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The MDM2 RING finger is required for cell cycle-dependent regulation of its protein expression

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Abstract The MDM2 oncoprotein is overexpressed in many human tumors and cancers. MDM2 functions as an E3 ligase for p53 and for itself. MDM2 also interacts with the retinoblastoma protein (RB) and the transcription factor E2F1 to promote cell cycle S-phase entry. Here, we report that MDM2 protein expression is cell cycle-regulated, which is dependent on its RING finger domain and requires Lys446. We show that MDM2 protein is stabilized at S phase. In addition, overexpression of MDM2 results in stimulation of E2F activity and accumulation of cells in S phase. These data suggest that ubiquitination of MDM2 is cell cycle-regulated and that MDM2 may play a role in cell cycle progression.

Key words: MDM2; Cell cycle; E3 ligase; RING finger; E2F; Protein expression

1. Introduction

The *Mdm2* gene was first identified as an amplified gene in a spontaneously transformed mouse 3T3 cell line. Overexpression of *mdm2* promotes tumorigenesis of NIH3T3 and Rat2 cells [1] and leads to transformation of primary rodent fibroblasts in cooperation with *ras* [2]. Importantly, overexpression of *MDM2* has been observed in a variety of human tumors/cancers, including approximately 30% of human sarcomas [3]. It has been well documented that MDM2 (murine double minute-2) functions as E3 ligase for p53 and for itself through its RING finger domain at the C-terminus [4–6]. MDM2 binds to p53 and targets p53 protein degradation via the ubiquitin-mediated pathway [4,7,8]. MDM2 can also directly inhibit p53-mediated transactivation [9,10]. On the other hand, p53 can directly activate MDM2 transcription [11]. In addition to the critical function on p53, MDM2 has been shown to interact with a group of cell cycle-related proteins, including the retinoblastoma protein (RB) and the transcription factor E2F1, to promote cell cycle G1–S transition [12–14].

A body of evidence indicates that MDM2 oncogenic function is associated with its ability in promoting cell cycle progression, in particular the S-phase entry. MDM2 inhibits the growth suppression at G1 imposed by p53 and RB [10,12]. Overproduction of MDM2 can overcome the TGF-β-imposed growth inhibition via the RB–E2F pathway [15]. Transgenic mice with targeted MDM2 expression in the mammary glands exhibit abnormal mammary gland development, high incidence of mammary tumors, and uncoupled S phase from mitosis and polyploidy in cells [16,17].

MDM2 protein has been shown to be phosphorylated at multiple sites in vivo [18] by ataxia telangiectasia-mutated kinase [19], DNA-dependent protein kinase [20], casein kinase 2 [21], or cyclin A-associated kinase [22]. Interestingly, MDM2 phosphorylation mediated by cyclin A-dependent kinase leads to reduced interaction between MDM2 and p53 [22]. Given that cyclin A-associated kinase is critical in promoting cell cycle progression, it is possible that the cell cycle-dependent phosphorylation can modulate MDM2 function and that MDM2 may in turn regulate cell cycle progression.

In this study, we show that MDM2 protein levels are cell cycle-regulated, which requires the intact RING figure domain and Lys446. In addition, our data show that MDM2 protein is accumulated in the S phase, which may play a role in cell cycle progression.

2. Materials and methods

2.1. Cell culture, synchronization and fluorescence-activated cell sorter (FACS) analysis

HeLa cells and U2-OS cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were synchronized by double thymidine block and release. Briefly, cells were treated with thymidine (2 mM) for 16 h. Cells were then washed three times, replaced with fresh medium and grown for 8 h prior to a second treatment with thymidine (2 mM). 16 h after the second thymidine treatment, cells were washed, refed with fresh medium, and collected at the indicated time points. For prometaphase arrest and release, cells were treated with nocodazole (100 ng/ml) for 16 h. Cells were washed, refed with fresh medium and collected at the indicated time. 1×10^6 cells were stained with propidium iodine and subjected to FACS analysis and data were analyzed using the Cell Quest program.

2.2. Western blot and Northern blot analyses

Cells were lysed in lysis buffer (50 mM Tris–HCl pH 8.0, 125 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin). Equal amounts of total protein were subjected to electrophoresis on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and subjected to Western blot analyses. Antibodies used were specific for MDM2 (SMP-14; Santa Cruz Biotechnology or a mixture of monoclonal antibodies 2A10, 4B1, and 3F3, gifts of Dr. J. Chen), cyclin B1 (GNS-1; Pharmingen), β-galactosidase (Promega), c-Myc (9E10, Santa Cruz Biotechnology), CD19

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Abbreviations: MDM2, murine double minute-2; RB, retinoblastoma protein

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(Pharmingen) and actin (C-11, Santa Cruz Biotechnology). To determine the mRNA levels, total RNA was isolated using the TRIZOL® Reagent (Gibco BRL) according to the manufacturer's instructions. 5 μg of total RNA was fractionated on a formaldehyde denaturing gel and subjected to Northern blot analysis, using a ^{32}P-labeled human *MDM2* cDNA probe. The membrane was rehybridized to a ^{32}P-labeled β-actin cDNA probe.

2.3. Transfection, cycloheximide treatment and cell cycle analysis

MDM2 mutants, MDM2C464A and MDM2K446A, were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagene) and confirmed by DAN sequencing. For experiments to examine ectopic protein expression, cells were transfected with pCMV-MDM2 plasmid DNA, and with pCMV-β-Gal where indicated, using FuGENE™ Transfection Reagent (Roch Molecular

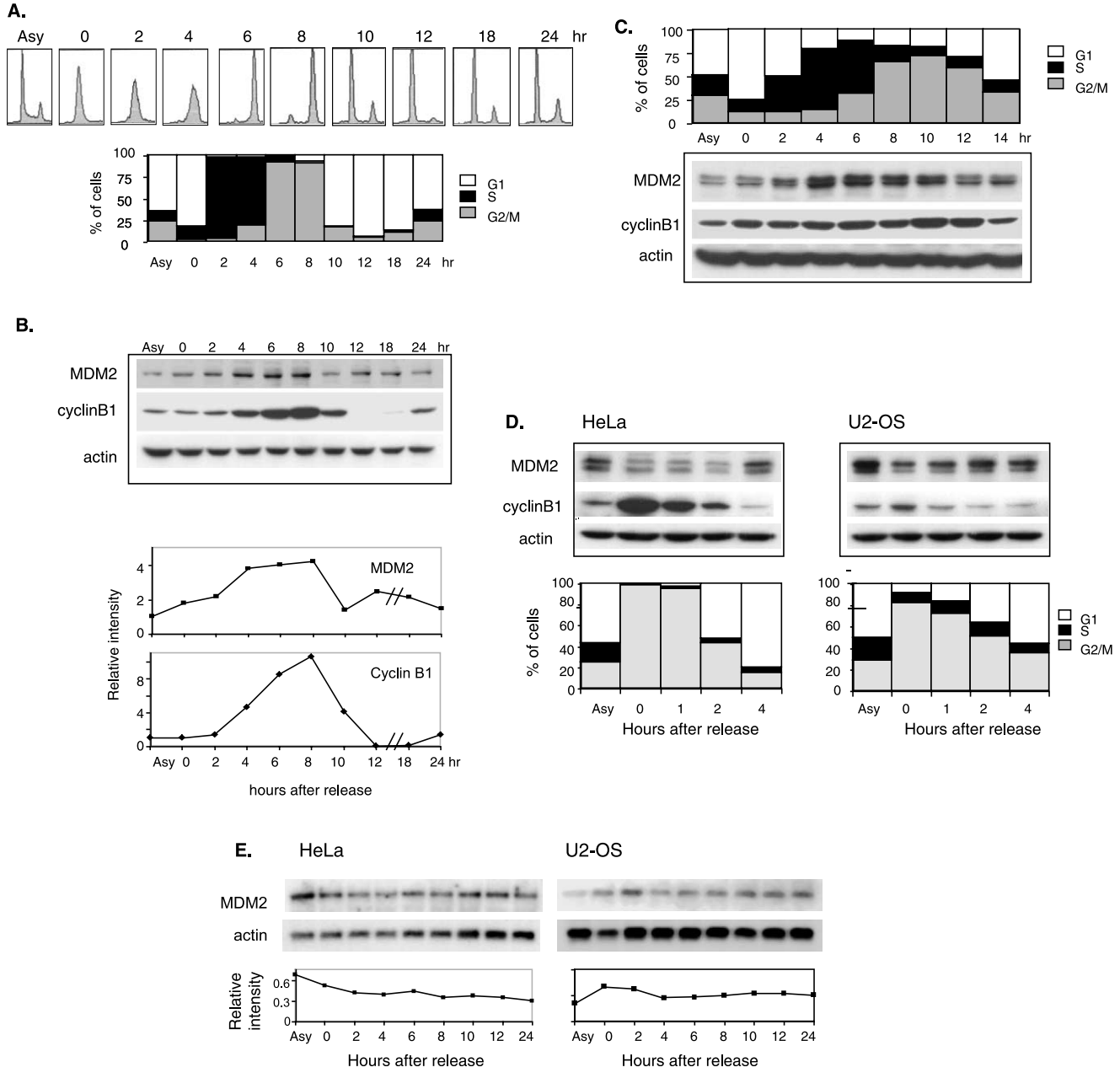

Fig. 1. Cell cycle-dependent regulation of MDM2 protein levels. A: HeLa cells were synchronized by double thymidine block and release, and collected at the indicated time. Cell cycle distribution was analyzed by FACS analysis and the percentage of cells in G1, S and G2/M is presented. B: Equal amounts of total proteins from synchronized HeLa cells were separated on 10% SDS–PAGE and subjected to Western blot analysis for MDM2, cyclin B1, and actin. MDM2 and cyclin B1 protein levels were normalized to the actin protein at each corresponding time point during the cell cycle. The relative intensity is presented, whereas the intensity of the MDM2 and cyclin B1 protein levels from asynchronized cells was arbitrarily set as 1.0. C: U2-OS cells were synchronized by double thymidine block and release, and collected at the indicated time. Cell cycle distribution was analyzed by FACS analysis and the percentage of cells in G1, S and G2/M is presented. Equal amounts of total proteins from synchronized U2-OS cells were separated on 10% SDS–PAGE and subjected to Western blot analysis. D: HeLa and U2-OS cells were synchronized by nocodazole treatment and release, and collected at the indicated time. Cells were subjected to FACS analysis and Western blot analysis for MDM2, cyclin B1 and actin. The percentage of cells in G1, S and G2/M is shown. E: *MDM2* mRNA levels are comparable during the cell cycle. HeLa and U2-OS cells were synchronized by double thymidine block and release. Total RNA was isolated and subjected to Northern blot analysis for *MDM2* and *β-actin*. The intensity of individual *MDM2* mRNA and *β-actin* mRNA bands was quantitated by densitometry scanning. The ratio of *MDM2* mRNA over *β-actin* mRNA at each time point is presented as relative intensity.

Biochemicals). 6 h after transfection, cells were trypsinized and reseeded for an additional 4 h prior to the treatment with double thymidine block (2 mM) and release. For cycloheximide treatment, cells synchronized by double thymidine block and release were treated by cycloheximide (25 μg/ml) for the indicated time. Cells were collected and subjected to FACS and Western blot analyses. To examine the effect on cell cycle by ectopic expression of MDM2 protein, U2-OS cells were co-transfected with pCMV-CD19 and pCMV-MDM2 derivative or vector DNA. 48 h after transfection, cells were collected, stained with a monoclonal antibody specific for CD19 followed by propidium iodine staining and FACS analysis. The cell cycle distribution of $CD19^+$ cells was analyzed by Cell Quest program.

3. Results

3.1. Cell cycle-dependent regulation of MDM2 protein expression

HeLa cells were synchronized at early S phase by double thymidine block as previously described [23,24] (Fig. 1A). After release from the double thymidine block, cells entered S phase at 2–4 h, G2/M phase at 6–8 h and exited M phase after 10 h (Fig. 1A). As expected, cyclin B1 protein levels increased in S phase and peaked at G2/M and began to decline at the onset of anaphase (Fig. 1B), via the APC-dependent degradation pathway [25,26]. Similarly, MDM2 protein appeared to be accumulated when cells entered S phase and remained at high levels through S and G2/M and declined thereafter (Fig. 1B). A similar pattern of MDM2 protein expression was observed in another human cancer cell line, U2-OS (Fig. 1C).

We then examined whether the decrease in MDM2 protein levels in M phase correlates with that of cyclin B1. We treated HeLa and U2-OS cells with nocodazole, which arrests cells at prometaphase [27]. After release from nocodazole treatment, cells proceeded through M phase and anaphase in a synchronized fashion (Fig. 1D). Whereas cyclin B1 protein levels were high in nocodazole-arrested cells and started to decrease at 30–60 min after release, as expected (Fig. 1D), MDM2 protein levels were down-regulated in nocodazole-arrested cells and recovered after several hours release from nocodazole treatment. These data suggest that down-regulation of MDM2 protein at M phase proceeds before cyclin B1 degradation.

Since MDM2 protein levels are up-regulated during S-G2 phase, we next examined whether the increased MDM2 protein level is due to increased MDM2 gene transcription. We therefore examined the steady-state levels of *MDM2* mRNA during the cell cycle. As shown by Northern blot analysis, the steady-state levels of *MDM2* mRNA appeared to be comparable during the cell cycle in both HeLa and U2-OS cells (Fig. 1E), suggesting that the cell cycle regulation of MDM2 protein expression is unlikely to be at the transcriptional level, nor at the mRNA stability level. Rather, the MDM2 protein stability could be regulated during the cell cycle.

3.2. The RING finger domain of MDM2 is required for cell cycle-dependent regulation of MDM2 protein

Since the RING finger domain of MDM2 has been shown to be important for the E3 ligase activity as well as for its self-

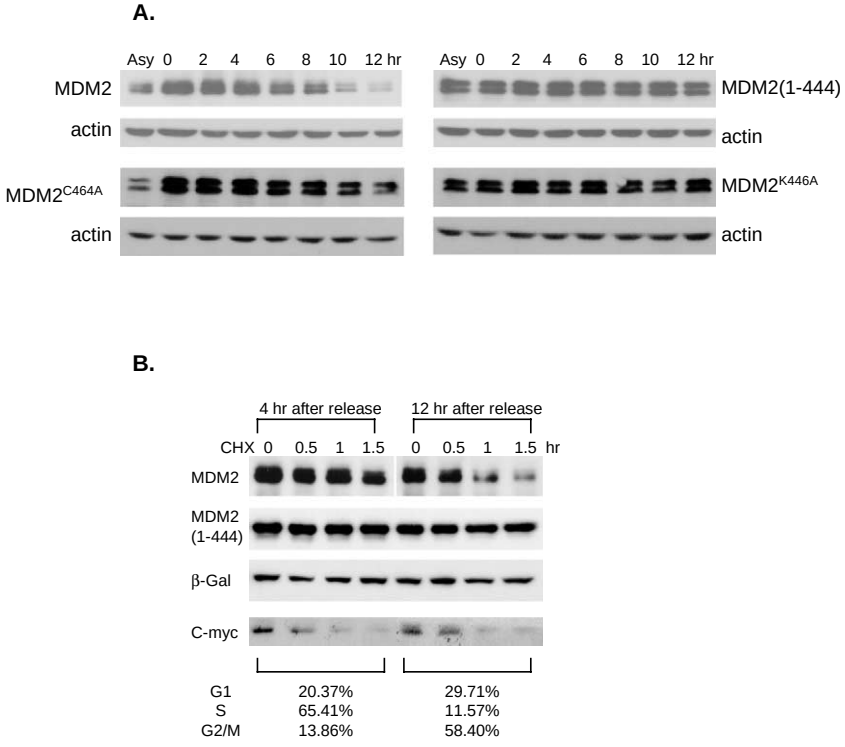

Fig. 2. The RING finger domain of MDM2 is required for cell cycle-dependent regulation. A: HeLa cells were transfected with pCMV-MDM2, pCMV-MDM2(1–444), pCMV-$MDM2^{C464A}$ or pCMV-$MDM2^{K446A}$. 6 h after transfection, cells were trypsinized and reseeded for 4 h. Cells were then synchronized by double thymidine block and release. Equal amounts of total proteins were separated on 10% SDS–PAGE and subjected to Western blot analysis for MDM2 or actin. B: MDM2 protein is stabilized at S phase. U2-OS cells were transfected with pCMV-β-Gal and pCMV-MDM2 or pCMV-MDM2(1–444). 6 h after transfection, cells were synchronized by double thymidine block and were then treated with cycloheximide (CHX) at either 4 h or 12 h after release of thymidine treatment. Cells were collected at the indicated time points. Aliquots of cells were subjected to FACS analysis, whereas equal amounts of total proteins derived from the cell lysates were separated on 10% SDS–PAGE and subjected to Western blot analysis for MDM2, β-galactosidase and C-Myc.

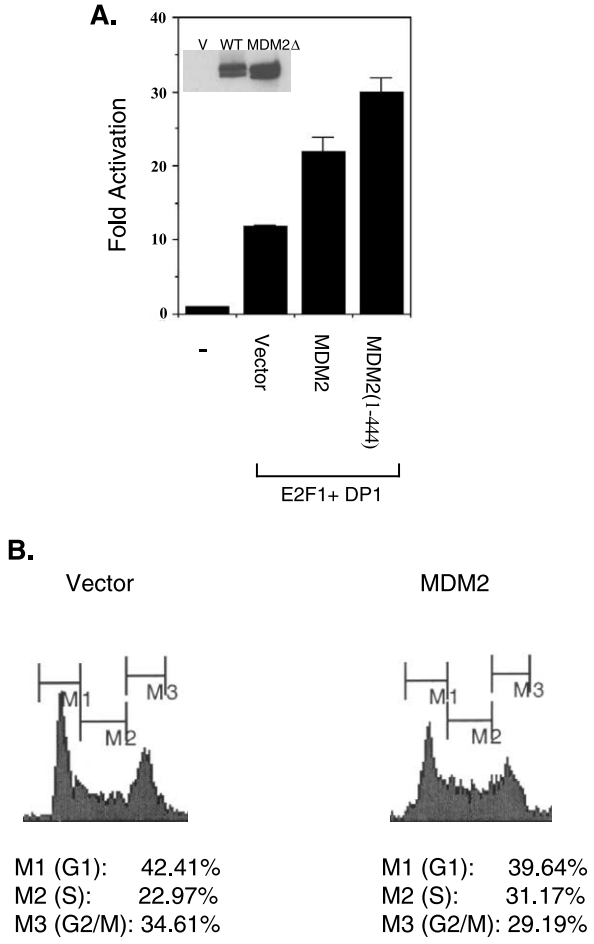

Fig. 3. Overexpression of MDM2 leads to enhanced E2F activity and increase in S-phase cell population. A: Saos-2 cells were co-transfected with pCMV-E2F1, pCMV-DP1 and either pCMV-MDM2, pCMV-MDM2(1–444) or vector DNA in the presence of pCMV-β-Gal and an E2F reporter (DHFR-luciferase). 24 h after transfection, cells were collected and lysed in the luciferase lysis buffer. The luciferase activity was normalized to β-galactosidase activity and presented as fold activation. Inset: Western blot analysis data showing the MDM2 protein levels in cells transfected with wild-type MDM2 (WT), MDM2(1–444) (MDM2Δ) or vector control (V). B: U2-OS cells were co-transfected with pCMV-CD19 and pCMV-MDM2 or vector DNA. 48 h after transfection, cells were harvested, stained for CD19 and propidium iodide, and subjected to FACS analysis. A representative of the cell cycle distribution of $CD19^+$ cells from three experiments is shown.

ubiquitination [4–6], we reasoned that the E3 ligase activity of MDM2 may play a role in the cell cycle-dependent regulation of MDM2 protein levels. We therefore examined the ectopic expression of MDM2 and its mutant MDM2(1–444), which lacks the RING finger domain and E3 ligase function, in synchronized HeLa cells. Cells transfected with either pCMV-MDM2 or pCMV-MDM2(1–444) were synchronized by double thymidine block and release. Western blot analysis showed that the levels of ectopically expressed wild-type MDM2 protein were high in thymidine-treated cells and remained high in S and G2/M phases, but significantly declined thereafter (Fig. 2A). However, the expression level of MDM2(1–444) was comparable throughout the cell cycle, suggesting that the RING finger domain plays a critical role in the cell cycle-dependent regulation of MDM2 protein stability. To address whether the E3 ligase activity of MDM2 is involved in cell cycle-dependent regulation on its protein, we examined the point mutant MDM2 protein, either defective in E3 ligase ($MDM2^{C464A}$) [4–6] or defective in auto-ubiquitination ($MDM2^{K446A}$) [28,29]. Interestingly, the mutant $MDM2^{C464A}$ protein was still subjected to cell cycle-dependent regulation. In contrast, the mutant $MDM2^{K446A}$ protein, devoid of auto-ubiquitination, appeared stable throughout the cell cycle (Fig. 2A). To further investigate the observed cell cycle effect on MDM2 protein expression, we examined the MDM2 protein half-life in S and G2/M phases. U2-OS cells were co-transfected with pCMV-β-gal and either pCMV-MDM2 or pCMV-MDM2(1–444) prior to synchronization by double thymidine block. At 4 h (S phase) or 12 h (G2/M phase) after release from the thymidine block, cycloheximide was added to the cells to block protein synthesis. As shown in Fig. 2C, the wild-type MDM2 protein was stable at S phase ($t_{1/2} > 90$ min) (data not shown), whereas the protein half-life was significantly shorter at M phase ($t_{1/2} < 45$ min). In contrast, unlike wild-type MDM2, MDM2(1–444) was stable in both S and G2/M phases ($t_{1/2} > 90$ min). As a control, the protein half-life of the co-expressed β-galactosidase remained comparable in both S and G2/M phases. Furthermore, the half-life of c-Myc protein (approximately 30 min) was similar in both S phase and M phase, in agreement with the published data [30–33]. These results indicate that the cell cycle-dependent regulation of MDM2 is likely through the ubiquitin-mediated proteasome degradation pathway and the C-terminal RING finger domain plays a critical role in this process.

3.3. *Overexpression of MDM2 leads to stimulation of E2F activity and an increase in S-phase cell population*

Given that MDM2 protein level is increased in S phase during the cell cycle, we reasoned that MDM2 may modulate the RB–E2F pathway in promoting the cell cycle S-phase entry [12,13]. Indeed, as shown in Fig. 3A, ectopic expression of wild-type MDM2 led to a clear stimulation of the E2F activity. Importantly, mutant MDM2(1–444) lacking the RING finger domain exhibited even higher activity in stimulating E2F activity (Fig. 3A), likely due to the stabilization of the mutant MDM2 protein. Furthermore, ectopic expression of MDM2 in U2-OS cells led to a clear increase in S-phase cell population (Fig. 3B). Taken together, these data suggest that the up-regulation of MDM2 protein at S phase may be important in modulation of the RB–E2F pathway in promoting cell cycle S-phase entry.

4. Discussion

Our data indicate that MDM2 protein is stabilized at S phase and declined at G2/M phases, suggesting that MDM2 protein degradation is regulated during the cell cycle. Interestingly, the degradation of MDM2 protein requires the RING finger domain but does not require its own E3 ligase activity since the point mutation inactivating the E3 ligase ($MDM2^{C464A}$), which leads to MDM2 protein stabilization, does not block the cell cycle-dependent degradation. However, a single amino acid substitution at Lys446, a site identified as auto-ubiquitination of MDM2 [28,29], does abolish the cell cycle-dependent regulation of the MDM2 protein. Together, these observations imply that E3 ligase activity of MDM2 is critical for MDM2 protein stability and another

E3 ligase, however, is responsible for cell cycle-dependent MDM2 protein degradation. It is interesting to note that inhibition of cdk by roscovitine results in reduced MDM2 protein levels [34] and that murine mdm2 protein is phosphorylated by cyclin A-associated kinase at Thr216, resulting in the increased ARF–MDM2 interaction [22]. The human MDM2 protein, although lacking the conserved Thr residue at the corresponding position, contains a cluster of conserved serines or threonines around this region and therefore may be regulated in a similar fashion. It is possible that specific cell cycle-dependent phosphorylation may serve as specific signal(s) to trigger the modulation of MDM2 protein stability. Further work is needed to elucidate the exact mechanism.

It has been well established that MDM2 oncogenic function is associated with its ability in promoting cell cycle progression and/or inhibit apoptosis. MDM2 inhibits RB and activates E2F activity [12,13]. Our data from this study also indicate that activation of E2F correlates well with the amount of MDM2 protein. Furthermore, overproduction of MDM2 can promote S-phase entry. These data suggest that MDM2 may play an important role in cell cycle progression.

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