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Rapamycin increases the p53/MDM2 protein ratio and p53-dependent apoptosis by translational inhibition of *mdm2* in cancer cells

Chia-Li Kao [a,1], Han-Shui Hsu [b,1], Hsin-Wu Chen [a,1], Tzu-Hao Cheng [a,*]

[a] Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan, Republic of China
[b] Institute of Emergency and Critical Care Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan, Republic of China

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ABSTRACT

Rapamycin, a potential anti-cancer agent, modulates activity of various factors functioning in translation, including eIF4E, an initiation factor selectively regulating expression of a subset of cellular transcripts. We show here that rapamycin suppresses levels of the p53-regulator MDM2 by translational inhibition without affecting *mdm2* mRNA expression or protein stability. Rapamycin inhibits translation of *mdm2* mRNA from the constitutive P1 promoter, which contains two upstream ORFs (uORFs) in the 5′UTR. Suppression is accompanied by increased hypo-phosphorylation of 4EBP-1, an inhibitory eIF4E binding protein. Ectopic expression of eIF4E abrogates rapamycin-mediated MDM2 inhibition, suggesting that eIF4E is crucial in modulating MDM2 expression in rapamycin-treated cells. Rapamycin administration also results in elevated PUMA expression and PARP cleavage, which is reproduced by siRNA knockdown of eIF4E or MDM2, suggesting that MDM2 suppression by rapamycin stimulates p53-mediated apoptosis. Together, our results define translational regulation of MDM2 expression by eIF4E and provide a molecular mechanism underlying rapamycin-induced p53-dependent apoptosis.

1. Introduction

Elevation of eIF4E protein is seen in several human cancers and is often correlated with genomic amplification in advanced disease stages [1,2]. *In vitro* ectopic expression of eIF4E promotes malignant transformation of immortalized rodent NIH3T3 fibroblasts [3]. More recently, evaluation of cell culture and animal models indicates that eIF4E functions in both the genesis and progression of tumor cells, and cells that become transformed due to eIF4E hyperactivity often exhibit attenuation of p53-dependent apoptotic response [4,5].

eIF4E generally functions as an initiation factor for cap-dependent translation; accumulated evidence indicates that it can also differentially regulate efficiency of protein synthesis of some mRNAs [6–8]. eIF4E forms a trimetric complex with eIF4G and eIF4A, termed eIF4F, which releases inhibitory secondary structure present in the cap-proximal 5′ untranslated region (5′UTR) of mRNA and enables binding of the 40S ribosomal subunit [9,10]. Most cellular mRNAs with short unstructured 5′UTRs are translated efficiently, even when eIF4F activity is low. However, long, GC-rich, highly structured 5′UTRs are prevented from being translated due to poor scanning and start codon recognition by the translation machinery. These mRNAs possess extensive 5′UTR secondary structure unfavorable for translation and therefore require higher levels of eIF4F or eIF4E [11]. In cells, the amount of active eIF4E available for translation is limiting [12] and negatively regulated by eIF4E binding proteins (4EBPs), which compete with eIF4G for an overlapping binding site on eIF4E and block formation of the eIF4F complex [13–15]. Interaction between eIF4E and the 4EBPs is controlled by phosphorylation: hypo-phosphorylated 4EBPs possess high affinity for eIF4E, an interaction decreased by hyper-phosphorylation [16].

* Corresponding author. Tel.: +886 2 28267331; fax: +886 2 28264843.
E-mail address: thcheng@ym.edu.tw (T.-H. Cheng).
[1] Contributed equally to this work.

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eIF4E also controls nuclear export of functionally related mRNAs [17]. The eIF4E mutant W73A, which cannot bind eIF4G and thus cannot function in translation [18], retains mRNA export activity, indicating uncoupling of translation and transport functions. Also eIF4E translational regulation occurs through the mRNA 5′UTR, while mRNA export requires secondary structure found in the 3′UTR [19]. Expression of several genes functioning in cellular proliferation, such as *cyclin D1* and *mdm2*, is modulated via eIF4E export activity [19,20].

MDM2 negatively regulates activity and abundance of the transcription factor and tumor suppressor p53 [21]. MDM2 binding to p53 both inhibits the latter's trans-activation activity and destabilizes it by functioning as an E3 ubiquitin ligase [22,23]. *mdm2* is transcriptionally controlled by p53, creating a feedback loop that monitors homeostasis of both proteins [24]. Under certain circumstance, this protein balance is disturbed and modulation of p53 signaling occurs upon change of MDM2 expression [25–27]. *mdm2* is transcribed from the constitutive P1 promoter and from a p53-dependent promoter P2. Both the P1- and P2-transcribed mRNAs give rise to full-length MDM2 protein but their distinct 5′UTRs control abundance of unique MDM2 isoforms and the rate of protein synthesis [28,29]. Translation efficiency of mRNAs from the P1 promoter is lower than from P2, due to the presence of two small upstream open reading frames (uORFs) missing from P2-derived transcripts [30]. However, *mdm2* transcripts derived from either P1 or P2, which have the same 3′UTR, show comparable mRNA nuclear export as levels of eIF4E increase [31]; the effect of eIF4E on translation of either of these mRNAs is not well understood.

Rapamycin, an FDA-approved antibiotic and immunosuppressant, is currently being tested in phase II and III clinical trials as an anti-tumor agent [32]. Rapamycin inhibits the kinase activity of mTOR, which functions as an upstream effecter of S6 ribosomal protein kinases and the 4EBPs [33]. Through de-phosphorylation and activation of the 4EBPs, rapamycin reduces active eIF4E, which, in turn, reduces the availability of the eIF4F complex for translation. In this study, we have analyzed the effect of rapamycin on MDM2 expression. We show that treatment with rapamycin selectively decreases MDM2 protein levels due to a deficiency in eIF4E and reduction of *mdm2* translation. This inhibition leads to an increased p53 to MDM2 protein ratio and induces expression of apoptotic effectors of p53.

2. Materials and methods

2.1. Plasmid construction

The full-length human eIF4E ORF was inserted into the pLLEXP1 vector [34] between the cytomegalovirus (CMV) promoter and the polyadenylation site to create CMV-eIF4E. As described [28], Exon1-MDM2-YFP and Exon2-MDM2-YFP produce transcripts containing the 5′UTR, but not the 3′UTR of the *mdm2* mRNA initiated at the P1 and P2 promoter, respectively. Exon1-MDM2-YFP$_{\triangle uORF1}$, Exon1-MDM2-YFP$_{\triangle uORF2}$ and Exon1-MDM2-YFP$_{\triangle uORF1,2}$ were modified from Exon1-MDM2-YFP using a QuickChange site-directed mutagenesis kit (Stratagene) to generate ATG to ATC point mutations at desired sites. Primer sets used for the uORF1 and uORF2 point mutation were: (5′-GTGTGTCGGAAAGATCGAGCAAGAAGCCG-3′) with (5′-CGGCTTCTTGCTCGATCTTTCCGACACAC-3′) and (5′-CCCGGAGAGTGGAATCGTCCCCGAGGC-3′) with (5′-GCCTCGGGGACGATTCCACTCTCCGGG-3′), respectively. The absence of additional mutations was confirmed by DNA sequencing. The CMV-GFP construct has been described previously [34].

2.2. Cell culture and transfection

Cells were culture in Dulbecco's modified Eagle's medium (DMEM) [Saos-2 cells], F-12 medium (H1299 cells), or McCoy's 5A medium (HCT116 cells) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Transfections were carried out using LipofectAMINE Plus (Invitrogen) [Saos-2] or LipofectAMINE 2000 (Invitrogen) [H1299 and HCT116] as per the manufacturer.

2.3. Rapamycin treatment

Saos-2 and HCT116 cells were seeded in six-well plates one day before rapamycin treatment to achieve appropriate confluency (~50% for HCT116 cells and ~70% for Saos-2 cells) at the time of treatment. After aspiration of medium, cells were washed with warm PBS (10 mM sodium phosphate, pH 7.4 and 0.9% NaCl) twice and cultured in Opti-MEMI medium (GIBCO) containing 1 μM rapamycin (Sigma) with 2% FBS for 24 h. Rapamycin was dissolved in DMSO.

2.4. Western blot analysis and antibodies

Cultured cells were lysed with RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS and 1% sodium deoxycholate) supplemented with 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Sigma). Equal quantities of protein were loaded and separated on SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. Membranes were blocked with PBS-T (1X PBS, 0.2% Triton X-100) containing 5% non-fat dry milk, probed with primary antibodies for 1 h, washed three times with PBS-T for 15 min, and incubated with an appropriate secondary antibodies conjugated with horseradish peroxidase for another 1 h. Following three 15 min PBS-T washes, signals were detected using ECL Plus Western Blotting Detection Reagents (Amersham) or Western Lighting (PerkinElmer Life sciences).

Antibodies against β-Tubulin (D-10, Santa Cruz), β-actin (AC-15, Abcam), p53 (DO-1, Oncogene), poly (ADP-Ribose) Polymerase (PARP) (Affinity BioReagents), PUMA (Sigma), eIF4E (BD Bioscience), phospho-eIF4E (Ser209) (Cell Signaling), 4EBP-1 (P-1, Santa Cruz) and phospho-4EBP-1 (Thr70) (Cell Signaling) were purchased. Polyclonal anti-GFP antibodies (Abcam) and monoclonal anti-MDM2 antibody (IF2, Calbiochem), used for the immunoprecipitation after [^{35}S]-methionine pulse-labeling, were also purchased. MDM2 antibody (4B11) was generously provided by Dr. A. Levine.

2.5. Semi-quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) and an equal amount (3 μg) of RNA from each sample was converted to cDNA by reverse transcriptase (Invitrogen). Equivalent volumes (3 μl) of cDNA products were amplified by PCR and products were resolved on 1% agarose gels to determine relative transcript levels. P1- and P2-transcribed *mdm2* mRNAs can be amplified using Exon1 primer (5′-GA GTGGAATGATCCCCGAGG-3′) with Exon6 primer (5′-GACTA CTACCAAGTTCCTGTAG-3′) and Exon2 primer (5′-CAGTGG CGATTGGAGGGTAG-3′) with Exon6 primer, respectively. Both *mdm2* mRNAs contain exon3 and can be analyzed together using Exon3 primer (5′-ATGTGCAATACCAACATGT CTG-3′) with Exon6 primer. The primer set, tubulin-f (5′-A TGAGGGAAATCGTGCACATC-3′) and tubulin-r (5′-AAGGT ATTCATGATGCGATCAG-3′), served to amplify a 500 bp β-Tubulin fragment. Numbers of amplification cycles for each primer set are reported in the text and figures.

2.6. Measurement of MDM2 protein half-life

HCT116 and Saos-2 cells were subjected to rapamycin (1 μM) treatment for 24 h prior to addition of cycloheximide (1 μg/ml, Sigma). Cellular lysates were collected at indicated times and analyzed by Western blotting using anti-MDM2 antibody (4B11). β-actin served as internal loading control. The MDM2 signal was quantified by densitometer and normalized to β-actin at each time point. Protein half-life was defined as the time required to observe 2-fold reduction in MDM2 level.

2.7. [^{35}S]-methionine pulse-labeling for MDM2

HCT116 cells were co-transfected Exon1-MDM2-YFP or Exon2-MDM2-YFP (9.5 μg) with CMV-GFP (0.5 μg) followed by 2 μM rapamycin treatment for 24 h. Cells were washed and incubated with warm methionine-free DMEM medium (GIBCO, 21013-024) for 30 min, and then supplemented with 400 μCi/ml [^{35}S]-methionine (Amersham, AG1094) for another 30 min. After washing twice with PBS, cells were lysed with RIPA buffer as described above. Lysates were pretreated with protein A agarose beads for 3 h at 4 °C, and then incubated with monoclonal anti-MDM2 antibody or polyclonal anti-GFP antibodies for immunoprecipitation overnight at 4 °C. Following incubation with protein A agarose beads for another 4 h, beads were washed with RIPA buffer twice and then three times with RIPA containing 500 mM NaCl. The immunocomplex was dissolved in SDS loading buffer and fractionated by SDS–PAGE. Gels were dried and exposed to X-ray film (Fujifilm) to visualize labeled proteins. A Personal Densitometer SI (Molecular Dynamics) and Image QuaNT Ver 4.2 Software were used to quantify the MDM2 and GFP signals on the X-ray films.

2.8. Subcellular fractionation

Subcellular fractionation was performed as described [35] with minor modifications. H1299 cells were suspended in hypotonic buffer (10 mM HEPES [pH 7.4], 5 mM KCl, 0.5 mM $MgCl_2$, 0.25% NP-40) with 1 mM DTT and protease inhibitor cocktail (Sigma) on ice for 20 min. After lysis, the nuclear pellet was collected by centrifugation at 220*g* for 5 min at 4 °C and the supernatant saved as the cytoplasmic fraction. The nuclear pellet was further rinsed in ice-cold PBS and re-suspended in RIPA buffer supplemented with 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF and the protease inhibitor cocktail. Following centrifugation at 14,000*g* for 25 min to remove insoluble material, the supernatant was saved as the nuclear fraction. B23 and β-Tubulin served as markers of the nuclear and cytoplasmic fractions, respectively.

2.9. siRNA knock down

HCT116 cells were transfected with siRNA using LipofectAMINE 2000. 300 nM of *mdm2* siRNA (5′-AGA-UCCUGCUGCUUUCGCAUU-3′ and 5′-UGCGAAAGCAGCAG-GAUCUUU-3′) synthesized by DHARMACON or 100 nM of *eIF4E* siRNA (DHARMACON, ON-TARGET plus SMART pool, L-003884-00) were used to inhibit expression of MDM2 and eIF4E, respectively. Transfection of an equivalent amount of annealed double-stranded oligonucleotides (5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUU-CGGAGAATT-3′) that do not target any gene served as a control.

3. Results

3.1. Rapamycin selectively down-regulates MDM2 expression

To assess the effect of rapamycin on MDM2 expression, we examined MDM2 protein levels in rapamycin-treated HCT116 cells (which contain functional p53 protein) and Saos-2 cells (which are p53-deficient). As shown previously, rapamycin inhibits mTOR, leading to hypo-phosphorylation of 4EBP-1. 4EBP-1 phosphorylation levels were greatly reduced following treatment of both cell lines with rapamycin (Fig. 1). Using β-Tubulin as a loading control, we found that endogenous MDM2 protein levels decreased in response to changes in 4EBP-1 phosphorylation (Fig. 1). However, rapamycin did not change the amount of 4EBP-1, eIF4E or the level of eIF4E phosphorylation (Fig. 1), suggesting that it preferentially suppresses MDM2 expression. Furthermore, we observed a more substantial reduction in MDM2 in Saos-2 cells, suggesting that rapamycin inhibition of MDM2 expression is p53-independent.

To determine the effect of rapamycin on global protein production, the level of *de-novo* protein synthesis, as reflected by incorporation of [^{35}S]-methionine into polypeptides during pulse-labeling, was examined. As shown in figure 1B, rapamycin slightly reduced the rate of global protein synthesis (less than 10%), suggesting decreased MDM2 levels can not be simply attributed to reduction of total protein synthesis in rapamycin-treated cells.

3.2. The effects of rapamycin on mdm2 mRNA level, MDM2 protein stability and the translation efficiency of mdm2 mRNA

Next we asked whether rapamycin-mediated MDM2 inhibition occurred transcriptionally, translationally or post-translationally. To analyze potential transcriptional suppression, we determined the amount of *mdm2* mRNAs produced from each of its two promoters (Fig. 2A) using semi-quantitative RT-PCR. Exon 1-6 and Exon 2-6 primer sets were used to amplify cDNAs copied from *mdm2* transcripts initiated at the P1 and P2 promoters, respectively. The numbers of amplification cycles for *tubulin* (21 and 23), Exon 1-6 (26 and 28), and Exon 2-6 (24 and 26) were chosen to ensure that the reactions were in the exponential phase of amplification. The quantity of Exon 1-6 PCR product in Saos-2 cells was unchanged in the presence or absence of rapamycin (Fig. 2B, row 1). Similarly, rapamycin treatment did not reduce levels of Exon 1-6 and Exon 2-6 PCR products in HCT116 cells (Fig. 2B, row 1 and 2). These observations suggest that MDM2 suppression is not the result of transcriptional regulation.

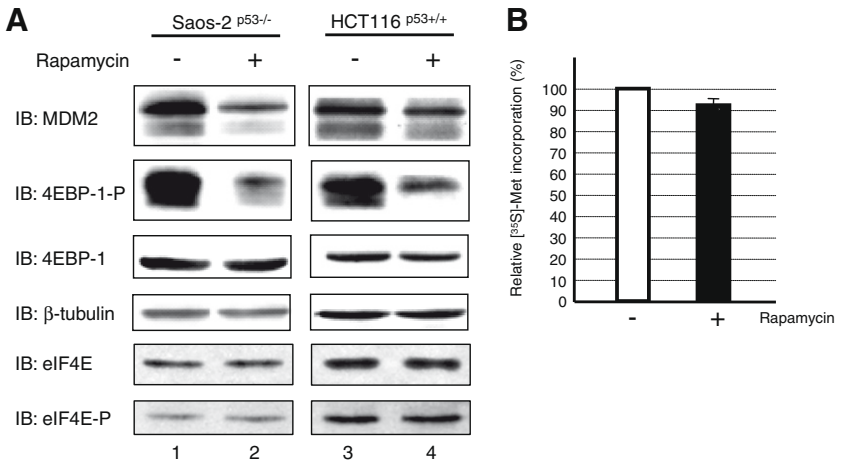

Fig. 1. MDM2 protein levels are selectively decreased in rapamycin-treated cells. (A) Following 24 h rapamycin treatment, Saos-2 (p53 deficient, left) and HCT116 (p53 functional, right) cells were collected and protein levels of MDM2, phosphorylated 4EBP-1, 4EBP-1, eIF4E and phosphorylated eIF4E were measured by Western blot. β-Tubulin served as loading control. (B) Incorporation of [^{35}S] -methionine into newly synthesized polypeptides in a 15 min pulse-labeling was determined in HCT116 cells treated with or without rapamycin as described previously [45]. Results are expressed as the relative [^{35}S] -methionine incorporation rate as compared with the un-treated sample. The values presented are the means ± s.d. for three independent experiments.

Next, we asked whether rapamycin affects MDM2 stability. MDM2 has been reported to be a protein with a short half-life [36]. Following rapamycin treatment, the amount of MDM2 protein in HCT116 cells was analyzed at indicated time points after the addition of the translation inhibitor cycloheximide (Fig. 2C). Using β-actin as a loading control, the MDM2 signal was quantified and the protein half-life was determined to be about 40 min, comparable to control values (Fig. 2C). In Saos-2 cells, rapamycin slightly increased the half-life of MDM2 protein (Fig. 2C). Therefore, under our experimental conditions, rapamycin treatment has negligible effect on MDM2 protein stability in either cell type.

To determine whether rapamycin interferes with protein synthesis from *mdm2* transcripts, we assayed MDM2 protein production by [^{35}S]-methionine pulse-labeling in HCT116 cells. HCT116 cells express functional p53 protein and thereby give rise to both *mdm2* transcripts, transcribed from the P1 and P2 promoters (Fig. 2B). To further evaluate the effect of rapamycin on each form of *mdm2* transcripts, Exon1-MDM2-YFP and Exon2-MDM2-YFP expression constructs, which produce transcripts mimicking *mdm2* mRNA transcribed from the P1 and P2 promoters, respectively, were individually transfected into cells and their corresponding MDM2-YFP protein synthesis rates assessed. A GFP-expressing construct was also introduced into cells and served as an internal control. In experiments normalized using isotope-labeled GFP, the amount of [^{35}S]-methionine-labeled endogenous MDM2 proteins was substantially decreased following rapamycin treatment (Fig. 2D, lane 2 vs. 1 and lane 4 vs. 3, bottom portion). In the presence of rapamycin, the rate of protein synthesis of full-length (90 kDa) MDM2 and truncated (74 and 76 kDa) forms was only about 40% of the un-treated control (Fig. 2D, bottom panel). This indicates that rapamycin inhibits protein synthesis from endogenous *mdm2* mRNAs. In addition, rapamycin also repressed translation of the transcript produced from Exon1-MDM2-YFP, which has a 5′UTR identical to *mdm2* mRNA initiated at P1 promoter (Fig. 2D, lane 2 vs. 1, top portion). The protein synthesis rate from Exon1-MDM2-YFP transcripts was decreased to approximately 50% of the control sample. In contrast, there was only a slight reduction in synthesis of the MDM2-YFP fusion protein in Exon2-MDM2-YFP expressing cells (Fig. 2D, lane 4 vs. 3, top portion; bottom panel). Collectively, these results suggest that rapamycin inhibits MDM2 expression due to translational repression, which is more significant when *mdm2* mRNA is transcribed from the P1 promoter.

3.3. eIF4E is a limiting factor for protein synthesis of mdm2 mRNAs

Rapamycin inhibits mTOR kinase activity, leading to hypo-phosphorylation of 4EBPs that sequester eIF4E. To determine the role of eIF4E in MDM2 repression, we introduced ectopic expression of eIF4E in Saos-2 cells and determined the effect of rapamycin on MDM2 protein levels. eIF4E overexpression rescued the decrease in MDM2 caused by rapamycin (Fig. 3A), indicating that eIF4E is a limiting factor in *mdm2* mRNA translation. Since only P1-transcribed *mdm2* mRNA is found in Saos-2 cells (Fig. 2B), it is likely that this particular *mdm2* transcript belongs to the specific group of transcripts sensitive to the amount of eIF4E for protein synthesis. Consistent with this notion, we found amounts of cellular MDM2 protein to be proportional to eIF4E protein levels in H1299 cells, which are p53-deficient and produce most *mdm2* transcripts from the constitutive P1 promoter. In H1299 cells, eIF4E elevation led to up-regulated MDM2 expression (Fig. 3B). Subcellular fractionation revealed that eIF4E protein accumulated primarily in the cytoplasm (Fig. 3C, lane 4 vs. 2, row 2) and that there was no change in phosphorylated eIF4E levels (Fig. 3C, row 1). More importantly, the total amount of *mdm2* transcripts and cytosolic *mdm2* mRNAs remained unchanged in the presence or absence exogenous eIF4E (Fig. 3D and E). Therefore, under these conditions, eIF4E overexpression increased protein synthesis of *mdm2* mRNA independent of transcription or nucleocytosolic transport.

3.4. uORFs in the mdm2 5′UTR regulate translation by eIF4E

Transcription from the *mdm2* P1 and P2 promoters produces mRNAs with exon1 and exon2 in their 5′UTRs, respectively. To investigate the role of the two 5′UTRs in translational regulation of *mdm2* mRNAs by eIF4E, we examined expression levels of exogenous MDM2 proteins from Exon1-MDM2-YFP and Exon2-MDM2-YFP constructs. The 3′UTR of *mdm2* mRNA, required for eIF4E to promote nuclear export, was replaced with RNA encoding yellow-fluorescent protein in the produced transcripts (Fig. 4A, top panel). Expression of Exon1-MDM2-YFP in the presence of ectopic eIF4E resulted in higher MDM2-YFP protein levels than in the absence of exogenous eIF4E (Fig. 4A, lane 3 vs. 2), as was the case with endogenous MDM2 proteins (Fig. 3B). Normalization with the loading control indicated a 2-fold increase in the amount of MDM2-YFP fusion protein in the presence of ectopic eIF4E (Fig. 4C). Similar experiments produced little change in protein expression of Exon2-MDM2-YFP (Fig. 4A, lane 6 vs. 5), suggesting that protein synthesis from the two transcripts is differentially regulated by eIF4E and that the transcript containing *mdm2* exon1 is subject to eIF4E translational control.

The 5′UTR of P1-transcribed *mdm2* mRNA exhibits two upstream open reading frames (uORFs) that could stall the ribosome and negatively regulate translation. To investigate whether uORFs regulate *mdm2* protein synthesis via eIF4E, we created constructs that disable one (either Exon1-MDM2-YFP$_{\triangle uORF1}$ or Exon1-MDM2-YFP$_{\triangle uORF2}$) or both (Exon1-MDM2-YFP$_{\triangle uORF1,2}$) uORFs. In each case, the translation start codon of uORF1 and/or uORF2 was mutated from ATG to ATC. Analysis of levels

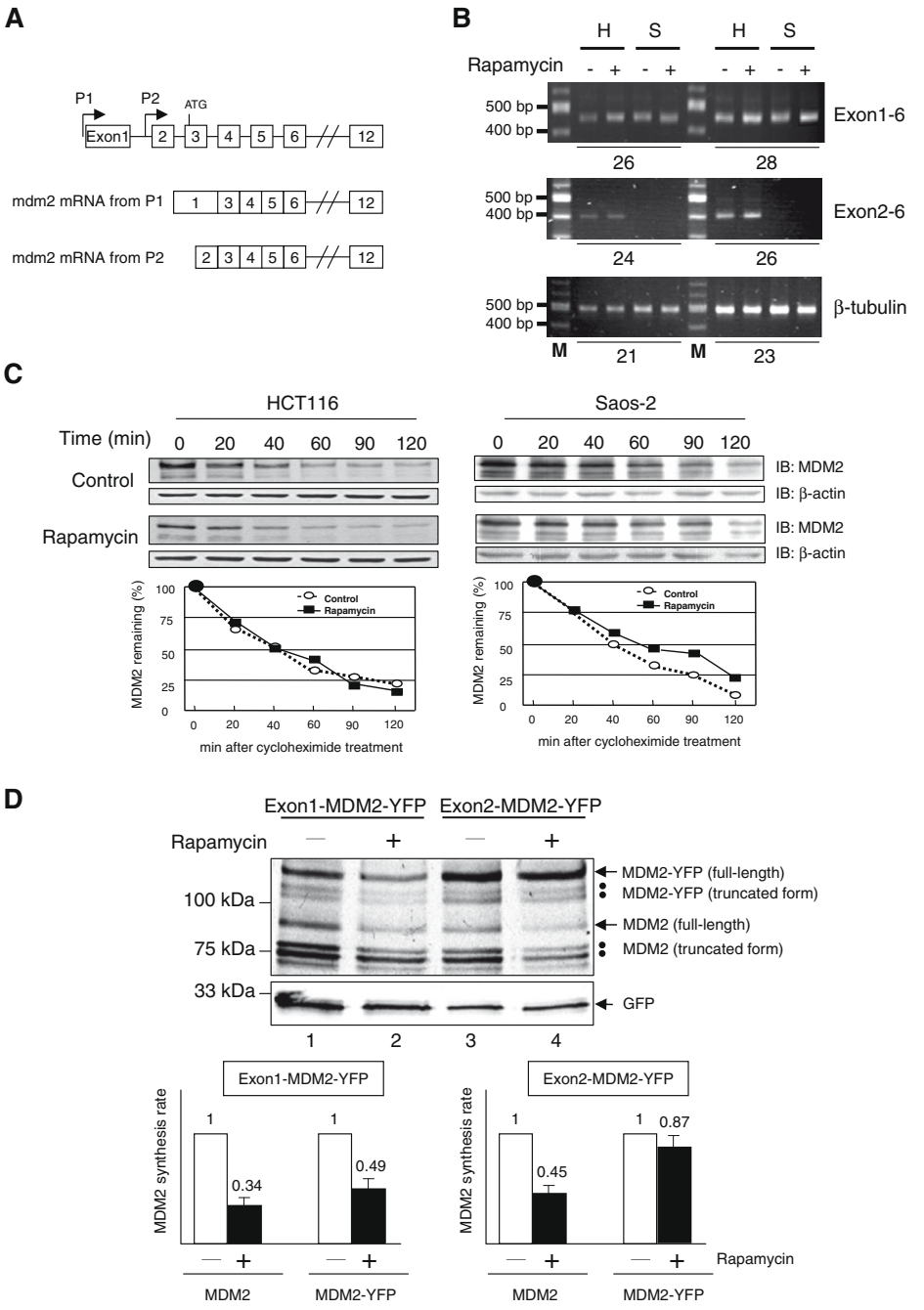

Fig. 2. Rapamycin-mediated decreases in MDM2 are caused by attenuated protein synthesis. (A) A schematic diagram showing genomic organization of the human *mdm2* gene with indication of the P1 and P2 promoters. P1 is a constitutive promoter, while P2 is located between exon1 and exon2 and is p53-activated. Also indicated is the position of the ATG codon in exon3 initiating synthesis of the full-length protein. (B) Expression of *mdm2* transcripts following rapamycin treatment of HCT116 (H) and Saos-2 (S) cells was analyzed by semi-quantitative RT-PCR. The primer set Exon1 and Exon6 together as well as the Exon2 and Exon6 primer set were used to detect *mdm2* mRNA from the P1 and P2 promoter, respectively. Sizes of PCR products are 452 bp for Exon1–Exon6 and 398 bp for Exon2–Exon6. β-Tubulin served as a control to ensure that equivalent amounts of RNA were analyzed. The number of amplification cycles is indicated at the bottom of the gel, and lanes M denote size markers. (C) HCT116 and Saos-2 cells pre-treated with rapamycin or control DMSO were treated with cycloheximide to block *de-novo* protein synthesis. At indicated times, cellular MDM2 protein levels were examined by Western blot analysis. MDM2 was normalized to β-actin and the relative amount of MDM2, compared to 0 min, is shown as a function of time in the bottom panel. (D) Synthesis rates of endogenous and exogenous MDM2 proteins were analyzed by [^{35}S]-methionine pulse-labeling in HCT116 cells expressing Exon1-MDM2-YFP or Exon2-MDM2-YFP following rapamycin treatment. Arrows and dots indicate position of migration of full-length and N-terminally truncated MDM2 proteins, respectively. In un-treated cells, the labeling signals of full-length and truncated MDM2 were normalized against that of GFP and set as 1. Similarly, the two forms of MDM2-YFP were pooled and also set to 1 as the exogenous control. Synthesis rates of either endogenous MDM2 or exogenous MDM2-YFP were measured relative to control cells and are illustrated in the bottom panel. Values represent means ± s.d. for cells expressing Exon1-MDM2-YFP or Exon2-MDM2-YFP from three independent experiments and are shown in the left and right panel separately.

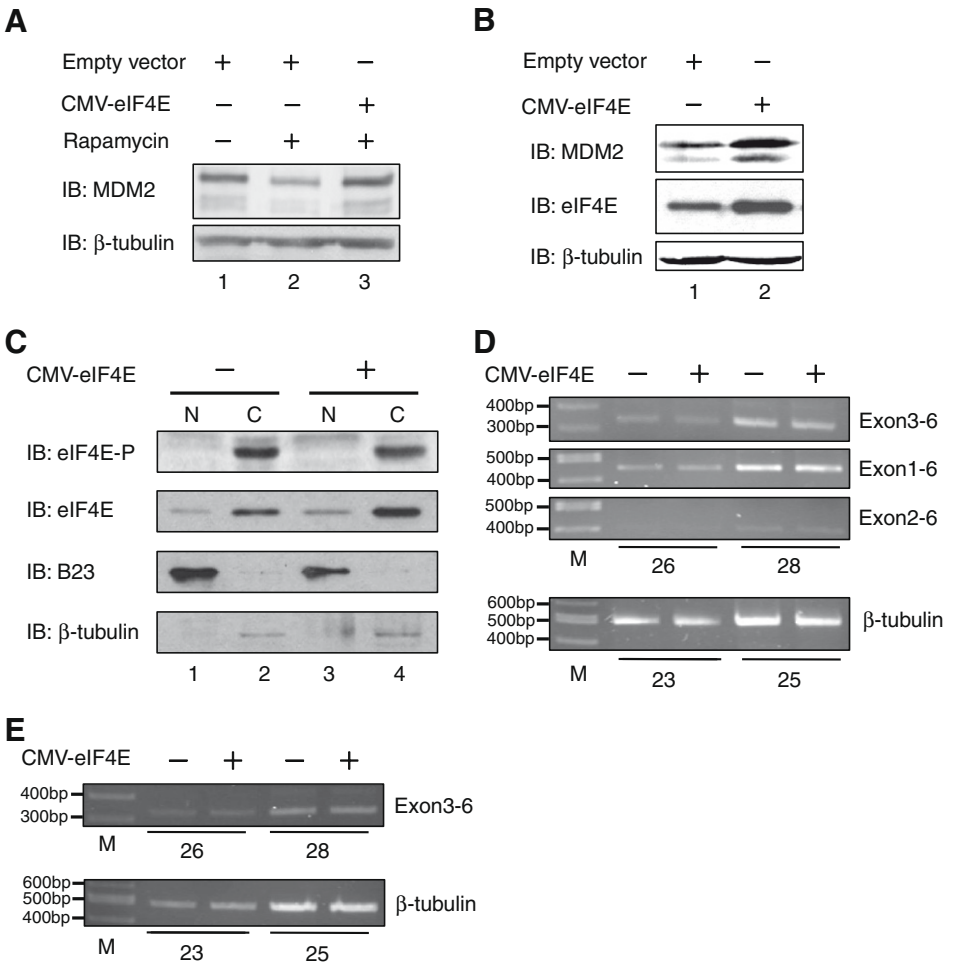

Fig. 3. Elevated eIF4E levels increase protein synthesis of *mdm2* mRNA and overcome the inhibitory effect of rapamycin on MDM2 expression. (A) Saos-2 cells transfected with the eIF4E expression construct CMV-eIF4E or empty vector were treated with rapamycin and the amount of MDM2 measured by Western blot. β-Tubulin served as loading control. (B) MDM2 protein levels in H1299 cells expressing the CMV-eIF4E construct were examined by immunoblotting using anti-MDM2, anti-eIF4E, and anti-β-Tubulin antibodies. Lysates of empty vector-transfected cells are included as controls in lane 1. (C) Levels of eIF4E and phosphorylated eIF4E were examined by Western analysis in nuclear (N) and cytoplasmic (C) fractions of H1299 cells transfected with CMV-eIF4E or empty vector. Fractionation controls are shown in lower panels using β-Tubulin as a cytoplasmic marker and B23 as the nuclear marker. (D) Following eIF4E overexpression, expression of *mdm2* transcripts in H1299 cells was analyzed by semi-quantitative RT-PCR. Similar to Fig. 2A, the Exon1–Exon6 and Exon2–Exon6 primer sets were used to detect *mdm2* mRNA transcribed from P1 and P2 promoters, respectively. Total amounts of *mdm2* transcripts were analyzed using the Exon3–Exon6 primer pair, which generates a 332 bp product from both *mdm2* mRNAs. β-Tubulin served an internal control; numbers of amplification cycles are indicated. (E) Cytoplasmic fractions of H1299 in (C) were examined to estimate amounts of *mdm2* mRNAs as described above.

of MDM2-YFP protein produced from these constructs indicated that inclusion of either uORF decreased protein synthesis from the *mdm2* transcript (Fig. 4B, lane 5 vs. 3 and 1) and that uORF1 had a more potent effect than uORF2, in agreement with a previous report [30]. When both uORFs were abolished, protein levels of Exon1-MDM2-YFP$_{\triangle uORF1,2}$ were not enhanced by eIF4E overexpression (Fig. 4B, lane 6 vs. 5) analogous to what was observed for Exon2-MDM2-YFP (Fig. 4A, lane 6 vs. 5). Relatively low expression of Exon1-MDM2-YFP$_{\triangle uORF2}$ was more efficiently up-regulated by ectopic eIF4E than was Exon1-MDM2-YFP$_{\triangle uORF1}$ (MDM2 induction fold: 1.84 vs. 1.31, Fig. 4C). This suggests that each uORF is susceptible to translational relief by eIF4E but to different extents.

3.5. Rapamycin enhances apoptosis by altering the p53/MDM2 balance

Rapamycin sensitizes tumor cells to DNA damage by increasing p53-dependent apoptosis [37]. Since MDM2 and p53 are mutually regulated, we examined selective MDM2 repression by rapamycin for a potential effect on p53 expression and activation of effectors triggering apoptosis. As expected, p53 and MDM2 protein levels were elevated in HCT116 cells treated with varying concentrations of the DNA damaging reagent adriamycin (Fig. 5A, lane 5 and 3 vs. lane 1). Intriguingly, the p53/MDM2 protein ratio remained almost constant in the presence or absence of adriamycin (Fig. 5A, right panel, white bars). In the presence of rapamycin, p53 induction by adriamycin was marginally affected, but MDM2 elevation was substantially suppressed (Fig. 5A, lane 6 vs. 5, lane 4 vs. 3), leading to an increase in p53 relative to MDM2 (Fig. 5A, right panel, black bars). This ratio change was also observed in cells lacking adriamycin treatment, indicating that rapamycin re-sets the p53/MDM2 balance in a manner that persists during DNA damage.

Next, we determined the consequences of rapamycin-induced changes in p53 signaling by analyzing activity of PUMA, a p53-responsive gene and proapoptotic member of the Bcl2 family [38]. In cells treated with rapamycin, PUMA expression was elevated and correlated with enhanced apoptotic response (Fig. 5A, row 4 and 5). Thus, changing the p53/MDM2 protein ratio or MDM2 protein level 2–3-fold is associated with increased apoptosis by rapamycin.

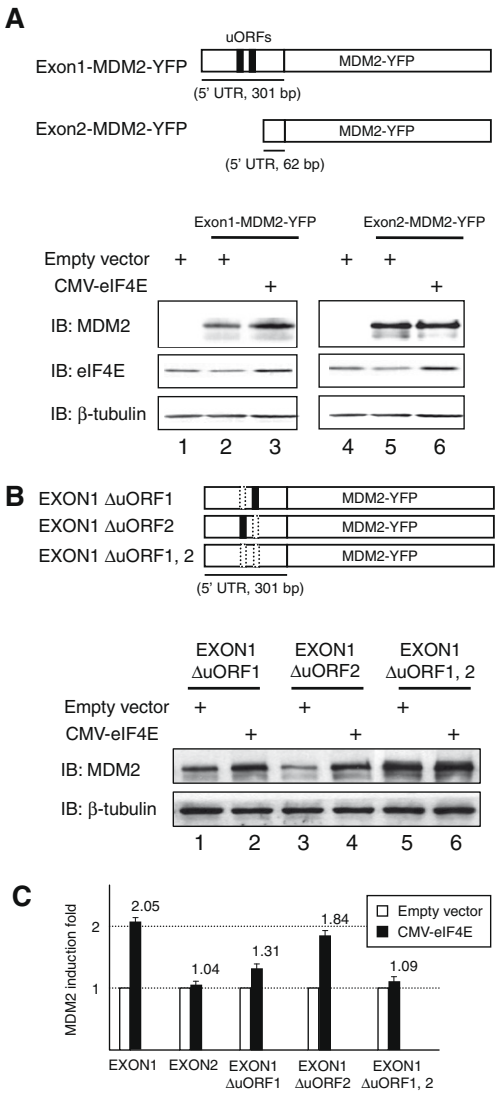

Fig. 4. uORFs within 5′UTR of P1-derived *mdm2* mRNA contribute to enhancement of MDM2 expression by eIF4E. (A) A schematic diagram of *mdm2-yfp* fusion transcripts is in the top panel. The transcript from Exon1-MDM2-YFP has a 301 bp 5′UTR containing 2 uORFs, as does endogenous *mdm2* mRNA transcribed from the P1 promoter. Similarly, Exon2-MDM2-YFP produces transcripts mimicking P2-derived *mdm2* transcripts. Lower panels, the amount of MDM2-YFP produced from Exon1-MDM2-YFP or Exon2-MDM2-YFP in H1299 cells in the presence or absence of exogenous eIF4E was analyzed by immunoblotting using antibodies against MDM2, eIF4E and β-Tubulin. (B) At top, schematic shows Exon1$_{\triangle uORF1}$, Exon1$_{\triangle uORF2}$ and Exon1$_{\triangle uORF1,\ 2}$ constructs in which the first, second or both uORFs in the 5′UTR of Exon1-MDM2-YFP, respectively, are mutated. The effect of exogenous eIF4E on MDM2 production in H1299 cells from Exon1-MDM2-YFP mutant constructs was analyzed by Western analysis. β-Tubulin served as control. (C) Quantitation of MDM2 induction by eIF4E using different MDM2-YFP constructs. MDM2 protein levels from each construct in cells co-transfected with empty vector are shown as a control and are set at a value of 1. Following eIF4E overexpression, the amount of MDM2-YFP was measured and compared to the corresponding control. Each experiment was performed in triplicate. The fold-change seen in the presence of exogenous eIF4E using Exon2-MDM2-YFP and Exon1$_{\triangle uORF1,\ 2}$ was analyzed by immunoblotting with a short exposure to prevent signal saturation.

As rapamycin negatively regulates eIF4E availability, we used small interfering RNAs targeting eIF4E to determine whether down-regulating eIF4E altered p53 and MDM2 expression or changed the p53/MDM2 protein ratio. In this experiment, eIF4E siRNA-transfected HCT116 cells showed significantly decreased amounts of eIF4E (Fig. 5B). Concurrently, there was a reduction in MDM2 but p53 protein levels remained unchanged (Fig. 5B), supporting the idea MDM2 is regulated by eIF4E in a specific manner. A change in the p53/MDM2 protein ratio was observed both in the presence and absence of DNA damage (Fig. 5B, right panel), as was the case in cells treated with rapamycin. Furthermore, this alteration was associated with elevated apoptosis, as indicated by an increase in the 86 kDa cleavage product of (ADP-ribose) polymerase (PARP) (Fig. 5B). Thus, the effect of rapamycin on MDM2 expression and the resulting cellular response was recapitulated when an eIF4E deficiency was induced.

To evaluate whether apoptosis is stimulated by altering the p53/MDM2 protein ratio, MDM2 expression was specifically inhibited by siRNA targeting the exon 1 region of *mdm2* transcripts. We observed increased levels of the 86 kDa form of PARP when MDM2 protein levels were decreased (Fig. 5C). Interestingly, p53 protein levels were unchanged by *mdm2* siRNA treatment in these experimental conditions. Thus the p53/MDM2 ratio increased, an occurrence linked to increased cellular apoptosis (Fig. 5C, right panels). Furthermore, in HCT116 p53$^{-/-}$ cells, proteins alteration as described above also occurred when the cells treated with *mdm2* or eIF4E siRNA. However, levels of cleavage PARP did not change (Fig. 5D), suggesting the apoptotic response caused by these siRNA treatments is dependent on p53.

4. Discussion

Cancer cells are often resistant to apoptosis induction by chemotherapeutic reagents. Although evidence suggests that rapamycin increases the sensitivity of cancer cells to DNA damage, which is p53-dependent and mediated by a defect in p21 function [37], we show here that changing the ratio of p53 to MDM2 protein represents another mechanism underlying increased apoptosis seen in rapamycin-treated cells. In this instance, rapamycin selectively down-regulates MDM2 expression through hypophosphorylation of 4EBP-1, which competes for binding of eIF4G on eIF4E and limits availability of eIF4E for translation [39]. Consequently, rapamycin increases the relative rather than absolute amount of cellular p53, thereby promoting expression of apoptosis-inducing genes such as PUMA. This hypothesis is supported by the finding that eIF4E and MDM2 expression can be inhibited in parallel by eIF4E siRNA knockdown, but that under these circumstances p53 levels remain unchanged, leading to an increased p53/MDM2 protein ratio and apoptosis. In addition, we found that down-regulating MDM2 using specific siRNA alters the p53 and MDM2 balance sufficiently to trigger apoptosis.

Reduction in steady-state MDM2 levels due to decreased stability mediated by rapamycin has been reported previously [40]. This investigation suggests that the rapamycin effect was mediated by inactivation of p70S6K1, which can enhance MDM2 phosphorylation and stability. However, in our study we did not observe significant changes in MDM2 half-life, which remained almost constant at about 40 min after rapamycin treatment of HCT116 and Saos-2 cells. It is noteworthy that the half-life of MDM2 protein in ovarian cancer cells (OVCAR-3) was 140 min prior to rapamycin treatment [40]. Since MDM2 is unstable with a half-life of around 30 min [36], the abnormally high stability observed in OVCAR-3 cells may

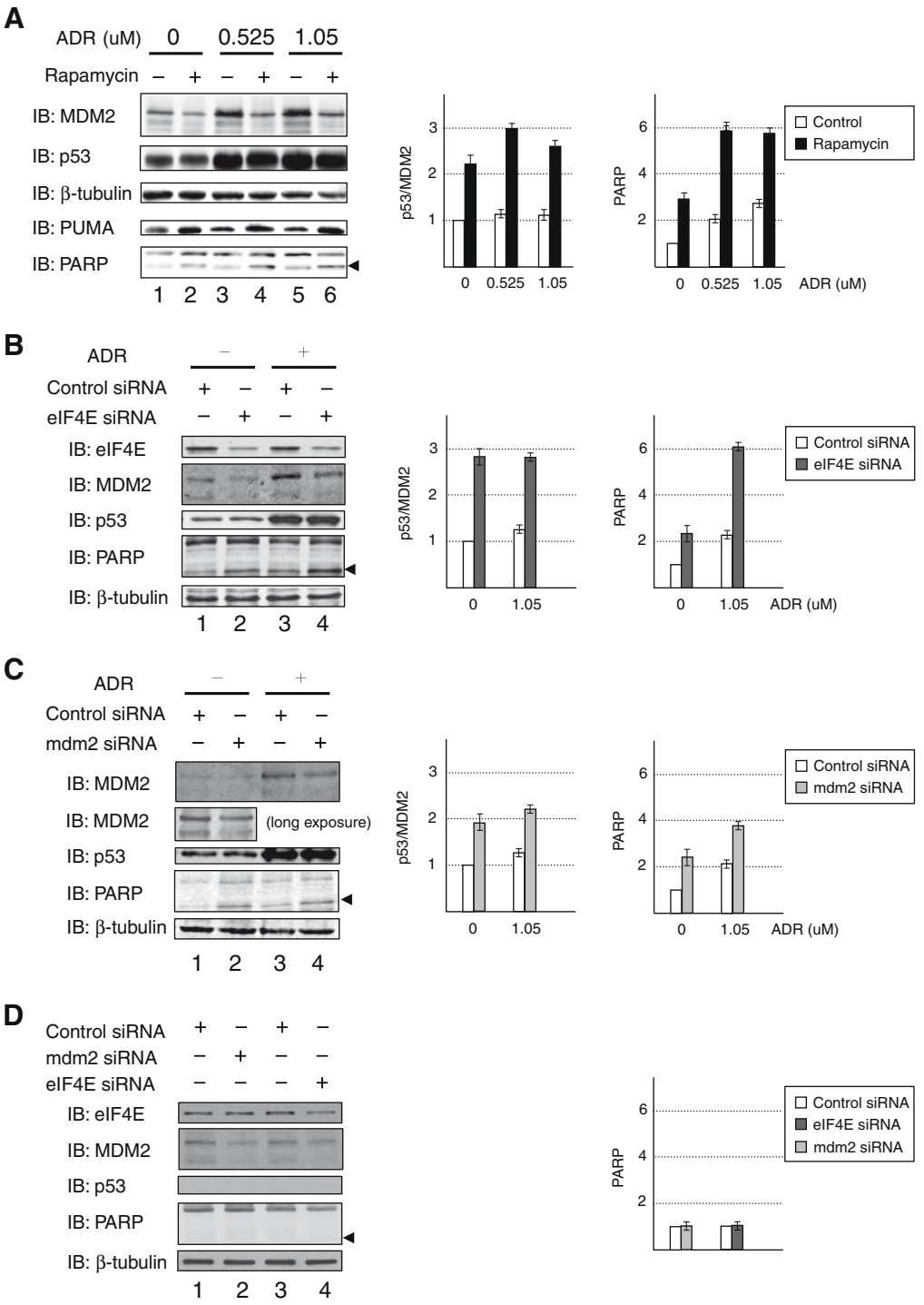

Fig. 5. Elevation in the p53/MDM2 protein ratio mediated by rapamycin, eIF4E knockdown or MDM2 knockdown promotes cellular apoptosis. (A) HCT116 cells pre-treated for 24 h with control DMSO or rapamycin were incubated with indicated adriamycin (ADR) concentrations for another 48 h. Adherent and suspended cells were collected for Western analysis using anti-MDM2, anti-p53, anti-β-Tubulin, anti-PUMA and anti-PARP antibodies. Arrowhead indicates position of 86 kDa PARP, which is the cleavage product of PARP by caspase 3 and serves as apoptotic index [37]. Quantitation of the p53/MDM2 protein ratio in each case is shown in right panel and is presented as the relative fold-change compared with the control sample, which did not treat with rapamycin and adriamycin. Similarly, the relative PARP (86 kDa) level in each case is quantified and illustrated. The values presented are the means ± s.d. for three separate experiments. (B) Following transfection with indicated siRNAs, HCT116 cells were incubated in growth medium without or with ADR (1.05 μM) for 26 h, and expression of eIF4E, MDM2, p53 and PARP was analyzed by Western blot. β-Tubulin served as loading control. The quantified data of p53/MDM2 protein ratio and processed PARP level are shown in right panels. (C) Expression of MDM2, p53 and PARP in HCT116 was analyzed as described in (B), except that *mdm2* siRNA was used. MDM2 immunoblotting with a long exposure for the first 2 lanes is shown in second row down. (D) Expression of cellular eIF4E, MDM2, p53, and PARP following treatment with indicated siRNAs was examined in HCT116 $p53^{-/-}$, HCT116 isogenic cells with p53 deficiency.

indicate a special circumstance in which MDM2 exhibits higher sensitivity to p70S6K1 inhibition by rapamycin. Rather than a change in protein stability or mRNA expression, we found that attenuation of MDM2 by rapamycin is due to selective reduction in *mdm2* protein synthesis. This conclusion was supported by labeling experiments showing decreased ^{35}S-methionine incorporation into MDM2 but not into a GFP control protein. We also found that the inhibitory effect of rapamycin on MDM2 can be rescued by eIF4E overexpression.

eIF4E functions as a cap-binding protein during translation initiation and also as an RNA regulon promoting nuclear transport of a subclass mRNAs including *mdm2*. Following activation of growth factor-regulated MAP-kinase kinases (MEKs), *mdm2* transcripts in the cytoplasm are increased by eIF4E phosphorylation, stimulating eIF4E transport activity [31,41]. We found that rapamycin decreases 4EBP-1 phosphorylation, but not that of eIF4E. Furthermore, by analyzing protein synthesis from transcripts that eliminate the 3′UTR required for eIF4E-mediated nuclear export, we found that expression of MDM2 proteins from EXON1-MDM2-YFP is suppressed in the presence of rapamycin as is the case with endogenous *mdm2* mRNAs. Thus, rapamycin's effect on MDM2 expression is primarily mediated through inhibition of the translation function that eIF4E exerts on *mdm2*, rather than by altering levels of eIF4E phosphorylation or amounts of cytosolic *mdm2* transcripts.

The main determinants of translation efficiency are features within the mRNA 5′UTR. Transcripts exhibiting high secondary structure or the presence of uORF(s) in 5′UTR are less efficiently translated and termed "weak" mRNAs [11]. It has been proposed that "weak" mRNAs are subject to preferential regulation by translation initiation factors such as eIF4E and that abnormal expression of proteins encoded by these mRNAs promotes neoplastic activity in cells showing elevated eIF4E levels [11]. *mdm2* mRNAs produced from the P1 promoter, which has two small uORFs in the exon1 sequence of *mdm2*, are translated less efficiently [30]. Here, we provide evidence showing that eIF4E overexpression can override translation deficits of *mdm2* mRNA. This effect is mediated through the uORFs, since protein synthesis from transcripts with disabled uORFs is unchanged when eIF4E levels are adjusted. These results support the notion that *mdm2* mRNA produced from the P1 promoter represents a "weak" mRNA and provides a molecular mechanism to account for specific translational control of *mdm2* by eIF4E. Translational regulation mediated through 5′UTR uORFs occurs similarly in the case of the *ODC* gene encoding ornithine decarboxylase [42]. We also observed that ornithine decarboxylase protein levels were decreased when eIF4E expression was inhibited by siRNA (data not shown). Therefore, these findings highlight the importance of uORFs as translational regulators and reinforce the idea that they mark a subgroup of mRNAs susceptible to translational regulation by changes in levels of eIF4E.

Translation de-regulation that leads to impaired p53/MDM2 feedback loop has been elegantly demonstrated in insulin-like growth factor 1 receptor (IGF-1R) deficient cells [43]. In these cells, reduction in assembly of translation initiation complex occurs and is accompanied with decreased global protein synthesis rate. However, the translation efficiency of both p53 and mdm2 mRNAs is affected to a greater extent than other transcripts. This gene-specific effect produces a significantly negative impact on the expression of proteins and disturbs the integrity of p53/MDM2 regulatory loop. During liver development, via an Akt and mTOR signaling pathway, embryonic hepatocyte apoptosis is suppressed by MDM2 up-regulation, which results from increased *mdm2* translation [44]. By blocking mTOR activity with rapamycin, this anti-apoptotic effect can be reversed by decreasing MDM2 expression. This finding supports our observations and underscores the biological significance of *mdm2* translational control. As we have found that *mdm2* transcripts, especially the one from the P1 promoter, are sensitive to eIF4E availability, reagents targeting the 4EBPs or eIF4E may represent a group of potential therapeutic molecules that render cancer cells more vulnerable to apoptosis.

Conflicts of interest

None declared.

Acknowledgments

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