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Knockdown of RNF2 induces apoptosis by regulating MDM2 and p53 stability

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Abstract

RNF2, also known as Ring1B/Ring2, is a component of the polycomb repression complex 1 (PRC1). RNF2 is highly expressed in many tumors, suggesting that it might have an oncogenic function, but the mechanism is unknown. Here we show that knockdown of RNF2 significantly inhibits both cell proliferation and colony formation in soft agar, and induces apoptosis in cancer cells. Knockdown of RNF2 in HCT116 $p53^{+/+}$ cells resulted in significantly more apoptosis than was observed in RNF2 knockdown HCT116 $p53^{-/-}$ cells, indicating that RNF2 knockdown-induced apoptosis is partially dependent on p53. Various p53-targeted genes were increased in RNF2 knockdown cells. Further studies revealed that in RNF2 knockdown cells, the p53 protein level was increased, the half-life of p53 was prolonged and p53 ubiquitination was decreased. In contrast, cells overexpressing RNF2 showed a decreased p53 protein level, a shorter p53 half-life and increased p53 ubiquitination. Importantly, we found that RNF2 directly binds with both p53 and MDM2 and promotes MDM2-mediated p53 ubiquitination. RNF2 overexpression could also increase the half-life of MDM2 and inhibit its ubiquitination. The regulation on p53 and MDM2 stability by RNF2 was also observed during the etoposide-induced DNA damage response. These results provide a possible mechanism explaining the oncogenic function of RNF2, and because RNF2 is important for cancer cell survival and proliferation, it might be an ideal target for cancer therapy or prevention.

Keywords

RNF2; PcG; p53; MDM2; ubiquitination

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

Polycomb group (PcG) proteins are epigenetic gene silencers that can suppress gene expression at the transcription level. These proteins were initially identified in *Drosophila* as transcriptional repressors required for correct expression of *homeotic (Hox)* genes (1). In mammals, two biochemically and functionally distinct PcG core complexes have been identified, and are referred to as polycomb repressive complex 1 and 2 (PRC1, PRC2) (2–3). PRC2, which is involved in the initiation of gene repression, consists of Ezh2/Kmt6, Suz12 and Eed, in which Ezh2/Kmt6 is a methyltransferase that trimethylates histone H3 at Lys27 (H3K27Me3) (4–6). The mammalian PRC1, which is an ubiquitin E3 ligase complex, consists of three ring domain-containing proteins termed RING1/Ring1A, RING2/Ring1B, and BMI-1/Bmi-1, among which RING2/Ring1B has been identified as the catalytic subunit (7). PRC1 and PCR2 do not physically interact, but the Ezh2-catalyzed histone mark H3K27Me3 is recognized by the PRC1 member Pc. This interaction was proposed to target PRC1 to the appropriate genomic locations, providing a mechanism of communication between the two complexes (8).

PcG proteins play essential roles in early embryonic development and stem cell self-renewal through transcriptional repression of many key transcription factors. In murine embryonic stem (ES) cells, genome-wide location analysis showed that PRC1 and PRC2 co-occupied 512 genes, many of which encode transcription factors with important roles in development (9). In human ES cells, the PRC2 subunit Suz12 was reportedly distributed across large portions of over two hundred genes encoding key developmental regulators, and *PRC2* target genes are preferentially activated during ES cell differentiation (10).

In addition to the known function in development and stem cell self-renewal, several members of the PcG protein family were shown to have oncogenic functions and they are overexpressed in different cancer types, among which the PRC2 member Ezh2 and the PRC1 member Bmi1 are most well studied (see review (11)). However, the mechanism explaining the means by which these PcG proteins perform their oncogenic function is not clear. Based on their transcriptional repressive function, several tumor suppressor genes were demonstrated to be under the transcriptional control of PcG proteins. For example, *p57, p27, RUNX3, E-cadherin* and *FBXO32* have been identified as targets of Ezh2 and they have been shown to be responsible for the oncogenic function of Ezh2 (12–17). Compared with the Ezh2 targets identified, very few PRC1 targets have been found. Based on the phenotype that removal of *Ink4a* can dramatically reduce lymphoid and neurological defects shown in *bmi-1* deficient mice, the *Ink4a* locus was first identified as a critical target of Bmi-1 (18–19).

RNF2, as a member of PRC1, is highly expressed in many different tumors, including gastric cancer, colon cancer, and lymphomas (20). Knockdown of RNF2 in HeLa cells results in morphological changes and inhibition of cell proliferation (7), suggesting an oncogenic function. Loss of *Rnf2* results in gastrulation arrest and genetic inactivation of the *Cdkn2a* (*Ink4a*/*ARF*) locus partially bypasses the early developmental arrest in *Rnf2*-null embryos, which suggests that Cdkn2a is also a target of *Rnf2* (21). However, the lethal

phenotype of *Rnf2* deficient mice cannot be rescued by Cdkn2a deletion, indicating that other important targets of *Rnf2* likely exist.

In order to determine a possible mechanism to explain how RNF2 exerts its oncogenic function, we knocked down RNF2 expression in HCT116 cells. The decreased expression of RNF2 not only inhibited cell proliferation, but was associated with induction of apoptosis. Because p53 plays an important role in cell proliferation and apoptosis, we determined whether p53 is involved in RNF2 knockdown-induced apoptosis. The result showed that RNF2 knockdown could induce significantly more apoptosis in p53 wildtype ($p53^{+/+}$) HCT116 cells compared to p53 deficient ($p53^{-/-}$) HCT116 cells. Further studies revealed that the p53 protein level was increased in RNF2 knockdown cells, but was decreased in cells overexpressing RNF2. We also investigated a possible mechanism to explain the effect of RNF2 on p53 and found that RNF2 directly binds with both p53 and MDM2. RNF2 can promote MDM2-mediated p53 ubiquitination and RNF2 can increase the half-life of MDM2 and inhibit its ubiquitination. In addition, we also showed that RNF2 plays an important role in MDM2 and p53 stability during DNA damage. These findings confirmed an oncogenic function of RNF2, and because RNF2 is vitally important for cancer cell survival and proliferation, it is an ideal target for cancer therapy or prevention.

Results

Knockdown of RNF2 inhibits cell proliferation

In order to study the function of RNF2 in cancer cells, we used lentivirus to express *shRNA* targeting RNF2 to suppress RNF2 expression in HCT116 colorectal cancer cells. Both shRNF2 #1 and #2 lentivirus (Figure 1a) showed good knockdown efficiency. Proliferation was assessed by MTS and soft agar assays. Results showed that both proliferation and soft agar colony formation of RNF2 knockdown cells were dramatically inhibited (Figures 1b and 1c). In addition, HCT116 cells freshly infected with *shMock* or *shRNF2* #2 lenvirus were injected subcutaneously into athymic nude mice and results indicated that *shRNF2* #2 infected cells could not form tumors *in vivo* (Figure 1d). The critical function of RNF2 in cell proliferation was also demonstrated in primary mouse embryo fibroblasts (MEFs) and a murine epidermal cell line, JB6 Cl41 cells (Supplementary Figures 1a–c and 2a–d).

Knockdown of RNF2 induces apoptosis

To determine whether knockdown RNF2 influences cell survival, cell viability was assessed by flow cytometry after Annexin V staining. Results indicated that cells expressing *shRNF2* underwent apoptosis beginning at day 2 after *shRNF2* virus infection and apoptosis continued to increase into day 3 after infection (Figure 2a, Supplementary Figure 3a). Cell cycle analysis showed that RNF2 knockdown cells had increased G1 phase, followed by obvious accumulation of these cells at sub-G1 (Figure 2b, Supplementary Figure 3b). Increased cleavage of caspase-3 and PARP was also detected in RNF2 knockdown cells (Figure 2c). To identify the apoptotic pathway that is activated after knockdown of RNF2, cleavage of caspase-8 and caspase-9 was also assessed. The cleavage of both caspase-8 and caspase-9 indicated that both intrinsic and extrinsic apoptosis pathways were activated (Supplementary Figure 3c). Knockdown of RNF2 also induced apoptosis, cell cycle arrest

and growth inhibition in other cancer cells tested, including HeLa and HepG2 cells (Supplementary Figures 4a–d). These results indicated that RNF2 is critically important for cell survival.

Apoptosis induced by knockdown RNF2 is partially dependent on p53

To elucidate the molecular mechanism as to how knockdown of RNF2 induces apoptosis, we knocked down the expression of RNF2 in HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells, because p53 plays an important role in apoptosis. HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells were infected with *shRNF2 #2* lentivirus and 3 days later apoptosis was detected by flow cytometry after annexin V staining. The results showed that apoptosis was significantly greater in RNF2 knockdown HCT116 p53$^{+/+}$ cells compared to RNF2 knockdown HCT116 p53$^{-/-}$ cells (Figure 3a, Supplementary Figure 5a). Cell cycle analysis also indicated that the sub-G1 population was substantially less in *shRNF2* -infected HCT116 p53$^{-/-}$ cells compared to *shRNF2*- infected HCT116 p53$^{+/+}$ cells (Figure 3b, Supplementary Figure 5b). Further results indicated that cleavage of PARP was also considerably greater in *shRNF2*-infected HCT116 p53$^{+/+}$ cells compared to *shRNF2*-infected HCT116 p53$^{-/-}$ cells (Figure 3c). To further investigate whether the p53 pathway is involved in apoptosis induced by RNF2 knockdown, we examined the expression level of a few p53 targets, and the result showed that p21 and GDF15 were more increased in RNF2 knockdown HCT116 p53$^{+/+}$ cells than in HCT116 p53$^{-/-}$ cells (Figure 3c). Their mRNA level was also examined and results showed that *CDKN1A* and *GDF15* transcription was increased in RNF2 knockdown cells (Figure 3d). These results indicate that apoptosis induced by knockdown of RNF2 is partially dependent on p53. Because RNF2 knockdown also can induce apoptosis in HCT116 p53$^{-/-}$ cells, although to a lesser degree, we concluded that RNF2 knockdown can induce apoptosis in both a p53-dependent and -independent manner.

Knockdown of RNF2 induces p53 up-regulation though increased p53 protein stability

Because knockdown of RNF2 induces p53-dependent apoptosis, we determined whether RNF2 down-regulation could influence p53 protein abundance. After *shRNF2* lentivirus infection, cells were harvested at different times and the level of p53 protein was determined. Results indicated that p53 protein abundance was increased in RNF2 knockdown cells (Figure 4a, Supplementary Figure 6). The increase in p53 protein abundance was also confirmed in RNF2 knockdown HeLa and HepG2 cells (Supplementary Figure 4d). Because RNF2 is a transcriptional repressor, we examined the possibility of whether RNF2 can regulate p53 transcription. Surprisingly, the results of RT-PCR showed that the mRNA level of *p53* was actually decreased in RNF2 knockdown cells (Figure 4a). The p53 protein has a short half-life and its abundance is mainly regulated by its stability. Therefore, we determined whether the increased p53 level in RNF2 knockdown cells was associated with increased stability. The p53 half-life was compared in *shMock* and *shRNF2*#2 lentivirus-infected HCT116 cells and the results confirmed that the half-life of p53 was longer in RNF2 knockdown cells (Figure 4b). Next we examined the ubiquitination of p53 in RNF2 knockdown cells, and the results indicated that p53 ubiquitination was decreased in these cells (Figure 4c). Based on these results, we conclude that RNF2 is important for p53 ubiquitination and stability.

Overexpression of RNF2 promotes p53 degradation

To investigate whether RNF2 directly regulates p53, HCT116 cells were infected with *RNF2* overexpressing lentivirus. The abundance of the p53 protein was detected and was found to be decreased in RNF2-overexpressing cells (Figure 5a). To evaluate whether the decreased p53 level was caused by increased protein degradation, HCT116 cells overexpressing RNF2 were treated with MG132. Results indicated that the decreased p53 protein level could be rescued, indicating that RNF2 overexpression decreased p53 protein abundance through increased protein degradation (Figure 5b). We next compared the half-life of p53 in *Mock*- or *RNF2*-overexpressing HCT116 cells and the results clearly showed that the half-life of p53 was shorter in RNF2-overexpressing cells (Figure 5c). Ubiquitination of p53 was also detected and was increased in RNF2-overexpressing HCT116 cells (Figure 5d). These results confirm that RNF2 is an important regulator of p53 ubiquitination and degradation.

RNF2 directly binds with p53 and MDM2, and promotes MDM2-mediated ubiquitination

Because RNF2 is important for p53 ubiquitination and degradation and RNF2 has been reported to exert E3 ligase activity (7, 22), we determined whether RNF2 directly binds with p53 and acts as an E3 ligase. We found that overexpressed RNF2 and p53 can bind with each other (Figure 6a). In the same overexpression system, we also found that RNF2 binds with both p53 and MDM2 to form a ternary complex (Figure 6b). Notably, the existence of the RNF2/p53/MDM2 complex was also detected under normal conditions (Figure 6c). The next question addressed was whether p53 might be a direct substrate of RNF2 for ubiquitination. This possibility was examined first in an overexpression system, but the results showed that overexpression of RNF2 alone could not induce p53 ubiquitination in 293T cells (Figure 6d). We also found that RNF2 could not mediate p53 ubiquitination *in vitro* (Supplementary Figure 7). This suggests that RNF2 does not directly mediate p53 ubiquitination. MDM2 is a very important E3 ligase that mediates p53 degradation and, therefore, of vital importance for regulating p53 stability (23–24). Therefore, we investigated whether RNF2 might regulate MDM2-mediated p53 ubiquitination. Results showed that when RNF2 was co-transfected, MDM2-mediated p53 ubiquitination was increased (Figure 6d). These data indicate that although RNF2 cannot directly serve as an E3 ligase for p53, it can promote MDM2-mediated p53 ubiquitination.

RNF2 can regulate MDM2 ubiquitination and stability

RNF2 can bind with both p53 and MDM2 to form a ternary complex (Figures 6b and 6c), suggesting that RNF2 might also be a regulator of MDM2. We then confirmed that RNF2 could directly bind with MDM2 after co-transfection in 293T cells (Supplementary Figure 8a). Then we determined whether RNF2 could regulate MDM2 stability. The MDM2 half-life was compared in *shMock* and *shRNF2*#2 lentivirus-infected HCT116 cells and the results showed that the half-life of MDM2 was shorter in RNF2 knockdown cells (Figure 7a). The influence of RNF2 on MDM2 stability was also confirmed in RNF2-overexpressing 293 cells, in which the MDM2 half-life was longer when RNF2 was co-transfected (Figure 7b). Next we examined the ubiquitination of MDM2 in RNF2-overexpressing cells, and results indicated that MDM2 ubiquitination was decreased in RNF2 overexpressing cells

(Figure 7c, Supplementary Figure 8b). These results showed that RNF2 is also an important regulator of MDM2 ubiquitination and stability.

RNF2 can regulate p53 and MDM2 stability during the DNA damage response

The p53 protein and MDM2 play important roles during the DNA damage response. Both the p53 and MDM2 protein levels are increased after DNA damage. To evaluate whether RNF2 is involved in the regulation of p53 and MDM2 during this process, we first compared the p53 and MDM2 protein level in RNF2-overexpressing and control HCT116 cells that were treated with etoposide. Results showed that during etoposide-induced DNA damage, the p53 protein level is dramatically increased in control HCT116 cells, but was not increased very efficiently in RNF2-overexpressing cells. Compared with the control group, no obvious change occurred in the level of phosphorylated p53 (Ser15) in RNF2-overexpressing cells (Figure 7d). We then compared the half-life of p53 and MDM2 and results showed that the MDM2 half-life was longer in cells overexpressing RNF2 (Figure 7e). These findings indicated that RNF2 plays an important role in the regulation of p53 and MDM2 during the DNA damage response.

Discussion

Accumulating evidence suggests that cancer development might involve epigenetic changes, which means that the observed, altered gene expression is caused by DNA or histone modification, without changing the DNA sequence. The polycomb group (PcG) proteins, which function as transcriptional repressors, have an important role in chromatin modification. Recent evidence showed that polycomb group (PcG) proteins are highly expressed in many kinds of tumors, and the expression level is associated with cancer progressiveness, indicating that PcG proteins might have oncogenic functions, but the mechanism as to how they exert their oncogenic function is still elusive.

RNF2 is reportedly highly expressed in many different tumors (7, 20) and is important for cell proliferation, but a clear mechanism is unknown. We studied the oncogenic function of RNF2 and according to our results, when RNF2 expression was knocked down in HCT116 cells, apoptosis occurred. Apoptosis induced by knocking down RNF2 is at least partially dependent on p53 because RNF2 knockdown could induce more apoptosis in HCT116 $p53^{+/+}$ cells compared to HCT116 $p53^{-/-}$ cells. Based on this inverse correlation between RNF2 and p53 observed in both RNF2 knockdown and RNF2-overexpressing cells, we hypothesized that RNF2 is an important regulator of p53. Because the p53 half-life is longer in RNF2 knockdown cells and p53 ubiquitination is decreased in RNF2 knockdown cells (and *vice versa*) (Figures 4b–c, 5c–d), we also suggest that RNF2 is very important for p53 stability and degradation.

Although RNF2 can exert E3 ligase activity, our further study showed that p53 is not a direct RNF2 target for ubiquitination. However our results showed that RNF2 could promote MDM2-mediated p53 ubiquitination (Figure 6d). Because the p53 protein level was also increased in RNF2 knockdown HeLa cells, in which MDM2 has been demonstrated to be inactive, and p53 degradation in HeLa cells is mainly mediated by the HPV E6 oncoprotein, we believe that MDM2-independent p53 regulation by RNF2 also exists. When RNF2

protein abundance was knocked down in HCT116 $p53^{-/-}$ cells, these cells still underwent some apoptosis, although substantially less than that observed in HCT116 $p53^{+/+}$ cells (Figures 3a–c, Supplementary Figures 5a and 5b). In addition, we also knocked down RNF2 in p53 null H1299 cells and results showed that apoptosis also occurred with RNF2 knockdown (data not shown). This supports the idea that a p53 independent pathway might also exist for apoptosis induced by RNF2 knockdown.

When examining the interaction between RNF2, p53 and MDM2, we found that RNF2 can bind with both p53 and MDM2 to form a ternary complex. Therefore, we further determined whether RNF2 might have a function in the regulation of MDM2 stability. Our results showed that RNF2 could increase the half-life of MDM2 and decrease MDM2 ubiquitination (Figures 7a–c). MDM2 and p53 have been shown to play an important role during the DNA damage response. Thus we also investigated whether RNF2 has a role in this process. Our results suggested that RNF2 is also involved in the regulation of p53 and MDM2 during the etoposide-induced DNA damage response (Figures 7d and 7e). Because *Ink4a* has been identified as a RNF2 target in mice, we also wondered whether p53 up-regulation might result from an increased $p14^{ARF}$ level, which has been determined to increase p53 stability by inhibiting the function of MDM2 (25–26). Our results showed that the level of $p14^{ARF}$ was slightly increased in RNF2 knockdown HeLa cells, but was not detectable in HCT116 or HepG2 cells (data not shown).

Recently, another group reported that Bim is up-regulated in Bmi-1 knockdown multiple myeloma cells. Bim deletion could partially rescue apoptosis induced by Bmi-1 loss, suggesting that Bim is one of the targets of Bmi-1 (27). In our experiments, we examined Bim expression, but did not observe its up-regulation in RNF2 knockdown HCT116 cells (data not shown). Down-regulation of Bmi-1 also has been reported to induce apoptosis in cancer cells (28). Interestingly, the p53 pathway has also been reported to be important for apoptosis induced by Bmi-1 down-regulation (29). However, because the protein level of p53 was not changed in Bmi-1 knockdown cells, Bmi-1 and RNF2 might use different mechanisms to regulate the p53 pathway to exert their oncogenic functions. As an important member of PRC1, RNF2 might also be able to inhibit transcription of some genes that are important for cell survival and proliferation. Thus, the identification of other potential targets of RNF2 and other possible mechanisms will be helpful to fully understand the oncogenic function of RNF2. However, herein we present strong evidence showing that RNF2 is very important for cancer cell survival and proliferation. We also provided one possible mechanism explaining the oncogenic function of RNF2. Because RNF2 regulates both p53 and MDM2, it might be an ideal target for cancer therapy and prevention.

Materials and methods

Construction of expression vectors

The *pCDNA3-Xpress-RNF2* plasmid was kindly provided by Dr. Seongman Kang (22) (Korea University, South Korea). The *pLKO.1-shMock* and *pLKO.1-shRNF2 #1, #2* lentivirus plasmids were purchased from Open Biosystem (Huntsville, AL). The *pSin-EF2-Flag-RNF2-puro, pRK5-HA-ubiquitin-WT, pCMV-MDM2, pCMV-Myc-MDM2* and *pCDNA3-Myc-p53* plasmids were constructed or maintained in our laboratory.

Cell culture, etoposide treatment, transfection and infection

The human colon cancer cell lines, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$, were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were grown in McCoy's 5A supplemented with 10% fetal bovine serum (FBS) and antibiotics. 293T, HeLa and HepG2 cells were grown in DMEM supplemented with 10% FBS and antibiotics. All cells were cultured in a 37°C, 5% CO_2 incubator.

HCT116 cells (1×10^6) were seeded in 10-cm dishes and treated with culture medium supplemented with 50 μM etoposide (Sigma-Aldrich, St. Louis, MO) for various time points. Cells were harvested at the indicated time points and cell lysates were prepared and used for Western blotting.

Transfection of plasmid DNA was performed using JetPEI (Polyplus transfection Inc., New York, NY) or LipofectAmine 2000 (Invitrogen, Carlsbad, CA). For lentiviral packaging, the packaging plasmid *psPAX.2*, the envelope plasmid *pMD2.G* and the *pSin-EF2-Flag-RNF2-puro* vector or the *pLKO.1-shMock or pLKO.1-shRNF2* vector were triple transfected into 293T producer cells and the supernatant fraction containing lentiviral particles was harvested at 36 and 60 h, respectively, after transfection. HCT116, HeLa and HepG2 cells were infected in the presence of 8 μg/mL polybrene (Millipore, Billerica, MA).

Antibodies, immunoprecipitation and immunoblotting

The antibodies against caspase-3, PARP, ubiquitin (P4D1) or β-actin were obtained from Cell Signaling Biotechnology, Inc. (Beverly, MA). The Myc-HRP, p53 and p53-HRP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MDM2 and RNF2 antibodies were obtained from Millipore (Billerica, MA) and Abcam (Cambridge, MA), respectively. Anti-Flag M2 was purchased from Sigma-Aldrich (St. Louis, MO). Anti-Rabbit IgG-HRP, anti-Mouse IgG-HRP and anti-Goat IgG-HRP were obtained from Santa Cruz Biotechnology, Inc.

For immunoprecipitation, 293T or HCT116 cells were harvested and lysates were prepared in NP-40 lysis buffer (50 mMTris-HCI [pH 7.4], 0.5% NP-40, 150 mMNaCl) with protease inhibitor cocktail (Roche Diagnostics, Quebec, Canada). Pre-cleared cell lysates were incubated with 1–2 μg of antibody overnight at 4 °C. They were then incubated with protein A/G-agarose (Santa Cruz) and rocked continuously at 4 °C for 2–4 h before 3 washes with NP-40 lysis buffer. Immunoprecipitates were suspended in SDS sample buffer and subjected to SDS-PAGE and Western blotting. For immunoprecipitation (IP) under denaturing conditions, proteins were extracted using regular IP buffer plus 1% SDS and heated at 95 °C for 5 min. The samples were diluted 1:10 in regular IP buffer before IP. The beads were washed, mixed with 6x SDS-sample buffer, boiled and then resolved by 10% SDS-PAGE. The proteins were visualized by immunoblotting.

Proliferation and anchorage-independent growth assay

For proliferation, cells were seeded (1×10^3/well) into 96-well plates and growth was measured using the Cell Titer 96 Aqueous One Solution cell proliferation assay at the

indicated times. For anchorage-independent growth, cells (8 ×10^3/well) were suspended in 1 mL of BME supplemented with 10% FBS and 0.33% agar and plated on 3 mL of solidified BME supplemented with 10% FBS and 0.5% agar in 6-well plates. Colonies were counted at day 5 after being seeded.

In vivo xenograft mouse model

Athymic nude mice [Cr:NIH(S), NIH Swiss nude, 6–8 weeks old] were purchased from Charles River. Animals were maintained under "specific pathogen free" conditions and all animal studies were conducted according to guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee. Animals were acclimated for 2 weeks before the study and had free access to food and water. The animals were housed in climate-controlled quarters with a 12-hour light/12-hour dark cycle. Animals were randomly assigned to two groups. HCT116 cells infected with *shMock* or *shRNF2 #2* lentivirus for 24 h were injected subcutaneously into the right flank of each mouse. Following injection, mice were weighed and tumors were measured by caliper 3 times per week. Tumor volume was calculated from measurements of 3 diameters of the individual tumor according to the following formula: tumor volume (mm^3) = (length × width × height × 0.52).

Flow cytometry analysis

For analysis of cell cycle profile/distribution, cells were fixed in ethanol and stained with propidium iodide before flow cytometry analysis. To determine the level of apoptosis, cells were labeled using the Annexin V FITC Apoptosis Detection kit (Medical & Biological Laboratories, Nagoya, Japan) according to the protocol provided. Briefly, cells were centrifuged, washed once with PBS containing serum, and incubated with Annexin V-conjugated FITC before analysis using a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

RT-PCR, qRT-PCR and primers

To examine the transcription of p53 target genes in RNF2 knockdown cells, total RNA was extracted from HCT116 cells infected with *shMock* or *shRNF2 #2* lentivirus for 36 h or after different time points indicated using the QiagenRNeasy kit (Qiagen) according to the manufacturer's instructions. RNA (3 μg) was reverse transcribed with the Superscript II reverse transcription kit (Invitrogen), cDNA was used as a template for PCRor real-time PCR. Primers used for real time PCR analysis: p21 (forward) 5′-CAGGGGACAGCAGAGGAAGA -3′ and (Reverse) 5′-TTAGGGCTTCCTCTTGGAGAA -3′;GDF15 (forward) 5′-CCCTGCAGTCCGGATACTC -3′ and (Reverse) 5′-GAACAGAGCCCGGTGAAG -3′; β-actin (forward) 5′-TCAAGATCATTGCTCCTCCTG -3′ and (Reverse) 5′-CTGCTTGCTGATCCACATCTG -3′.Primers used for PCR analysis: p53 (forward) 5′-ATGGAGGAGCCGCAGTCAGA-3′ and (Reverse) 5′-AGAAGCCCAGACGGAAACCG-3′; and β-actin (forward) 5′-CCGTGTGAACCATGTGACTT -3′ and (Reverse) 5′-CTAAGTTGCCAGCCCTCCTA -3′.

Cycloheximide chase and *in vivo* p53 or MDM2 ubiquitination assay

To examine p53 or MDM2 protein stability, HCT116 cells were infected with *shMock* or *shRNF2 #2* lentivirus for 36 h, or infected with *Mock* or *RNF2* overexpressing lentivirus for 3 days, or 293T cells were co-transfected with Myc-MDM2 and Flag-RNF2 plasmids for 36h. Cells were then treated with cycloheximide (CHX; 50 μg/mL; Sigma) and harvested at the indicated time points, and cell lysates were prepared and used for Western blotting.

For the *ex vivo* p53 or MDM2 ubiquitination study, 293T cells were transfected with combinations of expression vectors as described above. At 36 h after transfection, cells were treated with MG132 (30 μM) for 4 h before harvest. The proteins were extracted using SDS lysis buffer (50 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT) and boiled for 10 min before cellular debris was removed by centrifugation. The cell lysate was diluted 10 times with NP-40 lysis buffer, and immunoprecipitation was carried out with anti-Myc-Tag. After overnight incubation, protein A/G-agarose (Santa Cruz) was added and the lysate was rotated for 2–4 h before 3washes with NP-40 lysis buffer. Bound proteins were eluted by SDS sample buffer and analyzed by Western blotting.

For the endogenous p53 ubiquitination study, RNF2 knockdown or RNF2 overexpressing HCT116 cells were treated with MG132 (30 μM) for 4 h before harvesting. Proteins were extracted using SDS lysis buffer and immunoprecipitation was conducted with anti-p53. Eluted proteins were analyzed by Western blotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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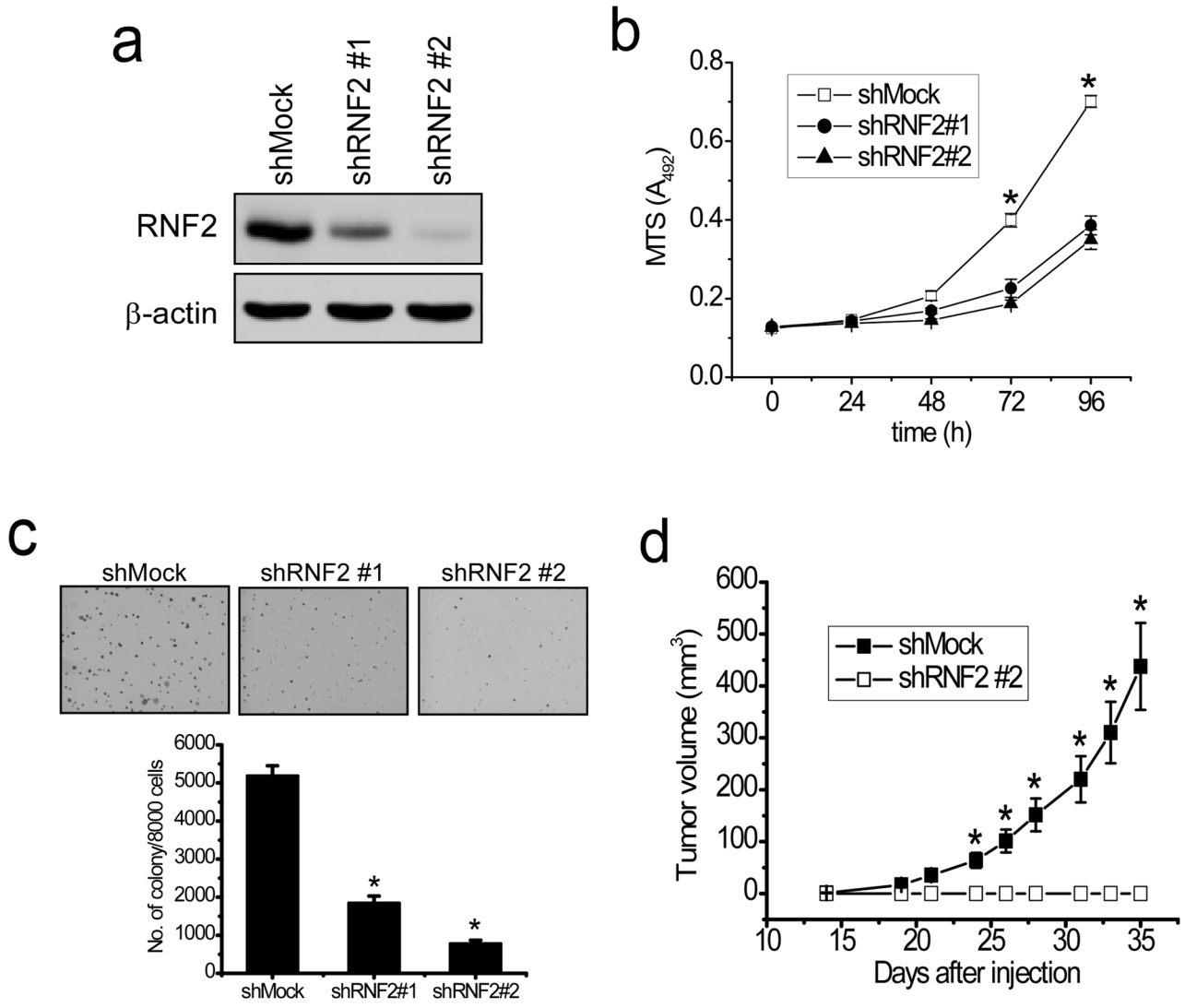

Figure 1.

Knockdown of RNF2 inhibits cell proliferation. (a) Efficient knockdown of RNF2 in HCT116 cell is shown by Western blot. HCT116 cells were infected with *shMock* or *shRNF2* lentivirus and at 2 days after infection, cell lysates were prepared and analyzed by Western blot. (b) Knockdown of RNF2 decreases cell proliferation. HCT116 cells infected with *shMock* or *shRNF2* lentivirus for 24 h were seeded (1×10^3 cells/well) in 96-well plates. Cell proliferation was measured at 24-h intervals up to 96 h. Data are expressed as means ± S.D. from 3 independent experiments, each conducted in triplicate, (*$p < 0.05$ versus *shMock* cells). (c) Anchorage-independent growth is decreased in RNF2 knockdown cells. HCT116 cells infected with *shMock* or *shRNF2* lentivirus for 24 h were grown in soft agar for 5 days. Data are expressed as means ± S.D. from 3 independent experiments, each conducted in triplicate, (*$p < 0.05$ versus *shMock* cells). (d) Knockdown of RNF2 inhibits tumor growth *in vivo*. HCT116 cells infected with *shMock* or *shRNF2* #2 lentivirus for 24 h were injected subcutaneously into the right flank of athymic mice, respectively. Data are expressed as means ± S.E. for each group of mice (n = 15 each).

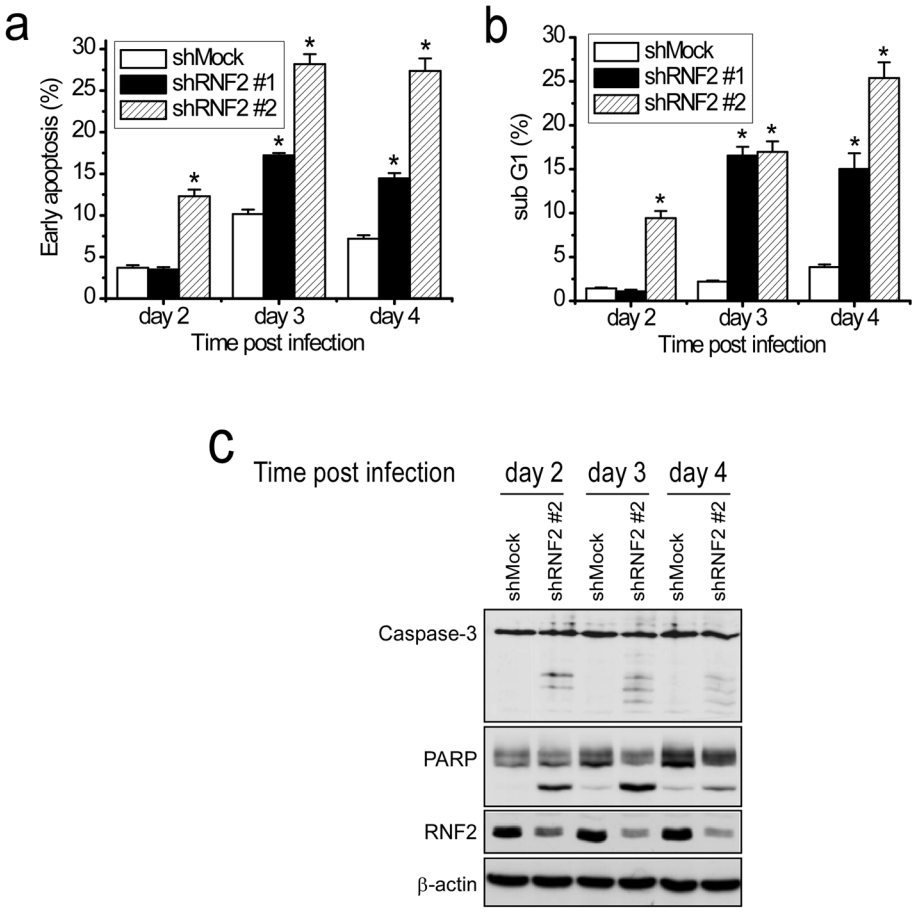

Figure 2.

Knockdown of RNF2 induces apoptosis. (a) Knockdown of RNF2 induces apoptosis in HCT116 cells. HCT116 cells were infected with *shMock* or *shRNF2* lentivirus at different times and apoptosis was analyzed by flow cytometry after Annexin V staining. Data are expressed as means ± S.D. from 3 independent experiments, (*$p < 0.05$ versus *shMock* cells). (b) Knockdown of RNF2 induces sub-G1 accumulation. HCT116 cells were infected with *shMock* or *shRNF2* lentivirus at different times before propidium iodide staining and flow cytometry analysis. Data are expressed as means ± S.D. from 3 independent experiments, (*$p < 0.05$ versus *shMock* cells). (c) Apoptosis of RNF2 knockdown HCT116 cells was detected by cleavage of caspase-3 and PARP. HCT116 cells were infected with *shMock* or *shRNF2* lentivirus and harvested at different times. Cell lysates were analyzed by Western blot and representative blots from 3 independent experiments are shown.

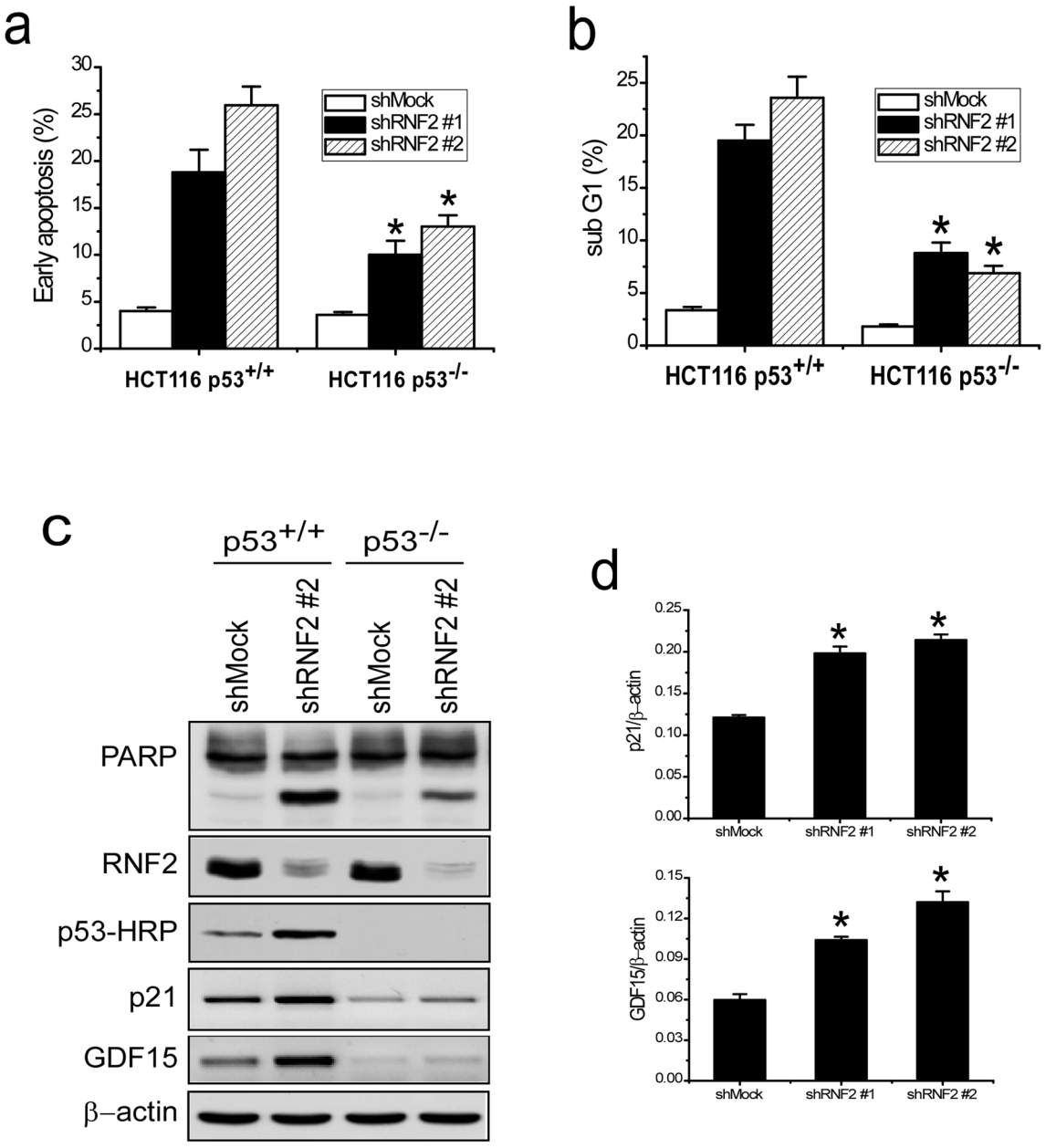

Figure 3.

Apoptosis induced by knocking down RNF2 expression is partially dependent on p53. (a) Knockdown of RNF2 induces more apoptosis in HCT116 $p53^{+/+}$ cells. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were infected with *shRNF2* #2 lentivirus and apoptosis was analyzed by flow cytometry at day 3 after infection. Data are expressed as means ± S.D. from 3 independent experiments, (*$p < 0.05$ versus HCT116 $p53^{+/+}$cells). (b) Knockdown of RNF2 induces more sub-G1 accumulation in HCT116 $p53^{+/+}$ cells. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were infected with *shMock* or *shRNF2* #2 lentivirus and cell cycle phase was assessed by flow cytometry at day 3 after infection. Data are expressed as means ± S.D. from 3 independent experiments, (*$p < 0.05$ versus HCT116 $p53^{+/+}$cells). (c) Knockdown of

RNF2 induces more cleavage of PARP in HCT116 $p53^{+/+}$ cells. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were infected with *shMock* or *shRNF2* #2 lentivirus and harvested at day 3 after infection. Cell lysates were analyzed by Western blot and representative blots from 3 independent experiments are shown. (d) qRT-PCR shows the up-regulation of typical p53-targeted genes in RNF2 knockdown HCT116 cells. HCT116 cells were infected with *shMock* or *shRNF2* #2 lentivirus, cells were harvested at 48h after infection. RNA was extracted and qRT-PCR was performed to detect the mRNA level of *p21*, *GDF15* and *β-actin*. Data are representative of 3 independent experiments.

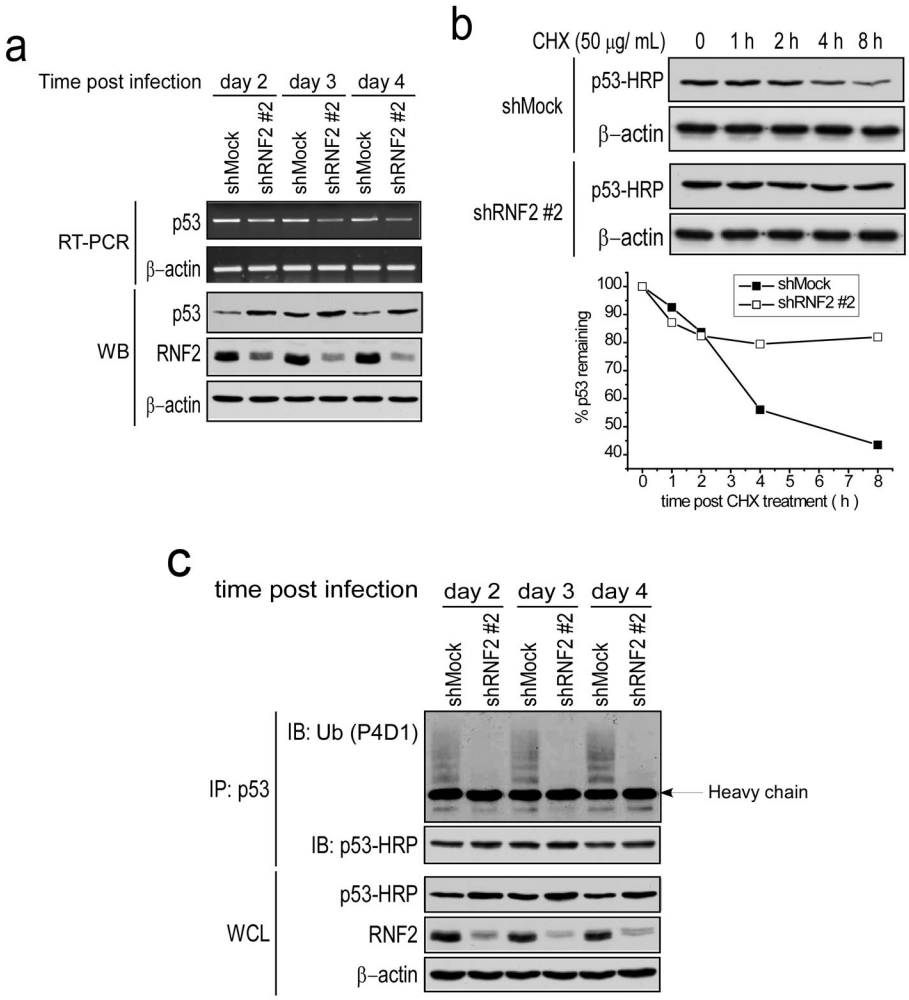

Figure 4.

Knockdown of RNF2 induces p53 up-regulation through increased p53 protein stability. (a) RNF2 knockdown cells exhibit increased p53 protein levels. HCT116 cells were infected with *shMock* or *shRNF2* #2 lentivirus and harvested at different times. Cell lysates were analyzed by Western blot. RNA was also extracted and RT-PCR was performed to detect the mRNA level. Data are representative of 3 independent experiments. (b) Knockdown of RNF2 increases the half-life of p53. HCT116 cells were infected with *shMock* or *shRNF2* #2 lentivirus for 36 h and then treated with CHX (50 μg/mL). Cells were harvested at different time points and lysates were used to detect p53. The graph (*lower panel*) shows the percentage of remaining p53 protein level after CHX treatment. (c) Knockdown of RNF2 decreases p53 ubiquitination. HCT116 cells were infected with *shMock* or *shRNF2* #2 lentivirus for different times and then treated with MG132 (30 μM) for 4 h. Cells lysates were prepared by using SDS lysis buffer and p53 proteins were immunoprecipitated to detect ubiquitination. The expression levels of p53 and RNF2 were also detected by Western blot.

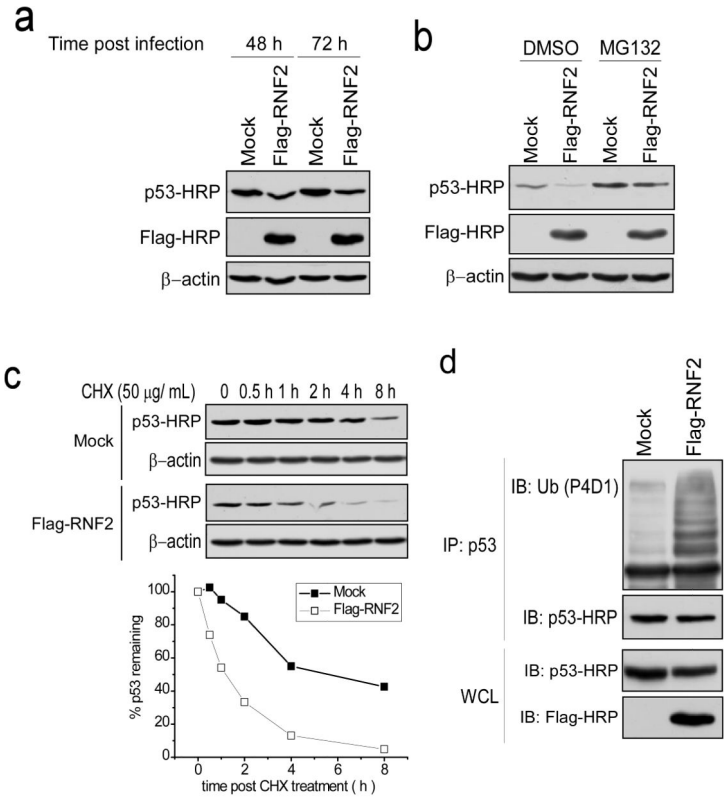

Figure 5.

Overexpression of RNF2 promotes p53 ubiquitination and degradation. (a) Overexpression of RNF2 decreases the endogenous level of the p53 protein. HCT116 cells were infected with *Mock* or *pSin-EF2-Flag-RNF2* lentivirus for 48 or 72 h and cell lysates were analyzed by Western blot. (b) MG132 rescues the decreased level of p53 protein induced by RNF2 overexpression. HCT116 cells were infected with *Mock* or *pSin-EF2-Flag-RNF2* lentivirus. At 72 h after infection, cells were treated with MG132 (30 μM) for 4 h and cell lysates were analyzed by Western blot. (c) Cells overexpressing RNF2 exhibit a decreased p53 half-life. HCT116 cells were infected with *Mock* or *Flag-RNF2* overexpressing lentivirus for 72 h and then treated with CHX (50 μg/mL). The level of p53 was detected by Western blot. The graph (*lower panel*) shows the percentage of remaining p53 protein after CHX treatment. (d) Cells overexpressing RNF2 show increased p53 ubiquitination. HCT116 cells were infected with *Mock* or *Flag-RNF2* overexpressing lentivirus for 72 h and then treated with MG132 (30 μM) for 4 h. Cell lysates were prepared in SDS lysis buffer and p53 proteins were immunoprecipitated to detect ubiquitination.

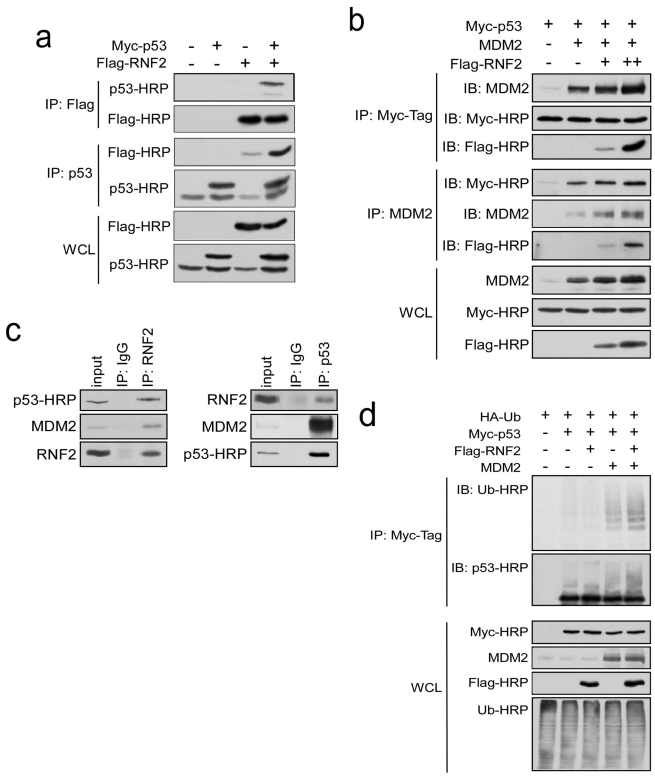

Figure 6.

RNF2 directly binds with both p53 and MDM2, and can promote MDM2-mediated p53 ubiquitination. (a) Exogenous RNF2 interacts with p53. 293T cells were co-transfected with *pSin-EF2-Flag-RNF2* and *pCDNA3-Myc-p53*. At 36 h after transfection, cells lysates were used for immunoprecipitation and immunoblotting. (b) Exogenous RNF2 binds with both p53 and MDM2 to form a ternary complex. 293T cells were co-transfected with *pSin-EF2-Flag-RNF2*, *pCMV-MDM2*and *pCDNA3-Myc-p53*. At 36 h after transfection, cells lysates were used for immunoprecipitation and immunoblotting. (c) Endogenous RNF2 binds with both p53 and MDM2. HCT116 cell lysates were used for immunoprecipitation and immunoblotting. (d) RNF2 can promote MDM2-mediated p53 ubiquitination *ex vivo*. 293T cells were co-transfected with *pRK5-HA-ubiquitin-WT*, *pCMV-MDM2*, *pCDNA3-Myc-p53* and *pSin-EF2-Flag-RNF2* plasmids. At 36 h after transfection, cells were treated with MG132 (30 μM) for 4 h. Cell lysates were prepared in SDS lysis buffer and used for immunoprecipitation and immunoblotting.

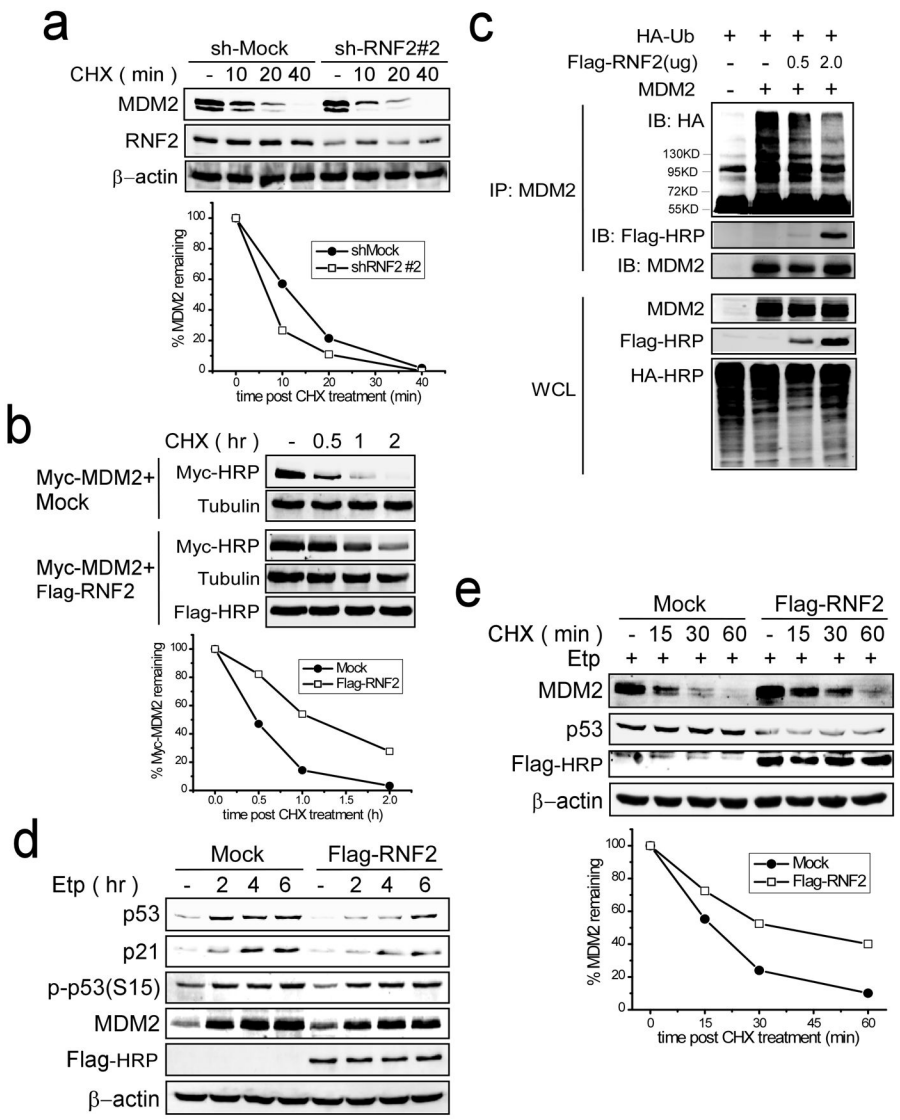

Figure 7.

RNF2 can regulate p53 and MDM2 stability during the DNA damage response. (a) Knockdown of RNF2 decreases the half-life of MDM2. HCT116 cells were infected with *shMock* or *shRNF2* #2 lentivirus for 48 h and then treated with CHX (50 μg/mL). Cells were harvested at different time points and lysates were used for Western blot analysis. (b) Cells overexpressing RNF2 exhibit an increased MDM2 half-life. 293 cells were co-transfected with *pCMV-Myc-MDM2* and *pSin-EF2-Flag-RNF2* or control vector. At 36 h after transfection, cells were treated with CHX (50 μg/mL). Cells were harvested at different time points and lysates were used for Western blot analysis. (c) RNF2 overexpression can decrease MDM2 ubiquitination *ex vivo*. 293T cells were co-transfected with *pRK5-HA-ubiquitin-WT, pCMV-MDM2* and *pSin-EF2-Flag-RNF2* plasmids. At 36 h after transfection, cells were treated with MG132 (30 μM) for 4 h. Cell lysates were prepared in 0.1% SDS lysis buffer and used for immunoprecipitation and immunoblotting. (d) The level of p53 in cells overexpressing RNF2 is not increased as efficiently as in control cells. HCT116 cells

were infected with *Mock* or *pSin-EF2-Flag-RNF2* lentivirus for 72 h, and then treated with 50 μM etoposide for various times before cell lysates were prepared and used for Western blotting. (e) MDM2 shows a decreased half-life in RNF2 overexpressing cells during the DNA damage response induced by etoposide. HCT116 cells were infected with *Mock* or *pSin-EF2-Flag-RNF2* lentivirus for 72 h and then treated with 50 μM etoposide for 4 h before further treatment with CHX (50 μg/mL). Cells were harvested at different time points and lysates were used for Western blotting.