p53, and protected p53 from MDM2-induced degradation. Because malignant cells with high-level expression of MDM2 are aggressive, and destabilization of MDM2 and stabilization of p53 are potentially significant approaches for cancer therapy, our research may provide an insight into the underlying mechanisms, revealing that TR3 may be a novel target for the development of new anticancer strategy that may restrict MDM2-induced tumor progression.

Materials and methods

Cell culture and transfection

HEK293 (human embryonic kidney cell), H1299 (p53-null, human non-small-cell lung carcinoma), and U2OS (osteosarcoma) cell lines were purchased from ATCC. HepG2 cell line (human hepatoma) was purchased from the Institute of Cell Biology, China. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U penicillin, and 100 μg/ml streptomycin. Transfection was performed using calcium phosphate precipitation method for 293 cell line, and liposomal transfection reagent (Roche, Fugene 6) for H1299, U2OS, and HepG2 cell lines according to the manufacturer's instructions.

Luciferase reporter assay

Cells were transfected with p53- or MDM2-luciferase reporter plasmid, β-gal, and other different expression vectors as required. After transfection, luciferase activity was normalized for transfection efficiency using corresponding β-gal activity. The ratios of luciferase/β-gal activity were used as indicators for transcriptional activity of p53 or MDM2 promoter.

RT–PCR and real-time-PCR

Cells were harvested and total RNA was extracted using RNeasy kit (Qiagen Inc.) and reverse transcribed by M-MuLV reverse-transcriptase (Fermentas). cDNA was amplified using MDM2 as a primer. Real-time PCR was carried out using Sybr Green-based detection in Roter-Gene according to the manufacturer's instruction. Levels of â-actin were used as normalization controls.

GST pull-down assay

Wild-type DNA was generated by PCR and then cloned into the corresponding sites of pGEX-4T-1. DNAs encoding the GST-tagged proteins were used to transform BL-21 bacterial cells and transformed cells were grown at 37°C. To purify the GST fusion proteins, cells were lysed by sonication in lysis buffer, and the resulting lysates were incubated for 1 h at 4°C with glutathione-Sepharose beads. The beads were pelleted by centrifugation and washed with lysis buffer. For p53 or TR3 binding, the GST-tagged proteins bound to beads were incubated with 500 μl of whole-cell lysate from transfected 293 cells for 5–6 h. Unbound p53 or TR3 protein was removed by washing with lysis buffer. Bound proteins were eluted by boiling for 10 min in 1 × loading buffer, resolved by SDS–PAGE, and examined by immunoblot analysis with anti-Myc or anti-FLAG antibody.

Yeast two-hybrid protein interaction assay

Interactions between p53 and TR3 in yeast were measured by activating the lacZ reporter. The yeast strain AH109 was transformed with appropriate plasmids encoding fusions of pGBKT7-BD to TR3 and plasmids encoding fusions of pGADT7-AD to p53. Colonies were selected on SD medium lacking Trp, Leu, His, and Ade at 30°C for 3 days, and the β-gal activity in the extracts prepared from the liquid culture was determined. Five independent colonies from each plate were grown overnight in 2 ml liquid medium as described above. The cells were harvested and β-gal activity was assayed according to the CLONTECH manual.

Immunoprecipitation

All immunoprecipitation procedures were carried out at 4°C. Cells were harvested and washed twice with PBS before lysis. Cells were lysed in lysis buffer. The lysates were then incubated with the appropriate antibody for 1 h, and were subsequently incubated with protein A-Sepharose beads for 1 h. The protein–antibody complexes that were recovered on beads were subjected to Western blot analysis after separation by SDS–PAGE.

Western blot analysis

Cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were lysed in lysis buffer, supplemented with protease inhibitors. Protein was separated by SDS–PAGE and electrically transferred to a polyvinylidene difluoride membrane. The membrane was probed with the appropriate primary antibody and with an HRP-conjugated secondary antibody. Blots were visualized by ECL.

Immunofluorescent staining and microscopic observation

Cells were transfected with GFP-p53, HA-MDM2, and/or Myc-TR3 expression vector as required. After 16 h, cells were fixed in 4% paraformaldehyde. For staining exogenous TR3 and MDM2 proteins, cells were incubated with anti-Myc or anti-HA antibody followed by Texas Red- or Alexa flour 350-conjugate secondary antibody. Stained cells were visualized under confocal microscope (Bio-Rad MRC-1024ES).

Acetylation assay

Cells lysates were extracted in cell lysis buffer and immunoprecipitated with appropriate primary antibody. The immunoprecipitates were separated by SDS–PAGE and blotted with specific anti-acetyllysine antibodies.

Ubiquitination assay

Cells were transfected with different expression vectors as indicated, and then lysed in Ni–agarose lysis buffer. His-ubiquitin-conjugated proteins were purified by nickel chromatography (Ni–NTA–agarose, Qiagen). The beads were washed successively 10 times with Ni–agarose wash buffer. To reduce nonspecific binding to beads, nickel-binding proteins were resuspended in 2 × sample buffer supplemented with 200 m mol/l imidazole and heated for 10 min at 100°C before being subjected to Western blotting with corresponding antibodies.

Fluorescence measurements

Fluorescence measurements were made with the use of a HITACHI model F-4500 (HITACHI Corp.) fluorescence spectrophotometer. Either 1 μM of MDM2 or TR3 protein was incubated with different concentration of p53 (from 26 to 182 nM). Protein quenching was monitored at 25°C by using 5 nm of excitation and 5 nm of emission slit-width. The excitation wavelength was 275 nm, and the emission spectra were measured between 285 and 400 nm. To estimate the affinity of MDM2 or TR3 protein to p53, fluorescence intensities at 310 nm with increasing concentrations of quencher were analyzed, and value of K_a was calculated according to formula (Jiang *et al*, 2004).

Apoptosis and cell cycle analysis

Cells were transfected with TR3 expression vector. After transfection, cells were irradiated with UV (50 J/m^2), and analyzed 8 h later. Cells were fixed with 70% ethanol, and incubated in DNase-free RNase A (50 μg/ml) at 37°C for 30 min, then immunostained with propidium iodide (Sigma, 50 μg/ml) for 30 min at 4°C against light. The stained cells were analyzed using the FACScater-plus flow cytometer.

Acknowledgements

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