

# The Candidate Tumor Suppressor ING1b Can Stabilize p53 by Disrupting the Regulation of p53 by MDM2<sup>1</sup>

Ka Man Leung, Lai See Po, Fan Cheung Tsang, Wai Yi Siu, Anita Lau, Horace T. B. Ho, and Randy Y. C. Poon<sup>2</sup>

Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

## Abstract

ING1b is a candidate tumor suppressor that can stimulate the transcriptional activity of p53 and inhibit cell proliferation. The molecular basis of how ING1b activates p53 function remains unclear. Here we show that ING1b could stimulate the activity of p53 by increasing the level and stability of the p53 protein. The stabilization and activation of p53 by ING1b could be reversed by MDM2 in a dose-dependent manner. Conversely, ING1b could reverse the inhibition and degradation of p53 caused by MDM2 in a dose-dependent manner. Furthermore, ING1b and MDM2 bound to p53 in a mutually exclusive manner. In agreement with these observations, we found that similarly to MDM2, ING1b binds to the NH<sub>2</sub>-terminal region of p53. These data suggest a model in which ING1b disrupts the interaction between p53 and MDM2, leading to the stabilization of p53 and growth inhibition.

## Introduction

Loss of the p53 tumor suppressor function is one of the most common steps in tumorigenesis (1). The functions of p53 are primarily mediated through the regulation of cell cycle checkpoints and apoptosis. The transcriptional activity and stability of p53 are highly regulated by posttranslational mechanisms involving protein-protein interactions, phosphorylation, acetylation, ubiquitination, and sumoylation. MDM2, one of the transcriptional targets of p53, binds to the transactivation domain of p53 and inhibits p53-mediated transcription, shuttles p53 out of the nucleus, and targets p53 for ubiquitin-mediated proteolysis (2). The control of p53 by MDM2 is disrupted after DNA damage. Protein kinases such as ATM or DNA-PK are activated after DNA damage and phosphorylate p53 at sites in the NH<sub>2</sub>-terminal region. These phosphorylations inhibit the binding of MDM2 to p53, leading to an activation of p53. ING1 was first identified as a candidate tumor suppressor through a functional screening based on selection of gene fragments that can block the activity of tumor suppressors (3). *ING1* encodes a *M<sub>r</sub>* 47,000 protein (ING1a), an alternatively spliced *M<sub>r</sub>* 33,000 protein (ING1b), and a *M<sub>r</sub>* 24,000 protein from an internal initiation site (ING1c; Ref. 4). ING1a is minimally expressed in human tissues in comparison with ING1b and ING1c (4). Overexpression of ING1 inhibits cell growth and enhances serum starvation-induced apoptosis (3, 5). Conversely, ING1 antisense constructs promoted transformation (3) and protection against apoptosis (5). The growth-inhibitory and apoptosis-inducing functions of ING1 involve p53 (6, 7). Transactivation of the p21<sup>CIP1/WAF1</sup> promoter by p53 is enhanced by ING1 but is eliminated by antisense ING1 constructs (6). Physical interaction between ING1 and p53 was demonstrated by

coimmunoprecipitation (6). An ING1-related protein ING2, but not ING1 itself, increases the acetylation of p53 at Lys382 (8). There is no indication as to how ING1 activates p53. In this report, we tried to shed light on the molecular basis of how ING1 activates p53 by examining the relationship with MDM2.

## Materials and Methods

**DNA Constructs.** Human ING1b in pCMV-SPORT6 was obtained from Genome Systems, Inc. (IMAGE:2186797). ING1b was amplified by PCR using the primer 5'-GGGAATTCATGTTGAGTCCTGCCAA-3' and Sp6 primer, cut with *EcoRI*, and put into pUHD-P1 to produce FLAG-ING1b in pUHD-P1. Human ING1a, ING1b, and ING1c in mammalian expression vectors were gifts from K. Rialowol (University of Calgary, Calgary, Canada). p53 in pRcCMV was a gift from A. Levine (Princeton University, Princeton, NJ). p53 was amplified by PCR with T7 and Sp6 primers that introduced *Clal* sites, and the *Clal*-cut PCR product was put into pLINX vector (a gift from T. Hunter, The Salk Institute, La Jolla, CA). The *NcoI* fragment of p53 was put into pUHD-P1 to produce FLAG-p53(CΔ161) in pUHD-P1. The p53 cDNA was amplified by PCR with primers 5'-CCAGCCGAATTCTGGCCCT-3' and 5'-TTTGAATTCTCAGTCTGAGTCAGGCC-3', cut with *EcoRI*, and put into pUHD-P1 to produce FLAG-p53(NΔ90) in pUHD-P1. Site-directed mutagenesis of L22Q and W23S mutation of p53 was constructed by a PCR method as described elsewhere (9), using T7 and Sp6 primers and the oligonucleotide 5'-TCAGACCAATCGAACTAC-3' and its antisense to introduce the mutation; the PCR product was cut with *NcoI* and *EcoRI* and ligated into pUHD-P1 to produce FLAG-p53(L22Q+W23S) in pUHD-P1. FLAG-p300 construct was a gift from Z. Wu (Hong Kong University of Science and Technology). MDM2 in pCMV<sup>3</sup> were gifts from B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). GST-MDM2 in pCAGGS was as described previously (10).

**Cell Culture.** H1299 (non-small cell lung carcinoma) was obtained from the American Type Culture Collection. Cells were transfected with the calcium phosphate precipitation method (11). Unless stated otherwise, 1 μg of p53-expressing plasmids and 10 μg of other plasmids were used to transfect cells in 10-cm plates. For binding assays, 5–10 μg of p53-expressing plasmids were used. The total amount of DNA for each transfection was adjusted to the same level using vectors with the same promoter. Cell-free extracts were prepared as described previously (12). Estimation of protein stability by promoter turn-off (13) or by cycloheximide (14) was performed as described previously.

**Luciferase and β-Galactosidase Assays.** Luciferase assays and β-galactosidase assays were performed as described previously (14).

**Antibodies and Immunological Methods.** Rat monoclonal antibody YL1/2 against mammalian tubulin and mouse monoclonal antibody A17 against CDC2 were from T. Hunt (Cancer Research United Kingdom). Monoclonal antibody M2 against FLAG tag was from Eastman Kodak, and rabbit polyclonal antibody against FLAG was a gift from K. Yamashita (Kanazawa University, Kanazawa, Japan). Goat antibodies raised against a COOH-terminal peptide derived from ING1 (sc-7566) and monoclonal antibody DO-1 against p53 (sc-126) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody 2A10 against MDM2 was a gift from A. Levine (Princeton University). Immunoblotting, immunoprecipitation, and binding to GSH-agarose were performed as described (12). Signals on immunoblots were

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<sup>2</sup> To whom requests for reprints should be addressed, at Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong. Phone: 852-23588718; Fax: 852-23581552; E-mail: bcrandy@ust.hk.

<sup>3</sup> The abbreviation used is: CMV, cytomegalovirus.

quantified using the NIH Image program with the appropriate dilution standards.

## Results and Discussion

**ING1 Enhances p53 Transcriptional and Growth-inhibitory Activities.** The p53-null H1299 cell line was used to study the effects of ING1 on p53 in the absence of endogenous p53. Immunoblotting of H1299 extracts with an antiserum that recognized all three forms of ING1 shows that only ING1b was readily detectable (Fig. 1A). Standards of ING1a, ING1b, and ING1c were expressed by transient transfection for side-by-side comparison. It is not clear whether the endogenous bands for ING1a were the *bona fide* protein because they are slightly larger than the standard. These data were in agreement with published results (15), which also suggest that ING1b is the major form of ING1 in the cell.

To see which isoform of ING1 could activate p53, the transcriptional activity of p53 was assayed using a MDM2 promoter-luciferase reporter (Fig. 1B). Activation of transcription by p53 was enhanced by coexpression of ING1b and ING1c (Fig. 1B, Lanes 7 and 8), whereas expression of ING1 alone did not activate transcription from the reporter (Lanes 2–4). We are not completely sure whether ING1a could activate p53 because ING1a was poorly expressed in comparison with other isoforms. As controls, MDM2 inhibited and p300 activated the transcriptional activities of p53 (Fig. 1B, Lanes 9 and 10). Stimulation of transcription by ING1 was dependent on functional p53, because no transcription was detected when ING1b was coexpressed with p53 mutants lacking the NH<sub>2</sub>-terminal transactivation domain or the COOH-terminal region, including part of the DNA binding domain (Fig. 1B, Lanes 11–14).

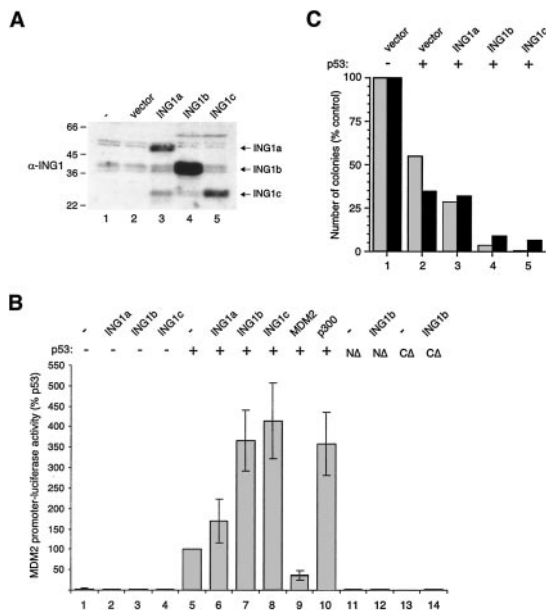


Fig. 1. Activation of p53 by ING1. **A**, the relative abundance of ING1 isoforms in H1299. Extracts of H1299 cells (Lane 1), cells transfected with control vector (Lane 2), or with constructs expressing ING1a (Lane 3), ING1b (Lane 4), or ING1c (Lane 5) were analyzed by immunoblotting with antibodies against ING1. *Left*, positions of the molecular size markers (in thousands). **B**, stimulation of p53 transcriptional activity by the ING1. Cells were transfected with plasmids expressing an MDM2-promoter luciferase reporter and  $\beta$ -galactosidase. Plasmids expressing wild-type p53, p53(N $\Delta$ 90), or p53(CA161) were cotransfected with ING1a, ING1b, ING1c, MDM2, and p300 as indicated. Cell extracts were prepared, and the luciferase and  $\beta$ -galactosidase activities were determined. The means of three independent experiments are shown; bars, SD. **C**, ING1 cooperates with p53 to inhibit cell growth. Plasmids encoding puromycin resistance gene were cotransfected with control vector, p53, or p53, together with ING1a, ING1b, or ING1c as indicated. After ~2 weeks of selection in medium containing puromycin, the cells were fixed, stained, and counted. The number of colonies is shown as a percentage of control. The results of two independent experiments are shown.

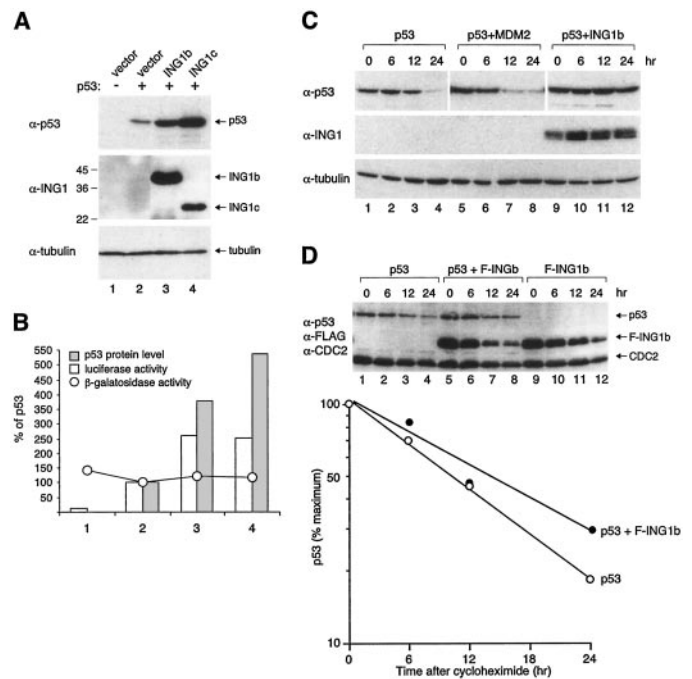


Fig. 2. ING1 increases p53 activity by alteration of the level of p53. **A**, cells were transfected with plasmids expressing p53, ING1b, and ING1c as indicated. Cell extracts were prepared and analyzed by immunoblotting with antibodies against p53, ING1, and tubulin. *Middle panel*, positions of the molecular size markers (in thousands). **B**, the p53 signals on the immunoblot in **A** were quantified with the appropriate dilution standards and plotted as a percentage of p53 alone. The activities from the MDM2-promoter luciferase reporter and  $\beta$ -galactosidase were determined and expressed as a percentage of p53 alone. **C**, cells were transfected with plasmids expressing p53, MDM2, or ING1b as indicated. Each transfection was divided into four plates, and the cells were allowed to grow for another 24 h. Doxycycline was added into the medium to suppress the transcription of p53, and cell extracts were prepared at the indicated time points. Immunoblotting was performed with antibodies against p53, ING1, or tubulin. Films with different exposures were used for the p53 blots to show a similar starting level for comparison. **D**, cells were transfected with plasmids expressing p53 and FLAG-ING1b as indicated. Each transfection was divided into four plates, and the cells were allowed to grow for another 24 h. Cycloheximide (25  $\mu$ g/ml) was added into the medium, and cell extracts were prepared at the indicated time points. Immunoblotting was performed with antibodies against p53, FLAG, and CDC2. The p53 signals on the immunoblots were analyzed and plotted in the *lower graph*.

Fig. 1C shows that expression of p53 caused a ~50% reduction in clonogenic survival in comparison with control (Fig. 1C). Coexpression of ING1b and ING1c with p53 further reduced the clonogenic survival to ~5% of control. These results indicate that the transcriptional activity and antiproliferation activity of p53 could be stimulated by ING1b and ING1c. We focused our attention on ING1b because of its relative abundance and the fact that ING1c is merely a truncated version of ING1b.

**ING1b Stimulates p53 Activity by Increasing the p53 Protein Level.** One possibility that ING1 could activate p53 is by increasing the level of p53. Fig. 2 shows a representational experiment in which the transactivation of the MDM2 promoter, the protein levels of p53 and ING1, and the transfection efficiency were measured simultaneously. As shown above, ING1b and ING1c stimulated the transcriptional activity of p53 (Fig. 2B). We noted, however, that the level of p53 was clearly elevated at the same time (Fig. 2A). The constant level of tubulin shows that similar amount of extracts were loaded in each lane. The variation in p53 expression was unlikely to be attributable to variation in transfection efficiency because the  $\beta$ -galactosidase activity from a cotransfected plasmid was maintained at a similar level (Fig. 2B). These data suggest that the activation of p53 by ING1 could be mainly attributed to an increase in p53.

**ING1b Stabilizes p53.** To evaluate whether ING1b affected the level of p53 by influencing its stability, the half-life of p53 was

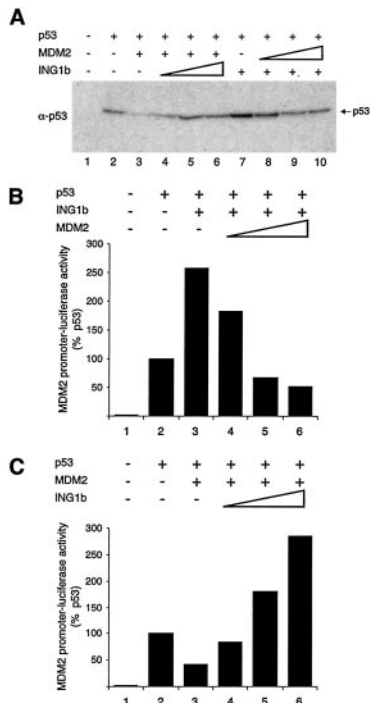


Fig. 3. ING1b antagonizes the effects of MDM2 on the level and activity of p53. *A*, ING1b and MDM2 alter the level of p53. Cells were transfected with control vector (Lane 1) or plasmids expressing p53 (Lanes 2–10). Plasmids expressing MDM2 (Lanes 3–6, 5  $\mu$ g; Lane 8, 10  $\mu$ g; Lane 9, 20  $\mu$ g; Lane 10, 30  $\mu$ g) and ING1b (Lane 4, 2  $\mu$ g; Lane 5, 4  $\mu$ g; Lane 6, 6  $\mu$ g; Lanes 7–10, 1  $\mu$ g) were cotransfected with p53. Cell extracts were prepared, and the amount of p53 was analyzed by immunoblotting. *B*, MDM2 inhibits the ING1b-activated p53 in a dose-dependent manner. Cells were transfected with plasmids expressing an MDM2-promoter luciferase reporter and  $\beta$ -galactosidase. Plasmids expressing p53, ING1b, and increasing dosage of MDM2 (Lane 4, 5  $\mu$ g; Lane 5, 10  $\mu$ g; Lane 6, 20  $\mu$ g) were cotransfected. Cell extracts were prepared, and the luciferase and  $\beta$ -galactosidase activities were assayed. The luciferase activities were normalized with the  $\beta$ -galactosidase activities and expressed as a percentage of p53 alone. *C*, ING1b stimulates the MDM2-inhibited p53 in a dose-dependent manner. The experiment was performed as in *B*, except that plasmids expressing p53, MDM2, and increasing dosages of ING1b (Lane 4, 2  $\mu$ g; Lane 5, 4  $\mu$ g; Lane 6, 6  $\mu$ g) were used.

estimated in the presence or absence of ING1b. After p53 was coexpressed with MDM2 or ING1b, the promoter driving p53 was turned off by the addition of doxycycline, and the level of p53 was detected by immunoblotting. Fig. 2C shows that coexpression of ING1b markedly stabilized p53 when compared with control. In contrast, MDM2 evidently decreased the stability of p53. The constant amount of tubulin indicated that the promoter turn-off was specific for p53. Similarly, experiments using cycloheximide to block *de novo* protein synthesis revealed a consistent increase in the half-life of the p53 in the presence of ING1b relative to that of control (Fig. 2D). Stabilization of p53 in these experiments was not as remarkable as that in the promoter turn-off experiments, probably because the synthesis of ING1b was also inhibited, diminishing its influence on p53. These data indicate that the stability of p53 could be increased by ING1b.

**ING1b Competes with MDM2 to Regulate the Level and Activity of p53.** MDM2 binds to the NH<sub>2</sub>-terminal transactivation domain of p53 and targets it for degradation. One obvious possibility is that ING1b could counteract MDM2-mediated degradation of p53. To address this possibility, the level of p53 was examined in the presence or absence of MDM2 and/or ING1b (Fig. 3A). MDM2 caused a reduction of p53 as expected, but this reduction was abrogated by ING1b in a dose-dependent manner, returning p53 to its original level. Conversely, ING1b increased the amount of p53, but this increase could be reversed by MDM2 in a dose-dependent manner.

In accordance with the idea that ING1b and MDM2 counteracted each other's effect on p53, we found that MDM2 abrogated the

stimulation of p53 transcriptional activity by ING1b in a dose-dependent manner (Fig. 3B). Similarly, ING1b abrogated the inhibition of p53 transcriptional activity by MDM2 (Fig. 3C). Taken together, these data indicate that ING1b could overcome the inhibition of MDM2 on p53, and MDM2 could overcome the stimulation of ING1b on p53.

**ING1b Competes with MDM2 for Binding to p53.** Given that ING1b and MDM2 regulate the level and activity of p53 in an opposite manner, one hypothesis is that ING1b and MDM2 could compete with each other for binding to p53. Fig. 4A shows that p53 could be coimmunoprecipitated with ING1b but not with control serum. One caveat here is that the efficiency of the p53-ING1b interaction was not very high under the washing conditions that we used. To see whether ING1b and MDM2 interact with p53 in a mutually exclusive fashion, the interaction between p53 and ING1b was examined either in the presence or absence of MDM2 (Fig. 4B).

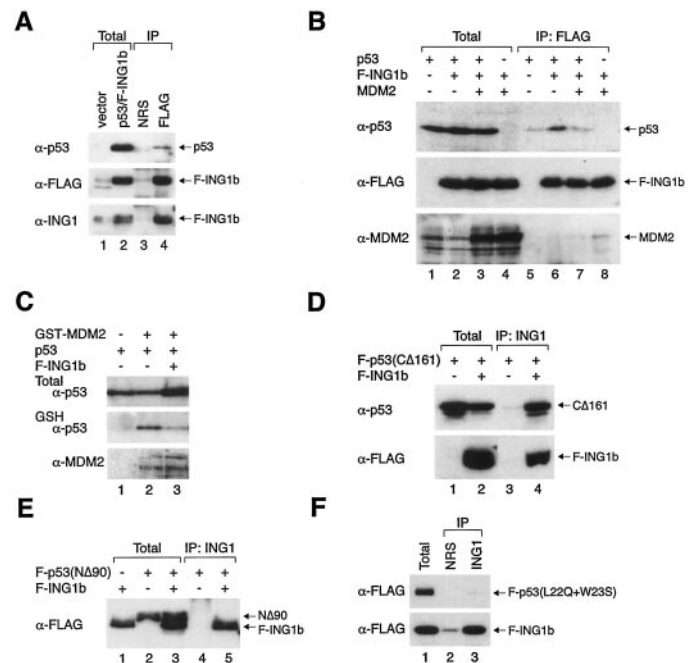


Fig. 4. ING1b binds to p53 and disrupts p53-MDM2 interaction. *A*, interaction between ING1b and p53. Cells were transfected with control vector (Lane 1) or p53 and FLAG-ING1b (Lane 2). Cell lysates from Lane 2 were immunoprecipitated with either normal rabbit serum (Lane 3) or anti-FLAG immune serum (Lane 4). The total cell lysates and immunoprecipitates were analyzed by immunoblotting with antibodies against p53, FLAG, or ING1 as indicated. *B*, disruption of p53-ING1b interaction by MDM2. Cells were transfected with plasmids expressing p53, FLAG-ING1b, and MDM2 as indicated. Cell extracts were immunoprecipitated with anti-FLAG immune serum. Total cell extracts (Lanes 1–4) and the immunoprecipitates (Lanes 5–8) were analyzed by immunoblotting with antibodies against p53 (top panel), FLAG (middle panel), or MDM2 (bottom panel). Note that more p53-expressing plasmids was used for binding assays than in other experiments to reduce the variation in p53 caused by ING1b and MDM2. *C*, disruption of p53-MDM2 interaction by ING1b. Cells were transfected with plasmids expressing GST-MDM2, p53, and FLAG-ING1b as indicated. Cell extracts were subjected to GSH-agarose binding. Total cell lysates (top panel) and GSH-agarose-bound proteins (lower panels) were analyzed by immunoblotting with antibodies against p53 and MDM2. *D*, ING1b can bind to a COOH-terminal truncated p53. Cells were transfected with plasmids expressing FLAG-p53(CΔ161) and FLAG-ING1b (Lanes 2 and 4). Cell extracts were immunoprecipitated with anti-ING1 immune serum. Total cell extracts (Lanes 1 and 2) and the immunoprecipitates (Lanes 3 and 4) were immunoblotted with antibodies against p53 (upper panel) or FLAG (bottom panel). *E*, ING1b does not associate with an NH<sub>2</sub>-terminal truncated p53. Cells were transfected with plasmids expressing FLAG-p53(NΔ90) and FLAG-ING1b as indicated. Cell extracts were immunoprecipitated with anti-ING1 immune serum. Total cell extracts (Lanes 1–3) and the immunoprecipitates (Lanes 4 and 5) were analyzed by immunoblotting with antibodies against FLAG. Transfection of FLAG-ING1b alone (Lane 1) was used to indicate the position of ING1b. *F*, disruption of p53-ING1b interaction by NH<sub>2</sub>-terminal mutation of p53. Cells were transfected with plasmids expressing FLAG-ING1b and FLAG-p53(L22Q+W23S). Cell lysates were immunoprecipitated with either normal rabbit serum (Lane 2) or anti-ING1 immune serum (Lane 3). The total cell lysates (Lane 1) and immunoprecipitates were analyzed by immunoblotting with antibodies against FLAG.

As before, p53 could be recovered in the immunoprecipitates of FLAG-ING1b (Fig. 4B, Lane 6). Significantly, the p53 that bound to FLAG-ING1b markedly diminished to background level in the presence of MDM2 (Fig. 4B, Lane 7). Immunoblotting confirmed that the same amount of FLAG-ING1b was expressed and immunoprecipitated in different samples. In a converse experiment, we showed that p53 could be recovered with a GST-MDM2 (Fig. 4C), and the amount of p53 that bound to GST-MDM2 was reduced in the presence of ING1b. Taken together, these data suggest that ING1b and MDM2 could compete for binding to p53.

A critical test of the hypothesis is to see whether ING1b and MDM2 bind to the same region on p53. MDM2 is known to bind to the NH<sub>2</sub>-terminal transactivation domain of p53 (16). Fig. 4D shows that a COOH-terminal truncation mutant of p53 that retained the first 161 residues could still bind ING1b. In fact, this interaction appears to be stronger than between full-length p53 and ING1b, suggesting that the COOH-terminal half of p53 could interfere with the interaction with ING1b. In contrast, no interaction was detected when the first 90 residues of p53 was deleted (NΔ90), leaving the DNA binding domain and the rest of the protein intact (Fig. 4E). Interestingly, we found that a p53 mutant (L22Q+W23S) that was defective in binding to MDM2 also has significantly lower affinity for ING1b (Fig. 4F). Thus, ING1b and MDM2 appear to bind to a similar region on p53.

**The Basis of Activation of p53 by ING1b.** ING1 is believed to be an important regulator of p53 because the activity of p53 can be eliminated by antisense ING1 constructs (6). We postulate that ING1b may activate p53 either directly or by dislodging an inhibitor such as MDM2. Our data support the model that ING1 attenuates the interaction between p53 and MDM2. We show that competition between ING1b and MDM2 could regulate the stability and activity of p53. One possibility is that MDM2 and ING1b bind to the same site on p53. This places ING1 as the first example of proteins that can disrupt the regulation of p53 by MDM2 by binding to p53. The proposed mechanism of ING1 on p53 is different from other positive regulators of p53. ARF, pRb, and MDMX activate p53 by direct interaction with MDM2 (17–19). Another regulator of p53, mSin3a, binds to the NH<sub>2</sub>-terminal, proline-rich region of p53 and stabilizes p53, but this stabilization occurs in an MDM2-independent manner (20). Note that we cannot exclude the possibility that ING1b binds to MDM2 directly and affects MDM2 level or blocks MDM2 from binding to p53. However, we do not favor this model because of the lack of significant interaction between MDM2 and ING1b.<sup>4</sup> Of course, it is also likely that other mechanisms are involved in the regulation of p53 by ING1.

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