

Mdm2 Mutant Defective in Binding p300 Promotes Ubiquitination but Not Degradation of p53*

EVIDENCE FOR THE ROLE OF p300 IN INTEGRATING UBIQUITINATION AND PROTEOLYSIS

Received for publication, March 23, 2001, and in revised form, April 19, 2001
Published, JBC Papers in Press, May 4, 2001, DOI 10.1074/jbc.M102634200

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Turnover of the p53 tumor suppressor protein is mediated by Mdm2 through the ubiquitin proteolysis pathway. p300, a co-activator for p53, also participates in this process by complexing with Mdm2. We now report that the mutant Mdm2, defective in p53 binding, does not promote p53 ubiquitination and degradation *in vivo* or inhibit p53 transcriptional activation. By contrast, the mutant Mdm2, defective in p300 binding, still retains its activity to promote p53 ubiquitination and to inhibit p53 transcriptional activation but fails in promoting p53 degradation. We also show that both wild-type Mdm2 and the mutant Mdm2, defective in p300 binding, can promote the ubiquitination of cancer-derived p53 mutants, but only wild-type Mdm2 can cause their degradation. Furthermore, adenoviral oncoprotein, 12S.E.1A, but not its deletion mutant that lacks p300 binding, was shown to decrease *in vivo* ubiquitination of mutant p53. Taken together, these results provide genetic evidence that p300 plays a pivotal role in the regulation of Mdm2-mediated p53 turnover by integrating the cellular ubiquitination and proteolytic processes.

The human tumor suppressor p53 plays a critical role in maintaining genomic stability and preventing tumorigenesis (1). Diverse mutations in the *p53* gene constitute the most common type of genetic alterations in human cancers (2). Li-Fraumeni Syndrome (LFS)¹ patients, suffering from a cancer-prone hereditary disorder, have been shown to harbor germ line mutations of the *p53* gene (3). Studies on *p53* knockout mice have provided supporting evidence for p53 functioning as a key tumor suppressor (4). In cellular response to DNA damage and other stresses, p53 protein accumulates and transcriptionally activates its target genes, which include *p21^{cip1/waf1}*, *GADD45*, *Mdm2*, *bax*, and other p53-inducible genes. The activation of these genes is believed to result in either cell cycle arrest or apoptosis (5, 6). Additionally, p53 has been demonstrated to have a role in the modulation of nucleotide excision repair (7–11).

* This work was supported by NIEHS, National Institutes of Health Grant ES2388. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: LFS, Li-Fraumeni syndrome; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein ligase; Ub, ubiquitin; HA, hemagglutinin; CBP, cAMP response element (CREB)-binding protein; CMV, cytomegalovirus; Mdm2, murine double minute; Wt, wild type.

It has been established that the turnover of p53 is regulated in part by the ubiquitin proteolysis pathway (12, 13). Ubiquitin is first sequentially transferred through the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme, and the ubiquitin-protein ligase (E3). The E3 enzyme then transfers the ubiquitin to one or more lysine residues in the substrate. Multiple ubiquitin molecules are attached to one another to form a polyubiquitin chain, which is deemed sufficient in targeting substrate proteins for destruction by the proteasome (reviewed in Ref. 14). It has been shown that Mdm2 can regulate the stability of p53 through the ubiquitin proteolysis pathway (15–17). More recent studies have demonstrated that Mdm2 has intrinsic E3 activity, which can ubiquitinate p53 and Mdm2 itself (18–21).

Although *in vitro* ubiquitination of p53 by Mdm2 only requires the addition of E1, ubiquitin-conjugating enzyme, and Ub (18, 21), participation of other cellular factor(s) can be envisaged in the complex processes of p53 ubiquitination and degradation *in vivo*. For example, adenoviral oncoprotein E1A has been shown to cause p53 stabilization in multiple cell types through interaction with p300/CBP or with Rb family proteins (22–25). The p53 polyubiquitination activity in cell extracts has demonstrated a significant decrease upon the induction of E1A expression (26). It has been reported recently that most endogenous Mdm2 is bound to p300 and that specific interactions between p300/CBP, p53, and Mdm2 are intimately involved in Mdm2-mediated p53 degradation (27).

Our previous studies have shown that interaction of p300/CBP with DNA repair protein hHR23A leads to the down-regulation of p53 (28). The current experiments investigated whether cellular p300 plays a role as a platform to bring together the necessary catalytic and regulatory factors needed for *in vivo* p53 ubiquitination and degradation (27). The data presented demonstrate that (i) the Mdm2 mutant defective in binding to p300/CBP can promote ubiquitination but not degradation of either wild-type or cancer-derived mutant p53, and (ii) wild-type E1A, but not its deletion mutant lacking p300/CBP binding, can decrease ubiquitination of p53 *in vivo*. These results provide evidence that p300 plays a pivotal role in Mdm2-mediated p53 degradation by integrating ubiquitination and proteolytic processes.

MATERIALS AND METHODS

Gene Constructs—Wild-type and mutant p53 expression vectors, p53(Wt), p53-143A (Val → Ala), p53-173H (Arg → His), and p53-248W (Arg → Trp) as well as pG13-*luc* were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Construct pG13-*luc* is a luciferase reporter containing 13 copies of synthetic p53 consensus binding sites derived from the native p21^{cip1/waf1} promoter (29). Mdm2 constructs, pCMV-*Mdm2*, pCMV-*Mdm2Δ4* and pCOC-*Mdm2ΔXXM*, were provided by Dr. Arnold Levine (Princeton University, Princeton, NJ), Dr. David Livingston (Harvard Medical School, Boston, MA), and

Dr. Moshe Oren (The Weizman Institute of Science, Rehovot, Israel), respectively. The pCMV-*Mdm2* encodes a full-length Mdm2 protein. The pCMV-*Mdm2Δ4* encodes a mutant Mdm2 protein with a deletion from 217 to 246 amino acid positions. The pCOC-*Mdm2ΔXM* contains an internal deletion and consequently encodes a truncated Mdm2 protein initiated at the AUG amino acid position 62 (30). The Wt 12S.E.1A and mutant E1A constructs were obtained from Dr. Michael Mathews (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The pBIND-p53-(1–71) and pBIND-p53-(19–71), encoding the corresponding p53 N-terminal domains, were constructed by in-frame fusion of the respective domains with yeast Gal4 DNA-binding domain. The specific p53 domains were generated by polymerase chain reaction, and individual fragments were inserted into the parent plasmid vector, pBIND (Promega). The pG5-*Luc* vector, containing five Gal4 binding sites upstream of a minimal TATA box followed by a firefly luciferase gene, was purchased from Promega. The expression vector for HA-Ub (31) was obtained from Dr. Dirk Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany).

Cell Culture, Transfection, Immunoprecipitation, and Immunoblotting for Ub-p53 Conjugates—The LFS 041 fibroblast strain MDAH041 (p53-null, harboring a codon 184 frameshift mutation that results in premature termination of translation of the p53 protein) was kindly provided by Dr. Michael Tainsky (M.D. Anderson Cancer Center, Houston, TX). These fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 37 °C in a humidified atmosphere of 5% CO₂. For the detection of Ub-p53 conjugates *in vivo*, exponentially growing LFS 041 fibroblasts were seeded at a density of 5–6 × 10⁵ cells/10-cm dish 18–20 h before transfection. The cells were transfected with expression vectors for p53, Mdm2 or mutant Mdm2, HA-Ub, and 12S.E.1A or mutant E1A in different combinations as indicated in each figure legend. Transfections were performed using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as described previously (28). The total amount of DNA for each transfection was kept constant by the addition of cognate empty vector. 24 h after transfection, cells were washed with PBS and then treated for 10 h with the proteasome inhibitor MG132 or lactacystin at 10 μM. Protein extracts were made from all cells including any detached cells in 750 μl of radioimmune precipitation buffer (2 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1.0% Nonidet P-40, 1.0% deoxycholate, 0.025% SDS, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture). The extracts were sonicated to reduce the viscosity. Immunoprecipitates were prepared by overnight incubation with 15 μl of anti-p53 monoclonal antibody DO6 (200 μg/ml, NeoMarkers) and 20 μl (bed volume) of protein A/protein G agarose beads (Oncogene Science) at 4 °C. The beads were washed four times with radioimmune precipitation buffer, and the bound proteins were boiled off in SDS sample buffer for 5 min and then separated by 8% SDS-polyacrylamide gel electrophoresis. To avoid any detection of mouse IgG (~55 kDa), separated proteins with molecular mass greater than the ~60-kDa range were transferred to a polyvinylidene difluoride membrane and probed with an anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), and proteins were detected as described previously (9, 32). The bound antibodies were removed from the filters with a stripper buffer (62.5 mM Tris-HCl, pH 6.7, 500 mM NaCl, 2% SDS, and 100 mM β-mercaptoethanol), and the membranes were rescued for second immunodetection of the Ub-p53 conjugates by anti-p53 antibody Ab-1801.

Western Blot Protein Analysis—To detect p53 and other proteins without immunoprecipitation, the expression vector for p53 or mutant p53 was co-transfected with Mdm2, Mdm2ΔXM, or Mdm2Δ4 expression vector. To evaluate the effects of E1A expression on Mdm2-mediated p53 degradation, the expression vector for p53 was similarly co-transfected with *Mdm2* in the absence and presence of increasing amounts of 12S.E.1A or E1AΔ-(2–36) expression vector. In these experiments, the LFS 041 fibroblasts (5–6 × 10⁵) were transfected for 24 h, the cells were washed and lysed in an SDS sample buffer, and proteins were quantitated and processed by SDS-polyacrylamide gel electrophoresis and immunoblotted for specific detection of p53, Mdm2, and E1A as well as actin proteins.

Reporter Assay—For the transient transfection/reporter assay, exponentially growing LFS 041 fibroblasts were seeded at a density of 1 × 10⁵ cells/35-mm dish 18–20 h prior to transfection. 0.5 μg each of p53 reporter pG13-*luc* and p53 expression vectors were co-transfected into LFS 041 cells in the absence or presence of 2 μg of expression vector for wild-type or mutant Mdm2 using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according manufacturer instructions. Empty vector, pcDNA3, DNA was used to maintain the total amount of DNA for each transfection. 24 h after transfection, cells were washed

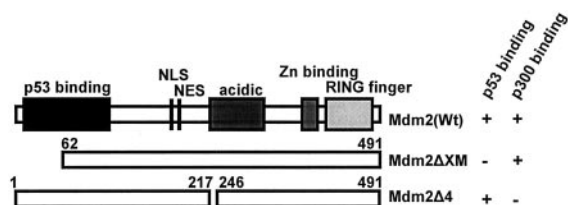


FIG. 1. Inherent properties of wild-type and mutant variants of Mdm2. The schematic presentation defines various elements identified in wild-type Mdm2 and the positions of different deletions in mutants used in the present work. +, presence; -, absence.

twice with PBS and lysed in 100 μl of cell culture lysis reagent. Luciferase activity was determined in 20 μl of cell lysates by using a luciferase substrate (Promega) and monitoring luminescence with a TD-20/20 luminometer (Turner Designs). To determine transcriptional activation by the p53 N-terminal transcription activation domain fused to Gal4 DNA binding domain, the pG5-*luc* reporter was substituted for the pG13-*luc* in the transfection assay. Relative luciferase activity for each time point was determined from three independently transfected plates.

RESULTS

Mutant Mdm2Δ4, Defective in p300 Binding, Retains Its Ability to Ubiquitinate p53 *In Vivo*—p53 degradation depends on its ability to specifically interact with the N-terminal domain of Mdm2 (15, 17). Furthermore, the p300 protein, as part of a complex with Mdm2, is also shown to be involved in Mdm2-mediated p53 degradation (27). In view of recent studies on Mdm2 as a ubiquitin ligase (18, 19, 21, 33), we sought to understand the contributions of the specific protein interactions of Mdm2 and p300 in p53 ubiquitination *in vivo*. Thus, the initial experiments determined the ability of two structurally different Mdm2 mutants to promote p53 ubiquitination. As shown in Fig. 1, Mdm2ΔXM contains an N-terminal deletion, and the truncated Mdm2 protein starts at amino acid position 62. Because of its inability to bind p53, this truncated Mdm2 protein fails to (i) inhibit p53-dependent transcriptional activation, (ii) abolish p53-mediated apoptosis, and (iii) promote *in vivo* p53 degradation (15, 30). However, Mdm2Δ4 contains an internal deletion and the protein, while retaining its ability to bind p53, is defective in binding p300 *in vivo*. Consequently, Mdm2Δ4 is also defective in promoting p53 degradation *in vivo* (27). Thus, *in vivo* ubiquitination of wild-type and various mutant p53 proteins was determined upon co-expression with these Mdm2 molecules and by immunoprecipitation and Western blotting. As can be seen in Fig. 2A, an anti-HA-reactive ladder of bands, appearing as a smear of apparent molecular mass of ~66–220 kDa and representing HA-Ub-p53 conjugates, was distinctly visible in lane 4 where p53 was co-transfected with *Mdm2(Wt)* and *HA-Ub*. No such conjugates were apparent in samples of various control transfections without *HA-Ub* (lanes 1, 2, and 3) or without *Mdm2* (lane 7). The data suggest that the conjugates result from specific interaction with Mdm2. As expected, co-transfection of p53 with mutant *Mdm2ΔXM* did not produce any conjugates (lane 5), indicating that because of its inability to bind p53, Mdm2ΔXM is defective in mediating p53 ubiquitination. This observation is consistent with the early finding that Mdm2ΔXM does not promote p53 degradation *in vivo* (15, 30). Ubiquitinated p53 conjugates appeared with an even higher intensity when p53 was co-transfected with mutant *Mdm2Δ4* (lane 6). Thus, this mutant, having an intact N terminus, can promote pronounced p53 ubiquitination. Overall results indicate that Mdm2 defective for p300 binding is not impaired in its ability to mediate p53 ubiquitination. This observation is novel and highly interesting, because as mentioned above, such mutant Mdm2 is defective in promoting p53 degradation (27).

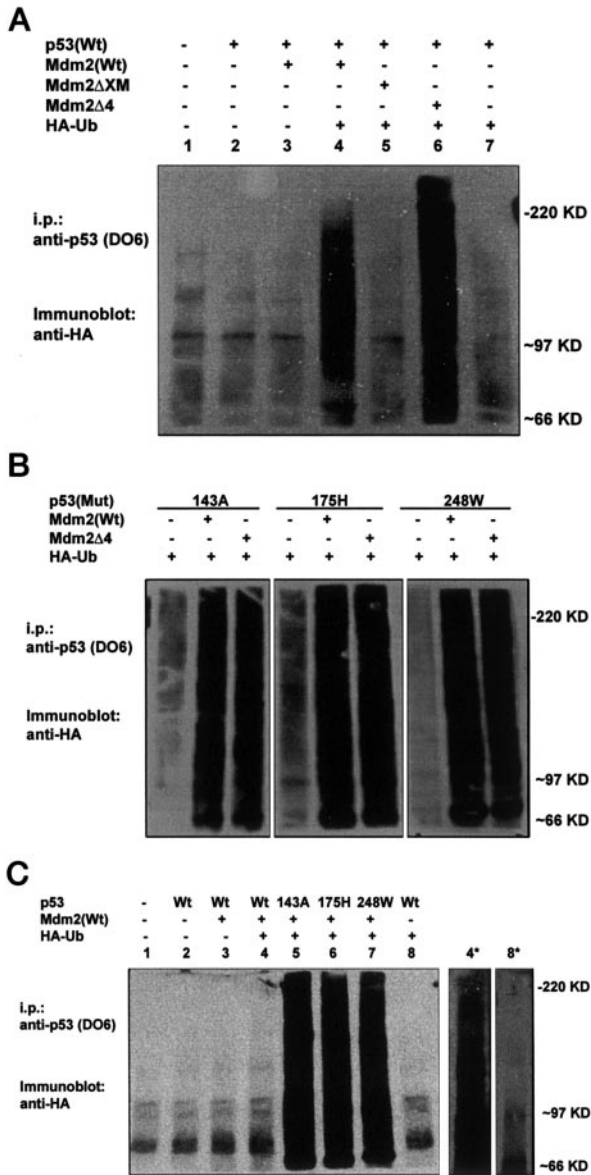


FIG. 2. In vivo ubiquitination of p53 by Mdm2. LFS 041 cells were transfected with the indicated expression vectors for p53 (1 μ g), Mdm2(Wt), or mutant Mdm2 (4 μ g) along with HA-Ub (4 μ g). The total amount of DNA for each transfection was kept at 10 μ g by the addition of empty vector. After 24 h of transfection, cells were treated for 10 h with the proteasome inhibitor MG132 (10 μ M). Whole-cell extracts were made in radioimmune precipitation buffer, and immunoprecipitates were prepared by using the anti-p53 monoclonal antibody DO6 and protein A/protein G agarose beads. Immunoprecipitates (*i.p.*) were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with an anti-HA antibody. *A*, p53(Wt) and wild-type or mutant Mdm2 were co-expressed with or without HA-Ub as indicated. *KD*, kilodalton. *B*, mutant p53 and the indicated wild-type or mutant Mdm2 were co-expressed with HA-Ub. *C*, wild-type and mutant p53 and Mdm2(Wt) were co-expressed, immunoprecipitated, and analyzed simultaneously. *Lanes 4** and *8** show the longer time exposure of HA-Ub-p53 conjugates seen in the corresponding *lanes 4* and *8*.

To confirm that Mdm2 Δ 4 specifically mediates ubiquitination of p53, various cancer-derived p53 mutants were tested for ubiquitination upon transfection by Mdm2 and Mdm2 Δ 4 (Fig. 2*B*). The p53 mutants 143A (Val \rightarrow Ala), 173H (Arg \rightarrow His), and 248W (Arg \rightarrow Trp) were seen to ubiquitinate to approximately equivalent levels when co-expressed with Mdm2(Wt). More importantly, mutant Mdm2 Δ 4, with an intact N terminus, also ubiquitinated the various p53 mutants tested to an extent comparable with Mdm(Wt). These results reproducibly demon-

strate that Mdm2 Δ 4 can ubiquitinate both wild-type and mutant p53 proteins. It should be noted that these mutant forms of p53 are devoid of sequence-specific DNA binding activity and were unable to activate the expression of a gene under the control of p53-responsive DNA binding site (29). Thus, these results suggest that the p53 transcriptional activation process is not associated with p53 ubiquitination.

For direct comparison, the ubiquitination of wild-type and cancer-derived mutant p53 by Mdm(Wt) in concomitant transfections and identical exposure conditions is shown in Fig. 2*C*. Surprisingly, ubiquitin conjugates of p53 mutants were found to accumulate *in vivo* much more efficiently than those of p53(Wt) (*lanes 5, 6, and 7 versus lane 4*). Nevertheless, ubiquitin conjugates of p53(Wt) (as shown above (Fig. 1*A*)), could be visualized with longer time exposures (*lane 4** and *8**). It should be noted that in all these experiments, the proteasome inhibitor MG132 had to be used to treat cells after transfection to allow ubiquitin conjugates to accumulate. Also, similar results were obtained using another highly specific and irreversible proteasome inhibitor, lactacystin (34) (data not shown). Thus, it would seem that the inhibitory effect of proteasome inhibitors was manifested more prominently on mutant p53 than on p53(Wt) ubiquitin-protein conjugates, perhaps also because mutant p53 is more easily ubiquitinated.

Mutant Mdm2 Δ 4, Defective in p300 Binding, Abrogates Transactivation by p53—Mdm2 is known to bind p53 and inhibit its transcriptional activation (30). Here we investigated the effect of mutant Mdm2 Δ 4 overexpression on the ability of p53 for transactivation. LFS 041 cells were co-transfected with p53(Wt) and the pG13-*luc* reporter in the presence of Mdm2(Wt), Mdm2 Δ XM, or Mdm2 Δ 4. Luciferase activity of the cell extracts from various transfections is shown in Fig. 3. As expected, p53(Wt) dramatically elevated luciferase activity relative to the control pCMV vector (Fig. 3*A*). Co-transfection of Mdm2(Wt) blocked the ability of p53 to transactivate the reporter construct. By contrast, mutant Mdm2 Δ XM failed to inhibit p53 activity, confirming that the inhibition requires the binding of Mdm2 to p53 (30). On the contrary, mutant Mdm2 Δ 4, in keeping with its ability to bind p53, was found to block transactivation by p53 to an extent comparable with that of Mdm2(Wt) (27). Similar experiments were then carried out to verify the effects of Mdm2 and its mutants on transcriptional activity of the p53 N-terminal activation domain. As shown in Fig. 3*B*, co-transfection of Mdm2(Wt) or mutant Mdm2 Δ 4 inhibited luciferase activity transcribed from reporter pG5-*luc* to a comparable extent. However, mutant Mdm2 Δ XM failed to exhibit any inhibitory effects but instead increased luciferase activity \sim 1.5-fold. Deletion of the Mdm2-binding site (from 1 to 19 amino acid positions) caused a dramatic decrease in the transactivation activity of Gal4-p53 to a level that was slightly above that achieved with Mdm2(Wt). Both Mdm2(Wt) and mutant Mdm2 Δ 4 were unable to exhibit a significant inhibitory effect on the transactivation activity of the truncated Gal4-p53-(19–71). These results show that the amino acids, spanning positions 1–19 in the p53 activation domain, contribute a very important component to the transactivation by p53 and provide the needed binding site for Mdm2 to cause inhibition of p53 activity. Taken together, it can be concluded that the binding of Mdm2 to p53, but not to p300, is responsible for the inhibition of p53-dependent transcriptional activation. Therefore, the binding of Mdm2 to p53 is required for both the ubiquitination and inactivation of p53.

Mutant Mdm2 Δ 4, Defective in p300 Binding, Fails to Promote Degradation of p53 in Vivo—Failure to promote *in vivo* p53 degradation by the mutants Mdm2 Δ XM and Mdm2 Δ 4 was confirmed by co-transfection experiments in LFS 041 cells and

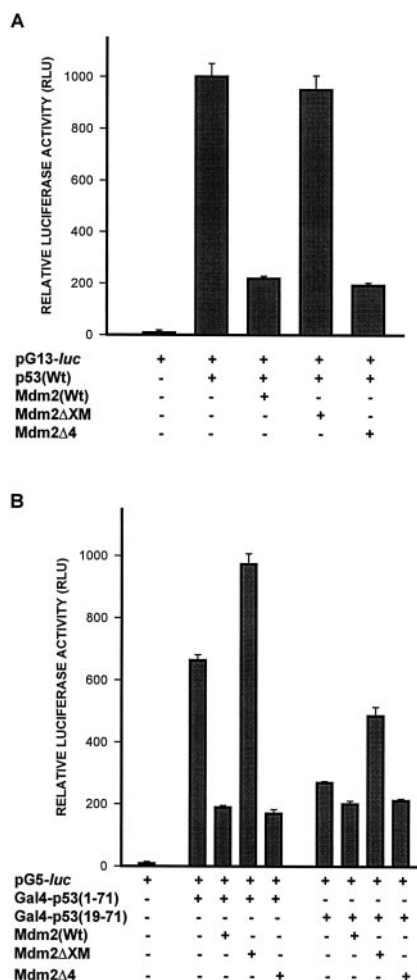


FIG. 3. Wild-type and mutant Mdm2 differentially inhibit the transcriptional activation by p53. *A*, p53 reporter pG13-*luc* and p53 expression vectors were co-transfected into LFS 041 cells in the absence (-) or presence (+) of expression vector for indicated wild-type or mutant Mdm2. Relative luciferase activity was measured as described under "Materials and Methods." The figure shows representative data of at least three independent experiments, with each transfection performed in triplicate. The data points indicate the mean \pm S.E. *B*, Gal4 reporter pG5-*luc* and expression vectors Gal4-p53(1-71) or Gal4-p53(19-71) were co-transfected into LFS 041 cells in the absence or presence of expression vectors for wild-type or mutant Mdm2 as indicated. Transfection, relative luciferase activity, and data presentation was as described for *A*.

analyzing the protein steady-state levels by Western blotting. As expected from previous studies (15, 27), the p53 steady-state level was reduced markedly in cells co-transfected with Mdm2(Wt). No such decrease was, however, seen in cells co-transfected with either the *Mdm2*ΔXM or *Mdm2*Δ4 mutants (Fig. 4A). In comparison to p53 levels, cells transfected with the mutant *Mdm2* showed appreciably higher levels of Mdm2 than those transfected with either the *Mdm2*(Wt) or the control vector. Similar patterns were exhibited in experiments extended to determine whether Mdm2 could promote degradation of cancer-derived mutant p53 proteins. The data indicate that these cancer-derived p53 missense mutants can efficiently, albeit with varying degrees, be down-regulated upon co-expression of Mdm2(Wt) but not mutants Mdm2ΔXM or Mdm2Δ4 (Fig. 4B). In conclusion Mdm2, to effectively maintain the cellular p53 protein level, requires the functional interaction of Mdm2 and p53 for the dual events, *i.e.* ubiquitination and degradation, of the p53 ubiquitin-proteasomal pathway. Ubiquitination of p53, however, does not necessarily lead to protea-

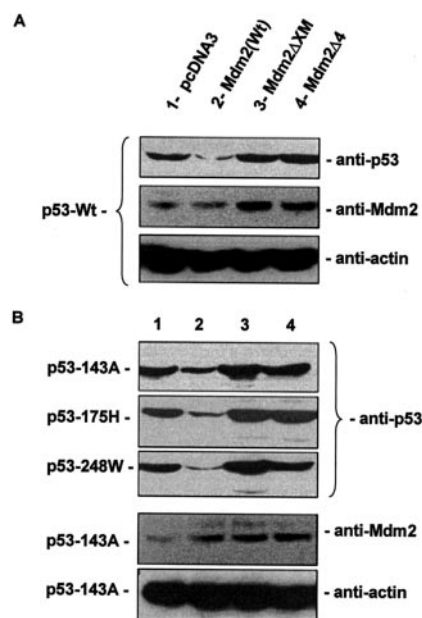


FIG. 4. Mdm2, defective in CBP/p300-binding, fails to diminish p53 steady-state level. 1.0 μ g of expression vector for wild-type (*A*) or various tumor-derived mutant p53 (*B*) proteins were transfected into LFS 041 cells together with 4.0 μ g of empty vector (control) or expression vectors for wild-type or the mutants Mdm2ΔXM or Mdm2Δ4. After 24 h of transfection, the cells were lysed in SDS sample buffer, and the proteins were quantitated and processed by SDS-polyacrylamide gel electrophoresis and Western blotting for p53, Mdm2, and actin as a loading control.

somal degradation, which depends on the additional binding interaction of Mdm2 with p300.

Stabilization of p53 Protein and Inhibition of p53 Ubiquitination by Adenoviral 12S.E.1A Protein—E1A can stabilize p53 via binding to p300-(22–25). This binding is known to disrupt the transcriptional co-activation mediated by CBP/p300 (35). In an effort to explore the role of p300 in the regulation of p53 stability, we next examined whether E1A could stabilize p53 protein in the presence of Mdm2. As shown in Fig. 5A, Mdm2 expression drastically decreased the steady-state level of p53. Co-expression of 12S.E.1A overcame the Mdm2-affected decrease of p53 steady-state levels in a dose-dependent manner and restored it to the level observed in control without any *Mdm2*. Interestingly, co-expression of E1AΔ-(2–36) lacking p300-binding also resulted in the restoration of the p53 expression level as reported earlier (36) (Fig. 5B). Because E1AΔ-(2–36) is known to possess the ability to bind several members of the Rb family (37), this result would seem to suggest the involvement of other cellular factor(s), *e.g.* Rb, in this process.

To examine the effect of E1A on the *in vivo* ubiquitination of p53 by Mdm2, we took advantage of our previous observation that the proteolysis of ubiquitin conjugates of p53 mutants is more susceptible to inhibition by proteasome inhibitors. Because detection of cellular ubiquitin conjugates of p53(Wt) is poor even in the presence of proteasome inhibitors (Fig. 2C, lane 4), the logical use of mutant p53 makes such an examination practical. Thus, expression vectors for p53-143A, Mdm2, and HA-Ub were co-transfected into LFS 041 cells along with increasing amounts of 12S.E.1A or E1AΔ-(2–36) expression vectors (Fig. 6). Transfected cells were either treated with MG132 or mock-treated with Me₂SO to allow the detection of p53 ubiquitin conjugates. The conjugates were immunoprecipitated with an anti-p53 antibody and then immunodetected in Western blots with an anti-HA antibody. No p53-ubiquitin conjugates appeared in lanes where mutant p53 or Mdm2 were transfected alone (lanes 1, 2, 8, and 9) or in lanes of mock-

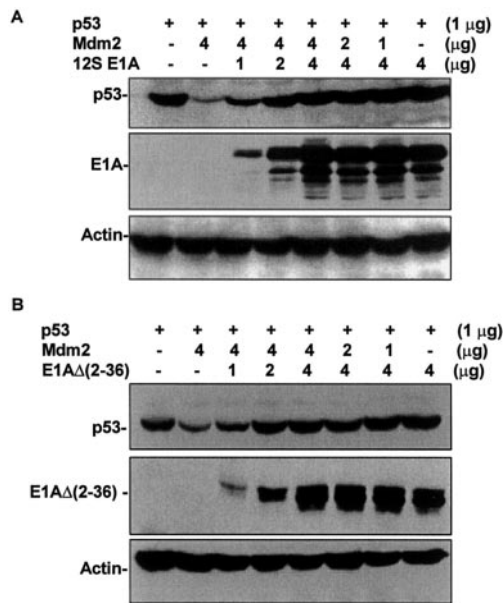


FIG. 5. Effect of oncoprotein 12S.E.1A on Mdm2-mediated p53 degradation. Expression vectors for p53 and Mdm2 along with either 12S.E.1A (A) or E1AΔ(2–36) (B) were co-transfected into LFS 041 cells in various combinations and concentrations as indicated. The total amount of DNA for each transfection was kept constant at 10 μg by adding the empty vector. Transfected cells were lysed and proteins processed as described in the Fig. 4 legend. Western blots were performed, and p53, E1A or E1AΔ(2–36), as well as actin (loading control) were visualized by using corresponding protein-specific antibodies.

treated cells without MG132 (lane 7 and 14). Compared with p53 and Mdm2 co-transfected positive controls, the inclusion of 12S.E.1A caused a significant concentration-dependent decrease in the appearance of p53-ubiquitin conjugates (lane 3 versus lanes 4, 5, and 6). Thus, the expression of 12S.E.1A clearly inhibits Mdm2-mediated p53 ubiquitination. On the contrary, co-transfection of E1AΔ(2–36) only caused a minimal, if any, decrease in p53-ubiquitin conjugates (lane 10 versus lanes 11, 12, and 13), indicating that E1AΔ(2–36) is incapable of inhibiting Mdm2-mediated p53 ubiquitination *in vivo*. These results are consistent with the earlier observations that p53 polyubiquitination activity in cell extracts *in vitro* is reduced greatly upon the induction of E1A expression (26). Therefore, the binding of E1A to p300 in effect stabilizes p53 protein by modulating the Mdm2-mediated ubiquitination.

DISCUSSION

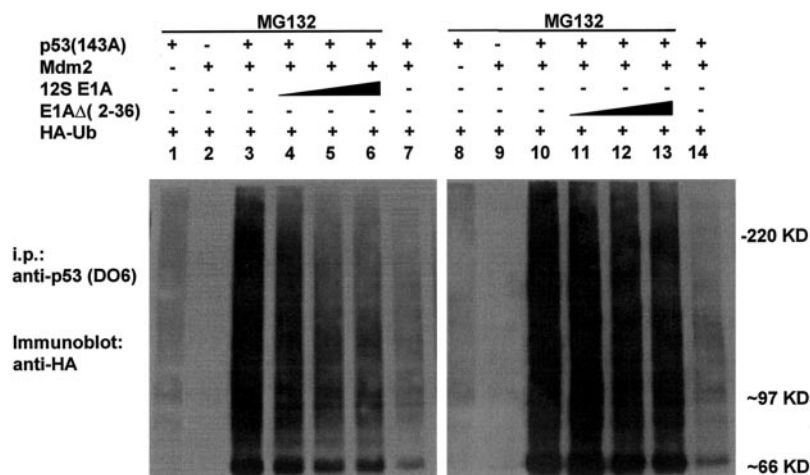
The regulation of p53 activity is in large part mediated through the changes in protein stability, which is of major significance in the response of cells to DNA damage and other cellular stresses. The tight control of metabolic stability of p53 is also critical for normal cell growth and development (38, 39). Therefore, it is of interest to identify cellular factors that contribute to the regulation of p53 turnover. In this study, we have described two deletion mutants of Mdm2 that differentially affect its ability to mediate p53 ubiquitination and degradation *in vivo*. These mutants differ from Mdm2(Wt) in regard to their binding to p53 or p300 (Fig. 1). We have also described the effects of 12S.E.1A on Mdm2-mediated p53 degradation by affecting p53 ubiquitination *via* binding to p300. These analyses provide considerable evidence that p300 plays a pivotal role in Mdm2-mediated p53 turnover by way of integrating ubiquitination and proteolytic processes.

There is now ample evidence that Mdm2 can function as a ubiquitin ligase (E3) for p53 *in vitro* (18, 21). Also, a more recent study has shown that Mdm2 can function as a ubiquitin ligase (E3) for p53 *in vivo* (20). Furthermore, we demonstrated

that Mdm2 can promote *in vivo* ubiquitination of both wild-type and specific DNA-binding defective p53 mutants. We have further demonstrated that the ubiquitination of p53 requires the binding of Mdm2 to p53 but not to p300. The capability of Mdm2 to mediate p53 ubiquitination, and its own ubiquitination *in vitro*, requires no other proteins except E1, ubiquitin-conjugating enzyme, and Ub (21). The *in vitro* E3 activity of Mdm2 has been shown to depend on its RING finger. In the same study, however, an Mdm2 mutant C449S, harboring a point mutation in the RING finger domain, was found to catalyze ubiquitination of p53 *in vitro* but did not target p53 for degradation *in vivo*. Therefore, it seems that ubiquitination and proteolysis are biochemically independent processes, and ubiquitination of p53 does not necessarily lead to proteasomal degradation (Figs. 2–4.). Because of the finding that p300-Mdm2 complexes participate in Mdm2-mediated p53 degradation, we speculated about the steps of the Mdm2-mediated ubiquitination/proteolysis pathway for p53 that could be affected such that Mdm2 mutants no longer promote p53 degradation *in vivo*. As such, the mutant Mdm2Δ4, defective in p300-binding, was demonstrated to retain the capability to mediate ubiquitination of both wild-type and mutant p53 and to inhibit p53-dependent transactivation *in vivo* but lost its capability in regard to p53 degradation *in vivo*. It should be mentioned that deletion within the 217–246 region in Mdm2Δ4 does not disrupt the nuclear export sequence, and Mdm2Δ4 can localize in the nucleus (27). Moreover, it has been shown that promoting the nuclear export of p53 by Mdm2 requires the nuclear export signal of p53 but not that of Mdm2 (40). This rules out the theory that the Mdm2Δ4 defect for p53 degradation is caused by an impaired nuclear export. Thus, the results from this report strongly support the idea that the ubiquitination and proteolysis processes are integrated by p300.

It has been hypothesized that p300 plays a role as a platform, bringing together the necessary catalytic and regulatory factors needed for p53 ubiquitination/degradation (27). In view of such a role, it can be envisaged that the p300 protein temporarily stabilizes the multiple protein complex of ubiquitination/degradation. Disruption of such multiple protein complexes through specific protein modification, *e.g.* phosphorylation or viral oncoprotein binding, would be expected to affect ubiquitination and/or degradation that in turn leads to the stabilization of p53. An examination of the effects of adenoviral 12S.E.1A on Mdm2-mediated p53 ubiquitination and degradation fully supports this scenario. Because the 12S.E.1A protein is known to disrupt p53-dependent transactivation by binding to the co-activator CBP/p300, a similar binding would disrupt the complexes of ubiquitination/degradation presumably assembled on p300. In the present study, both 12S.E.1A and E1AΔ(2–36) were shown to stabilize the p53 protein in the presence of Mdm2. However, consistent with its effects on p53 polyubiquitination activity *in vitro*, only the 12S.E.1A protein inhibited the Mdm2-mediated ubiquitination of p53 *in vivo*. It has been shown that the N-terminal transactivation domain of p53 is necessary and sufficient to drive Mdm2-mediated degradation (15). However, ubiquitination of p53 by Mdm2 does not require the direct binding of Mdm2 to p300. Biochemically then, the actual ubiquitination process must be occurring on p300 through a three-way interaction between p53, Mdm2, and p300. The binding of p53 to p300 is therefore important to such a ubiquitination process. Nevertheless, it is not fully clear as to how the E1A protein might stabilize p53. It is noted that in addition to interaction with p300 and Rb, E1A can also interact with Sug1 and the 19S proteasome and thereby reduce the ability of human papillomaviral E6 protein to target p53 for ubiquitin-mediated proteasomal degradation (41). Under con-

FIG. 6. 12S.E.1A but not E1A Δ (2-36) decreases *in vivo* Mdm2-mediated p53 ubiquitination. The LFS 041 cells were transfected with expression vectors for p53 (1.5 μ g), Mdm2 (6.0 μ g), HA-Ub (4.0 μ g), and varying concentrations of 12S.E.1A or E1A Δ (2-36) as indicated. The total amount of transfected DNA was maintained constant with an empty vector. After 24 h of transfection, cells were treated for 10 h with the proteasome inhibitor MG132 (10 μ M) or solvent control Me₂SO. Immunoblots for HA-Ub-P53 conjugates using an anti-HA monoclonal antibody were performed as described in the Fig. 2 legend. The results shown are representative of more than three independent experiments. *i.p.*, immunoprecipitate; *KD*, kilodalton.



ditions in which cells were transfected with the expression vector for 12S.E.1A and treated with a proteasome inhibitor, we observed a decrease instead of an increase in ubiquitin conjugates of p53 (Fig. 6.). It is possible that the effects of binding of E1A to Sug1 and the 19S proteasome were masked because of the efficient accumulation of ubiquitin conjugates under MG132 treatment. Thus, it seems that the binding of E1A to Sug1 and the 19S proteasome might not be the primary mechanism for E1A to stabilize p53.

The cancer-derived p53 mutants tested in the present study are unable to bind sequence-specific DNA to activate the expression of a gene adjacent to the p53 DNA binding site (29). These mutants are efficiently ubiquitinated and degraded by co-expression of *Mdm2*. The results suggest that the inability to transactivate the *Mdm2* gene is the main reason for protein accumulation of these mutants in cancer cells. We can not surmise why ubiquitin conjugates of these p53 mutants, compared with those of p53(WT), were easier to accumulate when transfected cells were treated with MG132. In a recent study, p53 mutants 143A and 248W were shown to be ubiquitinated by Mdm2 to an approximately equal level to p53(WT) without treatment by a proteasome inhibitor (33). One possibility is that the proteolysis of ubiquitin conjugates of these p53 mutants is more susceptible to the inhibition by proteasome inhibitors. However, the alternative that these mutants are inherently more prone to ubiquitination than the wild type cannot be ruled out.

Several lines of evidence converge in supporting the integration of ubiquitination and proteolysis through a complex assembled on the N-terminal end of p300. First, we have already established that hHR23A interacts with the CH1 region (aa 325-528) of p300/CBP to down-regulate p53 (28). Moreover, overexpression of wild-type hHR23A inhibits the p53 transcriptional activity and results in a decreased steady-state protein level of cellular p53 (28). Second, it is known that hHR23A/B interact with the 26S proteasome through its N-terminal ubiquitin-like domain and that these proteins co-purify with proteasome in human cells. Specifically, hHR23A/B is shown to interact with S5a, a subunit of the human 26S proteasome (42). Third, the ubiquitin-like domain of RAD23 is obviously responsible both for UV-damage response (43) and to interact with the 26S proteasome in yeast (44, 45). Last, our preliminary studies indicate that the p300-(1-595) segment is very unstable and co-expression of hHR23A causes a prominent decrease in the steady-state levels of p300-(1-595) and p300-(325-528) segments.² Therefore, a model is proposed to suggest that the

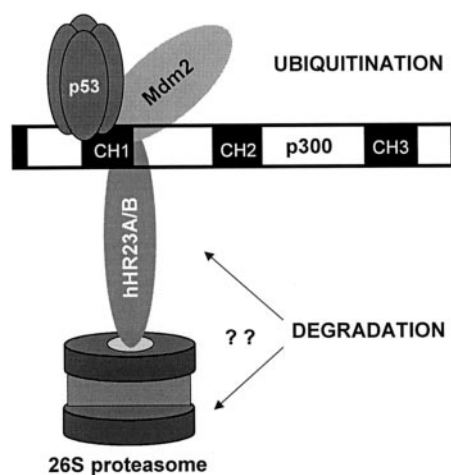


FIG. 7. A possible model for integrating ubiquitination and degradation. Three-way interaction between p53, Mdm2, and p300 at the N-terminal region of p300 is depicted schematically. The illustration shows the N terminus of tetrameric p53 interacting with the N terminus of the Mdm2 and CH1 region of p300. The C terminus of hHR23A interacts with the same region of p300 and links the assembled proteins with the 26S proteasome as part of a putative protein lysis complex.

hHR23A protein, through interaction with CH1 domain, enables the docking of proteasome onto p300 (Fig. 7).

The finding that p300 integrates ubiquitination and proteolysis underscores the complexity of the regulation of the metabolic stability of p53. However, it provides a rationale as to why p300 would be required for the stabilization of p53 in the cellular response to DNA damage (28, 46, 47). Interestingly, the p19ARF tumor suppressor is another participant in the regulation of Mdm2-mediated p53 degradation. It is noted that the p19ARF protein physically and functionally interacts with Mdm2 and p53 (48). The p19ARF stabilizes p53 and restores its transcriptional activity while promoting Mdm2 degradation (49). It will be worthwhile to explore whether p19ARF plays any part in p300/Mdm2-mediated p53 degradation. More importantly, it will be quite revealing to investigate how such an integrated process responds to DNA damage and cell cycle progression. Because p300 serves as a co-activator for a large group of transcription factors, more in-depth studies on the role of p300 in protein degradation are clearly warranted for understanding its diverse biological function.

² Q. Zhu, J. Yao, G. Wani, M. A. Wani, and A. A. Wani, unpublished observation.

Acknowledgments—We thank Drs. Vogelstein, Levine, Livingston, Oren, Mathews, Bohmann, and Tainsky for providing valuable reagents used in our various experiments. We are also thankful to Drs. Andrea

Doseff and Maqsood Wani for careful reading of the manuscript and helpful suggestions.

REFERENCES

- Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998) *J. Biol. Chem.* **273**, 1–4
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) *Science* **253**, 49–53
- Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. M., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., and Tainsky, M. A. (1990) *Science* **250**, 1233–1238
- Donehower, L. A., Harvey, M., Slagle, B. L., MaArthur, M. J., Montgomery, C. A., Butel, J. S., and Bradley, A. (1992) *Nature* **356**, 215–221
- Levine, A. J. (1997) *Cell* **88**, 323–331
- Ko, L. J., and Prives, C. (1996) *Genes Dev.* **10**, 1054–1072
- Ford, J. M., and Hanawalt, P. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8876–8880
- Ford, J. M., and Hanawalt, P. C. (1997) *J. Biol. Chem.* **272**, 28073–28080
- Wani, M. A., Zhu, Q. Z., El-mahdy, M., and Wani, A. A. (1999) *Carcinogenesis* **20**, 765–772
- Wani, M. A., Zhu, Q. Z., El-mahdy, M., Venkatachalam, S., and Wani, A. A. (2000) *Cancer Res.* **60**, 2275–2280
- Zhu, Q. Z., Wani, M. A., El-mahdy, M., and Wani, A. A. (2000) *J. Biol. Chem.* **275**, 11492–11497
- Chowdary, D. R., Dermody, J. J., Jha, K. K., and Ozer, H. L. (1994) *Mol. Cell. Biol.* **14**, 1997–2003
- Maki, C. G., Huibregtse, J. M., and Howley, P. M. (1996) *Cancer Res.* **56**, 2649–2654
- Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) *Bioessays* **22**, 442–451
- Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387**, 296–299
- Kubbutat, M. H., Ludwig, R. L., Levine, A. J., and Vousden, K. H. (1999) *Cell Growth Differ.* **10**, 87–92
- Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387**, 299–303
- Honda, R., Tanaka, H., and Yasuda, H. (1997) *FEBS Lett.* **420**, 25–27
- Honda, R., and Yasuda, H. (2000) *Oncogene* **19**, 1473–1476
- Maki, C. G. (1999) *J. Biol. Chem.* **274**, 16531–16535
- Fang, S. Y., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) *J. Biol. Chem.* **275**, 8945–8951
- Lowe, S. W., and Ruley, H. E. (1993) *Genes Dev.* **7**, 535–545
- Sanchez-Prieto, R., Leonart, M., and Cajal, S. R. Y. (1995) *Oncogene* **11**, 675–682
- Chiou, S. K., and White, E. (1997) *J. Virol.* **71**, 3515–3525
- Querido, E., Teodoro, J. G., and Branton, P. E. (1997) *J. Virol.* **71**, 3526–3533
- Nakajima, T., Morita, K., Tsunoda, H., Imajoh-Ohmi, S., Tanaka, H., Yasuda, H., and Oda, K. (1998) *J. Biol. Chem.* **273**, 20036–20045
- Grossman, S. R., Perez, M., Kung, A. L., Joseph, M., Mansur, C., Xiao, Z.-X., Kumar, S., Howley, P. M., and Livingston, D. M. (1998) *Mol. Cell* **2**, 405–415
- Zhu, Q., Wani, G., Wani, M. A., and Wani, A. A. (2001) *Cancer Res.* **61**, 64–70
- Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. (1992) *Science* **256**, 827–830
- Haupt, Y., Barak, Y., and Oren, M. (1996) *EMBO J.* **15**, 1596–1606
- Treier, M., Staszewski, L. M., and Bohmann, D. (1994) *Cell* **78**, 787–798
- Venkatachalam, S., Denissenko, M., and Wani, A. A. (1997) *Oncogene* **14**, 801–809
- Maki, C. G. (1999) *J. Biol. Chem.* **274**, 16531–16535
- Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268**, 726–731
- Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997) *Nature* **387**, 823–827
- Somasundaram, K., and El-Deiry, W. S. (1997) *Oncogene* **14**, 1047–1057
- Stein, R. W., Corrigan, M., Yaciuk, P., Whelan, J., and Moran, E. (1990) *J. Virol.* **64**, 4421–4427
- Montes de Oca, L. R., Wagner, D. S., and Lozano, G. (1995) *Nature* **378**, 203–206
- Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) *Nature* **378**, 206–208
- Geyer, R. K., Yu, Z. K., and Maki, C. G. (2000) *Nat. Cell Biol.* **2**, 569–573
- Turnell, A. S., Grand, R. J., Gorbea, C., Zhang, X., Wang, W., Mymryk, J. S., and Gallimore, P. H. (2000) *EMBO J.* **19**, 4759–4773
- Hiyama, H., Yokoi, M., Masutani, C., Sugasawa, K., Maekawa, T., Tanaka, K., Hoeijmakers, J. H., and Hanaoka, F. (1999) *J. Biol. Chem.* **274**, 28019–28025
- Watkins, J. F., Sung, P., Prakash, L., and Prakash, S. (1993) *Mol. Cell. Biol.* **13**, 7757–7765
- Young, P., Deveraux, Q., Beal, R. E., Pickart, C. M., and Rechsteiner, M. (1998) *J. Biol. Chem.* **273**, 5461–5467
- Schauber, C., Li, C., Tangoankar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998) *Nature* **391**, 715–718
- Yuan, Z. M., Huang, Y. Y., Ishiko, T., Nakada, S., Utsugisawa, T., Shioya, H., Utsugisawa, Y., Yokoyama, K., Weichselbaum, R., Shi, Y., and Kufe, D. (1999) *J. Biol. Chem.* **274**, 1883–1886
- Zhu, Q. Z., Wani, M. A., El-mahdy, M., Wani, G., and Wani, A. A. (2000) *Mol. Carcinog.* **28**, 215–224
- Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., and Sherr, C. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8292–8297
- Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) *Cell* **92**, 725–734