

ORIGINAL ARTICLE

FKBP12 enhances sensitivity to chemotherapy-induced cancer cell apoptosis by inhibiting MDM2

T Liu^{1,3}, J Xiong^{2,3}, S Yi¹, H Zhang¹, S Zhou², L Gu¹ and M Zhou¹

The FK506-binding protein 12 (FKBP12) is a cytoplasmic protein and has been reported to possess multiple functions in signaling transduction based on its interaction with different cellular targets. Here, we report that FKBP12 interacts with oncoprotein MDM2 and induces MDM2 degradation. We demonstrate that FKBP12 degrades MDM2 through binding to MDM2 protein, disrupting MDM2/MDM4 interaction and inducing MDM2 self-ubiquitination. The FKBP12-mediated MDM2 degradation was significantly enhanced when the transfected MDM2 was localized in the cytoplasm. The endogenous MDM2, when it was induced by p53 subjecting to DNA-damaging stimuli such as treatment with doxorubicin, was also significantly inhibited by FKBP12. This is due to translocation of p53-induced MDM2 from the nucleus to the cytoplasm, which facilitates interaction with cytoplasmic FKBP12. Furthermore, the enhanced level of MDM2 following p53 activation in nutlin-3 treated cells was also inhibited by FKBP12. The FKBP12-mediated downregulation of MDM2 in response to doxorubicin or nutlin-3 results in continuing and constitutive activation of p53, inhibition of XIAP and sensitization of cancer cells to apoptosis. These results identify a novel function for FKBP12 in downregulating MDM2, which directly enhances sensitivity of cancer cells to chemotherapy and nutlin-3 treatment.

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INTRODUCTION

FK506-binding protein 12 (FKBP12) is a 12 kDa enzyme which catalyzes the peptidyl prolyl *cis-trans* isomerization. FKBP12 is the smallest and most extensively studied protein among the 15 identified human FKBP. This protein consists of 108 amino acids with one single FKBP domain and is abundantly and ubiquitously expressed mainly in cytosol.^{1–3}

It was initially characterized that FKBP12 has the ability to bind the immunosuppressants FK506 and rapamycin. Binding of FKBP12 to FK506 and calcineurin forms a ternary complex to inhibit the serine/threonine phosphatase activity of calcineurin, which is important for several cellular processes such as T-cell activation.^{4,5} The complex of FKBP12 and rapamycin interacts mTOR (mammalian target of rapamycin) and inhibits its roles in regulating cancer progression and cell growth.^{6,7}

In addition, FKBP12 exhibits many other functions which involve binding to different cellular receptors or targets. For example, in the absence of FK506, FKBP12 binds to the ryanodine receptor, which is one of the major calcium-release channels in the sarcoplasmic and endoplasmic reticula. Interaction between FKBP12 and ryanodine receptor stabilizes the ryanodine receptor channel and modulates channel gating, leading to increased channel conductance levels and mean open-time.⁸ FKBP12 has also been shown to interact with transforming growth factor- β type I receptor to inhibit receptor-mediated signal transduction.⁹ Furthermore, FKBP12 has an inhibitory effect on the cellular activity of epidermal growth factor receptor by modulating the receptor's phosphorylation status.¹⁰

The human *MDM2* gene is an oncogene that is amplified or overexpressed in many sorts of human cancers.^{11,12}

Overexpression of MDM2 in cancer patients is associated with tumor progression and a poor treatment outcome. The oncogenic function of MDM2 protein is mainly through inhibition of the p53 tumor suppressor,^{13,14} hence, p53 function becomes inactivated in MDM2-overexpressing cells, leading to aberrant cell proliferation and growth. The MDM2 gene is itself a transcription target of p53, so MDM2 and p53 form a negative-feedback loop.^{15,16} When cells are in normal growing or in unstressed status, the MDM2-p53 negative-feedback loop keeps intact.¹⁷ However, when cells are subject to DNA-damaging stimuli such as exposure to radiation or treatment with certain chemotherapeutic drugs, p53 is rapidly accumulated and activated, resulting in increased transcription of MDM2. The p53-induced MDM2 expression will, in turn, inhibit p53, which we believe is an important mechanism for the development of resistance to radiation and chemotherapy, particularly for those cancers having a wild-type p53 phenotype and overexpressing MDM2.

MDM2 is an unstable protein that is degraded by a self-catalytic mechanism. MDM2, a member of the RING-finger-type family of E3 ubiquitin ligases, is also a substrate of its own RING domain E3 ligase; thus, MDM2 regulates ubiquitination of not only p53 but also MDM2 itself.^{18,19} The E3 ligase activity responsible for MDM2 self-ubiquitination is regulated by many cellular signaling pathways and molecular events. For instance, MDM4, a homolog of MDM2, binds to the RING domain of MDM2 and decreases the E3 ligase activity for MDM2 self-ubiquitination, resulting in increased MDM2 protein stabilization.^{20–22} Nucleic acids, such as polyA or polyG, and certain cellular small RNA such as XIAP IRES can also bind to the RING domain of MDM2 and suppress its self-ubiquitination activity.^{23,24} In contrast, the MDM2

¹Department of Pediatrics, Division of Hematology/Oncology, Aflac Cancer Center and Blood Disorders Service, Emory University School of Medicine, Atlanta, GA, USA and

²Institute of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Correspondence: Dr L Gu or Dr M Zhou, Department of Pediatrics, Division of Hematology/Oncology, Emory University School of Medicine, 1760 Haygood Drive, Atlanta, GA 30322, USA.

E-mail: mzhou@emory.edu or lgu@emory.edu

³These authors contributed equally to this work.

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self-ubiquitination and degradation is stimulated by FKBP25, although the mechanism and biological significance of FKBP25-mediated regulation remains unclear.²⁵

In this study, we demonstrate that FKBP12 can also stimulate MDM2 self-ubiquitination and degradation. Our discovery of an interaction between FKBP12 and MDM2 was based on our fortuitous observation of FKBP12 binding to MDM2, made in the course of studies of MDM2 regulation. This prompted us to further characterize the consequences of this binding, including the mechanism of action of FKBP12 in regulating MDM2 protein stability. In addition, we have explored the cellular consequences of this regulation in the response of cancer cells to treatment with doxorubicin and nutlin-3.

RESULTS

MDM2 is negatively regulated by FKBP12

To determine whether there is a link between FKBP12 and MDM2, we first tested four neuroblastoma cell lines (SH-EP1, NB-1691, SK-N-SH and NB-1643) and three lung cancer cell lines (PC9, BEAS-2B and A549) as well as HEK293 cells for expression levels of FKBP12 and MDM2. Western blot assay showed that SH-EP1, NB-1691 and A549 expressed lower level of FKBP12 and higher level of MDM2. In contrast, cell lines SK-N-SH, NB-1643, PC9, BEAS-2B and HEK293 expressed high level of FKBP12 and a relatively low level of MDM2 (Figure 1a), suggesting that high levels of FKBP12 are correlated with low levels of MDM2.

We next performed gene transfection of FKBP12 in SH-EP1, NB-1691 and A549. Enforced overexpression of FKBP12 resulted in downregulation of MDM2 in these cell lines (Figure 1b). This FKBP12-regulated downregulation of MDM2 is dose-dependent (Figure 1c). In order to rule out the possibility that the reduced

level of MDM2 is due to loss of antibody interaction using anti-MDM2 antibody SMP14, when MDM2 is phosphorylated,²⁶ we used both MDM2 antibodies SMP14 and 4B11 without loss of antibody reactivity to phosphorylated MDM2²⁶ in western blotting. The 4B11 antibody detected a similar reduced level of MDM2 as SMP14 in same FKBP12-transfected cells, suggesting that FKBP12 indeed downregulates MDM2. In addition, we used siRNA specifically for FKBP12 to inhibit the expression of FKBP12 in cell lines SK-N-SH and NB-1643. Silencing of FKBP12 led to upregulation of MDM2 (Figure 1d). Furthermore, we performed MDM2 transfection and knockdown in SK-N-SH and SH-EP1 cells, respectively, and no alteration of FKBP12 expression was detected in these MDM2 transfected or silenced cells (data not shown). These results clearly indicate that FKBP12 has a role in negatively regulating MDM2.

FKBP12 binds to MDM2 C-terminal RING domain

As FKBP12 regulates its cellular targets through protein-protein interaction, we tested whether FKBP12 binds to MDM2. We performed co-IP and western blot assays using NB-1643 and BEAS-2B cells, and results showed that FKBP12 and MDM2 bind with each other (Figure 2a). In contrast, no binding activity was detected between FKBP12 and MDM4. Because the C-terminal RING domain of MDM2 has critical role in regulating MDM2 self-degradation, we hypothesized that FKBP12 most likely binds to the MDM2 RING domain to regulate its function. To test our hypothesis, we performed pull-down experiments. As expected, FKBP12 bound to the MDM2 RING domain (Figure 2b). The binding between FKBP12 and MDM2 RING domain was further confirmed by ITC analysis, which showed a binding K_d of 1.94 μM (Figure 2c). No binding activity between FKBP12 and MDM2 with the RING domain deletion was detected (Figures 2b and d).

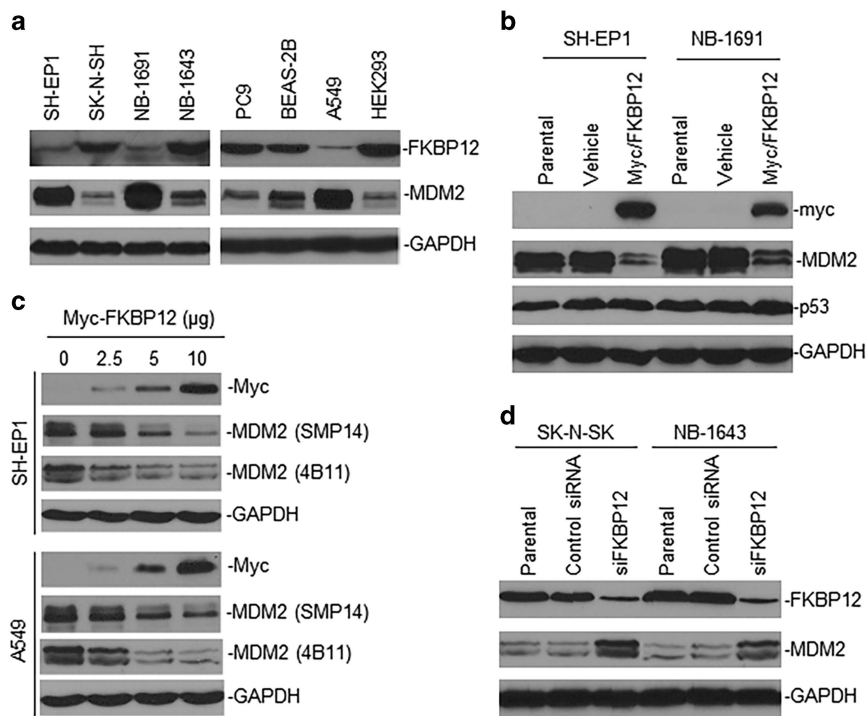


Figure 1. Inhibition of MDM2 expression by FKBP12 in cancer cells. (a) the levels of MDM2 and FKBP12 expression in 4 neuroblastoma (SH-EP1, SK-N-SH, NB-1691 and NB-1643), 3 lung cancer (PC9, BEAS-2B and A549) and HEK293 cell lines were detected by western blot assay. (b) Western blot results show the expression of transfected FKBP12 (with anti-Myc antibody) and endogenous MDM2 in cell lines as indicated. (c) Dose-response expression of MDM2 after transfection of FKBP12 in SH-EP1 and A549 cells. (d) expression of MDM2 in SK-N-SH and NB-1643 cell lines transfected with siFKBP12 or control siRNA, as detected by western blot assay. MDM2 antibodies used in a, b and d are either SMP14 or 2A10 (not indicated).

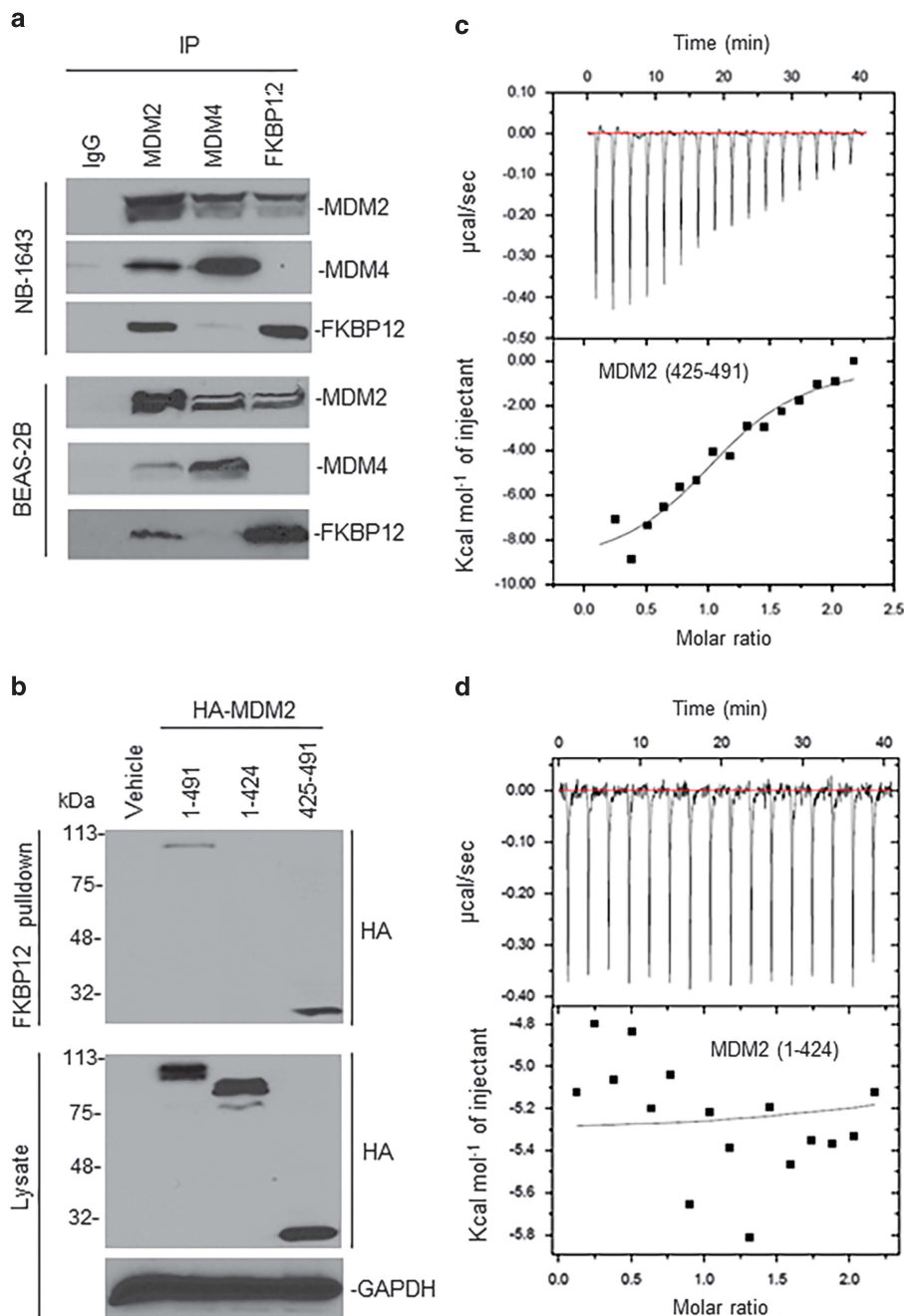


Figure 2. Binding of FKBP12 to MDM2 C-terminal RING domain. **(a)** co-IP and western blot assays using antibodies as indicated for binding between endogenous FKBP12 and MDM2 in NB-1643 and BEAS-2B cells. **(b)** Pull-down assays for binding between FKBP12 and transfected MDM2. GST-FKBP12 fusion protein was incubated with lysates of SK-N-SH cells transfected with full-length MDM2 (1–491) and MDM2 fragments 1–424 and 425–491 (RING domain) tagged with HA. Bead-purified proteins and proteins in cell lysates were detected by western blot with anti-HA antibody. **(c and d)** Confirmation of binding between FKBP12 and MDM2 (425–491) **(c)** and non-binding between FKBP12 and MDM2 (1–424) **(d)** as detected by ITC. The upper box shows the raw heating power over time and the lower box shows integrated energy values, normalized for each injection. All experiments were performed at least three times.

FKBP12 induces MDM2 self-ubiquitination and degradation
 Since FKBP12 binds to the MDM2 RING domain, we tested whether FKBP12 has a role in inducing MDM2 self-ubiquitination. We performed FKBP12 transfection and subsequently IP-western blot assay in SH-EP1 cells, and results showed that increased expression of FKBP12 resulted in enhanced self-ubiquitination of MDM2 (Figure 3a). In addition, we performed *in vivo* ubiquitination assay in SK-N-SH cells by co-transfection of His-Ub, FKBP12 and MDM2. Enforced expression of FKBP12 increased self-ubiquitination of MDM2 (Figure 3b). The FKBP12-regulated

MDM2 self-ubiquitination was inhibited in cells transfected with MDM2 C464A mutation. Furthermore, we tested the turnover of MDM2 protein by a CHX pulse-chase assay and found that FKBP12 induced MDM2 degradation. The half-life of MDM2 was > 60 min in control cells, whereas it decreased to < 60 min in FKBP12-transfected cells.

It has been demonstrated that MDM2 becomes stable when MDM4 binds to it. Therefore, we tested whether binding of FKBP12 to MDM2 disturbs the formation of the MDM2–MDM4 heterodimer, resulting in instability of MDM2. We performed co-IP

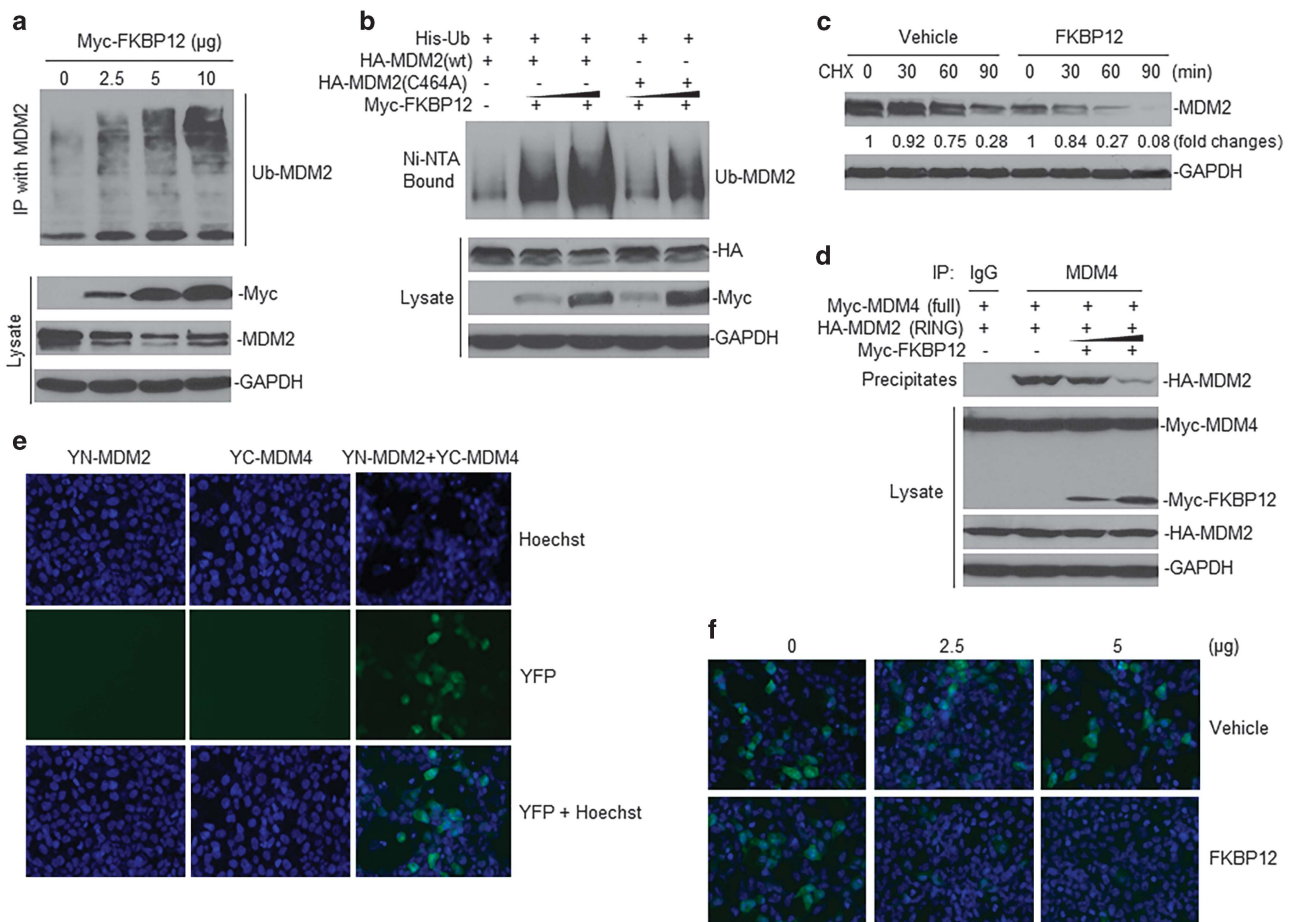


Figure 3. Effects of FKBP12 on MDM2 self-ubiquitination, protein stability and the interaction between MDM2 and MDM4. **(a)** SH-EP1 cells were transfected with different doses of FKBP12, as indicated. Cell lysates were immunoprecipitated with MDM2 antibody, and western blot performed for expression of ub-MDM2 with anti-ub antibody. The lower panel shows protein expression as detected by western blot. **(b)** *In vivo* ubiquitination assay for testing effect of FKBP12 on self-ubiquitination of transfected MDM2 (wt and mutation C464A). SK-N-SH cells were co-transfected with 5 µg of either MDM2 (wt) or MDM2 (C464A) and increasing concentrations of FKBP12 (1 and 5 µg). Proteins in Ni-NTA bound and cell lysate were detected by western blot. **(c)** Protein turnover of MDM2 in SH-EP1 cells transfected with FKBP12 and control plasmid (vehicle), as detected by CHX pulse-chase assay. Numerical labels under each band of MDM2 represent the expression levels after normalization for GAPDH, compared with untreated (0) samples (defined as 1 unit). **(d)** Effect of FKBP12 on MDM2–MDM4 interaction. SK-N-SH cells transfected as indicated were IP with anti-MDM4 antibody and precipitated MDM2 was detected with western blot using anti-HA antibody. Proteins were detected by western blot. **(e and f)** Representative photographs of YFP fluorescence signal in SK-N-SH cells co-transfected with YFP N/C fragments fused to the MDM2 RING and MDM4 RING domain (YN-MDM2 and YC-MDM4), respectively **(e)**, in the absence and presence of different amount of FKBP12 **(f)**.

and western blot assay in SK-N-SH cells co-transfected with MDM2, MDM4 and FKBP12. Results from this experiment showed that FKBP12 reduced the binding between MDM2 and MDM4 (Figure 3d). To confirm the role of FKBP12 in preventing MDM2–MDM4 interaction in living cells, we performed a bimolecular fluorescence complementation assay by fusing the MDM2 RING domain (425–491) and MDM4 RING domain (421–490) to the N- (1–154) and C- (155–238) terminal halves of YFP, respectively and transfecting the two constructs into cells. Transfection of YN-MDM2 RING or YC-MDM4 RING alone did not generate a signal, whereas co-transfection of the two constructs produced strong fluorescence in SK-N-SH cells due to interaction between MDM2 and MDM4 leading to a reconstitution of YN and YC (Figure 3e). The fluorescence generated by the interaction of the YN-MDM2 RING and YC-MDM4 RING significantly decreased in the presence of FKBP12 (Figure 3f), indicating that binding of FKBP12 to MDM2 prevents the formation of MDM2/MDM4 heterodimers.

Downregulation of MDM2 by FKBP12 activates p53 and inhibits XIAP

We evaluated the effect of FKBP12-mediated degradation of MDM2 on p53 activation in cancer cells. We performed FKBP12 transfection along with doxorubicin treatment in SH-EP1 cells and tested expression of MDM2 and activation of p53 as well as the p53-targeted genes p21 and PUMA. We found that doxorubicin-induced initial downregulation of MDM2 at about 1 h after treatment, followed by a mild upregulation between 3–8 h in control-transfected SH-EP1 cells. In contrast, MDM2 was remarkably and constitutively downregulated by doxorubicin in FKBP12-transfected SH-EP1 cells (Figure 4a). As also shown in Figure 4a, doxorubicin induced much higher activation of p53 and its target genes p21 and PUMA in FKBP12-transfected SH-EP1 cells than in control SH-EP1 cells. Interestingly, doxorubicin markedly reduced expression of the anti-apoptotic factor XIAP in FKBP12-transfected SH-EP1 cells, but not in control SH-EP1. Corresponding with

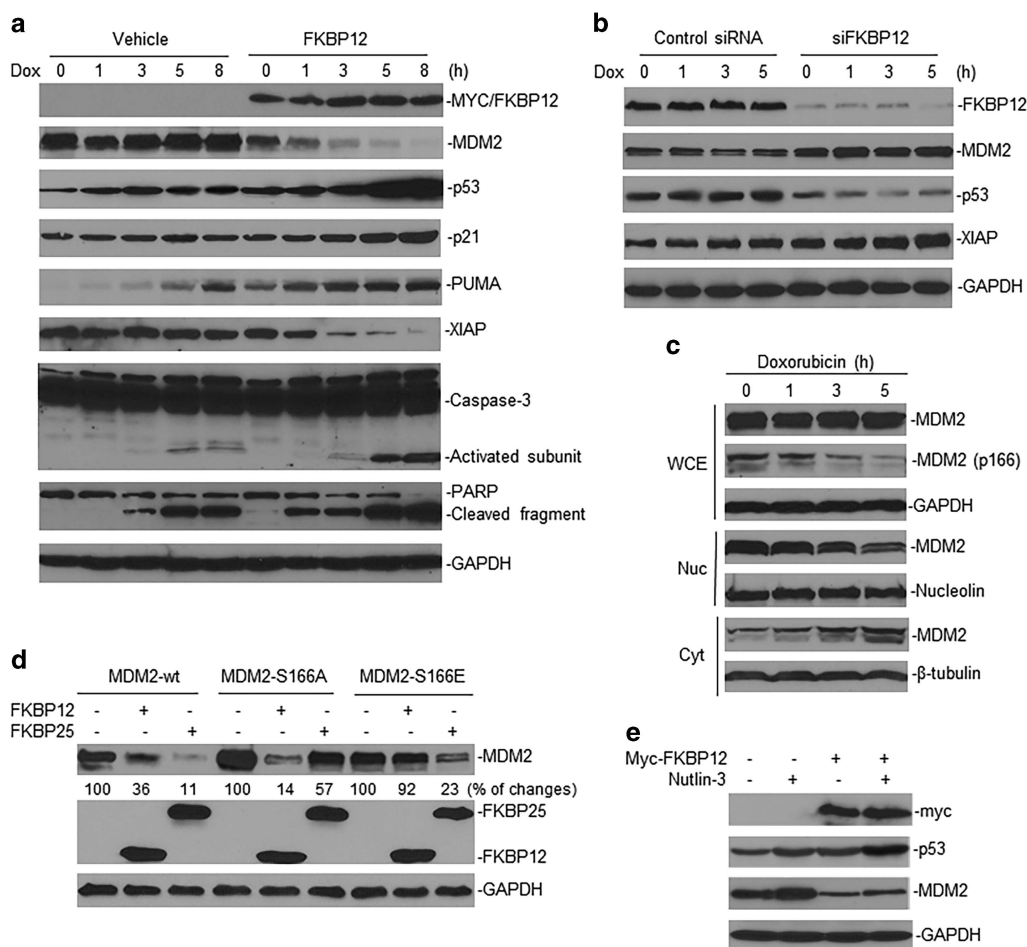


Figure 4. Effects of FKBP12 on MDM2 with different cellular localization, activation of p53 and expression of XIAP in response to doxorubicin treatment. **(a)** Protein expression in FKBP12-transfected or control (vehicle)-transfected SH-EP1 cells treated with 1 μM doxorubicin for different times was detected by western blot. **(b)** Western blot assay for expression of proteins in SK-N-SH cells transfected with siFKBP12 or control siRNA in treatment with 1 μM doxorubicin for different times as indicated. **(c)** SH-EP1 cells were treated with 1 μM doxorubicin for different times as indicated, and cell extracts were tested for the nuclear (Nuc) and cytoplasmic (Cyt) expression of MDM2 as well as for total MDM2 and MDM2 phosphorylation at site 166 in whole-cell extract (WCE) in western blot assays. **(d)** Differential downregulation of the cytoplasmic and nuclear MDM2 by FKBP12 and FKBP25. SK-N-SH cells were co-transfected with either FKBP12 or FKBP25 and different forms of MDM2 (wt, S166A and S166E) and transfected proteins were detected by western blot. **(e)** SH-EP1 cells transfected with FKBP12 were treated with nutlin-3 (10 μM for 24 h), and the expression of proteins as indicated was detected by western blot.

regulation of XIAP, higher activation of caspase-3 and cleavage of death substrate PARP was observed in FKBP12-transfected SH-EP1 than in control SH-EP1 after similar treatment of doxorubicin (Figure 4a).

In addition, we silenced FKBP12 using siRNA in SK-N-SH cells and tested expression of MDM2, p53 and XIAP in response to doxorubicin. Doxorubicin increased MDM2 in SK-N-SH/siFKBP12 cells but not in SK-N-SH/control siRNA cells. In contrast, doxorubicin induced higher activation of p53 in SK-N-SH/control siRNA than in SK-N-SH/siFKBP12. Consistent with increased MDM2 expression, upregulation of XIAP was detected in SK-N-SH/siFKBP12 cells but not in SK-N-SH/control siRNA cells (Figure 4b). Taken together, these results suggest that FKBP12 has a role in degrading and downregulating MDM2 in doxorubicin-treated cells, leading to activation of p53 and inhibition of XIAP.

In order to further investigate the mechanism by which FKBP12 downregulates MDM2 in response to doxorubicin-induced DNA damage, we examined the effect of enforced FKBP12 expression on the levels of nuclear and cytoplasmic MDM2. We have previously demonstrated that cellular stress and DNA damage

triggered by irradiation results in dephosphorylation and translocation of MDM2 from the nucleus to the cytoplasm.²⁷ As FKBP12 is a cytoplasmic protein, we hypothesize that FKBP12 efficiently degrades MDM2 when MDM2 is expressed in cytoplasm. Consistent with the response to irradiation, doxorubicin treatment of SH-EP1 cells induced export of nuclear MDM2 to the cytoplasm, accompanied by downregulation of MDM2 at serine 166 (Figure 4c). To further confirm that cytoplasmic MDM2 is more efficiently degraded by FKBP12, we performed co-transfection of FKBP12 or FKBP25 (control) and MDM2 S166A, which cannot be phosphorylated by PI3K/Akt; thus, it remains in the cytoplasm.²⁸ In this manner we were able to test whether FKBP12 induces a greater inhibition of cytoplasmic MDM2 S166A than it does with either wt-MDM2 or MDM2-S166E, which mimics phosphorylated MDM2 and is predominantly localized in the nucleus.²⁸ As expected, the degree of MDM2 S166A inhibition by FKBP12 was much greater than that seen with either wt-MDM2 or MDM2-S166E. As shown in Figure 4d, FKBP12 induced 86% reduction in MDM2-S166A, compared with 64% reduction in wt-MDM2 and an 8% reduction in S166E. In contrast, FKBP25, which is expressed in

the nucleus,²⁹ induced greater degradation of wt-MDM2 and MDM2-S166E than of MDM2 S166A.

Furthermore, we evaluated the effect of FKBP12 on nutlin-3 activity inducing p53 through blocking the interaction between MDM2 and p53. As expected, nutlin-3 induced activation of p53 and subsequently increased MDM2 expression in SH-EP1 cells. The increased MDM2 expression following activation of p53 by nutlin-3 was inhibited in same SH-EP1 cells with FKBP12 transfection (Figure 4e).

FKBP12 sensitizes cancer cells to doxorubicin and nutlin-3

As FKBP12 preferentially degrades cytoplasmic MDM2 following its translocation from the nucleus to the cytoplasm in response to doxorubicin, we tested whether the expression level of FKBP12 confers sensitivity to doxorubicin-induced cell growth inhibition and apoptosis. We performed clonogenic assays of SH-EP1

transfected with FKBP12 and SK-N-SH transfected with siFKBP12 in the presence of doxorubicin. We observed that there were no differences in colony formation between cells transfected with FKBP12 or siFKBP12 and cells transfected with control plasmids in the absence of doxorubicin. In the presence of doxorubicin, colony formation was greatly inhibited in FKBP12-transfected SH-EP1 as compared with control-transfected SH-EP1. In contrast, there was less inhibition of colony formation in siFKBP-transfected SK-N-SH than in control siRNA-transfected SK-N-SH after doxorubicin treatment (Figure 5a).

In addition, we measured the cytotoxic and apoptotic effect of doxorubicin on FKBP12-transfected SH-EP1 and siFKBP12-transfected SK-N-SH cells. Consistent with its growth inhibitory activity, doxorubicin exhibited greater cytotoxic and apoptotic effects on FKBP12-transfected SH-EP1 as compared with control-transfected SH-EP1, as detected by WST (Figure 5b) and flow cytometry (Figure 5d) assays respectively. Similarly, doxorubicin

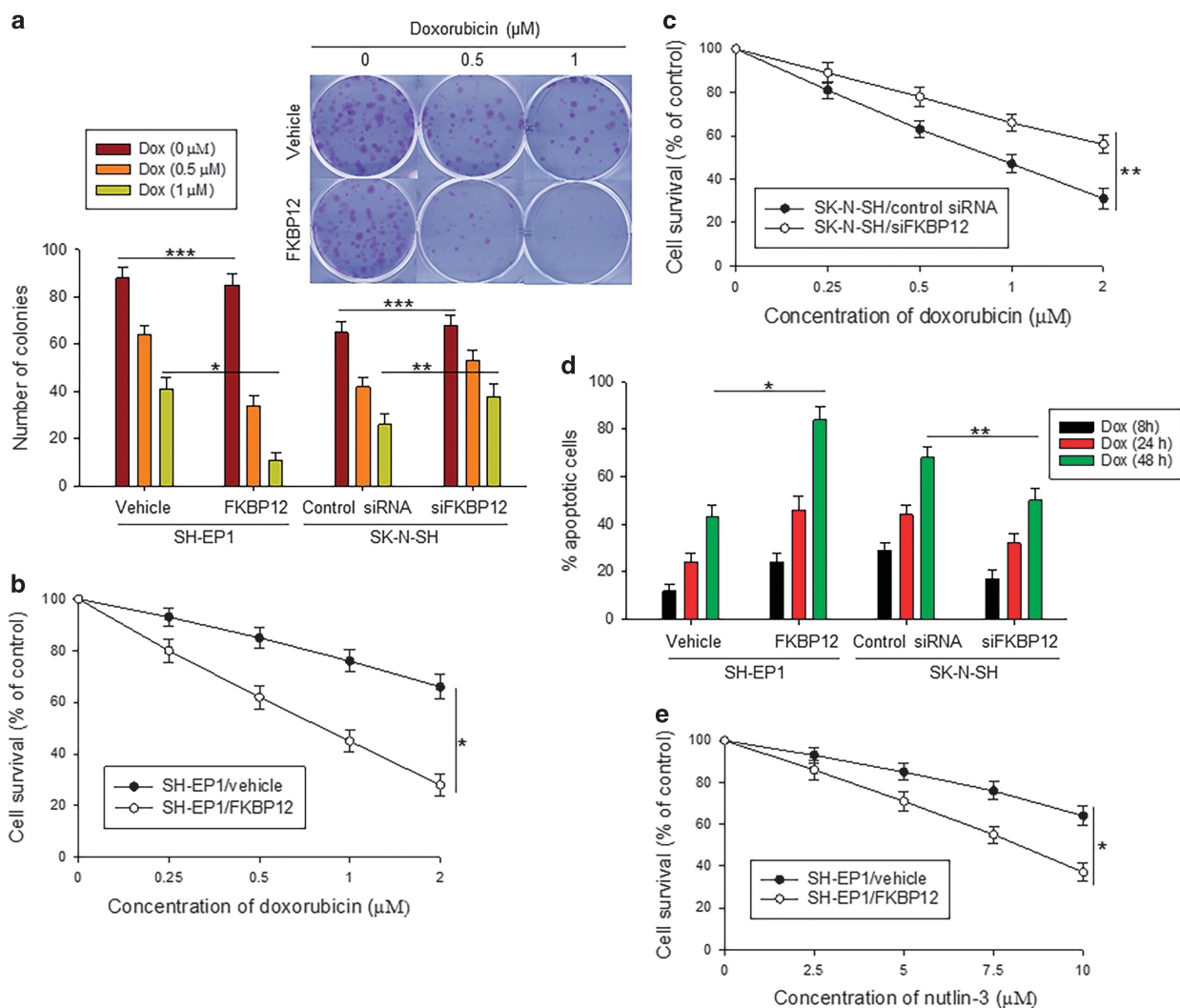


Figure 5. Effects of FKBP12 on cell growth and apoptosis after doxorubicin treatment. (a) Colony assay of SH-EP1 and SK-N-SH that were transfected with FKBP12 and siFKBP12, respectively, in the absence and presence of different concentrations of doxorubicin. Data represent mean of three independent experiments (bars \pm s.d.), * $P < 0.01$, ** $P < 0.05$, *** $P > 0.5$; inset: representative photographs of colony formation by SH-EP1 cells transfected with FKBP12 and treated with doxorubicin. (b and c) SH-EP1 transfected with FKBP12 (b) and SK-N-SH transfected with siFKBP12 (c) were treated with different concentrations of doxorubicin for 24 h and cell survival measured by WST analysis. Data represent mean \pm s.d. of three independent experiments, * $P < 0.01$, ** $P < 0.05$. (d) similar FKBP12-transfected SH-EP1 and siFKBP12-transfected SK-N-SH were treated with 1 μ M doxorubicin for different times as indicated and apoptotic cells quantitatively detected by flow cytometry, * $P < 0.01$, ** $P < 0.05$. (e) SH-EP1 transfected with FKBP12 were treated with different concentrations of nutlin-3 for 24 h and cell survival measured by WST analysis. Data represent mean \pm s.d. of three independent experiments, * $P < 0.05$.

showed less cytotoxic and apoptotic effects on siFKBP12-transfected SK-N-SH than on control-transfected SK-N-SH (Figures 5c and d).

Furthermore, we compared the cytotoxic activity of nutlin-3 in SH-EP1 transfected either with FKBP12 or control vehicle. Consistent with a significant activation of p53 in FKBP12-expressing SH-EP1 (Figure 4e), nutlin-3 exhibited greater cell killing in SH-EP1/FKBP12 than in SH-EP1/vehicle cells (Figure 5e).

DISCUSSION

A previous study using yeast two-hybrid system identified that a member of the FK 506-binding protein family, FKBP25, binds to MDM2 and stimulates its self-ubiquitination and proteasomal degradation.²⁵ In the present study, we found that FKBP12 also binds to MDM2 and induces MDM2 self-ubiquitination and degradation. We have further found that FKBP12 specifically binds to the MDM2 C-terminal RING (E3 ligase) domain to induce E3 ligase self-ubiquitination activity by disrupting the interaction between MDM2 and MDM4. Importantly, we demonstrate that the FKBP-mediated degradation of MDM2 should be clinically significant, as our results suggest that the expression levels of FKBP12 in cancer cells may predict a treatment outcome in radiotherapy and chemotherapy.

It has been well characterized that MDM2 and p53 are mutually regulated to form a negative-feedback loop, which is activated when cells undergo stress and DNA damage. Our results suggest that FKBP12 acts as an antagonist of the MDM2-p53-feedback loop during cellular stress and DNA damage. We found that, in the absence of doxorubicin treatment, p53 was not induced following inhibition of MDM2 by FKBP12 in FKBP12-transfected SH-EP1 and NB-1643 cells (Figure 1b). Following treatment with doxorubicin, p53 was only mildly induced, associated with a slight upregulation of MDM2 in control SH-EP1 cells (without FKBP12 overexpression); this suggests that the p53-MDM2 negative-feedback loop strongly operated in these cells leading to resistance to doxorubicin. In contrast, in SH-EP1 cells overexpressing FKBP12, doxorubicin treatment resulted in a marked activation of p53 accompanied by downregulation of MDM2, resulting in induction of apoptosis. This suggests a critical role of FKBP12-mediated degradation of MDM2 in inhibiting the p53-MDM2-feedback loop, resulting in sensitivity of cancer cells to doxorubicin.

In addition to regulation by FKBP12, MDM2 is also modulated by various other cellular signals. For example, the PI3K/Akt pathway regulates phosphorylation and subcellular distribution of MDM2. Cell growth/survival factor-induced activation of PI3K and its downstream target Akt induce phosphorylation of cytoplasmic MDM2 on serine 166. Phosphorylation of this site is required for translocation of MDM2 from the cytoplasm into the nucleus.²⁸ In contrast to survival signals that induce MDM2 phosphorylation, cellular stress and DNA damage invoke dephosphorylation of MDM2.^{30,31} Dephosphorylation of MDM2 at serine 166 leads to inhibition of nuclear entry of MDM2 or an increase in its translocation from the nucleus to the cytoplasm.³² Once MDM2 is localized in the cytoplasm, it binds to the IRES of XIAP mRNA to regulate its translational activity, which leads to elevated XIAP expression and IR resistance.²⁷

Unlike the nuclear protein FKBP25 that inhibits nuclear MDM2, the cytoplasmic FKBP12 preferentially inhibits MDM2 that is localized in the cytoplasm. Inhibition of the cytoplasmic MDM2 results in inhibition of XIAP translation and reduced expression of XIAP protein. Our results showed that after doxorubicin treatment, XIAP was indeed remarkably downregulated following the inhibition of MDM2 by FKBP12 in cancer cells that were transfected with FKBP12. Consistent with the strong downregulation of XIAP in FKBP12-overexpressing cells, caspase-3 was activated and the death substrate PARP was significantly cleaved. Thus, inhibition of XIAP following FKBP-mediated cytoplasmic MDM2 degradation may represent an

additional mechanism for sensitivity to anticancer treatment of wt-p53 cancer cells. Also, this mechanism may contribute to the sensitivity to anticancer treatment of p53-deficient cancer cells, as FKBP12-mediated MDM2 degradation and subsequent inhibition of XIAP are p53-independent.

Although FKBP12 is widely expressed in normal cells/tissues, its expression in cancer cells has not been extensively studied. A recent study reported that the expression level of FKBP12 varies significantly among different cancer cell lines. For example, cancer cell lines PC3 and C2C12 express high level of FKBP12, whereas HeLa and H460 express no or very low level of FKBP12.³³ The cancer cell lines examined in our study also express different levels of FKBP12. We found that cell lines SH-EP1, NB-1691 and A549 expressing low levels of FKBP12 had high levels of MDM2, and in contrast, cell lines SK-N-SH, NB-1643, PC9, BEAS-2B and HEK293 with high levels of FKBP12 expressed relatively low levels of MDM2. Our results support this negative correlation between the expression levels of FKBP12 and MDM2, which may determine the responsiveness of cancer cells to anticancer treatment.

In summary, our data suggest that cancer cells with FKBP12 overexpression are more sensitive to doxorubicin and nutlin-3 than cells expressing low level of FKBP12. Interestingly, these results are consistent with those in the study by Schreiber *et al.*, which showed that enforced reduction of FKBP12 abrogates sensitivity to rapamycin-mediated mTORC2 inhibition. Furthermore, they found that increased expression of FKBP12 can enhance sensitivity to rapamycin.³³ Our results support these observations and further indicate that the expression level of FKBP12 serve as a predictor of the responsiveness to anticancer treatment targeting the MDM2-p53 pathway.

MATERIALS AND METHODS

Cell lines and reagents

This study used total seven human tumor cell lines: 4 neuroblastoma lines (SH-EP1, SK-N-SH, NB-1691 and NB-1643) and 3 lung cancer cell lines (PC9, BEAS-2B and A549) as well as the human embryonic kidney cells HEK293. The neuroblastoma cell lines and lung cancer cell lines were obtained from Drs H Findley and DK Graham (Emory University), respectively, and HEK293 was purchased from the ATCC (Manassas, VA, USA). All cell lines were cultured in standard RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l of L-glutamine, 50 units penicillin and 50 µg/ml streptomycin at 37 °C with 5% CO₂ in air.

MDM2 antibodies (SMP14 and 2A10) and Ub (U5379) were purchased from Sigma (St Louis, MO, USA); MDM2 antibody (4B11) was purchased from Millipore (Billerica, MA, USA). FKBP12 (sc-28814), p53 (DO-1) and caspase-3 antibodies were purchased from Santa Cruz (Dallas, TX, USA); XIAP (2F1), PUMA (ab9346) and PARP (E-102) antibodies were purchased from Abcam (Cambridge, UK); p21 (12D1) and MDM2 (p166) were purchased from Cell Signaling (Danvers, MA, USA); MDM4 (2D10F4) was purchased from LifeSpan BioSciences (Seattle, WA, USA); HA and Myc antibodies were purchased from Covance (Indianapolis, IN, USA) and Clontech (Mountain View, CA, USA), respectively. Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Plasmids and transfection

We constructed HA or Myc-tagged FKBP12 and various (wt, truncated and mutated) MDM2 plasmids by RT-PCR and cloned into pCMV-HA or pCMV-myc expression vectors. Site-directed mutagenesis (Quick Change Mutagenesis Kit, Stratagene, Santa Clara, CA, USA) was used to create three MDM2 mutant forms (MDM2 C464A, Cys to Ala; MDM2 S166A, Ser to Ala; and MDM2-S166E, Ser to Glu). MDM2 C464A exhibits loss of E3 ligase activity,³⁴ whereas MDM2 S166A loses the Ser166 phosphorylation site and thus is confined to the cytoplasm. Conversely, MDM2-S166E mimics phosphorylated MDM2 and is constitutively localized in the nucleus.²⁸ YN-MDM2 RING and YC-MDM4 RING plasmids were generated by fusing the MDM2 RING domain (425–491) and MDM4 RING domain (421–490) into the N- (1–154) and C- (155–238) terminal halves of YFP, respectively. The GST-tagged FKBP12 was constructed by cloning FKBP12 into the pGEX expression system. The pCl-His-hUbi plasmid was purchased from Addgene (Cambridge, MA, USA). Lipofectamine 2000 reagents

(Invitrogen, Carlsbad, CA, USA) were used for gene transfection following the manufacturer's instructions. We purchased siFKBP12 and control siRNA from Santa Cruz, and transfected siRNA using the HiPerFect transfection reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's manual.

Immunoprecipitation and western blot assay

Immunoprecipitation was performed by lysing cells in a buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin. After centrifugation and discard of cell debris, about 500 µg was separated and incubated overnight with 15 µl Protein G plus/Protein A-agarose and 1 µg of antibodies at 4 °C. The beads were washed at least four times, and the protein was eluted and was subject to detection by western blot assay.

Western blot was performed using standard method. Briefly, whole-cell protein was prepared by lysing cells for 30 min at 4 °C in a buffer consisting 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin and 25 µg/ml leupeptin. In order to determine the cellular localization of MDM2, both nuclear and cytoplasmic fractions of SH-EP1 cells were isolated using the NE-PER kit (Pierce, Waltham, MA, USA) according to the manufacturer's instructions. The resulting cell lysates or protein from immunoprecipitation were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the gel contents were transferred to a nitrocellulose membrane and probed with antibodies as indicated. Finally, protein expression levels were visualized with a chemiluminescent detection system (Pierce).

GST pull-down assay

The FKBP12 protein was expressed as a GST fusion protein in the *E. coli* strain BL21 (DE3). Protein expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactoside for 8 h, at 18 °C. Fusion proteins were then affinity-purified with glutathione-sepharose 4B resin (GE Healthcare, Little Chalfont, UK), following the manufacturer's instructions. We mixed 20 µg of sepharose 4B beads conjugated to GST fusion protein with 2 mg of the lysates of SK-N-SH transfected with various MDM2 fragments in 3 ml of RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 and 0.25% sodium deoxycholate). After 4 h incubation at 4 °C, the beads were washed 4 times in RIPA buffer. The protein complex was eluted from the beads by boiling for 5 min in 50 µl of 2 × SDS sample buffer and then analyzed by SDS-PAGE.

Isothermal titration calorimetry assays

The binding property between FKBP12 and the MDM2 RING domains was also examined by ITC assay as described previously.²⁴ Briefly, 20 µM MDM2 proteins was placed in a 400 µl buffer containing 10 mM Hepes at pH 7.2 and 150 mM NaCl, in a loading 96 DeepWell PP plate (Nunc, Thermo Fisher Scientific). A 10-fold concentration of FKBP12 (200 µM) in 120 µl of the same buffer was automatically transferred by the auto-ITC200 instrument (MicroCal, GE) into the sample cell. FKBP12 solution (2 µl) was stepwise injected into the MDM2 sample cell using a syringe, and 16 injections were performed and each injection was 2 µl (except that the first injection was 0.4 µl) over a 10 s span with 210 s between injections. The binding stoichiometry (n), binding constant (K_d) and thermodynamic parameters (ΔH and ΔS) were determined by fitting the titration curve to a one-site binding mode, using Origin software (San Clemente, CA, USA) provided by the manufacturer.

Bimolecular fluorescence complementation assay

The bimolecular fluorescence complementation assay was performed using method as described previously,²⁴ Briefly, SK-N-SH cells were transfected with YN-MDM2 RING and YC-MDM4 RING along or co-transfected with the two plasmid in absence or presence of FKBP12. The transfected cells were then cultured at 30 °C for 10 h to allow the fluorophore maturation. After stained with Hoechst33258, the cells with visible YFP fluorescence (YN-MDM2 RING /YC-MDM4 RING dimer positive) were counted under fluorescence microscope and executed a comparison in a blinded fashion, of the different samples.

Ubiquitination assays

We performed *in vivo* ubiquitination assay to measure the effect of FKBP12 on MDM2 self-ubiquitination using method as previously described.²⁵

Briefly, we co-transfected MDM2, His6-ubiquitin and FKBP12 into SK-N-SH cells. After 24-h transfection, the cells were treated with MG132 for an additional 6 h, and then the cells were collected from each transfection into two aliquots. One aliquot (10%) was used for conventional western blotting to confirm the transfection efficiency of MDM2 and FKBP12. The remaining cells (90%) were used for the purification of His6-tagged MDM2, with the aid of Ni²⁺-NTA beads. Finally, the His-tagged MDM2 was then eluted and analyzed by western blot assay.

Pulse-chase assay

We performed a standard protein-synthesis inhibitor cycloheximide pulse-chase assay to test the effect of FKBP12 on turnover of MDM2 protein. Briefly, cells transfected with or without FKBP12 were treated with 50 µg/ml cycloheximide for different time points before lysis. The concurrent protein levels were analyzed by western blot as described above.

Clonogenic assays

The clonogenic assay, as described previously,³⁶ was used to detect the effect of FKBP12 on cell proliferation and growth. Briefly, FKBP12-transfected cells with or without doxorubicin treatment were prepared in a single-cell suspension, and then 200 cells were seeded into a 6-well plate and cultured for about 14 days to allow the cells form colonies. The colonies were then stained with a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for about 30 min, then carefully removed the dying solutions and rinsed with tap water. After that, the colonies were counted and calculated.

WST assay

The WST (water-soluble tetrazolium) salt assay was performed to determine the effect of FKBP12 on doxorubicin-induced cell death. Cells with or without FKBP12 or siFKBP12 transfection were cultured in 96-well plates and treated with different concentrations of doxorubicin for 20 h at 37 °C. WST-1 (25 µg/well) was then added and cells were incubated for an additional 4 h. The optical density was read with a microplate reader (set at a test wavelength of 450 nm and the reference wavelength of 620 nm).

Flow cytometry

Annexin-V staining followed by flow cytometry was performed to quantitatively detect the effect of FKBP12 on apoptotic cells induced by doxorubicin. Cells with or without FKBP12 gene transfection or knockdown and doxorubicin treatment were harvested and washed once with cold PBS, and then stained with FITC-annexin-V and 7-AAD, according to the manufacturer's instructions. The cells were detected by flow cytometry.

Statistical analysis

All data represent mean ± s.d. of three independent experiments. A two-tailed *t*-test was performed to compare the difference between two groups. A *P*-value < 0.05 is considered significantly different and *P* > 0.5 is considered as non significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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