MDM2 and MDMX Bind and Stabilize the Tumor Suppressor p53-Related Protein p73

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ABSTRACT

p73 is a close relative of the important tumor suppressor p53. Here we report that like p53, p73α and the alternatively transcribed p73β also bind to MDM2. Interaction between MDM2 and p53 represents a key step in regulation of p53, since MDM2 promotes the degradation of p53. However, in striking contrast to p53, we found that the half-life of p73 is increased by binding to MDM2. Like MDM2, the MDM2-related protein MDMX also binds to p73 and stabilized its level. Moreover, the growth suppression functions of p73 are enhanced in the presence of MDM2. In agreement with this, induction of the endogenous $p21^{Cip1/Waf1}$ by p73 is enhanced by MDM2. These differences between the regulation of p53 and p73 by MDM2/MDMX may highlight a physiological difference in their action.

INTRODUCTION

The p53 gene encodes one of the most important tumor suppressor in human cells, and undergoes frequent mutational inactivation in cancers (1) . The tumor suppressor function of p53 is believed to be partly mediated through its role as a transcriptional activator, which activates genes involved in the regulation of cycle checkpoints and apoptosis. p53 can activate the transcription of the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/WAF1}$ (2) , which is responsible for the inhibition of cyclin-dependent kinases and the cell cycle following DNA damage (3) . p53 can also activate the transcription of the apoptosis-promoting Bax gene. In addition, p53 can transcriptionally repress growth-promoting genes such as *fos* and *jun*, and may also act more directly to bind DNA and inhibit DNA replication.

Regulation of p53 protein levels within the cell is critical both for cell survival and for repression of inappropriate cell proliferation. Such tight control is provided, at least in part, by MDM2. The oncogene product MDM2 is another transcriptional activated target of p53. MDM2 can bind to p53 and can both inhibit p53-mediated transcription (4, 5) and target p53 for proteasome-mediated proteolysis (6, 7) . Hence MDM2 may act as a mechanism to limit the p53 response in a negative feedback loop (8) . Phosphorylation of Ser15 and Ser37 on p53 by ATM kinase and/or DNA-PK after DNA damage may inhibit the binding of MDM2 to p53 (9, 10, 11) , therefore leading to an increase in p53 level and activity.

The *CDKN2A* locus on human chromosome 9p21 encodes two distinct proteins translated from alternatively spliced mRNAs. One gene product is the CDK inhibitor $p16^{INK4A}$, which is specific inhibitor for CDK4 and CDK6; the other gene product is $p14^{ARF}$ ($p19^{ARF}$ in mouse). The induction of p53 degradation by MDM2 is inhibited by $p14^{ARF}/p19^{ARF}$ (12) . Whether $p14^{ARF}$ works by destabilizing MDM2 (13) or by stabilizing both p53 and MDM2 is not fully resolved, but the latter seems more likely since ternary p53-MDM2-$p14^{ARF}$ complexes can be detected (14) .

p73 has recently been identified as a close relative of p53 (15, 16) . Like p53, p73 can also induce the transcription of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$

and apoptosis. More recently, other p53-related proteins, like p51 (17, 18) and p63 (19) have also been described. The p73 gene is monoallelically expressed at chromosome 1p36, and p73 is itself implicated as a tumor suppressor in neuroblastoma and a variety of other cancers mapped to 1p36 (15, 16) . However, the mechanisms regulating p73 expression and activity are currently unknown. So far, little has been reported on the mutation of p73 in tumors (20) .

Here we show that like p53, p73 also binds to MDM2. Furthermore, p73 also interacts with the MDM2-related protein MDMX. But in striking contrast to p53, p73 protein is stabilized by binding to MDM2/MDMX. We further show that the anti-proliferative function of p73 is enhanced by MDM2. These results show that the regulation of p73 differs from that of p53, and may provide a mechanism for interplay between p53 and p73.

MATERIALS AND METHODS

DNA Constructs

Constructs of HA-tagged p73 in pcDNA3 were generous gifts from W.G. Kaelin Jr. (Dana-Farber Cancer Institute) (15) . FLAG-p73α.NΔ250 in pUHD-P1 and FLAG-p73β.NΔ250 in pUHD-P1 were constructed by putting the *EcoR* I-*Xba* I fragment of HA-p73α in pcDNA3 or HA-p73β in pcDNA3 respectively, into *EcoR* I and *Xba* I cut pUHD-P1 (21) . HA-p73α in pUHD-P1 and HA-p73β in pUHD-P1 were constructed by putting the *Sac* II-*EcoR* I of HA-p73α in pcDNA3 or HA-p73β in pcDNA3, into *Sac* II-*EcoR* I cut FLAG-p73α.NΔ250 in pUHD-P1 or FLAG-p73β.NΔ250 in pUHD-P1 respectively. MDM2 in pCMV, and p21 promoter-luciferase reporter construct were gifts from B.Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center). Murine p53 in pCMV was a gift from T. Hunter (The Salk Institute). FLAG-Smad4 in pcDNA3.1- was from H. Yam (R.Y.C.P.'s laboratory). Human MDMX was amplified by RT-PCR from A549 cells, using the primers 5'-TTCTTCACTACCCATATGACATCATTTTCC-3' and 5'-ATTTTTAGGATCCTACTACCATTAAGCTAT-3'. The fragment was subcloned into the *Hinc* II site of pBluescript vector. MDMX in pBluescript was digested with *Bam*H I and partially digested with *Nde* I, and subcloned into pBC vector (22) . The resulting GST-MDMX fragment was then put into the pCAGGS vector. GST-MDM2 in pCAGGS was also created as with GST-MDMX in pCAGGS. The β-galactosidase construct was a gift from Y. Chen (The Salk Institute). CD2 construct was a gift from C. Norbury (ICRF, University of Oxford), and CD20 construct was as described previously (21).

Cell culture and transfection

293 cells (transformed human embryonic kidney cells), H1299 cells (human non small cell lung caricinoma cells), and SAOS-2 cells (human osteogenic sarcoma) were obtained from the American Type Culture Collection (Rockville, MD). HtTA1 cells were gifts from H. Bujard. HtTA1 cells were HeLa cells (human cervical carcinoma cells) stabily transfected with pUHD15-1 expressing the tTA tetracycline repressor chimera (23) and can express

genes cloned into the pUHD-P1 vector in the absence of deoxycycline. Cells were grown in DMEM supplemented with 10% v/v calf serum (for 293 cells and HeLa cells) or 10% v/v fetal bovine serum (GIBCO-BRL) in a humidified incubator at 37°C with 5% CO_2.

Semi-confluent cells were transiently transfected with the calcium phosphate precipitation method (24) . Unless stated otherwise, semi-confluent cells were transfected with 4 μg of p73 DNA and 8 μg of MDM2 DNA for 60 mm plates. The total amount of DNA for each transfection was adjusted to the same level using vectors with the same promoter. Cells were grown for a further 24 hr for 293 cells, 36 hr for H1299 cells, or 48 hr for SAOS-2 cells after transfection before being harvested for cell extracts. To turn off gene expression in pUHD-P1 vectors, deoxycycline hydrochloride was added to the medium (to 1 μg/ml) at 24 hr after changing the medium.

Relative transfection efficiencies were measured by co-transfection of 1 μg of CMV-driven β-galactosidase expression plasmid and measurement of β-galactosidase enzymatic activity in cell extracts as described below, or by staining the cells for β-galactosidase activity and examining them under microscope (24) . In some experiments, transfection efficiencies were also measured by FACS analysis of cells stained with a FITC-conjugated anti-CD20 antibody for expression of co-transfected CD20 cell surface antigen as described previously (25) . Typical transfection efficiencies obtained were ~50% (293 and HeLa), 20% (H1299) and 5-10% (SAOS-2). Cell-free extracts were prepared as described previously (26) . The protein concentration of cell lysates was measured with bicinchoninic acid protein assay system (Pierce) using BSA as a standard.

Reverse transcription-PCR

To detect the relative levels of HA-p73 mRNA, RT-PCR was performed as described elsewhere (24) . Total RNA was extracted from transfected cells (RNeasy mini kit, Qiagen). The oligonucleotide to Sp6 promoter was used for reverse transcription, and Sp6 and T7 oligonucleotides were used for PCR. The PCR conditions were 94°C, 0.5 min; 50°C, 1 min; 72°C, 1.5 min for 40 cycles.

β-galactosidase assays

β-galactosidase assays were performed by incubating cell extracts in a buffer containing 45 mM Na_2HPO_4, 30 mM NaH_2PO_4, 7.5 mM KCl, 0.75 mM $MgCl_2$, 37.5 mM β-mercaptoethanol, and 1.5 mg/ml ortho nitro-phenyl β-D-galactopyanoside at 37°C. Yellow color development was assessed at OD420.

GSH-agarose binding

GST fusion proteins were recovered with 15 μl of glutathione (GSH)-agarose in 250 μl of bead buffer (25) . After incubation at 4° C with end-to-end rotation for 45 min, the beads were washed five times with 250 μl of bead buffer. The samples were then dissolved in 30 μl of SDS sample buffer, and the bound proteins were detected by SDS-PAGE followed by immunoblotting.

Colony formation assay

Different plasmids were co-transfected with a puromycin-resistant plasmid (pPUR) into H1299 cells ($5x10^4$ cells per 10 cm plate). The total amount of DNA added was adjusted to the same amount with single stranded DNA. After transfection, the cells were washed with phosphate-buffered saline and allowed to grow in medium containing serum for 48 hr. The cells were then incubated with medium containing serum and 2 μg/ml of puromycin. The cells were allowed to grow for another 2 weeks. Colonies were fixed with methanol:acetic acid (2:1 v/v) and visualized by staining with 2% (w/v) crystal violet. Around 500 colonies were scored for each experiment.

Antibodies and immunological methods

Monoclonal antibody 421 against p53, monoclonal antibody E72 against cyclin A, rat monoclonal antibody YL1/2 against mammalian tubulin, and rabbit anti-GST antibodies were gifts from J. Gannon and T. Hunt (ICRF, South Mimms). Monoclonal antibody 12CA5 against the HA-tag was a gift from T. Hunter (The Salk Institute). Monoclonal antibody 2A10 against MDM2 was a gift from A. Levine (Princeton University).

Monoclonal antibody M2 against FLAG tag was obtained from Eastman Kodak. Monoclonal antibody OX-34 against CD2 (DYNA) was a gift from C. Norbury (University of Oxford). Goat anti-p73α polyclonal antibodies were raised against a peptide corresponding to the C-terminus of human p73α (Santa Cruz Biotechnology, sc-7238). Rabbit anti-$p21^{Cip1/WAF1}$ antibodies were raised against a peptide corresponding to the last 19 amino acids of human $p21^{Cip1/WAF1}$ (Santa Cruz Biotechnology, sc-397). Immunoblottings were performed as described previously (3) , except for the anti-FLAG tag monoclonal antibodies M2, which was used according to the manufacturer's instructions. Immunoprecipitations were performed as described previously (3) .

For magnetic beads selection of transfected cells, cells were co-transfected with CD2-expressing plasmid and the indicated plasmids. After incubation for the indicated time, the CD2-positive cells were selected by magnetic bead prebound with anti-CD2 antibodies according to the manufacturer's instruction (DYNAL).

RESULTS

MDM2 destabilizes p53 but stabilizes p73

We set out to study whether like p53, the p53-related p73 is also regulated by binding to MDM2. To see whether there is any potential interaction between p73 and MDM2, plasmids expressing MDM2 were co-transfected with plasmids expressing HA-tagged p73α, and the alternatively spliced form HA-p73β (all driven by CMV promoters), into 293 cells. The expression levels of MDM2 and HA-p73 in these cells were detected by immunoblotting with antibodies against MDM2 and the HA-tag (Figure 1A). Surprisingly, we found that the levels of both p73α and p73β were much higher when co-transfected with MDM2-expressing plasmids (lanes 2 and 5), in comparison to cells transfected with p73-expressing plasmids alone (lanes 1 and 4). The expression of MDM2 was confirmed by immunoblotting the same membrane with an anti-MDM2 monoclonal antibody (2A10) (middle panel), which showed an increase expression of MDM2 over the endogenous MDM2, and the reduction of MDM2 level in cells transfected with the antisense plasmid. Consistent with the above result, co-transfection of HA-p73α with an antisense MDM2 plasmid did not elevate the level of p73α over control (lane 3). Immunoblotting the same membrane with an anti-tubulin monoclonal antibody showed that similar amount of extracts was loaded in each lane (bottom panel).

MDM2 did not simply increase the expression of any co-transfected constructs. As expected, co-expression of MDM2 with p53 reduced the level of p53 (Figure 1B). Immunoblotting of the same blot with both anti-p53 antibodies and anti-tubulin antibodies together clearly indicated the decrease in p53 in the presence of MDM2 in relation to the relatively constant level of tubulin. Furthermore, co-expression of MDM2 with an unrelated protein (FLAG-tagged Smad4 in this case) did not increase the level of FLAG-Smad4 (Figure 1C). This increase in p73 levels in the presence of MDM2 is in strong contrast to the previously reported effect of MDM2 in decreasing p53 level (6, 7) (see also Figure 1B), and suggests that the two closely related proteins can be differentially regulated.

The identity of the p73α bands was confirmed with an antibody raised against a peptide corresponding to the C-terminus of p73α. This antibody recognized HA-p73α, but not HA-p73β or p53 on immunoblots (Figure 1D). Identical anti-p73α antibody immunoreactive bands were also observed in binding to MDM2 (see later).

High transfection efficiency was achieved in the 293 cells used above, but these cells contained endogenous p53 that are inactivated by adenoviral proteins. To rule out the involvement of p53 in these experiments, we also performed the experiments in the p53-null cell lines SAOS-2 and H1299. As with 293 cells, co-expression of MDM2 significantly increased the level of p73 in SAOS-2 cells (Figure 2A, only p73α is shown here). Equal loading of the samples was shown by immunoblotting with antibodies against cyclin A and tubulin. Moreover, a progressive increase in p73 level was seen when increasing amount of MDM2-expressing DNA was co-transfected with p73-expressing DNA into H1299 cells (Figure 2B, only p73β is shown here). Interestingly, transfection of p73α alone into H1299 cells increased the level of endogenous MDM2 (Figure 2A, lane 2), suggesting p73α can also activate the transcription of MDM2. These results clearly demonstrate that p73 can be stabilized by MDM2 under conditions where p53 is destabilized.

Association of p73 and transcriptional-inactive mutants of p73 with MDM2

Given that co-expression of MDM2 can stabilize p73α and p73β, and that the sequence of the MDM2-binding domain of p53 (TFSDLW) (27, 28) is similar to that in p73 (TFEHLW) (Figure 3C), we next investigated whether MDM2 can form a complex with p73. Human 293 cells were transfected with plasmids expressing HA-p73α or HA-p73β individually, or in combination with plasmids expressing MDM2. The expression of MDM2 and HA-p73 in these cells was detected by immunoblotting the total cell lysate with antibodies against MDM2 and the HA-tag respectively (Figure 3A). Cell extracts were immunoprecipitated with the anti-MDM2 monoclonal antibody 2A10, and the HA-p73 that associated with MDM2 was detected by immunoblotting the immunoprecipitates with anti-HA antibody. Figure 3A shows that both HA-p73α and HA-p73β can be

detected in the MDM2-immunoprecipitates, suggesting that MDM2 and p72 can physically associate with each other. This result was verified in the reciprocal experiment, where HA-p73α or HA-p73β were co-expressed with MDM2 in 293 cells, and cell lysates were immunoprecipitated with either the anti-HA monoclonal antibody 12CA5 or a control antibody. Immunoblots of the immunoprecipitates were probed for the presence of both HA-p73 (anti-HA antibody 12CA5) and MDM2 (antibody 2A10). Figure 3B shows that MDM2 was complexed with HA-p73, since it was present in 12CA5 immunoprecipitates but not in the control antibody immunoprecipitates. Figure 3B also shows that the transcriptionally inactive mutants of p73α and p73β (R292H) can bind to MDM2, suggesting the transcriptional activity of p73 is not required for binding to MDM2. These results indicate that p73 and MDM2 can associate with each other.

To see whether binding of p73 to MDM2 is involved in the stabilization of p73 (see later), we created N-terminal truncation mutants of p73α and p73β and tested whether they can bind to MDM2. A GST-tagged MDM2 (GST-MDM2) was co-expressed with full length p73α, or NΔ250 mutants of p73α and p73β in HtTA1 cells. The GST-MDM2 and associated proteins were selectively precipitated using GSH-agarose, and the full length or truncation mutants of p73 were detected by immunoblotting. Figure 3D shows that whereas full length p73α can be detected to bind to GST-MDM2, no N-terminal truncation mutants of p73α and p73β can be detected to bind to GST-MDM2 under similar conditions. We also found that the N-terminal truncation mutants of p73 were not stabilized by MDM2 (see later).

Binding and stabilization of p73 by MDMX

MDMX is a structural homologue of MDM2, but it is not subject to transcriptional activation by p53 (29, 30) . It was therefore of interest to investigate whether MDMX behaves similarly to MDM2 in associating with and stabilizing p73. MDMX was fused with GST (GST-MDMX) and expressed with HA-p73α or HA-p73β in mammalian cells. The expression of GST-MDMX and HA-p73 was detected by immunoblotting with antibodies against GST and HA respectively. Figure 4 shows that both HA-p73α and HA-

p73β protein levels were elevated when co-expressed with GST-MDMX, while levels of tubulin remained relatively constant. Thus, MDMX, like MDM2, appears to promote an elevation in p73 protein levels. Since MDMX is a MDM2-related protein that also associates with p53, and the sequences of the p53-binding domains of MDM2 and MDMX are conserved, we investigated whether this stabilization may arise from a direct association between p73 and MDMX. Transfected cell extracts (above) were incubated with GSH-agarose to precipitate GST-MDMX, and any associated HA-p73α or HA-p73β was detected by immunoblotting with anti-HA monoclonal antibody. From Figure 4, it is apparent that HA-p73α and HA-p73β were only precipitated when GST-MDMX was co-expressed. This suggests that MDMX, like MDM2, can bind to p73, and that this binding may result in stabilization of the p73 protein.

MDM2 affects the half-life of p73

MDM2 regulates p53 level by targeting p53 for proteasome-dependent degradation (6, 7) . To have an idea whether the observed elevation in p73 levels by MDM2 or MDMX expression was due to increased transcription of p73, or a post-transcriptional effect, we assessed the levels of the transfected HA-p73α mRNA by reverse transcription-PCR (RT-PCR). Figure 5A shows that the relative level of the transfected HA-p73α messages was similar in cells transfected with HA-p73α alone or in cells co-transfected with MDM2. Thus it seems unlikely that MDM2 plays a major role in altering transfected p73 transcription or message stability, and that its effect in elevating p73 protein levels may be post-translational. We therefore asked whether p73 is normally subject to proteasome-mediated degradation, by analyzing p73 protein levels in the presence or absence of the proteasome/calpain inhibitor LLnL (also called MG101) (Figure 5B). Higher amount of HA-p73α was detected in HA-p73α-transfected H1299 cells after treatment with LLnL, suggesting p73α may be degraded by the proteasome pathway. As before, co-transfection with MDM2-expressing plasmid increased the level of HA-p73α (lane 3). But significantly, treatment with LLnL did not further increase the level of p73α in the presence of MDM2 (lane 4), suggesting that MDM2 and LLnL may both act on the same

target to promote p73 stability, i.e. MDM2 can prevent proteasome-mediated degradation of p73. Equal amount of tubulin was detected in all the samples (bottom panel).

To have an initial idea of the half-life of p73, cells were transfected with plasmids expressing p73 before treated with cycloheximide to turn off protein expression (Figure 5C). We found that when p73 was co-expressed with MDM2, p73 has a higher stability than when p73 was co-transfected with control plasmids. To have a better indication of the half-life of p73, full length p73 and NΔ250 truncation mutants of p73 were subcloned into vectors which expression is under inducible control of tetracycline (or deoxycycline). HtTA1 cells were allowed to express full length p73β and the NΔ250 mutant together, then their expression was turned off by addition of deoxycycline to the medium (Figure 5D). The repression of the expression of p73β and NΔ250 mutant was tight since no expression of the two proteins can be detected 12 hours after addition of deoxycycline (lanes 6 and 12). Figure 5D shows that when co-expressed with MDM2 (constant expression not under deoxycycline control), the half-life of p73β is appreciably longer than when p73β was co-transfected with control plasmids. In contrast, in exactly the same transfection where full length p73β showed changes in stability in the presence of MDM2 above, the half-life of p73β NΔ250 mutant was not affected by the presence or absence of MDM2. This suggests that association with MDM2 is required for the increase in half-life of p73. Taken together, these data suggest that the rate of degradation of the p73 protein can be decreased by binding to MDM2.

MDM2 enhances the anti-proliferative function of p73

Given that the total level of p73 was elevated in the presence of MDM2, we next investigated whether the biological functions of p73 were influenced by MDM2. One of the transcriptional targets of p73 is the CDK inhibitor $p21^{Cip1/WAF1}$. We studied the induction of the endogenous $p21^{Cip1/WAF1}$ after p73 was transfected into H1299 cells. Cells were co-transfected with a CD2 surface marker for the subsequent selection of transfected cells. Figure 6A shows that the endogenous $p21^{Cip1/WAF1}$ was induced after

transfection of p73α but not the transcriptional-inactive mutant R292H. Significantly, co-expression of MDM2 with p73α enhanced the induction of $p21^{Cip1/WAF1}$.

We next investigated the growth potential of cells expressing p73 and MDM2 by colony formation assays. H1299 cells were transfected with a puromycin-resistant plasmid, and stabily transfected cells were selected by growing in medium containing puromycin. The number of colonies formed was measured after different expression plasmids were co-transfected with the puromycin-resistant plasmid. Expression of HA-p73α reduced the number of colonies formed to about 40% of the control, confirming the anti-proliferative potential of p73α (Figure 6B) Co-expression of p73α with MDM2 further reduced the number of the colonies formed (to about 15%). In comparison, expression of MDM2 alone produced similar number of colonies as the control. Note that the inhibition of colony formation by p73 was not 100% because the puromycin-resistant gene and the p73 DNA were co-transfected on different plasmids, and only the integration of the puromycin-resistant plasmid was selected for. We assume the inhibition of colony formation was due to the different transient levels of p73 protein and/or activity in the presence or absence of MDM2. Hence consistent with the stabilization of the p73 protein by MDM2, and the increase in $p21^{Cip1/WAF1}$ level, these results indicate that the anti-proliferative functions of p73 are enhanced in the presence of MDM2.

DISCUSSION

Loss of p53 is believed to be an important event in the tumorigenesis of many cancers, but many cancers do not contain p53 mutation and therefore the loss of other tumor suppressors may be involved. p73 is regarded as the putative tumor suppressor mapped to 1p36, because deletions of 1p36 are common in a variety of cancers (31) .

Different domains within p53 (transactivating domain, DNA binding domain, and oligomerization domain) are highly conserved in p73. Given that the sequence in p53 that binds MDM2 is also similar in p73, it is important to see whether p73 can indeed associate with and is regulated by MDM2. Here we demonstrate that MDM2 and the related protein MDMX can bind to p73α and p73β. In contrast to p53, the stability of p73 on the protein level is increased by MDM2/MDMX. These data points to a distinct difference in the roles and regulation of these two related proteins.

Consistent with the stabilization of p73 by MDM2, MDM2 also enhanced the growth inhibitory functions of p73 as indicated by colony formation and $p21^{Cip1/WAF1}$ induction. Our unpublished data also indicate that expression of p73α increased the sub-G1 DNA population in HeLa cells, suggesting an induction of apoptotic cell death in these cells by p73α. Co-expression of p73α and MDM2 significantly increased the sub-G1 DNA population, suggesting that the induction of apoptosis by p73 may also be enhanced by MDM2. We found that MDM2 increased the half-life of p73β (Figure 5) and p73α (data not shown) in cycloheximide and promoter turn off assays. Importantly, N-terminal truncation mutants of p73 that do not bind to MDM2 (Figure 3) are not stabilized by MDM2 (Figure 5). Similarly, we have created N-terminal truncation mutants of MDM2 (NΔ115 and NΔ297). These MDM2 mutants did not bind p53 or p73, and failed to affect the protein level of p53 and p73 (unpublished data).

There are two distinct ways in which MDM2 can inactivate p53. MDM2 can inhibit p53-mediated transcription by masking the transactivating domain (4, 5) , as well as targeting p53 for proteasome-mediated proteolysis (6, 7) . We found that the endogenous $p21^{Cip1/WAF1}$ was induced by expression of p73, and more $p21^{Cip1/WAF1}$ was induced when MDM2 was present (Figure 6). However, it is possible that binding of

MDM2 to p73 could also mask the transactivating domain of p73. Our preliminary evidence using p53-responsive promoters-driven luciferase reporter indicated that luciferase activity was strongly induced in the presence of transfected p73α, compared to control plasmids or the transcriptional-inactive mutant (R292H) of p73α (unpublished data). On co-expression of MDM2, however, there are variations on the luciferase activity depending on the promoter and the cell line used (unpublished data). It is currently unclear regarding the relative importance of the stabilization of p73 protein level and the transcriptional masking by MDM2. We also have preliminary data indicating that the increase in growth inhibition effects of p73 in the presence of MDM2 may not be solely dependent on the transactivating activity of p73. Transcriptional-independent functions are not unprecedented in this family of proteins since p53 can induce apoptosis in the absence of transcription (32) .

The exact molecular mechanisms of how MDM2 differentially regulates p53 and p73 stability is unclear at the moment. One possibility is to do with the involvement of other proteins participating in p53 degradation like $p14^{ARF}$. It would be interesting to see whether $p14^{ARF}$ is also present in the p73-MDM2 complexes. Another possibility that may explain the different effect of MDM2 on p53 and p73 is to do with other regions of p73 that are not involved in binding to MDM2. Both the N-terminal MDM2 binding domain as well as the C-terminus of p53 are reported to be important for the MDM2-mediated degradation (33) . It is possible that the C-terminal elements of p53 important for MDM2-mediated degradation are not conserved in p73, hence the p73-MDM2 is stable. One of the key questions about p73 is how the protein level is normally regulated in the cell. It is unclear how p73 is normally induced, but unlike p53, p73 is not induced by DNA damage (16) . The DNA damage-dependent ATM kinase or DNA-PK phosphorylation site in p53 (Ser15) that inhibits MDM2 binding is not conserved in p73 (9, 10, 11) . The precise physiological functions of the two forms of p73 are currently unknown (34, 35) , although some difference in their transactivating potential has been suggested (36) . We found that both forms of p73 can be stabilized by MDM2 and MDMX.

The difference in the regulation of protein stability by MDM2/MDMX for p53 and p73 suggests that there may be interplay between the two proteins. p73 can only form weak oligomers with p53 (16) , so direct synergistic action between p53 and p73 on transcription may not play an important role. However, it has recently been suggested that p73 may form a stronger complex with mutant forms of p53 (36) . Another potential interplay suggested by the results presented here is that since p73-MDM2 complexes were not degraded, p73 can in theory enhance the effects of p53 by acting as a reservoir for MDM2 or related proteins, hence sparing p53 for inhibition and degradation by MDM2. Hence loss of p73 gene would not merely obliterate p73-transcription dependent events, it may also allow the extra MDM2 or related proteins to bind to p53 and leads to p53 inactivation. At least under transient expression conditions, we found that the MDM2 that associated with p53 was reduced when p73 was co-expressed (unpublished data). Furthermore, it is conceivable that other p53-related proteins like p51 and p63 can also interact functionally with p73 and p53 through MDM2 or related proteins. We therefore suggest that a subtle interplay between the members of the p53 family and MDM2 family. It will be interesting to further investigate the competition between p53 and p73 for MDM2 and to determine how such competition may play a role in regulating cell proliferation.

Based on animal studies, the early embryonic lethality in MDM2 null mice is rescued by deletion of p53 (37, 38) . This implies that the lethality of the MDM2 null mutants is due to overactivation of the p53 pathway. This also suggests that p73-MDM2 regulation may not be essential for early development. But other subtle functions of p73-MDM2 interaction cannot be ruled out. Furthermore, it is unclear about the role of MDMX, since its transcription is not induced by p53. It is possible that MDMX or other yet unidentified MDM2-related proteins may have differential specificity towards different members of the p53 family.

It would be interesting to see which other regulations of p53 are also distinct from p73 and other p53-related proteins. It was recently shown that none of the viral proteins that are known to bind p53 - adenovirus E1B 55 kDa protein, SV40 large T antigen, and

HPV16 E6 - binds to p73 (39) , but it has been shown that the adenoviral protein E4orf6 can bind to p73 (40) . In this connection, an interesting parallel to the differential effect of MDM2 on p73 and p53 described here is that while the HPV16 E6 targets p53 for degradation, the similar proteins in SV40 (large T antigen) and adenovirus (E1B-55 kDa protein) stabilize p53 (41, 42) . The striking difference between the regulation of p53 and p73 by MDM2/MDMX described here may in the future shed more light on the distinct roles of this family of proteins in the cell.

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FIGURE LEGENDS

Figure 1. MDM2 destabilizes p53 but stabilizes p73 in 293 cells.

(A) 293 cells were transfected with plasmids expressing HA-p73α (lanes 1-3), HA-p73β (lanes 4 and 5), MDM2 (lanes 2 and 5), and antisense-MDM2 (lane 3). Cells were grown for a further 24 hr after transfection before being harvested. Cell extracts were prepared, applied onto a 7.5% SDS-PAGE and immunoblotted with the anti-HA monoclonal antibody (12CA5) (top panel), 12CA5 and the anti-MDM2 monoclonal antibody (2A10) (middle panel), or 12CA5 and the anti-tubulin monoclonal antibody (YL1/2) (bottom panel). The positions of HA-p73α, HA-p73β, MDM2, and tubulin are indicated.
(B) Plasmids expressing p53 were transfected with control plasmids (lane 1), or MDM2-expressing plasmids (lane 2) into 293 cells. Cell extracts were applied onto 7.5% SDS-PAGE and immunoblotted with the anti-MDM2 monoclonal antibody (2A10) (top panel), or the anti-p53 monoclonal antibody (421) (middle panel). The blot was probed with monoclonal antibodies against p53 and tubulin together in the bottom panel.
(C) Plasmids expressing FLAG-tagged Smad4 were transfected with control plasmids (lane 1), or MDM2-expressing plasmids (lane 2) into 293 cells. Extracts were applied onto 7.5% SDS-PAGE and immunoblotted with the anti-MDM2 monoclonal antibody (2A10) and the anti-FLAG monoclonal antibody (M2).
(D) Cell extracts from 293 cells transfected with control plasmids (lane 1), plasmids expressing p53 (lane 2), HA-p73α (lane 3), or HA-p73β (lane 4) were applied onto 7.5% SDS-PAGE and immunoblotted with antibodies against p73α.

Figure 2. Stabilization of p73 by MDM2 in p53-null SAOS-2 and H1299 cells.

(A) Cell extracts from SAOS-2 cells transfected with plasmids expressing HA-p73α (lanes 2 and 3) and MDM2 (lane 3) were applied onto 7.5% SDS-PAGE and immunoblotted with monoclonal antibody against MDM2 (2A10) (top panel), or 2A10 and monoclonal antibody against HA-tags (12CA5) (second panel). Equal loading of the samples was indicated by immunoblotting with the anti-cyclin A monoclonal antibody (E72) (third panel), and the anti-tubulin monoclonal antibody (YL1/2) (bottom panel).

(B) H1299 cells were transfected with plasmids expressing HA-p73β (4 μg), and an increasing amount of plasmids expressing MDM2 (lane 1, 0 μg; lane 2, 2 μg; lane 3, 4 μg; lane 4, 10 μg; lane 5, 12 μg). Cell extracts preparation and immunoblotting with the antibodies against HA (12CA5) (top panel), MDM2 (2A10) (middle panel), and tubulin (YL1/2) (bottom panel) were as described above.

Figure 3. Interaction between p73 and MDM2.

(A) 293 cells were transfected with plasmids expressing HA-p73α (lanes 1,2, and 5), HA-p73β (lanes 3 and 4), MDM2 (lanes 2 and 4), and antisense-MDM2 (lane 5). Cell extracts were prepared, applied onto a 7.5% SDS-PAGE gel and immunoblotted with the anti-MDM2 monoclonal antibody (2A10) (top panel), or 2A10 and the anti-HA monoclonal antibody (12CA5) (middle panel) as described above. Cell extracts (100 μg) were immunoprecipitated with the antibody against MDM2 (2A10), and immunoblotted with the anti-HA monoclonal antibody (12CA5) (bottom panel). The positions of HA-p73α, HA-p73β, MDM2, and IgG heavy chains from the immunoprecipitation are indicated.

(B) 293 cells were transfected with plasmids expressing MDM2 and HA-p73α (lanes 1 and 2), HA-p73β (lanes 3 and 4), HA-p73α.R292H (lanes 5 and 6), HA-p73β.R292H (lanes 7 and 8). Cell extracts (100 μg) were immunoprecipitated with either the anti-HA monoclonal antibody (12CA5) (odd numbered lanes) or an unrelated monoclonal antibody (even numbered lanes). The immunoprecipitates were subjected to immunoblotting with the anti-MDM2 monoclonal antibody (upper panel), or 2A10 and 12CA5 together (bottom panel).

(C) Alignment between the N-terminal amino acid sequences of human p53, p73α, and p73β. Identities between p53 and p73 are enclosed in shaded boxes, and the MDM2-binding domain of p53 is indicated by the solid line.

(D) HtTA1 cells were transfected with plasmids expressing GST-MDM2, together with plasmids expressing HA-p73α (lanes 1 and 5), control plasmids (lanes 2 and 6), FLAG-p73α.NΔ250 (lanes 3 and 7), or FLAG-p73β.NΔ250 (lanes 4 and 8). Cell extracts (100 μg) were incubated with GSH-agarose to isolate the GST-MDM2 and associated proteins.

Total cell lysates (lanes 1-4) and the GSH-agarose precipitates (lanes 5-8) were immunoblotted with antibodies against HA-tags (12CA5) and FLAG-tags (M2) together.

Figure 4. Interaction between p73 and MDMX.

(A) 293 cells were transfected with plasmids expressing HA-p73α (lanes 1 and 2) and GST-MDMX (lanes 2 and 3). Cell extracts were prepared, applied onto a 7.5% SDS-PAGE gel and subjected to immunoblotting with anti-GST polyclonal antibodies (top panel), the anti-HA monoclonal antibody (12CA5) (second panel), and the anti-tubulin monoclonal antibody (YL1/2) (third panel). Cell extracts (100 μg) were incubated with GSH-agarose and unbound proteins were washed off. The precipitates were immunoblotted with 12CA5 (bottom panel).

(B) 293 cells were transfected with plasmids expressing HA-p73β and GST-MDMX (lane 2). Cell extracts were prepared, applied onto a 7.5% SDS-PAGE gel and subjected to immunoblotting with antibodies against GST and HA-tags (12CA5) (top panel), and the against tubulin (YL1/2) (middle panel). Cell extracts (100 μg) were incubated with GSH-agarose and unbound proteins were washed off. The precipitates were immunoblotted with 12CA5 (bottom panel).

Figure 5. Regulation on the protein level of p73 by MDM2.

(A) Constant level of transfected p73 mRNA. 293 cells were transfected with plasmids expressing HA-p73α (lanes 2, 3, 5, and 6), and MDM2 (lanes 3 and 6). Total RNA was extracted from the cells, and the relative amount of HA-p73α mRNA transcribed from the transfected plasmids was measured by RT-PCR as described in Materials and Methods. Reverse transcriptase was excluded in the control reactions in lanes 4-6.

(B) Stabilization of p73 by proteasome inhibitor. Plasmids expressing HA-p73α were co-transfected with control vectors, or plasmids expressing MDM2 into H1299 cells. After transfection, the cells were divided into two identical plates (vector: lanes 1 and 2; MDM2: lanes 3 and 4) and allowed to grow for 24 hr. Buffer (lanes 1 and 3) or LLnL (100 μM) (lanes 2 and 4) was added to the medium, and the cells were incubated for a

further 12 hr before being harvested for cell extracts preparation. Extracts were applied onto 7.5% SDS-PAGE and immunoblotted with antibodies against HA-tags (12CA5) (upper panel), or tubulin (YL1/2) (bottom panel).

(C) Extension of half-life of p73 in the presence of MDM2. Plasmids expressing HA-p73β were co-transfected with control vectors (lanes 1-4), or plasmids expressing MDM2 (lanes 5-8) into H1299 cells. Cycloheximide was added to the medium (final 10 μg/ml), and the cells were harvested for cell extracts preparation at the indicated times. Extracts were applied onto SDS-PAGE and immunoblotted with the anti-HA monoclonal antibody (12CA5).

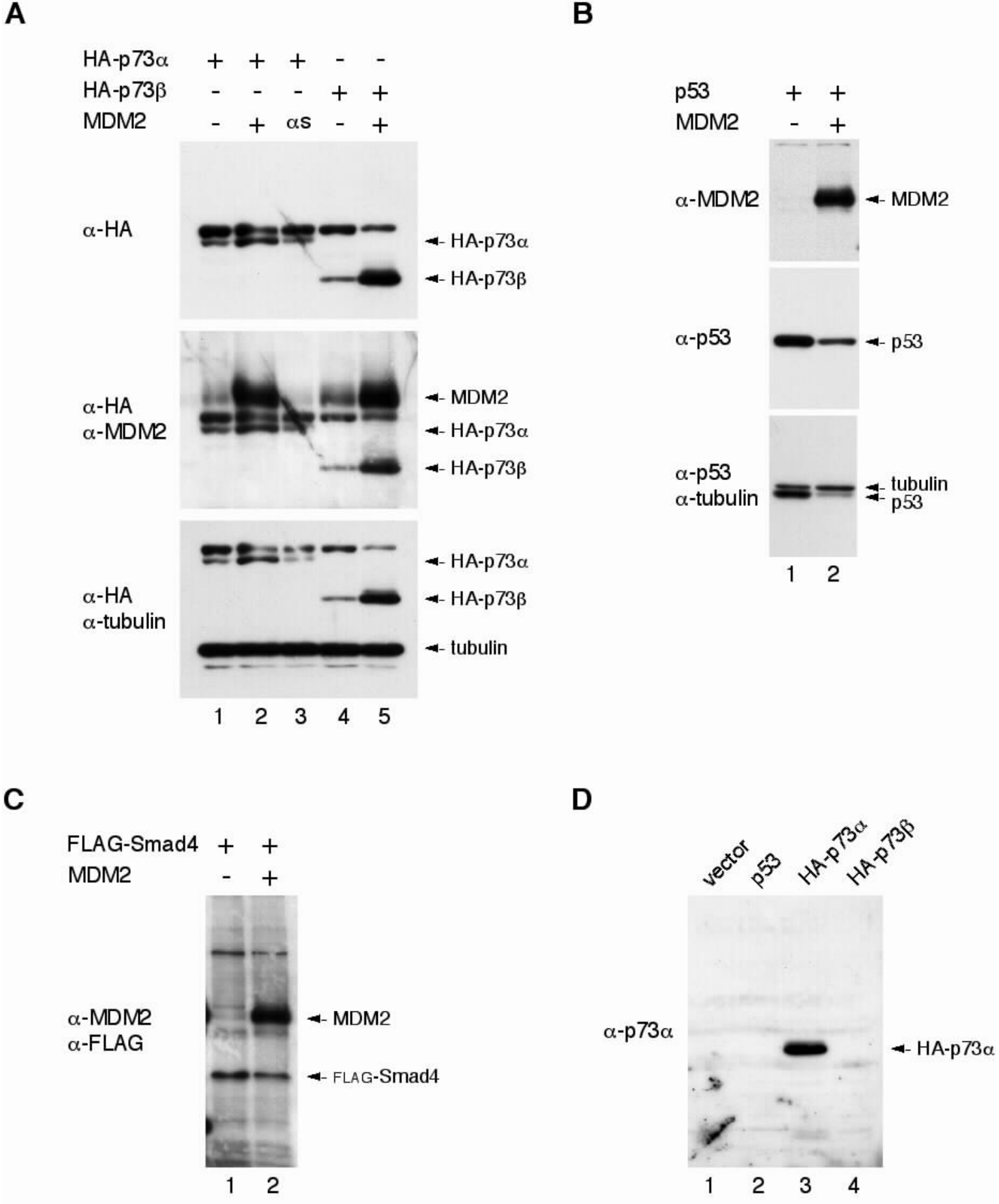
(D) Longer half-life of full length p73, but not N-terminal truncation mutant, in the presence of MDM2 *in vivo*. Plasmids containing HA-p73β and FLAG-p73β.NΔ250 under inducible control of deoxycycline were co-transfected into HtTA1 cells. Control plasmids (lanes 1-6), or plasmids expressing MDM2 (lanes 7-12) were also co-transfected. At 24 hr after transfection, deoxycycline was added to the medium to turn off the expression of p73β and NΔ250 as described in Materials and Methods. The cells were harvested at the indicated times for extracts preparation, and the extracts were immunoblotted with antibodies against HA-tags (12CA5) (upper panel) or FLAG-tags (M2) (lower panel).

Figure 6. Inhibition of cell proliferation by p73 and MDM2.

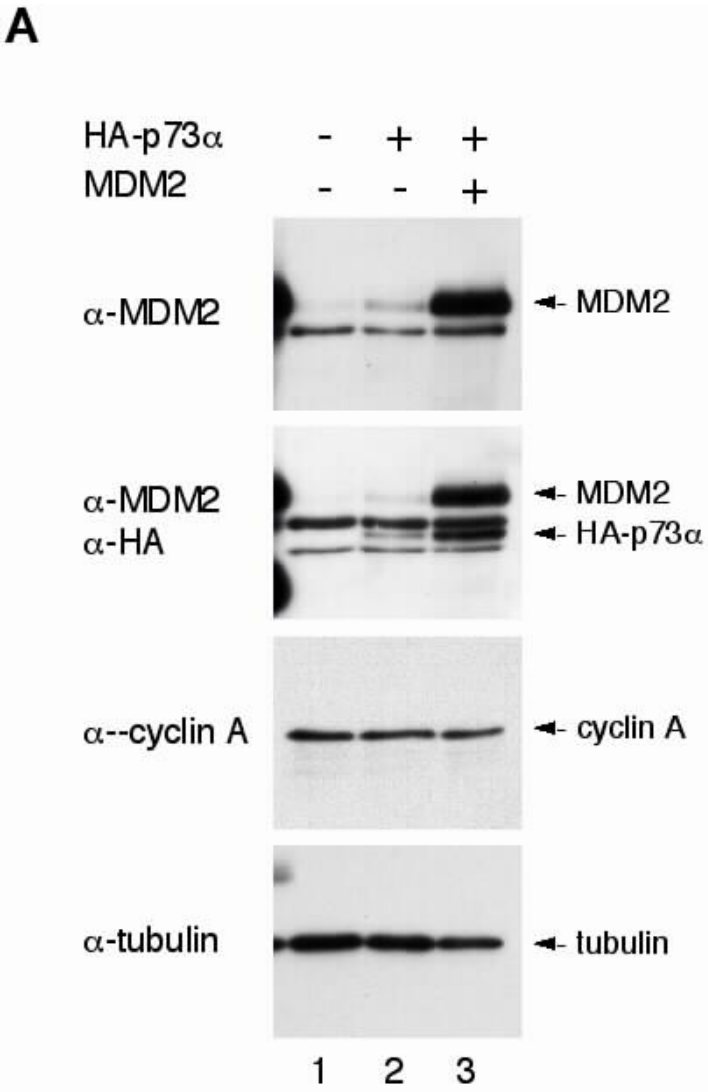
(A) Induction of endogenous $p21^{Cip1/WAF1}$ by p73 and MDM2. H1299 cells (~$5x10^5$ cells per 10 cm plate) were co-transfected with plasmids expressing CD2 surface marker (5 μg), together with plasmids expressing HA-p73α (5 μg, lanes 2 and 3), HA-p73α R292H (5 μg, lanes 4 and 5), and MDM2 (10 μg, lanes 3 and 5). Cells were harvested 30 hr after transfection and transfected cells were isolated by anti-CD2 magnetic beads selection as described in Materials and Methods. Cell extracts were prepared, applied onto a 7.5% SDS-PAGE gel and subjected to immunoblotting with antibodies against $p21^{Cip1/WAF1}$ (upper panel) or tubulin (bottom panel).

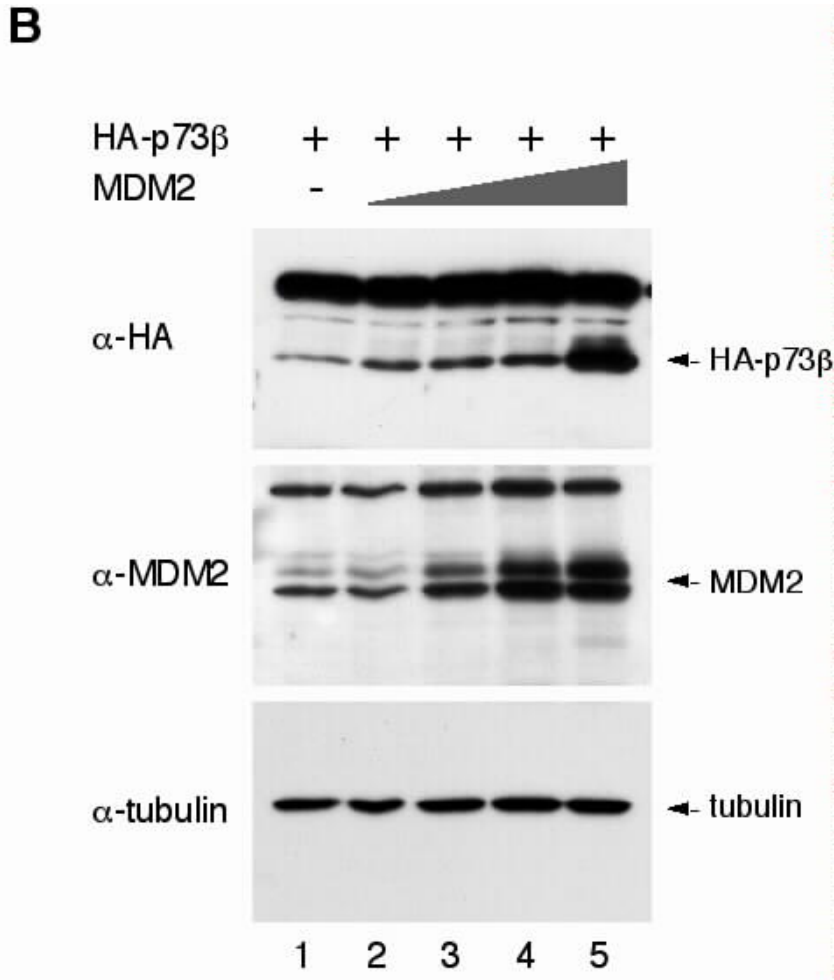
(B) Colony formation assays. H1299 cells were transfected with 2 μg of the plasmid pPUR that conferred puromycin-resistance, together with the vector pcDNA3, plasmids

expressing MDM2 (10 μg), HA-p73α (6 μg), or HA-p73α and MDM2 together as indicated. The total amount of DNA added was adjusted to the same amount with pcDNA3 vector. The cells were then grown in medium containing 1 μg/ml of puromycin for 2 weeks. Colonies were fixed and visualized by staining with crystal violet. The average of three independent experiments and the standard deviation are shown.

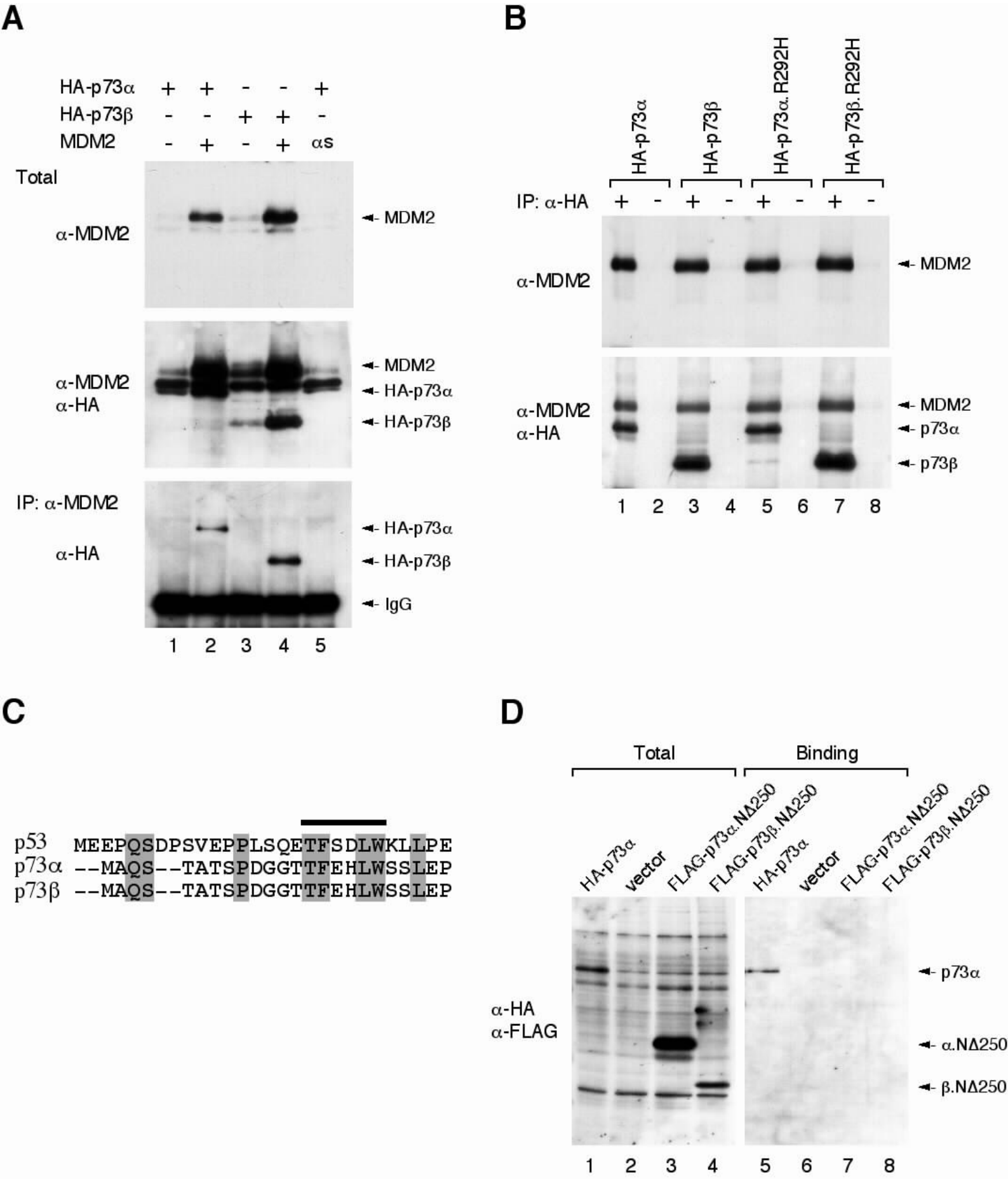


Ongkeko *et al.* Figure 1





Ongkeko *et al*. Figure 2



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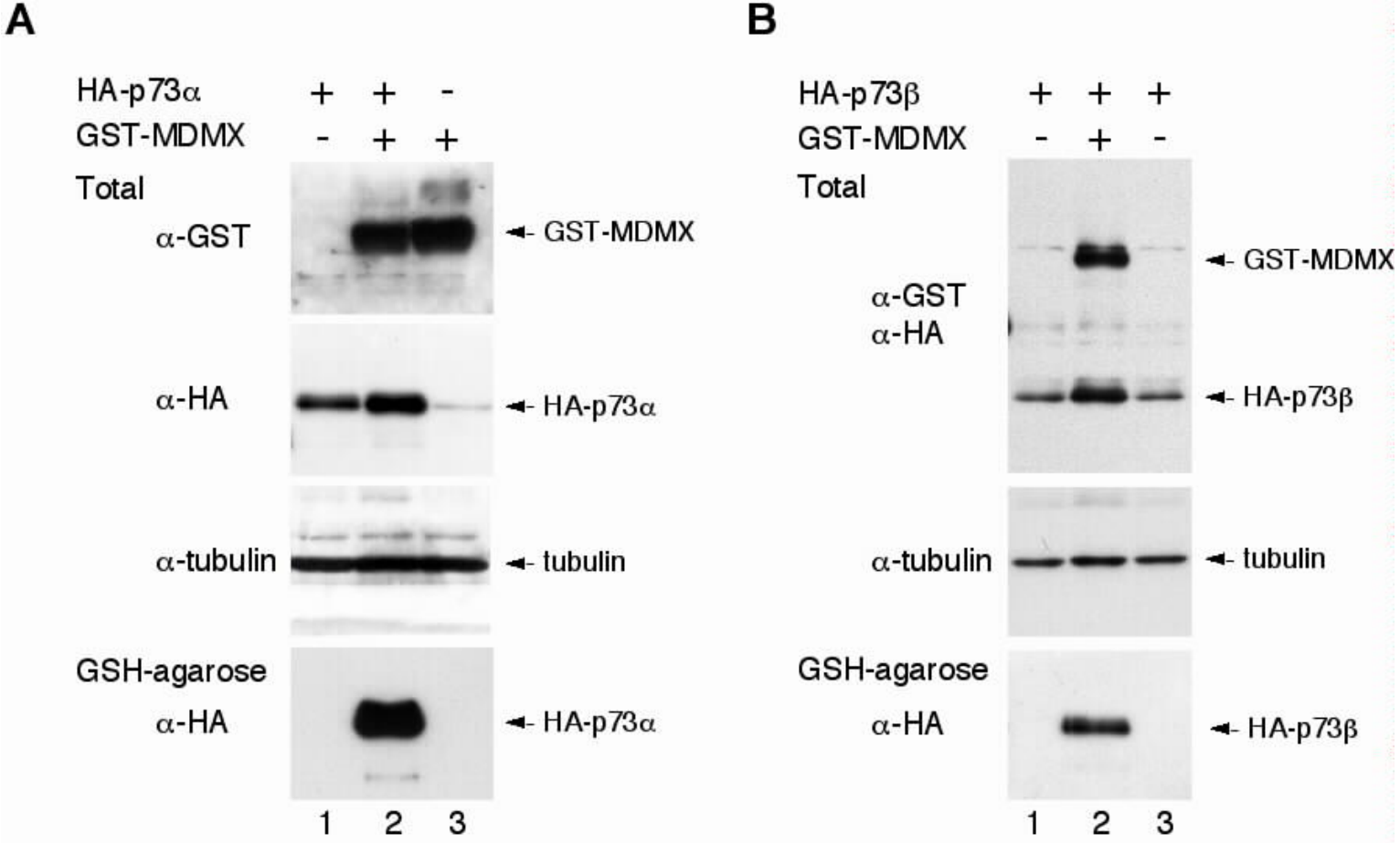
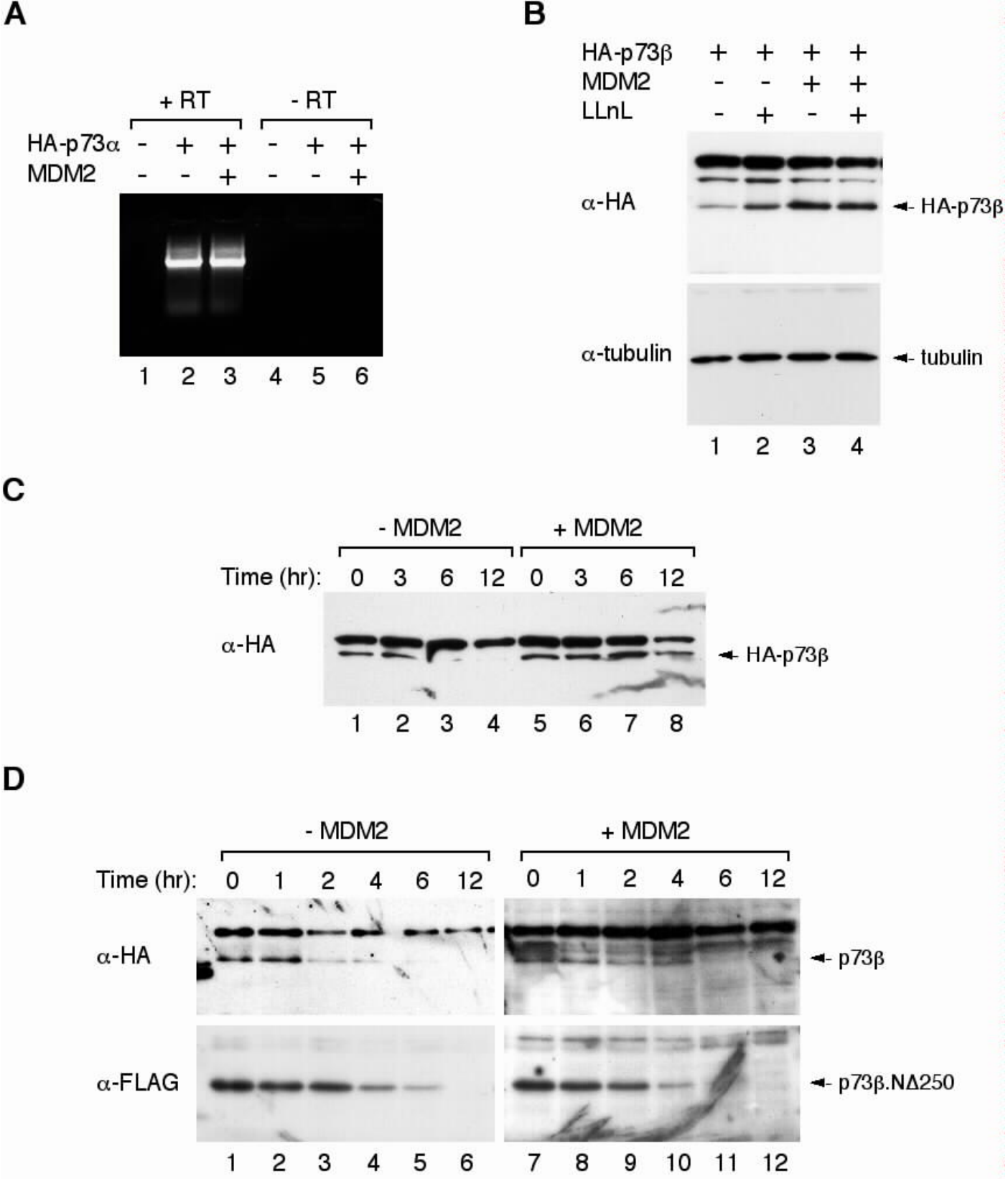
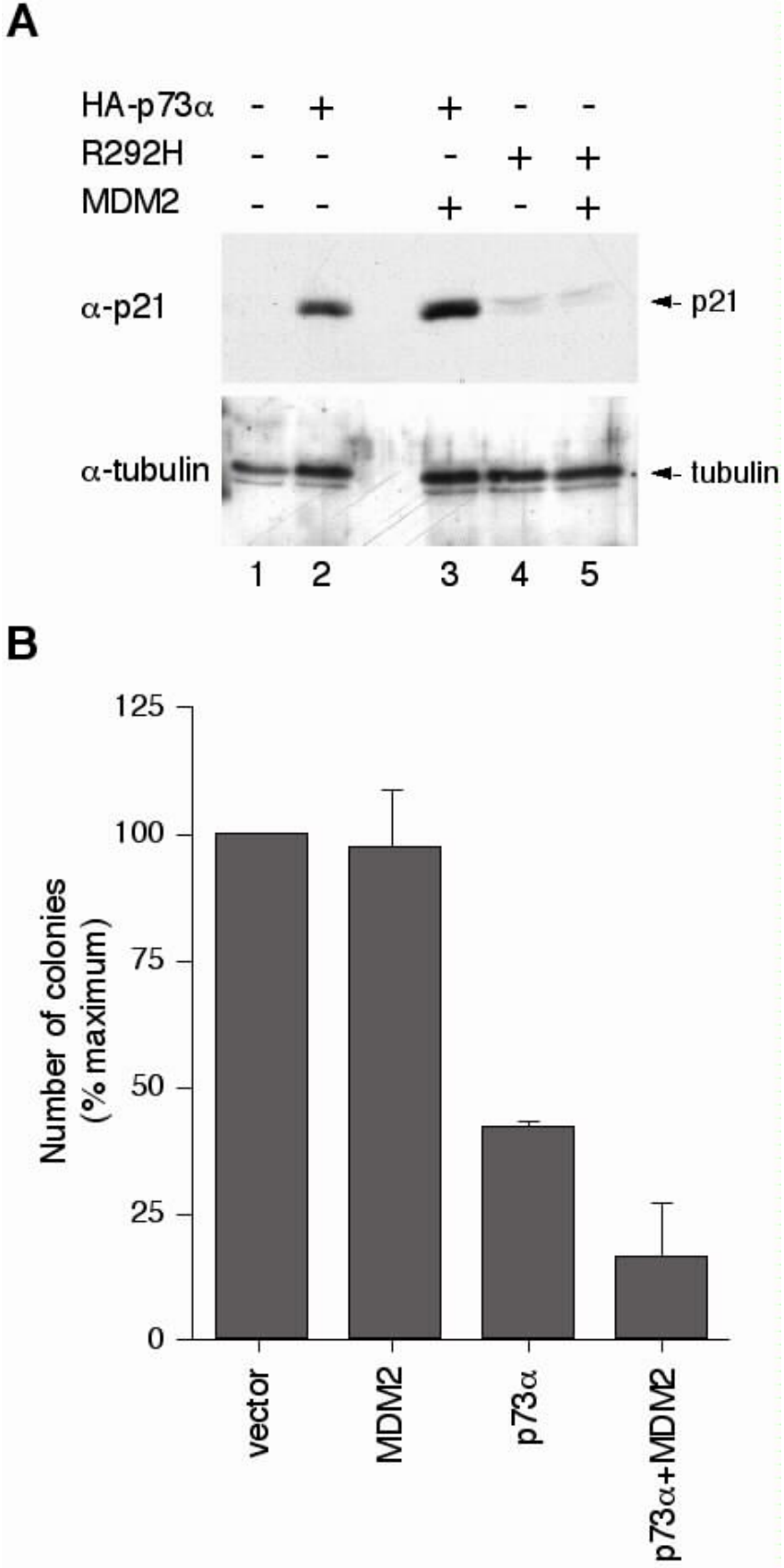


Ongkeko *et al*. Figure 4



Ongkeko *et al.* Figure 5



Ongkeko *et al*. Figure 6