

AD _____

Award Number: DAMD17-01-1-0054

TITLE: Functional Analysis of p53 Acetylation in Prostate Tumor
Suppression

PRINCIPAL INVESTIGATOR: Tso-Pang Yao, Ph/D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, NC 27710-3813

REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20031126 031

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 2002 - 30 Jun 2003)	
4. TITLE AND SUBTITLE Functional analysis of p53 Acetylation in Prostate Tumor Suppression		5. FUNDING NUMBERS DAMD17-01-1-0054	
6. AUTHOR(S) Tso-Pang Yao, Ph.D.		8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, NC 27720-3813 E-Mail: Yao00001mc.duke.edu			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The tumor suppressor p53 is stabilized and activated in response to cellular stress through posttranslational modifications including acetylation. p300/CBP-mediated acetylation of p53 is negatively regulated by MDM2. We show that MDM2 can promote p53 deacetylation by recruiting a complex containing HDAC1. The HDAC1 complex binds MDM2 in a p53-independent manner and deacetylates p53 at all known acetylated lysines <i>in vivo</i> . Ectopic expression of a dominant negative HDAC1 mutant restores p53 acetylation in the presence of MDM2, whereas wild-type HDAC1 and MDM2 deacetylate p53 synergistically. Fibroblasts over-expressing a dominant negative HDAC1 mutant display enhanced DNA damage-induced p53 acetylation, increased levels of p53, and a more pronounced induction of p21 and MDM2. These results indicate that acetylation promotes p53 stability and function. As the acetylated p53 lysine residues overlap with those that are ubiquitinated, our results suggest that one major function of p53 acetylation is to promote p53 stability by preventing MDM2-dependent ubiquitination, while recruitment of HDAC1 by MDM2 promotes p53 degradation by removing these acetyl groups. We also found that acetylation regulates p53 subcellular localization and MDM2 function. Our results indicate that acetylation regulates p53 function at multiple levels.			
14. SUBJECT TERMS P53, acetylation, ubiquitination, MDMD2, HDAC			15. NUMBER OF PAGES 41
17. SECURITY CLASSIFICATION OF REPORT Unclassified			16. PRICE CODE
			20. LIMITATION OF ABSTRACT Unlimited
18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		

Table of Contents

Cover.....	
SF 298.....	1
Table of Contents.....	2
Introduction.....	3
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	
Appendices.....	7
Manuscripts (2)	

Title: Functional analysis of p53 acetylation in prostate tumor suppression

Introduction:

The long-term goal of this research proposal is to understand the regulation and the functional importance of specific p53 acetylation in tumor suppression. The activation of p53 is a critical step in preventing oncogenic transformation and multiple types of tumor formation. The importance of p53 in prostate cancer has been illustrated by its critical role in the prostate gland apoptosis induced by androgen-ablation, a powerful therapy used to treat early stage, androgen-dependent prostate cancer. Further supporting the dominant role of p53 in suppressing prostate tumor progression, mutations in p53 are prevalent in majority of tumors that have progressed to a more advanced and hormone-refractory stage. Understanding the role of p53 acetylation could provide critical information on how activation of p53 is achieved. The data we have obtained in the last 12 months provide evidence that acetylation regulates p53 at several levels. We also demonstrated that MDM2, the key negative regulator for p53, is also subject to regulation by acetylation.

Body:

In the last twelve months, we have made progresses in characterizing the function of p53 acetylation and MDM2 acetylation. Briefly, we have obtained evidence that acetylation can promote p53 stability by preventing MDM2-mediated ubiquitination and subsequent degradation. Furthermore, we found that acetylation also regulates p53 subcellular localization. This surprising finding indicates that reversible acetylation regulates p53 function at multiple levels. The detailed description of the research progress can be found in the Appended manuscripts (1 and 2).

By Mass Spectrometry, we have also successfully identified lysine residues in MDM2 that are subject to acetylation. By generating anti-acetylated MDM2 specific antibodies and site directed mutagenesis, we confirmed that MDM2 is acetylated at specific lysines *in vivo* (Figure 1). We are currently analyzing the function of MDM2 acetylation.

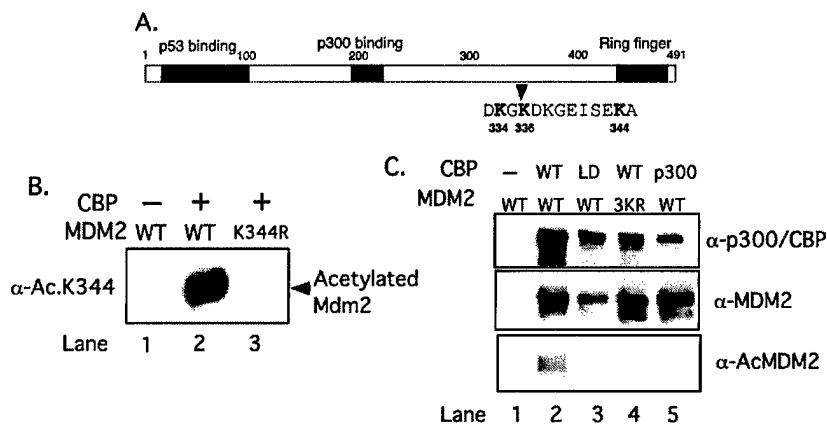


Figure 1. MDM2 is modified by acetylation. (A) A schematic diagram of MDM2. The three acetylated lysine residues identified by Mass Spectrometry are marked. (B) The generation of acetylated MDM2 specific antibodies. Peptides (14 amino acids) containing at either lysine 334, 336

or 344 (Ac-K334, Ac-K336 or Ac-K344) were synthesized and used to generate antibodies in rabbits. The acetylated MDM2 specific antibodies were obtained by sequential affinity purification using the unacetylated peptide column to remove immuno-reactivity to unmodified MDM2, followed by an acetylated peptide column. Anti-Ac-K344 was used here as an example. Unmodified recombinant MDM2 (without HAT), wild type or mutant MDM2, whose K344 was converted to arginine, were subject to *in vitro* acetylation by CBP (plus HAT) followed by immuno-blotting with the anti-Ac-K344. Note that this antibody only recognized wild type MDM2 that was acetylated by CBP (compare Lane 1 and 2) and it failed to react with the MDM2 mutant of which lysine 344 was converted to arginine (K344R, Lane 3). Thus, this antibody specifically recognizes MDM2 acetylated at lysine 344. Similarly, antibodies against acetylated MDM2 at lysine 334 (α -Ac-K334) or 336 (α -Ac-K336) were found to specifically recognize the acetylated MDM2 (data not shown). (C) MDM2 is acetylated *in vivo*. Expression plasmids for wild type or mutant MDM2 with lysine 334, 336 and 344 converted to arginine (3KR) were co-expressed with wild type CBP, acetyltransferase inactive CBP mutant (CBP-LD) or wild type p300. The acetylation status of MDM2 was then determined by immunoprecipitating with an antibody against MDM2 followed by immuno-blotting with a mixture of anti-acetylated lysine MDM2 antibodies (α -AcMDM2: a mixture of α -Ac-K334, α -Ac-K336 and α -Ac-K344). Note that these acetylated MDM2 specific antibodies recognized MDM2 when wild type CBP (Lane 2) but not its enzyme-deficient LD mutant (Lane 3) or p300 (Lane 5) was co-expressed. Importantly, these antibodies did not react with 3KR mutant in the presence of ectopically expressed CBP (Lane 4), further demonstrating the specificity of the antibody toward acetylated MDM2 at

specific lysines. Similar results on MDM2 acetylation *in vivo* were obtained using antibodies against individual acetylated lysine residues (data not shown). The expression of p300, CBP and MDM2 were confirmed by immuno-blotting with their respective antibodies.

Key Research Accomplishment

1. We have identified the key acetylation sites in p53 and showed that these lysines are also the targets of MDM2 mediated ubiquitination. We have obtained evidence that acetylation promotes p53 stability, possibly, by competing with the MDM2-mediated ubiquitination
2. We have found that full acetylation of p53 can lead to its nuclear exit.
3. We have identified lysine residues in MDM2 that are subject to acetylation.

Reportable Outcome:

1. Ito, A. Kawaguchi, Y., Lai, C.-H, Kovacs, J. J., Higashimoto, Y., Appella, E., and Yao, T.-P#. MDM2-HDAC1 mediated-deacetylation of p53 is required for its degradation. *EMBO. J.* 22. 6236-6245 (2002)

2. Kawaguchi, Y., Ito, A., Appella, E., and Yao. T.-P#. Control of p53 subcellular localization by charge modification at C-terminus lysine residues (submitted)

Conclusion:

Our study has provided the first in vivo evidence that acetylation controls p53 stability and that MDM2 is also subject to regulation by acetylation.

MDM2–HDAC1-mediated deacetylation of p53 is required for its degradation

Akihiro Ito, Yoshiharu Kawaguchi, Chun-Hsiang Lai, Jeffrey J. Kovacs, Yuichiro Higashimoto¹, Ettore Appella¹ and Tso-Pang Yao²

Department of Pharmacology and Cancer Biology, Duke University, Durham, NC 27710 and ¹Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

²Corresponding author
e-mail: yao00001@mc.duke.edu

The tumor suppressor p53 is stabilized and activated in response to cellular stress through post-translational modifications including acetylation. p300/CBP-mediated acetylation of p53 is negatively regulated by MDM2. Here we show that MDM2 can promote p53 deacetylation by recruiting a complex containing HDAC1. The HDAC1 complex binds MDM2 in a p53-independent manner and deacetylates p53 at all known acetylated lysines *in vivo*. Ectopic expression of a dominant-negative HDAC1 mutant restores p53 acetylation in the presence of MDM2, whereas wild-type HDAC1 and MDM2 deacetylate p53 synergistically. Fibroblasts overexpressing a dominant negative HDAC1 mutant display enhanced DNA damage-induced p53 acetylation, increased levels of p53 and a more pronounced induction of p21 and MDM2. These results indicate that acetylation promotes p53 stability and function. As the acetylated p53 lysine residues overlap with those that are ubiquitylated, our results suggest that one major function of p53 acetylation is to promote p53 stability by preventing MDM2-dependent ubiquitylation, while recruitment of HDAC1 by MDM2 promotes p53 degradation by removing these acetyl groups.

Keywords: acetylation/HDAC1/MDM2/p53/ubiquitylation

Introduction

The activation of the tumor suppressor p53, which triggers growth arrest and apoptosis in cells that are in danger of becoming cancerous, involves the regulation of p53 stability. In unstressed cells, p53 is maintained at low levels by its key negative regulator MDM2 (Freedman *et al.*, 1999). Although p53 is required for active tumor suppression, the negative regulation of p53 by MDM2 is fundamentally important. This point is best illustrated by a genetic study in mice, where the loss of MDM2 led to early embryonic lethality due to uncontrolled p53 levels and activity (Montes de Oca Luna *et al.*, 1995). The importance of this negative regulation is further supported by the observation that expression of MDM2 is positively regulated by p53 (Wu *et al.*, 1993), and thus establishes

a tight negative feedback loop. MDM2 is an E3 ligase that ubiquitylates a defined set of lysine residues at the C-terminus of p53 (Honda *et al.*, 1997; Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). The MDM2-mediated ubiquitylation of p53 is believed to trigger rapid degradation of p53 by proteasomes (Freedman *et al.*, 1999), or to promote its nuclear export (Boyd *et al.*, 2000; Geyer *et al.*, 2000). Thus, MDM2 functions as a key negative regulator for p53, at least in part, by controlling the p53 ubiquitylation status (Zhang and Xiong, 2001).

p53 is transiently stabilized and activated in response to various cellular insults. The stabilization and activation of p53 are thought to be mediated by post-translational modification events, such as phosphorylation (Giaccia and Kastan, 1998; Appella and Anderson, 2001), which was proposed to interfere with the ability of MDM2 to negatively regulate p53 (Shieh *et al.*, 1997; Unger *et al.*, 1999). Although multiple serine residues are phosphorylated in response to genotoxic stress (Shieh *et al.*, 1997; Canman *et al.*, 1998; Hirao *et al.*, 2000), p53 activation is not always accompanied by the specific phosphorylation events proposed to be important for its function (Ashcroft *et al.*, 2000). In contrast to phosphorylation, we have reported that p53 invariably becomes acetylated in response to a wide variety of cellular insults that are known to be potent p53-activating agents (Ito *et al.*, 2001). Importantly, stress-induced p53 acetylation is transient and reversible, suggesting the existence of negative regulators that keep p53 acetylation in check (Ito *et al.*, 2001). Supporting this hypothesis, we and others have found that MDM2 negatively regulates p53 acetylation (Kobet *et al.*, 2000; Ito *et al.*, 2001). This result suggests a functional link between MDM2, an E3 ubiquitin ligase, and acetylation. The importance of this observation is further supported by the finding that the inhibitory effect of MDM2 on p53 acetylation can be reversed by the tumor suppressor p14^{ARF} (Ito *et al.*, 2001), which binds to MDM2 and inhibits its activity (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998b; Honda and Yasuda, 1999). Together, these observations support the notion that the regulation of acetylation is a central target of the p53–MDM2–p14^{ARF} feedback loop. However, the mechanism by which MDM2 controls p53 acetylation is not completely understood. It was previously shown that MDM2 can suppress CREB binding protein (CBP) acetyltransferase activity *in vitro*. This result supports the idea that MDM2 inhibits p53 acetylation, at least in part, by binding and reducing p300/CBP acetyltransferase activity (Kobet *et al.*, 2000; Ito *et al.*, 2001). However, the observation that the deacetylase inhibitor TSA can restore p53 acetylation in the presence of MDM2 suggests a more complex mechanism, wherein MDM2 might also utilize a deacetylase pathway to control p53 acetylation level (Ito *et al.*, 2001). Uncovering this pathway will be essential to understand-

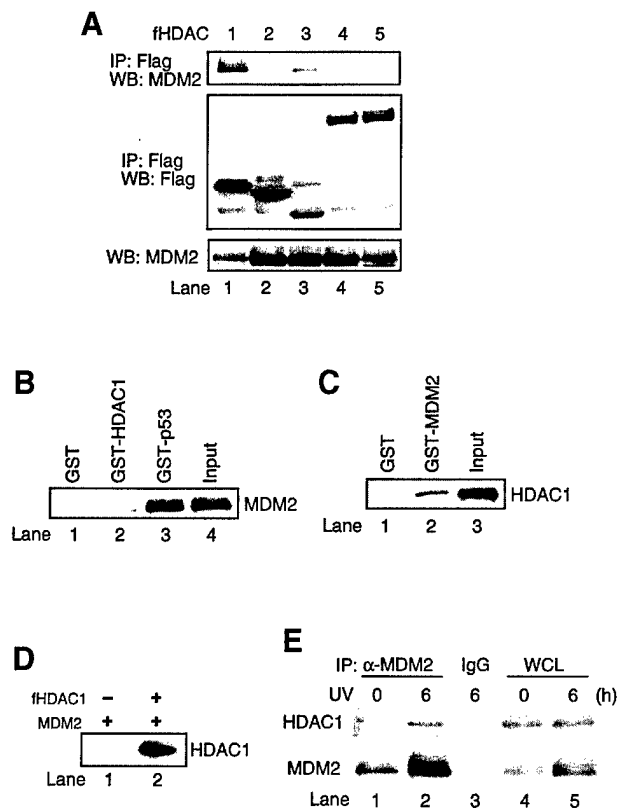


Fig. 1. Interaction between MDM2 and HDAC1. (A) 293T cells were cotransfected with 10 μ g of MDM2 and 5–10 μ g of either Flag-tagged HDAC1 (lane 1), HDAC2 (lane 2), HDAC3 (lane 3), HDAC4 (lane 4) or HDAC5 (lane 5). Cellular extracts were immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-MDM2 antibody (top panel) or anti-Flag antibody (middle panel). Total MDM2 protein was detected with anti-MDM2 antibody (bottom panel). (B) Either GST (lane 1), GST-HDAC1 (lane 2) or GST-p53 (lane 3) were incubated with recombinant MDM2 protein followed by immunoblotting with anti-MDM2 antibody. (C) H1299 cells were transfected with Flag-tagged HDAC1 and the cellular extracts were incubated with either GST (lane 1) or GST-MDM2 (lane 2) followed by immunoblotting with anti-Flag antibody. (D) H1299 cells were transfected with MDM2 alone (lane 1) or cotransfected with Flag-tagged HDAC1 (lane 2) and cellular extracts were immunoprecipitated with anti-MDM2 antibody followed by immunoblotting with anti-Flag antibody. (E) A549 cells were exposed to UV-B irradiation (75 J/m²) for 6 h and cellular extracts were immunoprecipitated with either anti-MDM2 antibody (lanes 1 and 2) or mouse IgG as a control (lane 3) followed by immunoblotting with anti-HDAC1 antibody (top panel) or anti-MDM2 antibody (bottom panel). Total HDAC1 and MDM2 protein were detected with either anti-HDAC1 antibody (top panel, lanes 4 and 5) or anti-MDM2 antibody (bottom panel, lanes 4 and 5).

ing how p53 acetylation is regulated and is the subject of this study.

The acetylation of p53 is mainly catalyzed by the acetyltransferases p300 and CBP *in vivo* (Ito *et al.*, 2001). Interestingly, acetylation occurs at multiple lysine residues (six in total, see below) clustered at the C-terminus of p53 (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). It is not known why there are so many lysine residues targeted for acetylation. The precise function of acetylation and the mechanism by which acetylation controls p53 also remain to be established. Acetylation was previously correlated with p53-dependent senescence and its ability to induce apoptosis (Luo *et al.*, 2000; Pearson *et al.*, 2000). As acetylation of p53 enhances its

DNA-binding activity *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998), it has been widely assumed that acetylation functions by enhancing p53 transcriptional activity. However, there may be additional mechanisms by which acetylation could control p53 function. For example, since the activation of p53 involves its stabilization, we have previously suggested that acetylation might control the stability of p53. Indeed, our observation that inhibition of p53 deacetylation by the deacetylase inhibitor trichostatin A (TSA) is accompanied by an increase in p53 stability is consistent with this hypothesis (Ito *et al.*, 2001). However, how acetylation controls p53 stability is not known.

In this report, we present evidence that acetylation controls p53 stability by potentially interfering with MDM2-mediated ubiquitylation. We found that MDM2 and the deacetylase HDAC1 form a complex that controls p53 acetylation in a cooperative fashion, thus providing a molecular link between the ubiquitylation and the deacetylation machinery. We provide evidence that stable overexpression of a dominant-negative HDAC1 mutant in fibroblasts leads to markedly enhanced p53 acetylation and p53 stability in response to DNA damage, suggesting that one function of acetylation is to promote p53 stability. Consistent with this conclusion, we found that acetylation and ubiquitylation occur at an overlapping set of lysine residues in p53. Our results suggest a simple model wherein acetylation promotes p53 stability by competing with MDM2-mediated ubiquitylation. The MDM2-HDAC1 interaction thus provides an efficient coupling of deacetylation and ubiquitylation machinery to negatively regulate p53 function.

Results

HDAC1 specifically interacts with MDM2

Our previous studies have shown that MDM2 can inhibit p300-induced p53 acetylation, in part, by repressing p300 acetyltransferase activity. However, the inhibitory effect of MDM2 on p53 acetylation can also be reversed by a pan-HDAC deacetylase inhibitor TSA (Ito *et al.*, 2001), suggesting the involvement of active deacetylation. To further address the potential mechanism by which MDM2 negatively regulates p53 acetylation, we first considered the possibility that MDM2 may induce p53 deacetylation by recruiting a putative p53 deacetylase, since MDM2 itself does not possess TSA-sensitive deacetylase activity (data not shown). To test this hypothesis, we investigated whether MDM2 interacts with any of the known HDAC family members. Through a co-immunoprecipitation assay, we found that among HDAC1–7, only HDAC1 strongly co-immunoprecipitated with MDM2, while the others did not (Figure 1A; data not shown). This result indicates that MDM2 selectively interacts with HDAC1. To determine whether MDM2 binds HDAC1 directly, the ability of bacterially-expressed recombinant GST HDAC1 and MDM2 proteins to interact was investigated by pull-down assay (Figure 1B). As expected, recombinant MDM2 bound GST-p53 (Figure 1B, lane 3); however, MDM2 failed to bind GST-HDAC1 under the same conditions (Figure 1B, lane 2). Thus, these results indicate that MDM2 specifically interacts with HDAC1 via an

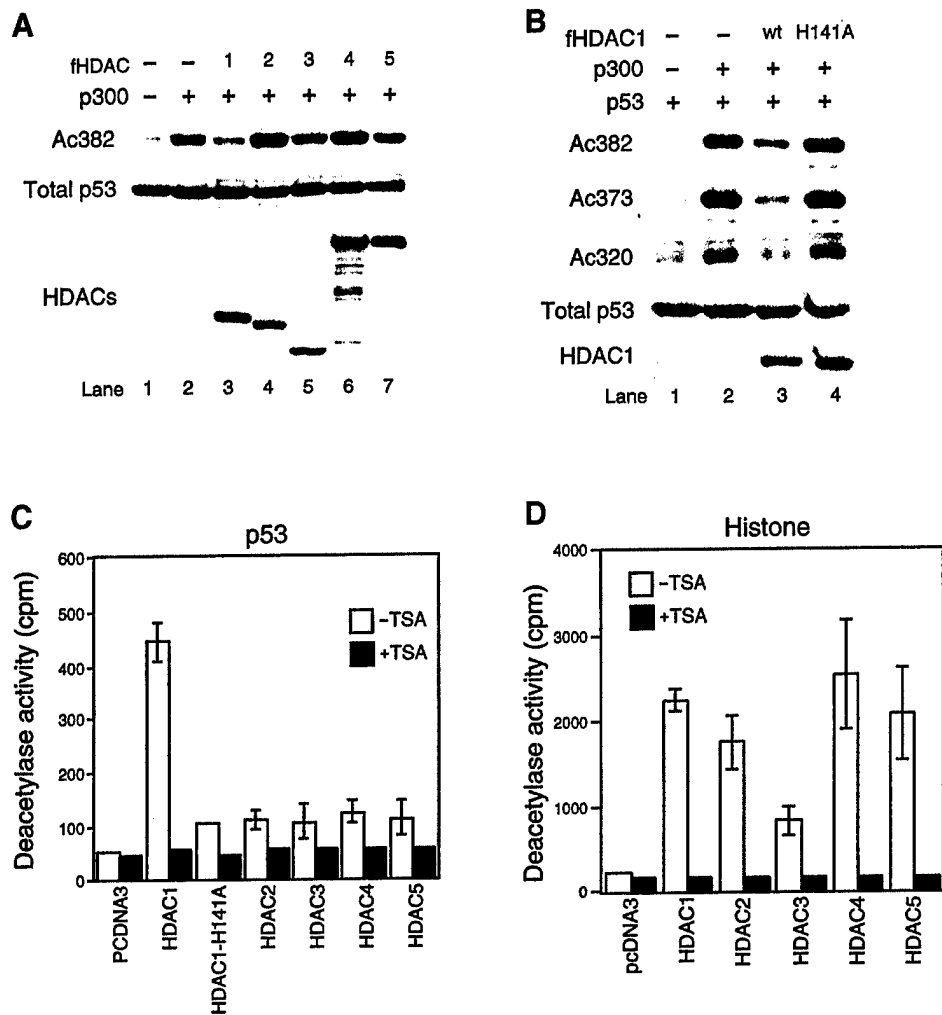


Fig. 2. Deacetylation of p53 by HDAC1. (A) 293T cells were transfected with an empty vector (lane 1) or cotransfected with either 2 μ g of p300 alone (lane 2) or cotransfected with 2 μ g of Flag-tagged HDAC1 (lane 3), 2 μ g of HDAC2 (lane 4), 2 μ g of HDAC3 (lane 5), 4 μ g of HDAC4 (lane 6) or 4 μ g of HDAC5 (lane 7). The levels of endogenous acetylated p53, total p53, and each HDAC were detected by immunoblotting with anti-acetylated p53 (Lys382) (top panel), anti-p53 antibody (middle panel) and anti-Flag antibody (bottom panel). (B) H1299 cells (p53^{-/-}) were transfected with 0.2 μ g of p53 alone (lane 1) or cotransfected with either 2 μ g of p300 alone (lane 2), with 2 μ g of HDAC1 wild-type (lane 3) or 2 μ g of enzyme-dead H141A mutant (lane 4). The level of acetylated p53 was assessed using either antibody specific for acetylated Lys382 (Ac382), acetylated Lys373 (Ac373) or acetylated Lys320 (Ac320). The levels of total p53 and Flag-tagged HDAC1 were detected as described in (A). (C and D) 293T cells were transfected with empty vector or Flag-tagged HDACs and cellular extracts were prepared as described in Materials and Methods. Deacetylase activity was measured against acetylated GST-p53 (C) or histone H4 peptide (D) in the presence or absence of TSA. Results are representative of three independent experiments. Note that all HDAC family members possess deacetylase activity towards histones, but only HDAC1 can efficiently deacetylate p53.

indirect mechanism or that a specific modification of MDM2 or/and HDAC1 is required for this interaction.

As MDM2 binds p53 directly, we investigated whether the binding of MDM2 and HDAC1 was mediated by p53. To test this possibility, an HDAC1 expression plasmid was transfected into H1299 cells (p53^{-/-}) and the interaction of MDM2 and HDAC1 was determined by pull-down assay using GST-MDM2, followed by immunoblotting to visualize the associated HDAC1. As shown in Figure 1C, GST-MDM2, but not GST, interacts with HDAC1 expressed in H1299 cells. A similar conclusion regarding HDAC1-MDM2 interaction in H1299 cells was obtained by co-transfecting HDAC1 and MDM2 expression plasmids, which were assayed by co-immunoprecipitation (Figure 1D). These results demonstrate a specific and p53-independent interaction between MDM2 and HDAC1.

We investigate further the interaction between the endogenous HDAC1 and MDM2. As shown in Figure 1E, at the basal state in which the MDM2 level is low, a co-immunoprecipitation assay revealed a weak but reproducible interaction of MDM2 and HDAC1. However, upon UV irradiation, which induces MDM2 expression (~4-fold), a marked increase of MDM2 and HDAC1 association (~3-fold) was observed. These results demonstrate an endogenous MDM2-HDAC1 interaction that can be stimulated by UV irradiation, likely due to an increase of the MDM2 protein level in response to DNA damage.

MDM2-HDAC1 deacetylates p53 cooperatively

The physical interaction of MDM2 and HDAC1 supports the hypothesis that MDM2 recruits HDAC1 to deacetylate p53. Consistent with this hypothesis, it was previously

proposed that HDAC1 could function as a p53 deacetylase (Luo *et al.*, 2000). However, although the ectopic expression of HDAC1-associated MTA2/PID can induce p53 deacetylation, whether or not HDAC1 is a p53 deacetylase *in vivo* was not directly tested, nor did the study address whether other HDAC family members could deacetylate p53. To investigate these issues and determine whether the specific association with MDM2 is functionally correlated with the ability of an HDAC to function as a p53 deacetylase, expression plasmids for p300, HDAC1-5 were cotransfected into 293T cells and the p53 acetylation status on lysine 382 was assessed using an acetyl-Lys382-specific antibody. As shown in Figure 2A, consistent with its unique association with MDM2, only HDAC1 but not other HDACs efficiently induced p53 deacetylation on lysine 382 (Figure 2A, lanes 3-7). Importantly, ectopic expression of HDAC1 also led to deacetylation at lysine

320 and 373 (Figure 2B), both of which are also known to become acetylated upon p53 activation (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). Thus, *in vivo*, HDAC1 can deacetylate all three known acetylated lysine residues in p53. An identical conclusion that HDAC1, but not other HDAC members, possesses strong p53 deacetylase activity can be demonstrated directly by an *in vitro* assay using HDACs immunoprecipitated from cells transfected with the HDAC expression plasmids (Figure 2C), while all HDAC family members possess a deacetylase activity toward histones (Figure 2D). Thus, among the HDAC members tested, HDAC1 uniquely associates with MDM2 and can specifically function as a p53 deacetylase, supporting its role for mediating MDM2-dependent p53 deacetylation.

To determine whether MDM2 is required for HDAC1 to function as a p53 deacetylase, we investigated the ability of HDAC1 to deacetylate p53 in MDM2-deficient cells. As shown in Figure 3A, overexpression of HDAC1 still results in p53 deacetylation in MDM2^{-/-};p53^{-/-} mouse embryonic fibroblasts (MEFs). Although MDM2 is not required for high levels of HDAC1 to deacetylate p53, it is possible that the interaction with MDM2 could facilitate HDAC1's activity as a p53 deacetylase. To test this possibility, low concentrations of MDM2 and HDAC1 were co-expressed with p53 and p300 in MDM2^{-/-};p53^{-/-} MEFs, and the p53 acetylation status was assessed. Under these conditions, neither MDM2 nor HDAC1 alone had an appreciable effect on the level of p53 acetylation (Figure 3B, lanes 2 and 4). However, co-expression of both MDM2 and HDAC1 dramatically reduced the level of p53 acetylation (Figure 3B, lane 5). These results demonstrate that MDM2 and HDAC1 function synergistically to induce p53 deacetylation. To elucidate the potential mechanism underlying this synergistic activity, we asked whether MDM2 affects the interaction between p53 and HDAC1. As shown in Figure 3C, in MDM2-deficient MEFs, HDAC1 only interacts weakly with p53 (Figure 3C,

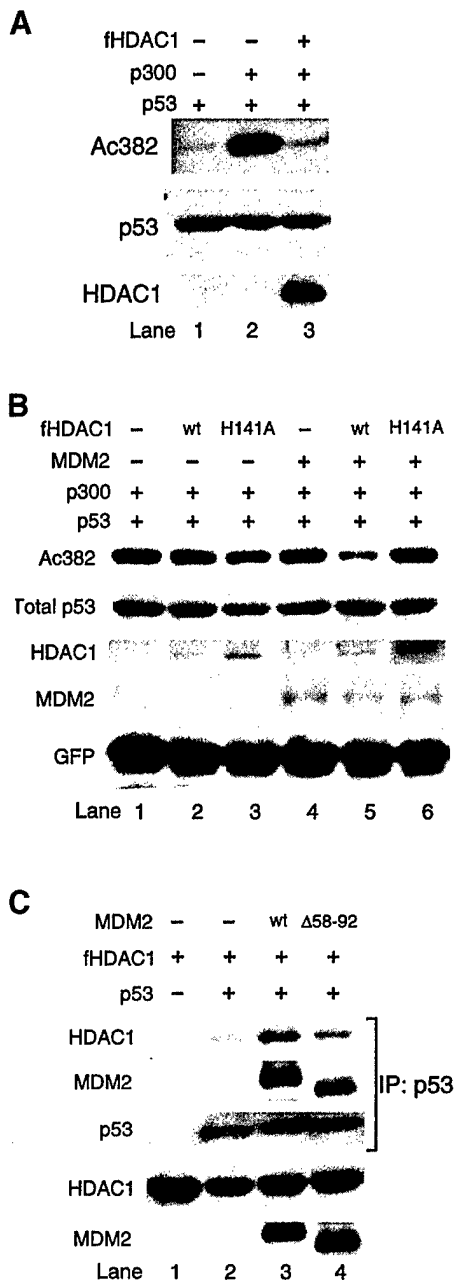


Fig. 3. HDAC1 and MDM2 work cooperatively to deacetylate p53. (A) MEF (p53^{-/-};MDM2^{-/-}) cells were transfected with 0.1 μg of p53 (lane 1), or cotransfected either with 2 μg of p300 alone (lane 2) or with p300 and 2 μg of Flag-tagged HDAC1 (lane 3). The level of total p53 (middle panel) and acetylated p53 (top panel) were detected as described in Figure 2. (B) MEF (p53^{-/-};MDM2^{-/-}) cells were transfected with either 0.1 μg of p53, 2 μg of p300 and 0.5 μg of internal control GFP (lane 1), or cotransfected either with 12.5 ng of Flag-tagged HDAC1 wild-type (lane 2), with 12.5 ng of Flag-tagged HDAC1 H141A mutant (lane 3), with 0.5 μg of MDM2 (lane 4), with MDM2 and 12.5 ng of Flag-tagged HDAC1 wild-type (lane 5) or 0.5 μg of MDM2 and 12.5 ng of Flag-tagged HDAC1 H141A mutant (lane 6). The levels of indicated proteins were determined by immunoblotting. Of note, we used 160× the amount of Flag-tagged HDAC1 expression vectors in (A) when compared with (B). (C) MEF (p53^{-/-};MDM2^{-/-}) cells were transfected with either 1 μg of Flag-tagged HDAC1 wild-type alone, or cotransfected either with 0.3 μg of p53, with 0.3 μg of p53 and 4 μg of MDM2 wild-type, or 0.3 μg of p53 and 4 μg of p53-binding-deficient MDM2 mutant (Δ58-92). Cells were also treated 24 h post-transfection with the protease inhibitor LLnV (10 μM) for 4 h to inhibit MDM2-mediated p53 degradation. Cellular extracts were immunoprecipitated with anti-goat p53 antibody followed by immunoblotting with Flag antibody (top panel), anti-MDM2 antibody (second panel) or anti-p53 antibody (third panel). Total HDAC1 and MDM2 protein were detected with either anti-Flag antibody (fourth panel) or anti-MDM2 antibody (bottom panel). Of note, the interaction between p53 and p53-binding-deficient MDM2 mutant (Δ58-92) is likely mediated by HDAC1 through the ternary complex formation (second panel, lane 4).

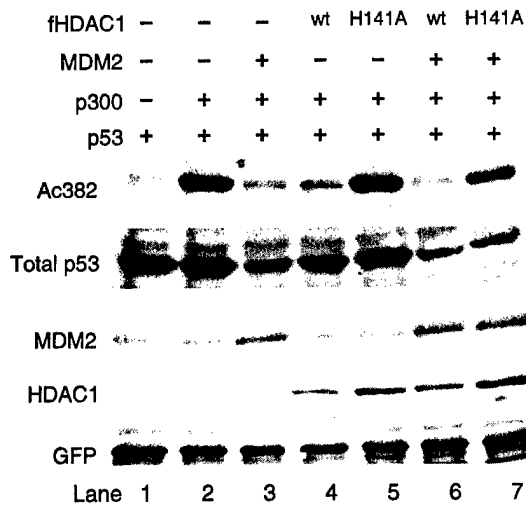


Fig. 4. MDM2 induces p53 deacetylation through HDAC1. H1299 cells were transfected with 0.2 μ g of p53 wild-type and 0.5 μ g of GFP (all lanes), and cotransfected with one or more of the following; 2 μ g of p300 (lanes 2–7), 2 μ g of MDM2 (lanes 3, 6 and 7), 2 μ g of Flag-tagged HDAC1 wild-type (lanes 4 and 6), 2 μ g of Flag-tagged HDAC1 H141A mutant (lanes 5 and 7). The levels of indicated proteins were determined by immunoblotting.

lane 2). However, this interaction is dramatically stimulated upon the re-introduction of wild-type MDM2 (Figure 3C, lane 3). The p53-binding-deficient MDM2 mutant is less efficient in facilitating the p53–HDAC1 interaction. (Figure 3C, lane 4). Thus, the reduced p53 binding is correlated with reduced recruitment of HDAC1 by MDM2. Together, these results indicate that MDM2 promotes the interaction between HDAC1 and p53 and forms a ternary complex, allowing efficient p53 deacetylation.

HDAC1 mediates MDM2-dependent p53 deacetylation

We next examined whether or not HDAC1 was responsible for MDM2-mediated p53 deacetylation. The observation that HDAC1 binds MDM2 indirectly (Figure 1) and that bacterially-expressed recombinant HDAC1 does not possess p53 deacetylase activity (data not shown) suggests that the p53 deacetylase activity of HDAC1 requires additional factors. We reasoned that as HDAC1 normally functions in a multi-protein complex (Zhang *et al.*, 1998a), a catalytically inactive (enzyme-dead) HDAC1 could potentially titrate away the cofactors needed for the active complex and thus function as a dominant-negative mutant. To this end, we asked whether a catalytically inactive HDAC1 mutant (H141A) could reverse the effect of MDM2 on p53 acetylation. As shown in Figure 4, overexpression of MDM2 causes a dramatic reduction of p53 acetylation induced by p300 (Figure 4, lane 3). However, upon co-expression of the HDAC1 H141A mutant, the effect of MDM2 is partially lost and p53 acetylation restored (Figure 4, lane 7). This observation supports the idea that HDAC1 H141A acts as a dominant-negative mutant and that MDM2-induced p53 deacetylation is mediated by HDAC1. Together, these observations provide evidence that MDM2 recruits HDAC1 into a multi-protein complex, which promotes p53 deacetylation.

A common set of lysines are modified by acetylation and ubiquitylation

To begin to address the potential function of p53 acetylation, we first generated p53 mutants that can not be acetylated (Figure 5A). By analyzing the acetylation pattern of recombinant p53 acetylated by CBP, we found that mutation of all three known acetylated lysines (320, 373 and 382) does not eliminate p53 acetylation completely (Figure 5B, lane 2). However, additional mutations of lysines 370, 372 and 381 essentially abolished the acetylation by CBP (Figure 5B, lane 3). Thus, there are at least six lysine residues in p53 that can be acetylated by CBP *in vitro*. To generate the non-acetylatable p53 mutant, all six lysine residues were mutated to arginine (6KR), in order to minimize the structural impact by maintaining the positive charges at these residues.

Our previous study established a positive correlation between p53 acetylation and its total protein level (Ito *et al.*, 2001); we therefore examined the potential function of acetylation in regulating p53 stability. As both acetylation and ubiquitylation modify the ϵ amino group of lysine residues, we first evaluated whether there is a relationship between p53 acetylation and ubiquitylation. Recent studies on p53 ubiquitylation have identified several lysine residues important for MDM2-mediated ubiquitylation (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). When compared with the acetylated lysine residues identified in this report, we found that lysine residues targeted for ubiquitylation overlap those that can be acetylated *in vitro* (Figure 5C). Consistent with this idea, the acetylation-deficient 6KR mutant is completely resistant to MDM2-mediated degradation (Figure 5D and E). Thus, a common set of lysine residues is targeted by both p300/CBP-mediated acetylation and MDM2-mediated ubiquitylation. This result suggests the possibility that the acetylation of C-terminal lysine residues might prevent their ubiquitylation and consequently lead to p53 stabilization.

A dominant-negative HDAC1 mutant promotes both p53 acetylation and stability in response to DNA damage

Despite its ability to affect p53 acetylation levels, HDAC1 and its H141A mutant showed little effect on p53 protein levels when acetylation was induced by overexpression of p300 (Figure 4). It is possible that there may be only a very small portion of total p53 becoming acetylated under this artificial condition, and consequently, the balance of acetylation and ubiquitylation is not faithfully reflected in this system. To circumvent this problem and to investigate the role of p53 acetylation in a physiological setting, we generated NIH 3T3 lines that stably expressed HDAC1 or its dominant-negative mutant by retrovirus-mediated gene transfer. A physiological level of p53 acetylation can be induced by DNA-damaging agents in these cell lines, and p53 stability and activity can then be evaluated. To this end, control NIH 3T3 cells and their derivatives overexpressing wild-type HDAC1 and dominant-negative HDAC1 were irradiated with UV, and the levels of p53 and its acetylation were determined. Importantly, overexpression of HDAC1 or its dominant-negative mutant had no effect on the basal level of p53 or its acetylation (Figure 6A). This indicates that there is little

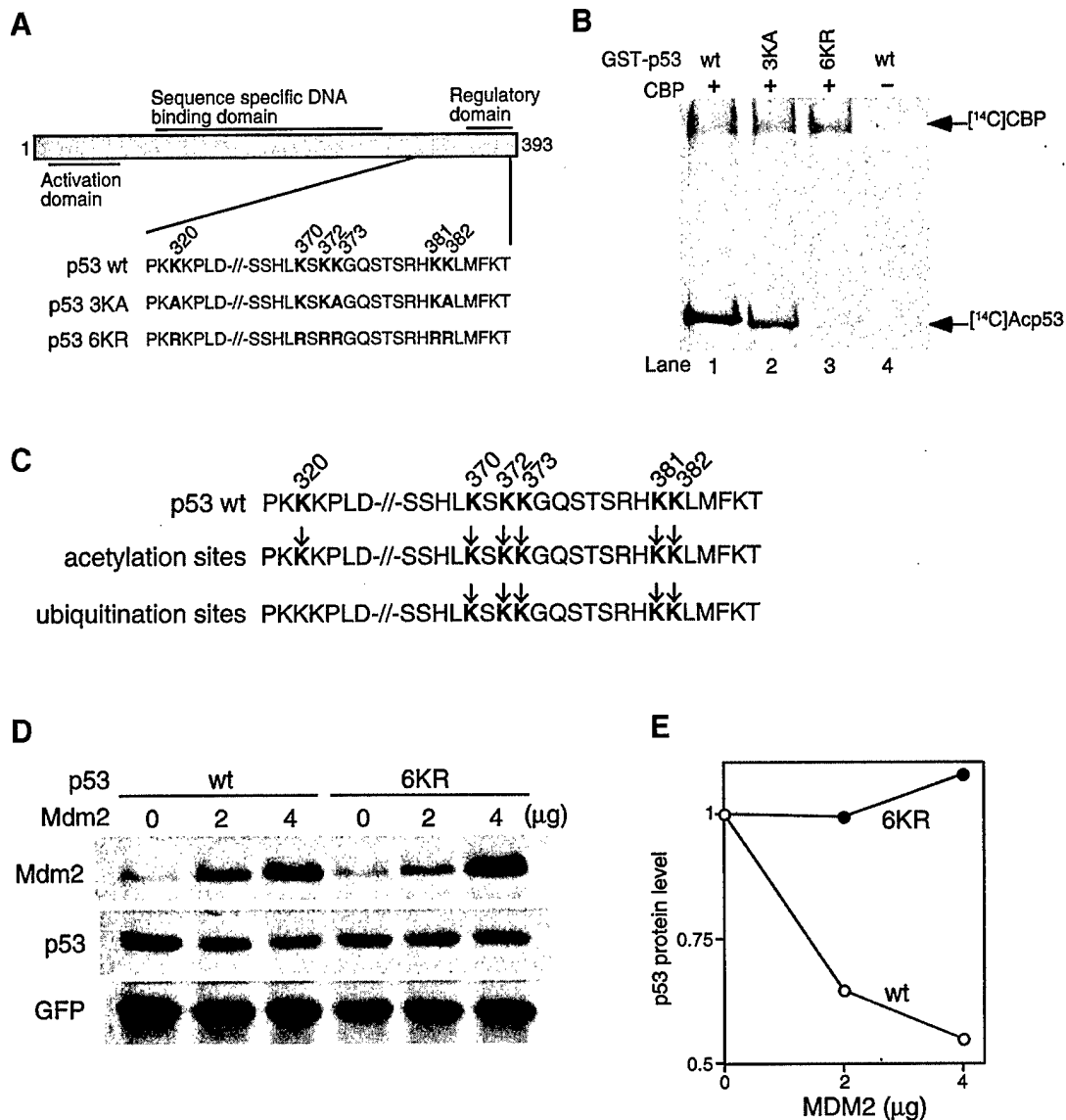


Fig. 5. Mutations of lysine residues of acetylation sites prevent MDM2-mediated p53 degradation. **(A)** Schematic structure of p53 mutations of C-terminal lysines to alanines or arginines. **(B)** GST-p53 wild-type (lane 1), GST-p53 3KA mutant with mutated lysine residues 320, 373 and 382 to alanine residues (lane 2), or GST-p53 6KR mutant with mutated lysine residues 320, 370, 372, 373, 381 and 382 to arginine residues (lane 3) were acetylated by recombinant CBP in the presence of the [¹⁴C]acetyl-CoA and analyzed by SDS-PAGE followed by autoradiograph. Acetylated p53 and CBP are indicated by arrows. As a negative control, wild-type GST-p53 was incubated without recombinant CBP in the presence of the [¹⁴C]acetyl-CoA (lane 4). **(C)** Acetylation sites of p53 overlap with ubiquitination sites. The lysine residues susceptible to acetylation and ubiquitination in the C-terminus of p53 are indicated by arrows. **(D)** H1299 cells were transfected with 0.5 μg of an expression plasmid encoding GFP as an internal control, 0.2 μg of wild-type p53 or p53 6KR mutant, together with empty pcDNA3 vector or the indicated amount of MDM2 vector. Thirty-six hours after transfection, cell extracts were prepared and analyzed by immunoblotting with anti-p53, anti-MDM2 and anti-GFP antibodies. **(E)** The band intensity of p53 and GFP protein levels was measured with NIH imaging software. p53 (empty circles) and 6KR mutant (filled circles) levels were normalized to GFP levels and were set to 1 in the absence of MDM2.

non-specific effect on p53 activation caused by HDAC1 or its mutant. After UV treatment, however, the p53 acetylation level was significantly enhanced in NIH 3T3 cells overexpressing dominant negative HDAC1 and reduced in the wild-type HDAC1-expressing cells (Figure 6A and C). This result demonstrates that HDAC1 can modulate p53 acetylation status in response to DNA damage. Consistent with the hypothesis that acetylation promotes p53 stability, the level of stabilized p53 protein is dramatically increased in the H141A mutant cell line in

response to UV irradiation (Figure 6A and B). This conclusion is further supported by the observation of an increase in p53 half-life in mutant HDAC1 H141A-expressing cells and a decrease in wild-type HDAC1-expressing cell when compared with control cell lines (Figure 6D and E). Importantly, the UV-induced, p53-dependent induction of p21 and MDM2 also reaches a much higher level in HDAC1 H141A mutant cell lines than in control and wild-type HDAC1 lines, demonstrating that an increase in p53 acetylation is accompanied by both

p53 stabilization and enhanced function (Figure 6A and B). Together, these results support the idea that HDAC1 regulates p53 stability and function by modulating its acetylation levels.

Discussion

We have demonstrated previously that acetylation of p53 is invariably and transiently induced upon its activation (Ito *et al.*, 2001). The transient nature of p53 acetylation suggests the presence of negative regulators for p53 acetylation. In this report, we identify MDM2 and HDAC1 as the key components that function cooperatively to control p53 deacetylation. We found that acetylation functions, at least in part, by promoting p53 stability. The realization that p300/CBP-mediated acetylation and MDM2-mediated ubiquitylation occur at a common set of lysine residues provides a potential molecular mechanism by which acetylation controls p53 stability by competing with ubiquitylation. The identification of an MDM2-HDAC1 interaction thus provides a novel mechanism to couple the regulation of acetylation and ubiquitylation for the efficient control of p53 levels.

We have previously demonstrated that p53 acetylation is negatively regulated by MDM2 and that this activity can be reversed by p14^{ARF} (Ito *et al.*, 2001). We and others have shown that MDM2 suppresses p53 acetylation, at least in part, by inhibiting the acetyltransferase activity of p300 and CBP (Kobet *et al.*, 2000; Ito *et al.*, 2001). However, we also observed that the inhibitory activity of MDM2 toward p53 acetylation can be reversed by the deacetylase inhibitor TSA (Ito *et al.*, 2001). This observation led us to propose that MDM2 must utilize additional mechanisms to regulate p53 acetylation. The identification of a specific interaction between MDM2 and HDAC1 now provides evidence for a second mechanism employed by MDM2 to control p53 acetylation. Consistent with the idea that MDM2 recruits HDAC1 to downregulate p53 acetylation, HDAC1, but not other members of the HDAC family (2, 3, 4, 5 and 7), has the capacity to function as a p53 deacetylase towards all three known acetylated lysine residues of p53 *in vivo* (Figure 2; data not shown). Interestingly, HDAC2 has been found to co-exist with HDAC1 in several complexes (Zhang *et al.*, 1998a), but does not interact with MDM2 appreciably and has no significant p53 deacetylase activity. This surprising observation raises an interesting possibility that HDAC1 might reside in a different complex to function as a p53 deacetylase. An earlier report demonstrated that HDAC1 acts as a p53 deacetylase based on the observation that overexpression of HDAC1-associated PID/MTA-2 protein can induce p53 deacetylation (Luo *et al.*, 2000). Our study, however, provides the first direct evidence that HDAC1 indeed functions as a p53 deacetylase *in vivo* (Figure 2). It is worth noting that our data suggest that MDM2 and HDAC1 may interact via an intermediate factor; however, given the important role of PID/MTA-2 in p53 deacetylation (Luo *et al.*, 2000), it remains to be tested whether PID plays a role in mediating the HDAC1-MDM2 interaction. The important role of HDAC1 in MDM2-mediated p53 deacetylation is further substantiated by the finding that a dominant-negative HDAC1 mutant (H141A) can restore p53 acetylation levels in the presence

of MDM2 (Figure 4). The identification of an MDM2-HDAC1 complex not only provides a novel mechanism by which p53 acetylation is regulated, but it also revealed an unexpected link between the acetylation and ubiquitylation machinery (see below for more discussion).

Although high levels of HDAC1 can induce p53 deacetylation in the absence of MDM2, low levels of HDAC1, which likely reflect physiological conditions, fail to do so (Figure 3A and B). However, the ability of HDAC1 to function as a p53 deacetylase, at low levels, is dramatically induced in the presence of MDM2 (Figure 3B). This result suggests that MDM2 facilitates the functional interaction between HDAC1 and p53. In support of this hypothesis, we showed that the physical association between HDAC1 and p53 was enhanced in the presence of MDM2 (Figure 3C). As the level of HDAC1 itself appears to be constant and not subject to regulation (Figure 1E; A.Ito, unpublished observation), the interaction with MDM2, whose level is regulated by p53 in response to various stresses, provides a means to control the activity of HDAC1 toward p53. Under this scenario, the p53 deacetylase activity of HDAC1 becomes activated when the MDM2 levels are increased by active p53 in response to stress. Consistent with this idea, the endogenous interaction between HDAC1 and MDM2 is enhanced after DNA damage when the level of MDM2 is induced (Figure 1E). Thus, together with MDM2, HDAC1 becomes a key component of a p53 negative feedback loop. Supporting this idea, inhibition of HDAC1 activity by an HDAC1 dominant-negative mutant leads to a dramatic enhancement of DNA damage-induced p53 acetylation, p53 stability and activity (Figure 6). The cooperative activity of MDM2 and HDAC1 toward p53 could be achieved through the stimulation of p53-HDAC1 complex formation as shown in Figure 3C. Another interesting possibility is that, as MDM2 can ubiquitylate substrates other than p53 (Shenoy *et al.*, 2001), MDM2 might regulate the activity of HDAC1 by ubiquitylating HDAC1 or a component of the HDAC1 complex. Such a possibility is consistent with our previous observation that p14^{ARF}, which is known to inhibit the MDM2 E3 ligase activity toward p53 (Honda and Yasuda, 1999), can reverse MDM2-mediated p53 deacetylation (Ito *et al.*, 2001).

Regardless of the mechanism, our results support the idea that the recruitment of HDAC1 by MDM2 plays an important role in regulating p53 deacetylation and function. However, it is likely that HDAC1 is not the only deacetylase that regulates p53 acetylation. We have evidence that a portion of p53 deacetylation could be carried out in the cytoplasm whereas HDAC1 resides in the nucleus (A.Ito, Y.Kawaguchi and T.P.Yao, unpublished observation). Furthermore, recent studies have shown that the NAD-dependent and TSA-insensitive histone deacetylase Sir-2, can also deacetylate p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001). However, unlike HDAC1, which is able to deacetylate p53 at all three known acetylated Lys residues, Sir-2 was shown to mainly deacetylate Lys 382 (Vaziri *et al.*, 2001). Further studies will be required to elucidate the individual role of these deacetylases in regulating p53 function. However, the participation of multiple deacetylases further supports the

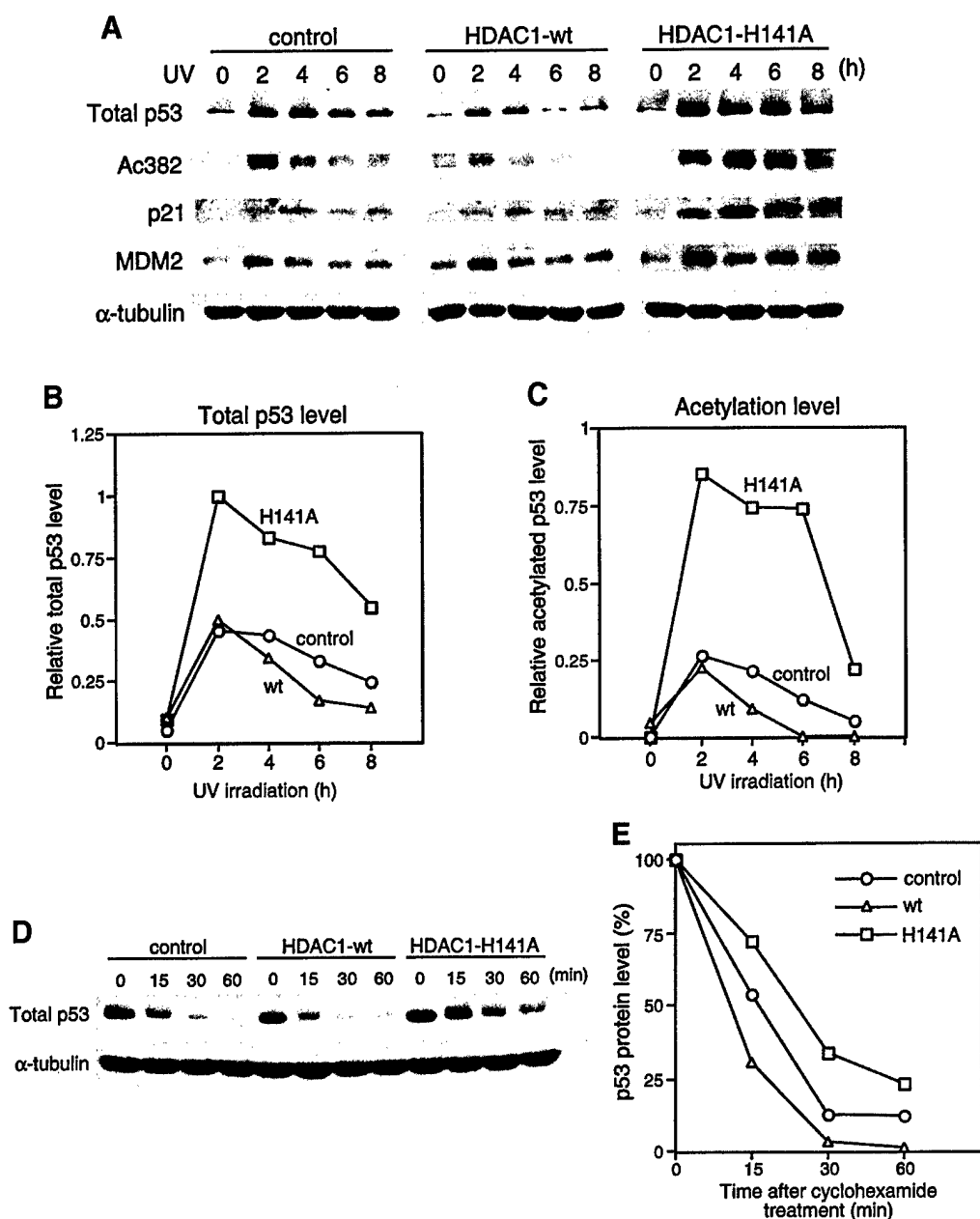


Fig. 6. Effect of HDAC1 on p53 acetylation, stability and activity in response to DNA damage. (A) NIH 3T3 cells infected with mock vector (control), pBabe-HDAC1 wild-type (HDAC1-wt), or pBabe-HDAC1 H141A mutant (HDAC1 H141A) were exposed to UV-B (75 J/m^2). Cells were harvested at the indicated times. The levels of total p53 (panel 1), acetylated p53 (panel 2), p21 (panel 3), MDM2 (panel 4) and the internal control α -tubulin (panel 5) were assessed by immunoblotting. (B and C) The band intensity of p53, acetylated p53 and α -tubulin protein levels in all three cell lines were measured with NIH imaging software. The levels of p53 (B) and acetylated p53 (C) were normalized to α -tubulin and the highest intensity levels of p53 or acetylated p53 were set to 1. (B) and (C) are representative results of three (B) and two (C) independent experiments. (D) All three stable cell lines were exposed to UV-B (75 J/m^2) and 2 h post-irradiation, cyclohexamide ($10 \mu\text{g/ml}$) was added to inhibit new p53 protein synthesis (designated 0 h). Cells were harvested at the time points indicated after cyclohexamide treatment. The level of total p53 (upper panel) and α -tubulin (lower panel) was determined. (E) The band intensity of p53 and α -tubulin protein levels were measured by NIH imaging software. p53 levels were normalized to α -tubulin levels and calculated against the amount of p53 present at time point 0, which was set at 100%. Results are representative of three independent experiments.

idea that acetylation is a critical mechanism for regulation of p53.

We have previously shown that acetylation appears to be a critical modification, as it invariably accompanies p53 activation and is the target of key p53 regulators, like MDM2, p14^{ARF} and p300/CBP (Ito *et al.*, 2001). However, the exact function and mechanism by which acetylation

controls p53 activation remains unclear. As acetylation stimulates p53 DNA-binding activity *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998), it was hypothesized that acetylation of p53 promoted its transcriptional activity. Supporting this idea, a recent study shows that, although it does not play a major role on p53 association with target promoters *in vivo*, p53 acetylation is involved

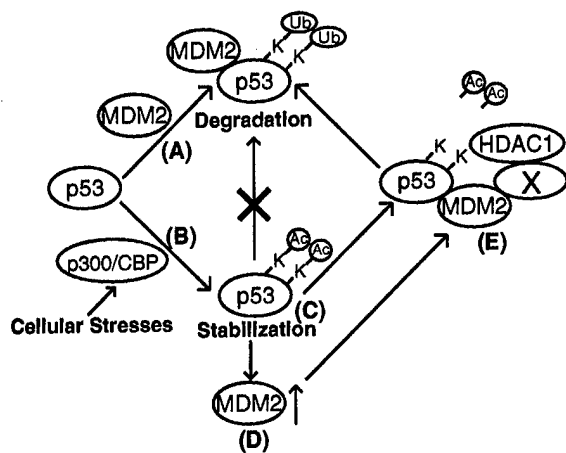


Fig. 7. A model for the regulation and interplay of p53 acetylation and ubiquitylation. Under unstressed conditions, p53 is ubiquitylated on lysine residues by MDM2 and targeted for degradation (A). Upon its activation by various cellular insults, p53 becomes acetylated by p300/CBP at the same set of lysine residues also targeted by MDM2 (B). Acetylation on lysine residues thus prevents MDM2-mediated ubiquitylation and leads to p53 stabilization (C). The stabilized p53 functions as a tumor suppressor and also induces MDM2 (D). The high level of MDM2, in turn, recruits the p53 deacetylase HDAC1 and triggers p53 deacetylation (E). The deacetylated p53 with unmodified lysine residues is now ready to be ubiquitylated by MDM2 and ultimately degraded.

in the recruitment of transcriptional co-activators (Barlev *et al.*, 2001). Our current study provides evidence that, in addition to a role in p53 transcriptional activity, there exists another novel function for p53 acetylation. We have reported previously the parallel kinetics of p53 acetylation and stabilization and the enhancement of p53 stability by inhibition of its deacetylation (Ito *et al.*, 2001). Both observations are consistent with the idea that one function of acetylation is to promote p53 stability. By assessing the p53 status in fibroblasts stably overexpressing a dominant-negative mutant of HDAC1, we have obtained further evidence that enhanced acetylation is associated with an increase in p53 protein stability in response to DNA damage (Figure 6). Although the complete *in vivo* acetylation sites in p53 still remain to be established, the *in vitro* mapping effort reveals that both p300/CBP-mediated acetylation (Figure 5B) and MDM2-mediated ubiquitylation (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000) occurred at an overlapping set of lysine residues (Figure 5C). This observation offers a further link between p53 acetylation and stability. The potential competition for acetylation and ubiquitylation of these lysine residues provides a plausible molecular mechanism by which acetylation promotes p53 stability. In this model (Figure 7), at the p53 basal state, a set of C-terminal lysine residues of p53 are subject to MDM2-mediated ubiquitylation, which leads to p53 degradation. Upon its activation by stress signals, these lysine residues become acetylated by p300/CBP and are no longer available for MDM2-dependent ubiquitylation, leading to p53 stabilization. Stabilized p53 functions as a tumor suppressor and induces high levels of MDM2, which in turn, promotes p53 deacetylation by recruiting a p53 deacetylase, HDAC1. The unmodified lysine residues can then serve

as the substrates for MDM2-mediated ubiquitylation resulting in p53 degradation. The interaction of MDM2 and HDAC1 provides a novel molecular mechanism for an efficient coupling of deacetylation and ubiquitylation of p53 that allows MDM2 to effectively inactivate and degrade p53 and complete the negative feedback loop. The potential functional interaction between the acetylation and ubiquitylation machinery described in this model would also suggest a broader and more general role for reversible acetylation in the regulation of protein stability and other ubiquitylation-dependent biological processes.

Materials and methods

Cell lines and transfection

293T, H1299, A549 and MDM2-/-;p53-/- double null MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, while NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂. All transfections were performed by either the calcium phosphate method as described previously (Yao *et al.*, 1992) or the lipofectamine method (Invitrogen). Retroviruses were produced by transient transfection of a pBabe Puro vector construct into phoenix cells. NIH 3T3 cells were infected with retrovirus containing media in the presence of 8 µg/ml of polybrene overnight. Thirty-six hours later, cells were selected in the presence of 1.5 µg/ml puromycin and kept under selection in medium containing 1.5 µg/ml puromycin during experiments.

Plasmids

p53, MDM2, and p300 vectors were described previously (Ito *et al.*, 2001). HDAC1 cDNA was provided by Dr F.Dangond (Harvard Medical School) and cloned into the *EcoRI/NotI* sites of pcDNA3 vector with a Flag tag. HDAC4 and 5 cDNAs were provided by Dr S.L.Schreiber (Harvard University) and described in Grozinger *et al.* (1999). HDAC1 H141A mutant cDNA (provided by Dr S.L.Schreiber) was subcloned into the *BamHI/EcoRI* sites of pcDNA3 vector. For retroviral constructs, HDAC1 wild-type and H141A mutant cDNAs were cloned into the *BamHI/EcoRI* sites of the pBabe Puro vector. p53-3KA mutant cDNA, mutated lysines 320, 373 and 382 to alanine, 6KR mutant, and mutated lysines 320, 370, 372, 373, 381 and 382 to arginine cDNAs were generated by site-directed mutagenesis and cloned into the pcDNA3 or the pGEX vector (Amersham).

DNA-damage treatment

DNA damage was performed by exposing cells to a 310 nm wavelength UV source and cells were harvested at indicated time points.

Immunoprecipitation and immunoblotting

Cells were lysed in buffer (20 mM Tris-HCl pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT) supplemented with 5 µM TSA and protease inhibitors. For immunoprecipitation with anti-goat p53 antibody, equal amounts of lysate (containing 100–300 µg of total cellular protein) were incubated with 1 µg of goat anti-p53 antibody (FL-393; Santa Cruz) and protein G-Sepharose (Pharmacia) for 3 h at 4°C. To detect acetylated mouse p53, equal amounts of lysate (containing 300–500 µg of total cellular protein) were incubated with agarose-conjugated anti-p53 antibody (Pab421) overnight at 4°C. To detect endogenous MDM2 and HDAC1 interaction, 2 mg of cellular lysate was incubated with 1 µg of anti-MDM2 antibody (SMP14; Santa Cruz) and protein A and G-Sepharose mixtures (Pharmacia) overnight at 4°C. When immunoprecipitation was not performed, 20–50 µg of total extracts were analyzed. Proteins were detected by chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-human p53 antibody (Ab-6; Calbiochem), anti-p53 antibody for detecting mouse p53, anti-human acetylated (Lys320) p53 antibody (Sakaguchi *et al.*, 1998), anti-human acetylated (Lys373) p53 antibody, anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-mouse acetylated (Lys382) p53 antibody, anti-human MDM2 antibody (SMP14; Santa Cruz), anti-Flag antibody (M2; Sigma), anti-HDAC1 antibody (H-11; Santa Cruz), anti- α -tubulin antibody (DM1A; Sigma), anti-p21 antibody (H164; Santa Cruz) or anti-GFP antibody (Boehringer Mannheim).

In vitro deacetylation assay

The expression vectors for Flag-tagged HDACs (10–15 µg) were transfected into 293T cells and the cells were lysed in low stringency buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) in the presence of protease inhibitors. After pre-clearing with protein A beads, the extracts were immunoprecipitated with anti-Flag antibody in the presence of rabbit anti-mouse antibody and protein A beads for 5 h at 4°C and then the beads were washed three times with low stringency buffer, twice with low stringency buffer containing 0.5 M NaCl, and twice with deacetylase buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 10% glycerol). For inhibition studies, the immune complexes were pre-incubated with 400 nM of TSA in deacetylase buffer for 30 min at 4°C. The immune complexes were incubated with 10 000 c.p.m. of [¹⁴C]-labeled acetylated GST-p53 or ³H-labeled acetylated histone 4 peptide in 200 µl of deacetylase buffer for 2 h at 37°C and the release of [¹⁴C] or [³H]acetate was quantified by scintillation counting.

In vitro acetylation assay

The *in vitro* acetylation assay was performed as described previously (Ito *et al.*, 2001). Briefly, recombinant CBP protein (1 µg), purified from sf9 insect cells infected with baculovirus expressing CBP, was incubated with 1 µg of wild-type GST-p53 or GST-p53 mutants in the presence of 50 nCi [¹⁴C]acetyl-coenzyme A in 30 µl of reaction buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM dithiothreitol, 100 µM EDTA, 1 mM PMSF) for 1 h at 37°C. Acetylation was analyzed by SDS-PAGE followed by autoradiography.

Acknowledgements

We thank Dr S.L.Schreiber for his generous gift of the HDAC1 H141A, HDAC4 and HDAC5 cDNAs, Dr F.Dangond for the HDAC3 cDNA, Dr C.W.Wu for the GST-HDAC1 construct and Dr G.Lozano for the MDM2-/-;p53-/- double null MEF cells. We are grateful to Drs C.Anderson, B.Harvat, A.R.Means, X.F.Wang, Ms A.Guardiola and Mr T.A.Bolger for critically reading the manuscript. This work is supported by funding from the Damon Runyon-Walter Winchell Cancer Foundation (DRS 20), National Institutes of Health (CA85676-01A1) and Department of Defense (DAMD 17-01-1-0054) to T.-P.Y.

References

Appella,E. and Anderson,C.W. (2001) Post-translational modifications and activation of p53 by genotoxic stresses. *Eur. J. Biochem.*, **268**, 2764–2772.

Ashcroft,M., Taya,Y. and Vousden,K.H. (2000) Stress signals utilize multiple pathways to stabilize p53. *Mol. Cell. Biol.*, **20**, 3224–3233.

Barlev,N.A., Liu,L., Chehab,N.H., Mansfield,K., Harris,K.G., Halazonetis,T.D. and Berger,S.L. (2001) Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol. Cell*, **8**, 1243–1254.

Boyd,S.D., Tsai,K.Y. and Jacks,T. (2000) An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. *Nat. Cell Biol.*, **2**, 563–568.

Canman,C.E., Lim,D.S., Cimprich,K.A., Taya,Y., Tamai,K., Sakaguchi,K., Appella,E., Kastan,M.B. and Siliciano,J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281**, 1677–1679.

Freedman,D.A., Wu,L. and Levine,A.J. (1999) Functions of the MDM2 oncoprotein. *Cell. Mol. Life Sci.*, **55**, 96–107.

Geyer,R.K., Yu,Z.K. and Maki,C.G. (2000) The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nat. Cell Biol.*, **2**, 569–573.

Giaccia,A.J. and Kastan,M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.*, **12**, 2973–2983.

Grozinger,C.M., Hassig,C.A. and Schreiber,S.L. (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl Acad. Sci. USA*, **96**, 4868–4873.

Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.

Hirao,A., Kong,Y.Y., Matsuoka,S., Wakeham,A., Ruland,J., Yoshida,H., Liu,D., Elledge,S.J. and Mak,T.W. (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science*, **287**, 1824–1827.

Honda,R. and Yasuda,H. (1999) Association of p19(ARF) with Mdm2

inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.*, **18**, 22–27.

Honda,R., Tanaka,H. and Yasuda,H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.*, **420**, 25–27.

Ito,A., Lai,C.H., Zhao,X., Saito,S., Hamilton,M.H., Appella,E. and Yao,T.P. (2001) p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. *EMBO J.*, **20**, 1331–1340.

Kobet,E., Zeng,X., Zhu,Y., Keller,D. and Lu,H. (2000) MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. *Proc. Natl Acad. Sci. USA*, **97**, 12547–12552.

Liu,L., Scolnick,D.M., Trievel,R.C., Zhang,H.B., Marmorstein,R., Halazonetis,T.D. and Berger,S.L. (1999) p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. *Mol. Cell. Biol.*, **19**, 1202–1209.

Luo,J., Su,F., Chen,D., Shiloh,A. and Gu,W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature*, **408**, 377–381.

Luo,J., Nikolaev,A.Y., Imai,S., Chen,D., Su,F., Shiloh,A., Guarente,L. and Gu,W. (2001) Negative control of p53 by Sir2α promotes cell survival under stress. *Cell*, **107**, 137–148.

Montes de Oca Luna,R., Wagner,D.S. and Lozano,G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203–206.

Nakamura,S., Roth,J.A. and Mukhopadhyay,T. (2000) Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. *Mol. Cell. Biol.*, **20**, 9391–9398.

Pearson,M. *et al.* (2000) PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature*, **406**, 207–210.

Pomerantz,J. *et al.* (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, **92**, 713–723.

Rodriguez,M.S., Desterro,J.M., Lain,S., Lane,D.P. and Hay,R.T. (2000) Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Mol. Cell. Biol.*, **20**, 8458–8467.

Sakaguchi,K., Herrera,J.E., Saito,S., Miki,T., Bustin,M., Vassilev,A., Anderson,C.W. and Appella,E. (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.*, **12**, 2831–2841.

Shenoy,S.K., McDonald,P.H., Kohout,T.A. and Lefkowitz,R.J. (2001) Regulation of receptor fate by ubiquitination of activated β2-adrenergic receptor and β-arrestin. *Science*, **294**, 1307–1313.

Shieh,S.Y., Ikeda,M., Taya,Y. and Prives,C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, **91**, 325–334.

Unger,T., Juven-Gershon,T., Moallem,E., Berger,M., Vogt Sionov,R., Lozano,G., Oren,M. and Haupt,Y. (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.*, **18**, 1805–1814.

Vaziri,H., Dessain,S.K., Eaton,E.N., Imai,S.I., Frye,R.A., Pandita,T.K., Guarente,L. and Weinberg,R.A. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell*, **107**, 149–159.

Wu,X., Bayle,J.H., Olson,D. and Levine,A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.*, **7**, 1126–1132.

Yao,T.P., Segreaves,W.A., Oro,A.E., McKeown,M. and Evans,R.M. (1992) *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell*, **71**, 63–72.

Zhang,Y. and Xiong,Y. (2001) Control of p53 ubiquitination and nuclear export by MDM2 and ARF. *Cell Growth Differ.*, **12**, 175–186.

Zhang,Y., LeRoy,G., Seelig,H.P., Lane,W.S. and Reinberg,D. (1998a) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell*, **95**, 279–289.

Zhang,Y., Xiong,Y. and Yarbrough,W.G. (1998b) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725–734.

Received January 17, 2002; revised and accepted September 30, 2002

**Acetylation at multiple C-terminal lysine residues regulates p53 subcellular
localization**

Yoshiharu Kawaguchi, Akihiro Ito, Ettore Appella¹ and Tso-Pang Yao²,

Department of Pharmacology and Cancer Biology, Duke University, Durham, North
Carolina 27710, USA.

¹ Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health,
Bethesda, Maryland 20892, USA

² Corresponding author. Email: yao00001@mc.duke.edu

Manuscript information: The number of text pages, 19; that of figures, 5

Word and character counts: The number of words in the abstract, 178

The total characters in paper, 47,500

Abbreviations footnotes: NES, nuclear export signal; LMB, leptomycin B

Abstract

The basal level of the tumor suppressor p53 is regulated by MDM2- mediated ubiquitination at specific lysines, which leads to p53 nuclear exclusion and degradation. Upon cellular stresses, however, these lysines become acetylated by p300/CBP resulting in p53 stabilization. Here we report an unexpected finding that p300-mediated acetylation also promotes cytoplasmic localization of p53. Over-expression of p300, but not an acetyltransferase-deficient mutant, stimulates the cytoplasmic accumulation of p53, an effect negated either by inactivating its C-terminal nuclear export signal (NES), or by the nuclear export inhibitor, LMB. p300-dependent nuclear exit of p53 does not involve MDM2, but requires multiple lysines targeted by p300. Conversion of a minimum of four lysines to alanines but not arginines mimics the effect of MDM2 and p300 on the cytoplasmic accumulation of p53 by preventing p53 tetramerization and thus potentially exposing its NES. Our results suggest that acetylation and ubiquitination regulate p53 transport by neutralizing the lysine charge, which in turn controls p53 oligomerization and the accessibility of the NES. Thus, the charge patch created by C-terminal lysines is critical for the regulation of p53 nuclear-cytoplasmic distribution.

Introduction

The tumor suppressor p53 plays critical roles in regulating cell growth and protecting cells from malignant transformation. Due to its growth inhibitory and proapoptotic activity, p53 is kept inactive at low concentrations in unstressed cells and any activation of p53 must be transient. In general, the negative regulation of p53 requires the oncogene MDM2 (1). MDM2 can bind p53 directly and suppress p53 transcriptional activity (2-4). MDM2 also regulates p53 protein stability by serving as an E3 ligase for p53 ubiquitination, which triggers the rapid degradation of p53 (5-8). The induction of MDM2 by p53 is believed to form the basis of a negative feedback loop that ensures that p53 activation is transient (9). However, a recent study showed that, in response to specific stresses, the activation of p53 does not lead to MDM2 induction (10). This observation suggests the existence of an alternative mechanism that is capable of terminating p53 activity.

The activity of p53 is also regulated by its subcellular localization. p53 actively shuttles between the nucleus and cytoplasm via a mechanism that is mediated by a nuclear export signal (NES) and its receptor Crm-1 (reviewed in (11)). Although its importance for p53 function is apparent, little is known about how p53 nuclear export is regulated. Recently, p53 export was reported to be activated by MDM2-mediated ubiquitination (12-15). However, the exact mechanism by which ubiquitination promotes p53 nuclear export is not known. It has been also proposed that the C-terminal NES is normally exposed in the inactive monomeric or dimeric forms of p53, which are subject to active nuclear export. Upon activation, however, p53 forms tetramers, in which the C-terminal NES is buried and inaccessible to the Crm1 export machinery, resulting in p53 nuclear retention (16). At present, it is not understood how the oligomerization status of p53 is regulated in response to specific stress signals or modifications, such as ubiquitination, that control p53 function.

In contrast to ubiquitination, acetylation has been generally believed to play a positive role in p53 function (reviewed in (17)). We have shown that various p53-activating agents promote p53 acetylation, which is correlated with increased stability (18). The acetylation of p53, which is catalyzed by the p300/CBP acetyltransferases, occurs on at least six lysine residues clustered at the C-terminus (18-21) (A.I. and T.P.Y,

submitted). It is unclear why so many lysines are modified by acetylation. Nor is it known whether the acetylation of the lysines provides a functional moiety for protein interaction or serves to modify the conformation of p53. One clue as to the function of p53 acetylation lies in several potentially interesting links between acetylation and ubiquitination. For example, both the acetylation and ubiquitination machinery modify the ϵ amino group of the lysine residue. Furthermore, acetylation and ubiquitination occur on a common set of lysine residues at the C-terminus of p53 (A.I. and T.P.Y. submitted). Lastly, MDM2 negatively regulates p53 acetylation (18, 22). From these observations, we hypothesize that MDM2-mediated ubiquitination and p300/CBP-mediated acetylation target the same set of lysine residues in p53 to control its function.

Here, we report new data demonstrating that, in contrast to its known positive role in p53 function, p300-mediated acetylation also promotes p53 nuclear exit leading to p53 accumulation in the cytoplasm. We show that over-expression of p300 stimulates cytoplasmic accumulation of p53 in an acetylation-dependent but MDM2-independent manner. Mechanistically, we provide evidence suggesting that both acetylation and ubiquitination promote redistribution of p53 to the cytoplasm by neutralizing the positive charge of the C-terminal lysine residues. We further show that the charge neutralization of four or five C-terminal lysines prevents p53 oligomerization, which would lead to the exposure of the p53 NES, and thus allows efficient p53 nuclear export. The requirement of multiple modified lysines for efficient export suggests a potential novel threshold mechanism wherein the acetylation level of p53 serves as a signal that terminates p53 function by activating its nuclear export.

Results

Acetylation regulates subcellular localization of p53

We have previously shown that p53 becomes acetylated by p300/CBP upon cellular stresses (18). As p53 activation is accompanied by its accumulation in the nucleus, we asked whether p300-mediated acetylation regulates p53 subcellular localization. To test this, we transfected p53 null H1299 cells with expression plasmids for p53 and p300, and then examined p53 subcellular localization. As shown in Figure 1A(a), when expressed alone, p53 resides almost exclusively in the nucleus. To our surprise, upon co-expression with p300, p53 localized to both the nucleus and the

cytoplasm in the majority of the cells expressing both transfected p53 and p300 (Figure 1A(b), and B). Cell counts demonstrated that up to 84% of the cells that expressed both p53 and p300, showed cytoplasmic accumulation of p53 (Figure 1B). To determine if the accumulation of p53 in the cytoplasm involved p53 acetylation, we first examined whether an acetyltransferase-deficient p300DY mutant (C.H. Lai, T.P.Y. in preparation) could affect the subcellular localization of p53. As shown in Figure 1A(c) and B, unlike wild type p300, the acetyltransferase deficient p300DY mutant did not stimulate p53 accumulation in the cytoplasm. Importantly, neither p300 nor the p300DY mutant had an effect on the levels of p53 under our experimental conditions (Figure 1D, and (18)). Together, these results indicate that p300-mediated cytoplasmic accumulation of p53 requires p300-acetyltransferase activity.

As p53 subcellular localization is regulated by active nuclear export, we investigated whether acetylation-dependent p53 accumulation in the cytoplasm is dependent on the activation of the nuclear export machinery. As shown in Figure 1A(d) and B, the nuclear export inhibitor leptomycin B (LMB) efficiently blocked the cytoplasmic accumulation of p53 induced by p300. We also evaluated the subcellular localization of a p53 mutant whose C-terminal nuclear export signal (NES) is inactivated (16). As shown in Figure 1A(f) and B, the p53 nuclear export mutant no longer responded to p300 and remained in the nucleus. These results demonstrate that p300-mediated cytoplasmic accumulation of p53 requires the C-terminal NES and suggest that acetylation enhances p53 nuclear export.

To further extend our studies beyond the immuno-localization analysis, we determined the subcellular localization of p53 in response to p300 by biochemical fractionation. As shown in Figure 1C, p53 is normally a nuclear protein (Lane 1-2). However, co-expression of wild type p300, but not the p300DY mutant, induced a marked accumulation (25 % of the total p53) of the p53 protein in the cytoplasmic fraction (compare lane 2, 4 and 6). The p300-dependent cytoplasmic accumulation of p53 could again be largely reversed by LMB treatment (Lane 8). These results are in agreement with the data obtained from the immuno-localization, providing further evidence that p300-mediated acetylation affects subcellular localization of p53. Importantly, the presence of acetylated p53 in the cytoplasmic fraction suggests that p53

can be exported to the cytoplasm after being acetylated (Figure 1C). These results are consistent with the idea that p300-mediated p53 acetylation promotes p53 nuclear export.

p300-mediated cytoplasmic accumulation of p53 is independent of MDM2

It was reported that MDM2 stimulates p53 nuclear export by promoting p53 ubiquitination (12, 13). As p300 functionally interacts with MDM2 (23), we determined whether p300-induced cytoplasmic accumulation of p53 requires MDM2. To test this idea, we expressed p53 alone or together with p300 in MDM2-deficient MEF cells (24), and assessed p53 subcellular localization. As shown in Figure 2A, in the absence of MDM2, p300 is still capable of stimulating the cytoplasmic accumulation of p53. In fact, more than 90% of cells over-expressing p300 showed p53 in cytoplasm, which is similar to the effect induced by MDM2 (Figure 2B). This result indicates that p300-mediated cytoplasmic accumulation of p53 is independent of MDM2.

C-terminal lysine residues are required for efficient acetylation-induced cytoplasmic accumulation of p53

The results presented so far support the idea that p300 affects subcellular localization of p53 in an acetylation-dependent manner, likely by directly acetylating p53. To further investigate this possibility, we determined whether lysine (K) residues known to be acetylated by p300 are required for acetylation-induced cytoplasmic accumulation of p53. We, therefore, mutated multiple lysines to arginines (5KR and 6KR, Figure 3A) that are known targets of acetylation by p300 (A.I. and T.P.Y. submitted) and evaluated the subcellular distribution of these mutants in response to p300. As shown in Figure 3B, when expressed alone, the localization of these p53 KR mutants is almost entirely nuclear and indistinguishable from wild type p53. However, in response to ectopically expressed p300, the number of cells that show a cytoplasmic accumulation of the 5KR (51%) and 6KR (30%) p53 mutants is markedly reduced compared to that of wild type p53 (84%). These data indicate that the lysine residues acetylated by p300 are required to mediate a maximal p53 nuclear exit in response to p300.

Neutralization of positively charged lysine residues in the C-terminus regulates subcellular localization of p53

Our data suggests that, similar to ubiquitination, the acetylation of p53 also leads to its accumulation in the cytoplasm. As previously mentioned, acetylation and ubiquitination modify an over-lapping set of lysine residues (Figure 3 and A.I. and T.P.Y. submitted). Although, ubiquitin and acetyl groups have little in common structurally, they both modify the ϵ amino group of the lysine residue and neutralize its charge. We hypothesize that acetylation and ubiquitination promote p53 nuclear exit by neutralizing the charge of the targeted lysine residues. To test this hypothesis, we generated charge-neutralizing mutations by converting lysine (K) residues known to be acetylated and ubiquitinated to alanine (A), either individually or in combination, and evaluated their subcellular localization. As shown in Figure 4, the subcellular localization of the 2KA and several 3KA (3KA-1, 3KA-2 and 3KA-3) mutants with different combination of mutated lysine residues (Figure 4A), is similar to that of wild type p53 and is mostly nuclear (Figure 4B and C). In contrast, when four lysine residues are mutated in three different combinations (4KA-1, 4KA-2 and 4KA-3), these p53 mutants clearly began to accumulate in the cytoplasm. The conversion of five lysine residues (5KA) lead to a further increase in the number of cells with cytoplasmic p53 staining (Figure 4B and C). The cytoplasmic localization of the 4KA-1, 4KA-2, 4KA-3 and 5KA p53 mutants was observed in 51%, 52%, 48% and 64% of transfected cells, respectively, compared with 19% for wild type p53 (Figure 4C). Furthermore, the intensity of the wild type p53 protein detected in the cytoplasm was much weaker than that of the 4KA and 5KA mutants (data not shown). Importantly, the population of cells showing cytoplasmic p53 4KA and 5KA mutants can be effectively eliminated upon treatment with LMB (Figure 4B and C), supporting the idea that the 4KA and 5KA mutations lead to a more efficient p53 nuclear export. Together, these observations indicate that the degree of p53 cytoplasmic accumulation is proportional to the number of lysine residues neutralized. Therefore, these results suggest that ubiquitination and acetylation modulate p53 subcellular localization by modifying the positive charge of specific lysine residues at the C-terminus of p53.

C-terminal lysine charge determines the oligomerization status of p53

The accessibility of the C-terminal NES to the export machinery has been demonstrated to be regulated by the oligomerization status of p53 (16). We, therefore,

assessed whether modification of the lysine charge activates p53 export by regulating p53 oligomerization status. The observation that the conversion of four but not three lysines to alanines activates p53 export provides us with the ability to address this question. To test this possibility, recombinant wild type, 3KA, and 4KA (4KA 1-3, Figure 4A) mutant polypeptides encompassing the entire p53 tetramerization domain and lysine rich C-terminus (amino acids 326-393) were tested for their ability to oligomerize. A mutant p53 with four lysines converted to arginines instead of alanine (4KR), which prevents ubiquitination and acetylation but preserves the charge of the lysine, was used as an additional control. As shown in Figure 5, the wild type and 3KA polypeptides dimerized and tetramerized readily (Lanes 2 and 4). In contrast, the 4KA-1, 4KA-2 and 4KA-3 polypeptides completely failed to do so (Lane 6, 8 and 10). This observation is consistent with their respective sub-cellular localizations (Figure 4). Importantly, polypeptides from the 4KR mutant, which is a nuclear protein (data not shown), showed a wild type capacity to oligomerize (Lane 12). This result further supports the hypothesis that charge modifications are one of the key elements in the regulation of p53 oligomerization. Together, these results demonstrate that the oligomerization status of p53 can be controlled by the charge conferred by a defined number of lysine residues.

Discussion

In this report, we provide evidence that acetylation regulates p53 subcellular localization, at least in part, by activating its nuclear export. This novel finding suggests that acetylation might have an unsuspected role in the termination of p53 signaling. Our attempt to understand how two unrelated modifications, namely acetylation and ubiquitination, both stimulate p53 nuclear export led to the discovery that the charge of specific C-terminal lysine residues may play a critical role in regulating p53 subcellular localization. Modification of at least four lysine residues by acetylation neutralizes their charge, which in turns prevents p53 oligomerization and exposes its nuclear export signal, leading to efficient export. Our data thus provide a model for understanding how p53 nuclear export is regulated by ubiquitination and acetylation.

The regulation of p53 subcellular localization is believed to be controlled, in part, by its oligomerization status. Interestingly, a dominant C-terminal NES is located in the oligomerization domain of p53 and it was previously suggested that its oligomerization

status is involved in the accessibility of the p53 NES (16). In fact recent reports have shown that the active form of p53 assumes a tetrameric conformation that will mask the NES and allow efficient p53 nuclear accumulation ((25) (26) (27) (28)). In addition, NMR and crystal structures of the oligomerization domain demonstrate that the C-terminal NES is exposed in the monomeric or dimeric conformation but it is buried in the p53 tetramers (29-31). The inter-conversion between the tetrameric and monomeric or dimeric states would, therefore, determine the availability of the NES and, consequently, the efficiency of p53 export. However, the biochemical mechanism that controls p53 oligomerization and its regulation is not known. In this report, we provide experimental evidence that the charge of specific lysine residues in the C-terminus may be a key determinant of p53 oligomerization, which could in turn control the efficiency of its nuclear export. This conclusion is supported by the observation that mutations that neutralize four or five specific lysine residues result in p53 that cannot oligomerize and localize to the cytoplasm. Importantly, this cytoplasmic accumulation can be reversed by LMB treatment, further suggesting a dominant role of p53 nuclear export in response to the charge modification (Figure 4 and 5). Our data, however, do not exclude the possibility that the charge neutralization of lysine residues might also affect p53 nuclear import as well. The importance of lysine 320, which is part of the p53 nuclear import signal (NLS) (32), in acetylation-dependent p53 cytoplasmic accumulation is consistent with this possibility (Figure 3). Regardless of which mechanism might play a more dominant role, our results support the idea that a charge modification of the lysine residues may dictate the subcellular localization of p53.

If the C-terminal charge of lysine residues plays a critical role in p53 subcellular localization, how is this charge patch regulated? Evidence suggests that MDM2-mediated ubiquitination, which was recently shown to promote p53 export (14, 15), is one key regulator. In theory, the ubiquitin moiety itself could be the signal that activates p53 nuclear export, as is the case for its role in receptor endocytosis (reviewed in (33)). However, our data indicate that acetylation, which shares no structural similarity with ubiquitin, can nonetheless promote cytoplasmic accumulation of p53 by activating the export machinery. Moreover, our results and data from others demonstrate that mutation of specific C-terminal lysines to alanines or isoleucine, but not arginines, leads to p53

cytoplasmic accumulation (Figure 4 and 5), further indicate that ubiquitination as well as acetylation promote p53 export by neutralizing the charge of specific lysine residues (14,15,34).

Interestingly, although the conversion of three different combinations of four lysines known to be targets of acetylation and ubiquitination to alanines (4KA mutants) promotes cytoplasmic accumulation of p53, the conversion of only three lysines with different combinations (3KA) has little effect (Figure 4). This result suggests that there might be a threshold for the activation of p53 export, which is determined by the overall charge provided by the lysine cluster at the C-terminus. In support of this possibility we show that the p53 4KA mutant fails to tetramerize, while the 3KA mutant oligomerizes as efficiently as the wild type (Figure 5). This result correlates well with the respective subcellular localization of these mutants and further supports the proposed model that the p53 NES is not accessible in the tetrameric configuration but exposed in the monomeric or dimeric form (16). The NES in the 4KA p53 mutant is therefore more accessible to Crm1 than that in the wild type or 3KA mutant, resulting in more active export of the p53 4KA but not 3KA mutant. The idea that acetylation regulates p53 export by neutralizing the lysine charge is in agreement with a recent study on histone H2A.Z in which acetylation was shown to regulate the histone tails by modifying the charge patch of multiple lysines (35).

We note, however, that the p53 4KA and 5KA mutants are more resistant to MDM2-mediated degradation (33, and data not shown). Thus, although K to A mutations can mimic the effect of ubiquitin-induced p53 nuclear export, they do not confer the ubiquitin-mediated p53 degradation. This observation has two implications. First, p53 nuclear export and degradation are not necessarily linked events. A similar conclusion can be drawn from a recent study showing that a p53 mutant deficient in nuclear export can nonetheless be degraded by MDM2 (15). Second, it suggests that although the p53 species targeted for degradation and nuclear export are both modified by ubiquitin, they likely represent two distinct p53 populations that are modified differently. As it was recently demonstrated that MDM2 can promote p53 mono-ubiquitination *in vitro* (36), an interesting possibility is that mono-ubiquitinated p53 is exported while the poly-ubiquitinated species is targeted to the proteasome for degradation. How MDM2

promotes two different types of ubiquitination on p53 is a critical issue to be addressed in future studies.

The finding that acetylation regulates the subcellular localization of p53, at least in part, by activating nuclear export, seems, at first, paradoxical, as previous evidence suggests that acetylation positively regulates p53 function (reviewed in (17)). However, a recent study on the role of ubiquitination in transcription has concluded that ubiquitination not only serves to degrade and inactivate transcriptional activators, but it is also essential for their transcriptional activity (37). In that study, therefore, ubiquitination functions as both a positive and a negative regulator, providing an efficient coupling of the activation and inactivation of specific transcription events. By analogy, our findings raise the interesting possibility that acetylation not only activates p53 function initially (18), but also serves as a signal to terminate p53 function by activating its export. In this speculative scenario, the threshold for p53 export is established by the level of p53 acetylation such that when four or more lysine residues are acetylated, p53 will be efficiently exported. Indeed, we have observed that fully acetylated p53 can be exported from the nucleus (Figure 1C). We note, however, that although p300 mediated-acetylation can promote p53 cytoplasmic accumulation under our experimental conditions, it remains to be established whether this mechanism is sufficient to trigger p53 nuclear exit during the normal p53 activation cycle. Further experiments will be required to demonstrate the importance of acetylation in regulating p53 subcellular localization under physiological conditions.

Our results provide evidence that the charge status presented by a specific set of lysine residues is a key determinant and regulatory target for the regulation of p53 subcellular localization. The regulation is achieved by the modification of these lysine residues by the MDM2-mediated ubiquitination and/or p300/CBP-mediated acetylation machinery. As MDM2 is not always induced upon p53 activation (10), our finding that acetylation can promote p53 nuclear exit could provide another potential feedback mechanism to ensure that the activation of p53 is transient and properly terminated.

Acknowledgements

We thank Dr. G. Lozano for MDM2^{-/-};p53^{-/-} null MEF cells. We are grateful to Dr. D. Lew, Ms. C. Hubbert and A. Guardiola for critically reading the manuscript. This work is supported by funding from the Damon Runyon-Walter Winchell Cancer Foundation (DRS 20), National Institute of Health (CA85676-01A1), and Department of Defense (DAMD 17-01-1-0054) to T.P.Y.

Materials and methods

Cell lines and transfection

H1299 human cells and p53(-/-), MDM2(-/-) mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All cells were grown at 37 °C in the presence of 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO₂. All transfections were performed by the calcium phosphate method as described previously (38).

Plasmids

Wild-type human p53 cDNA, wild-type human MDM2, wild-type human myc-p300 and human p300DY mutants were described previously (18). p53-5KR and -6KR mutants were generated using site-directed mutagenesis, changing lysines 320, 370, 372, 373, 381 and 382 to arginines. P53-2KA, -3KA, -4KA and -5KA mutants were also constructed using site-directed mutagenesis to exchange the lysines to alanines. p53-NES(-) mutants were also constructed using site-directed mutagenesis to change the leucines (a.a 348 and 350) to alanines.

Immuno-fluorescence

For immuno-fluorescence staining, cells grown on a glass coverslip were transfected with 0.1 µg p53 and 1µg myc-epitope tagged p300 expression plasmids. Immunostaining was performed as described (39) using anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz), anti-myc monoclonal antibody 9E10 and anti-MDM2 monoclonal antibody SMP-14 (Santa Cruz).

Fractionation, Immunoprecipitation and immunoblotting

The cells were homogenized using a dounce homogenizer in buffer [25 mM HEPES-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA, 5mM MgCl₂, 50 mM NaF, 1mM dithiothreitol (DTT)] supplemented with 5 µM of deacetylase inhibitor TSA (Sigma) and protease inhibitors. After centrifugation at 960g for 5 min, nuclei pellets were washed with the homogenizer buffer, and then lysed in buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DDT] supplemented with 5 µM TSA and protease inhibitors. Lysates were centrifuged at 5000g for 5 min to obtain supernatants of nuclear fraction. While, cytoplasmic supernatants were added NaCl and NP-40 to bring up to 150 mM and 1%, respectively. Immunoprecipitation and immunoblotting were performed as described

previously (18). Proteins were detected with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys320) p53 antibody, anti-human acetylated (Lys373) p53 antibody, anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti- α -tubulin antibody (DM1A, Sigma) or anti-green fluorescent protein (GFP) antibody (Boehringer Mannheim).

Protein production and oligomerization assay

Wild-type, 3KA, 4KA or 4KR p53 C-terminal DNA (amino acid 326-393) including the tetramerization domain was cloned into pGEX-6P-1 vectors. GST fusion constructs were expressed in *E.coli*, and then purified by Glutathione Sepharose 4B (Amersham) and cleaved with prescission protease (Amersham). The oligomerization assay was performed as described previously (16).

Figure legend

Figure 1. Acetylation regulates p53 nuclear export. **(A)** Subcellular localization of p53 and p300 following transfection into p53 (-/-) H1299 cells. Cells were transfected with p53wt alone (a, g), p53wt and myc-p300 (b, d, h, j), p53wt and myc-p300DY (c, i), p53 NES(-) mutant alone (e, k), or p53 NES(-) mutant and myc-p300 (f, l) as indicated. The localization of p53, p300, and p300DY was determined as described in materials and methods. 10 ng/ml of LMB was added 8h prior to immunostaining as indicated (d, j). **(B)** Acetylation by p300 increases the percent of cells with cytoplasmic p53. 100-200 cells from each transfection were scored. Results are an average of three independent experiments. In the case of co-expression with p300 or p300DY, only anti-myc antibody positive cells were scored. **(C)** Cytoplasmic p53 is acetylated. Levels of acetylated p53 in the nuclear (N) and cytoplasmic (C) fractions were determined by immunoprecipitation with anti-p53 polyclonal antibody, followed by immunoblotting with anti-acetylated p53 antibody. Levels of total p53 and α -tubulin were assayed by blotting with anti-p53 monoclonal antibody (Ab-6) and anti-tubulin monoclonal antibody. **(D)** p300 dose not change the expression level of total p53. GFP was used as internal control, and detected by anti-GFP monoclonal antibody.

Figure 2. Acetylation-mediated p53 nuclear export is not MDM2-dependent. **(A)** Subcellular localization of p53, p300 and MDM2 following transfection into p53(-/-) / MDM2(-/-) MEF cells. p53 (a,b,c) and p300 (d) were detected as described in Figure 1A. MDM2 (e) was detected by immunostaining with the anti-MDM2 monoclonal antibody (SMP-14). **(B)** The percent of cells with cytoplasmic p53 was determined as described in Figure 1B.

Figure 3. The C-terminal lysines of p53 are involved in the acetylation-mediated nuclear export of p53. **(A)** Schematic representation of wild-type p53 and an expanded view of the C-terminus outlining the lysine (K) to arginine (R) mutations of p53, 5KR, and 6KR mutants. Abbreviations are as follows: TAD, transactivation domain (1-43 amino acids); DNA-BD, DNA binding domain (102-292 amino acids); OLIGO, oligomerization domain (326-358 amino acids) **(B)** The loss of available acetylation sites results in a reduction in the percentage of cells with cytoplasmic p53. Wild-type p53 or the KR

mutants were transfected into H1299 cells alone or co-transfected with p300 as indicated. The percent of cells with cytoplasmic p53 was determined as in Figure 1B.

Figure 4. Neutralization of positively charged lysine residues in the C-terminus of p53 regulates subcellular localization of p53. **(A)** Schematic representation of the p53 lysine (K) to alanine (A) mutants. **(B)** Subcellular localization of wild-type p53 and KA mutants transfected into H1299 cells. LMB treatment was performed as described in Figure 1A. **(C)** An increase in the number of KA mutations results in an increase in the percent of cells with cytoplasmic p53. Results were determined as in Figure 1B. **(D)** The level of cytoplasmic p53 is proportional to the number of lysine residues neutralized. Levels of p53 in the nuclear (N) and cytoplasmic(C) fraction were determined as in Figure 1C. The ratio of cytoplasmic p53 is calculated by dividing cytoplasmic p53 by total p53 (nuclear plus cytoplasmic p53).

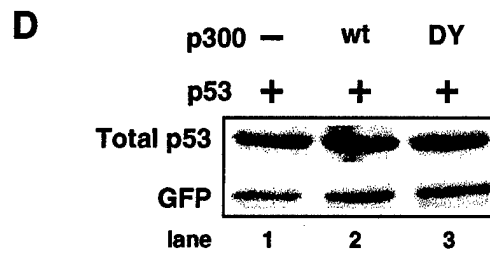
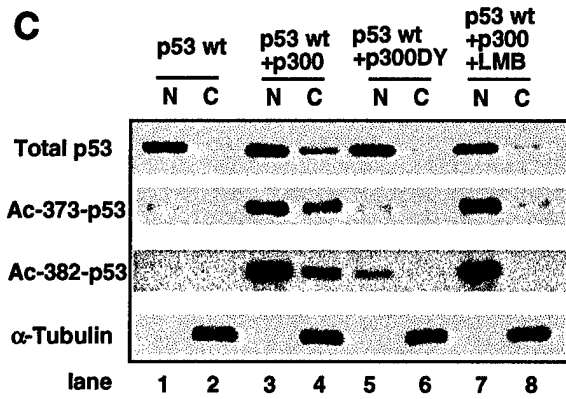
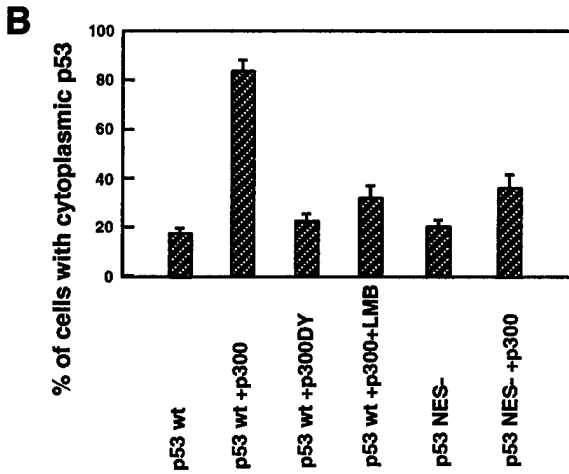
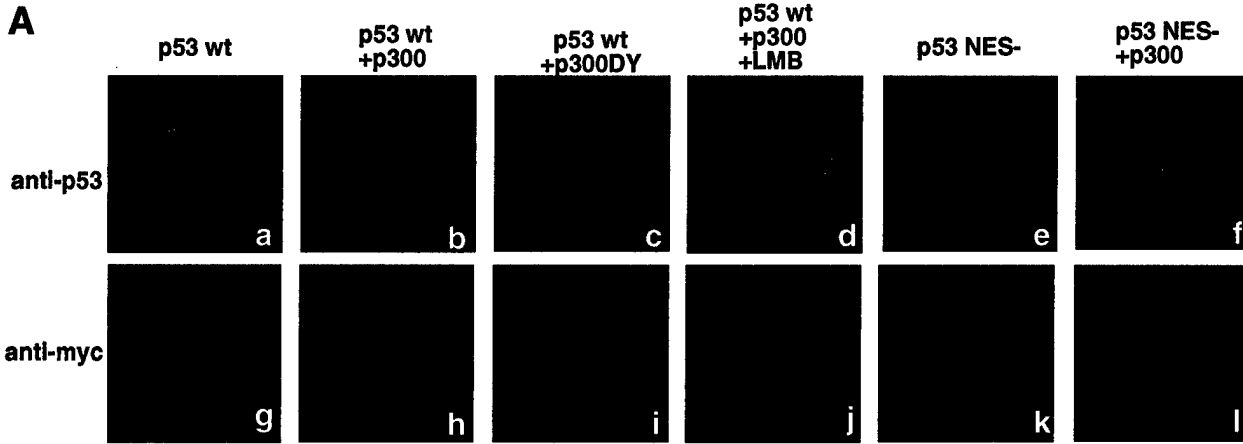
Figure 5. Neutralization of positively charged lysine residues in the C-terminus determines the oligomerization status of p53. Wild-type (lane 1, 2), 3KA (lane 3, 4), 4KA (lane 5, 6) or 4KR (lane 7, 8) mutant p53 polypeptides consisting of the tetramerization domain (amino acid 326-393) were expressed in *E.coli* as GST fusions. Ten micrograms of GST-cleaved protein were incubated at 37 °C with or without 0.1% glutaraldehyde for 15 min, then analyzed by a 20% SDS-PAGE to separate the p53 monomer from the oligomers.

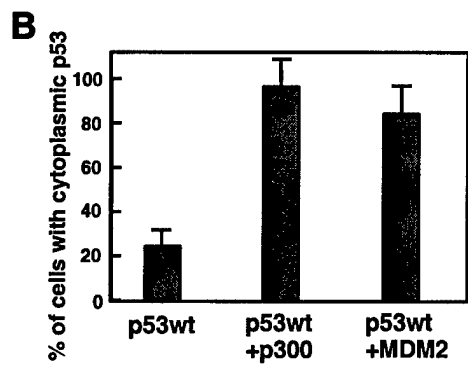
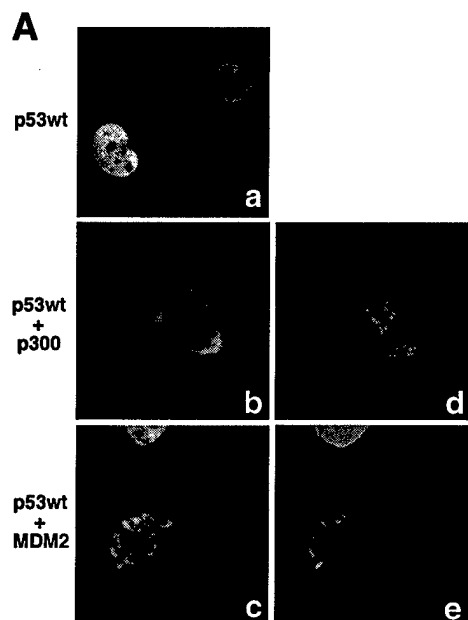
References

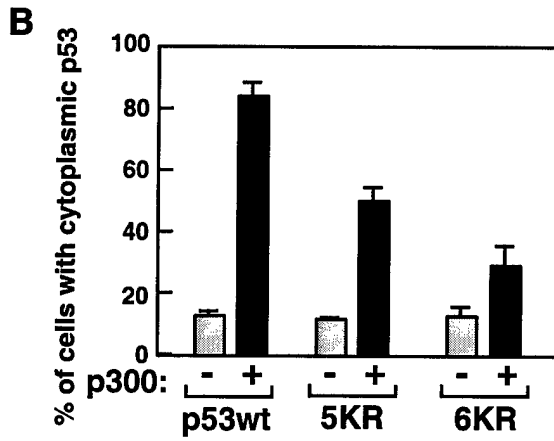
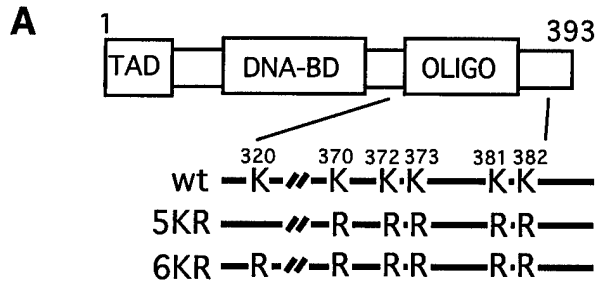
1. Freedman, D. A., Wu, L. & Levine, A. J. (1999) *Cell Mol Life Sci* **55**, 96-107.
2. Chen, J. L., J; Levine, A J (1995) *Molecular Medicine* **1**, 142-152.
3. Momand, J., Zambetti, G. P., Olson, D. C., George, D. & Levine, A. J. (1992) *Cell* **69**, 1237-45.
4. Oliner JD, P. J., Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. (1993) *Nature* **362**, 857-860.
5. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. (1997) *Nature* **387**, 299-303.
6. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. (1997) *Nature* **387**, 296-9.
7. Honda, R. & Yasuda, H. (1999) *Embo J* **18**, 22-7.
8. Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H. & Weissman, A. M. (2000) *J Biol Chem* **275**, 8945-51.
9. Wu, X., Bayle, J. H., Olson, D. & Levine, A. J. (1993) *Genes Dev* **7**, 1126-32.
10. Ashcroft, M., Taya, Y. & Vousden, K. H. (2000) *Mol Cell Biol* **20**, 3224-33.
11. Yoshida, M. & Horinouchi, S. (1999) *Ann N Y Acad Sci* **886**, 23-36.
12. Geyer, R. K., Yu, Z. K. & Maki, C. G. (2000) *Nat Cell Biol* **2**, 569-73.
13. Boyd, S. D., Tsai, K. Y. & Jacks, T. (2000) *Nat Cell Biol* **2**, 563-8.
14. Gu, J., Nie, L., Wiederschain, D. & Yuan, Z. M. (2001) *Mol Cell Biol* **21**, 8533-46.
15. Lohrum, M. A., Woods, D. B., Ludwig, R. L., Balint, E. E. & Vousden, K. H. (2001) *Mol Cell Biol* **21**, 8521-32.
16. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J. & Wahl, G. M. (1999) *Embo J* **18**, 1660-72.
17. Prives, C. & Manley, J., L. (2001) *Cell* **107**, 815-818.
18. Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E. & Yao, T. P. (2001) *Embo J* **20**, 1331-1340.
19. Gu, W. & Roeder, R. G. (1997) *Cell* **90**, 595-606.

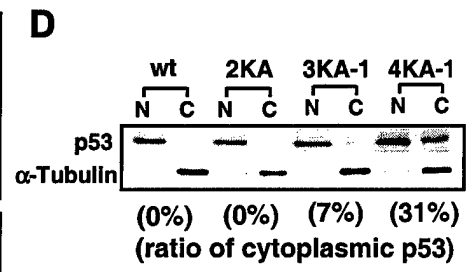
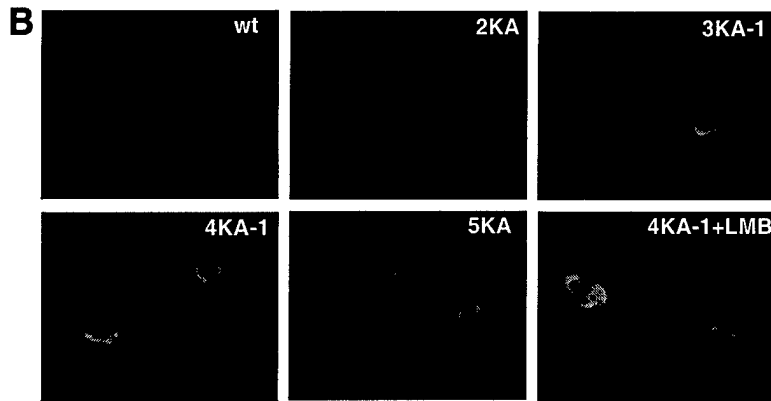
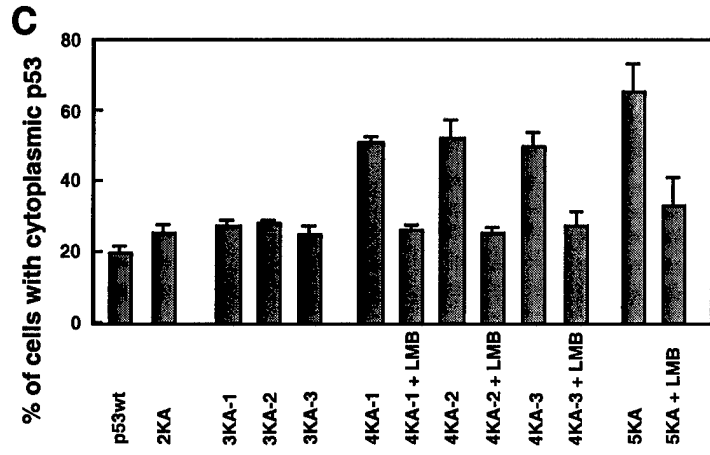
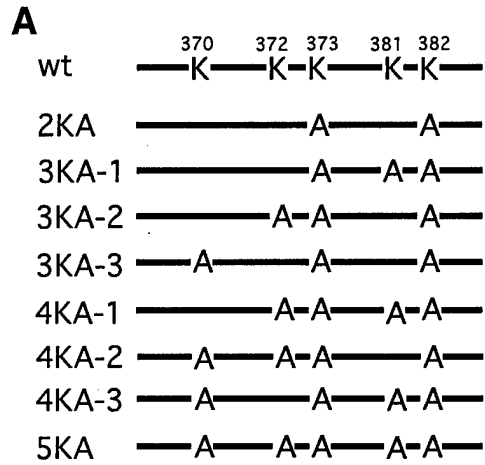
20. Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W. & Appella, E. (1998) *Genes Dev* **12**, 2831-41.
21. Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D. & Berger, S. L. (1999) *Mol Cell Biol* **19**, 1202-9.
22. Kobet, E., Zeng, X., Zhu, Y., Keller, D. & Lu, H. (2000) *Proc Natl Acad Sci U S A* **97**, 12547-52.
23. Grossman, S. R., Perez, M., Kung, A. L., Joseph, M., Mansur, C., Xiao, Z. X., Kumar, S., Howley, P. M. & Livingston, D. M. (1998) *Mol Cell* **2**, 405-15.
24. McMasters, K. M., Montes de Oca Luna, R., Pena, J. R. & Lozano, G. (1996) *Oncogene* **13**, 1731-6.
25. Friedman, P. N., Chen, X., Bargonetti, J. & Prives, C. (1993) *Proc Natl Acad Sci U S A* **90**, 3319-23.
26. Hupp, T. R. & Lane, D. P. (1994) *Curr Biol* **4**, 865-75.
27. Tarunina, M., Grimaldi, M., Ruaro, E., Pavlenko, M., Schneider, C. & Jenkins, J. R. (1996) *Oncogene* **13**, 589-98.
28. McLure, K. G. & Lee, P. W. (1998) *Embo J* **17**, 3342-50.
29. Lee, W., Harvey, T. S., Yin, Y., Yau, P., Litchfield, D. & Arrowsmith, C. H. (1994) *Nat Struct Biol* **1**, 877-90.
30. Clore, G. M., Ernst, J., Clubb, R., Omichinski, J. G., Kennedy, W. M., Sakaguchi, K., Appella, E. & Gronenborn, A. M. (1995) *Nat Struct Biol* **2**, 321-33.
31. Jeffrey, P. D., Gorina, S. & Pavletich, N. P. (1995) *Science* **267**, 1498-502.
32. Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A. & Rotter, V. (1990) *Mol Cell Biol* **10**, 6565-77.
33. Hicke, L. (2001) *Cell* **106**, 527-30.
34. Nakamura, S., Roth, J. A. & Mukhopadhyay, T. (2000) *Mol Cell Biol* **20**, 9391-8.
35. Ren, Q. & Gorovsky, M. A. (2001) *Mol Cell* **7**, 1329-35.
36. Lai, Z., Ferry, K. V., Diamond, M. A., Wee, K. E., Kim, Y. B., Ma, J., Yang, T., Benfield, P. A., Copeland, R. A. & Auger, K. R. (2001) *J Biol Chem* **276**, 31357-67.

37. Salghetti, S. E., Caudy, A. A., Chenoweth, J. G. & Tansey, W. P. (2001) *Science* **293**, 1651-3.
38. Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M. & Evans, R. M. (1992) *Cell* **71**, 63-72.
39. Eckner, R., Yao, T. P., Oldread, E. & Livingston, D. M. (1996) *Genes Dev* **10**, 2478-90.









Kawaguchi et. al, Figure5

