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Regulation of p53 and MDM2 Activity by MTBP

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p53 is a critical coordinator of a wide range of stress responses. To facilitate a rapid response to stress, p53 is produced constitutively but is negatively regulated by MDM2. MDM2 can inhibit p53 in multiple independent ways: by binding to its transcription activation domain, inhibiting p53 acetylation, promoting nuclear export, and probably most importantly by promoting proteasomal degradation of p53. The latter is achieved via MDM2's E3 ubiquitin ligase activity harbored within the MDM2 RING finger domain. We have discovered that MTBP promotes MDM2-mediated ubiquitination and degradation of p53 and also MDM2 stabilization in an MDM2 RING finger-dependent manner. Moreover, using small interfering RNA to down-regulate endogenous MTBP in unstressed cells, we have found that MTBP significantly contributes to MDM2-mediated regulation of p53 levels and activity. However, following exposure of cells to UV, but not γ-irradiation, MTBP is destabilized as part of the coordinated cellular response. Our findings suggest that MTBP differentially regulates the E3 ubiquitin ligase activity of MDM2 towards two of its most critical targets (itself and p53) and in doing so significantly contributes to MDM2-dependent p53 homeostasis in unstressed cells.

p53 is a critical coordinator of a wide range of cellular stresses ranging from myocyte stretch-induced apoptosis to increased global DNA repair in fibroblasts exposed to UV (30, 44). To facilitate a rapid response to stress, cells have evolved a mechanism that relies upon stabilization and activation, by posttranslational modification, of existing constitutively expressed p53 protein. In normal cells it has been found that p53 is both functionally inhibited and, moreover, maintained in an unstable state by the action of MDM2 (35).

Originally discovered as one of three genes amplified on double minute chromosomes in a tumorigenic derivative of NIH 3T3 cells (5), *MDM2* was later shown to possess oncogenic potential when overexpressed (10, 12). High-level expression of *MDM2* has also been shown to confer tumorigenic potential upon nontransformed rodent fibroblasts in athymic nude mice (10, 12). MDM2 can immortalize rat embryo fibroblasts and can cooperate with activated RAS to transform these cells (12). Elevated levels of MDM2 protein have been found in a variety of human tumors, most notably in soft tissue sarcomas where up to 30% of primary tumors contain multiple copies of the *MDM2* gene (27). One mechanism by which *MDM2* overexpression promotes tumor development is through its ability to bind to the p53 tumor suppressor (36, 38), thereby blocking the transactivation (36, 39), cell cycle arrest (6), and apoptotic functions of p53 (17). MDM2 can inhibit p53 activity in a number of ways including preventing p53 from recruiting TAFs (45), promoting nuclear export (13), inhibiting p53 acetylation (20), and perhaps most importantly by virtue of its function as an E3 ubiquitin ligase with specificity for, among others, p53 (19). In addition to regulating p53 levels by targeting p53 for proteasomal degradation (18, 25), MDM2 also transfers ubiquitin to itself (11), MDMX (8, 40), the β2 adrenergic receptor (43), glucocorticoid receptor (24), TIP60 (28), and PCAF (21).

Induction of p53's transcriptional activity leads to increases in *MDM2* mRNA and MDM2 protein (1, 47), and thus an autoregulatory feedback loop exists between these two proteins. The importance of this feedback loop has been confirmed by studies of transgenic animals. Homozygous deletion of *MDM2* is lethal in mice, whereas mice that possess homozygous deletion of both *MDM2* and *p53* are viable and develop normally (22, 37). Studies of the role of *p53* in human cancer have also been informative in this regard. Inactivation of the p53 tumor suppressor protein is a key event in carcinogenesis, as illustrated by the fact that more than 50% of all human malignancies harbor mutations of *p53* (reviewed in reference 42). It has been found that *p53* is rarely mutated in primary tumors in which *MDM2* is amplified, although there is increasingly good evidence of exceptions to this (16, 32). Thus, *MDM2* overexpression blocks p53 function in vivo and this contributes to the development of tumors (27). Together, these results demonstrate that a primary function of *MDM2*, at least during development, is to regulate p53 function.

A critical question remains: how is the E3 ligase activity of MDM2 towards itself and p53 regulated? Clearly there is a need for differential regulation of MDM2's ubiquitin ligase activity towards itself and p53 to facilitate efficient degradation of p53, without concomitant destruction of MDM2, in a normal unstressed cell, while this balance would then be reversed following a p53-activating stress response. We have investigated the effect on MDM2 activity of the MDM2 binding protein MTBP, which we originally identified as part of a systematic search for MDM2 binding proteins (3, 46). We report here that MTBP increases ubiquitination and degradation of p53, while reducing autoubiquitination and thereby stabilizing MDM2. Thus, MTBP has the ability to differentially regulate the ubiquitin ligase activity of MDM2 towards itself and p53.

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MATERIALS AND METHODS

Plasmids and antibodies. The p53 (pCEP4-hp53) and pMBP10 (pCEP4-mMTBP) expression constructs were described previously (3). hMDM2:pCMV-neobam was a kind gift from B. Vogelstein. The human MDM2 RING finger mutant (Cys464Ala):pCMVneobam3 was a kind gift of D. Xirodimas. The human Δ1–49 MDM2 clone was constructed from hMDM2:pCMVneobam by PCR and cloned into the BamHI site of pCMVneobam using the following primers with flanking BamHI restriction endonuclease sites and incorporating a Kozak consensus sequence: 5′-GAG AGG ATC CCC CGC CGC CCA CCA TGA AAG AGG TTC TTT TTT ATC TTG G and 5′-GAG AGG ATC CCT AGG GGA AAT AAG TTA GCA CAA TC. Primers were supplied by MWG. The β-galactosidase plasmid used as a transfection efficiency control has been described previously (3). Human MTBP was cloned and sequenced from a human placental cDNA library, and the construct for human MTBP expression was created by PCR with primers containing flanking NotI restriction endonuclease sites to amplify the full-length open reading frame: 5′-GAG AGC GGC CGC ATC TCT GCG GCG ATG GAT CGG TAC and 5′-GAG AGC GGC CGC TCA TTT CTT GCT TGT CTT TTC TAA TAC. Human MTBP was then subcloned into the NotI site of pCEP4 essentially as described elsewhere for the murine MTBP clone (3).

Mouse monoclonal antibodies against human Mdm2 (Ab-1 and Ab-2), p53 (Ab-6), and β-galactosidase (Ab-1, used as a transfection efficiency control) were purchased from Oncogene Research Products. The anti-actin antibody (C-2, used as a total protein loading control), anti-MDM2 (SMP14) antibody, and antiubiquitin (P4D1) used for immunoprecipitation were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-p53 antiserum (Ab2433) was obtained from Abcam. Leu-16 antibody against CD20 used as an isotype control for immunoprecipitation was purchased from Becton Dickinson, and the antihemagglutinin (anti-HA) antibody used to detect HA-tagged MTBP (12CA5) was purchased from Roche Molecular Biochemicals. A rabbit polyclonal antibody (antisera 1) was raised against a peptide fragment from human MTBP (CSSDWQEIHFDTE) and this recognizes both human (hMTBP) and murine MTBP (mMTBP).

Cell culture and transfection. H1299 (*p53*-null, human non-small cell lung carcinoma) and MCF-7 (mammary adenocarcinoma, ARF-null) cells were maintained in RPMI 1640 or Dulbecco's minimal essential medium, respectively, in the presence of 10% fetal calf serum with penicillin-streptomycin. *MDM2*/*p53* double-null mouse embryo fibroblasts were maintained in high-glucose DMEM medium in the presence of 10% fetal calf serum, 0.4% β-mercaptoethanol, and penicillin-streptomycin.

Cells were transiently transfected using 3 μl of GeneJuice reagent (Novagen) per microgram of DNA, and empty vector was used to ensure equal DNA content in transfections. In some experiments transfected cells were treated with the proteasome inhibitor, MG132 (100 μM) (Affiniti Research Products), 3 h prior to harvest or with an inhibitor of de novo protein synthesis, cycloheximide (50 μg/ml) (VWR International), 2 h before harvesting. Small interfering RNA (siRNA) was delivered to cells by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs for MTBP (5′ GGC UCAUUUGCACUCAAUU 3′), a scrambled control for MTBP (5′ GGACGC AUCCUUCUUAAUU 3′), MDM2 (5′ GCCACAAAUCUGAUAGUAU 3′), and a lamin control (5′ CUGGACUUCCAGAAGAACA 3′) were synthesized by Dharmacon.

In some experiments, cells were subjected to 5-Gy γ-irradiation from a ^{137}Cs source (Gammacell 1000; MDS Nordion) or 40-J/m^2 UV-irradiation from a 30-W UV lamp (Philips) calibrated using a Black-Ray model J-225 shortwave UV measuring meter (UVP).

Western analysis. Cells were harvested by trypsinization after the indicated times and pelleted by centrifugation. Cell pellets were lysed in SLIP buffer (50 mM HEPES [pH 7.5], 10% glycerol, 0.1% Triton X-100, 150 mM NaCl) in the presence of the following protease inhibitors: aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), pepstatin A (1 μg/ml), soybean trypsin inhibitor (100 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). After 10-min incubation on ice, lysates were centrifuged at 20,000 × *g* and protein concentrations in the supernatant were determined using Bradford reagent (Bio-Rad).

Fifty-microgram samples of total protein in 1× protein sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.25% β-mercaptoethanol, bromophenol blue [1 mg/ml]) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were blocked in phosphate-buffered saline–Tween 20 (0.1%, vol/vol) containing nonfat dry milk (Bio-Rad) (5%, wt/vol) for 1 h at room temperature before incubation with primary antibodies (each at 3 μg/ml, except anti-p53 at 1 μg/ml and anti-MTBP at 1:1,000). Membranes were washed three times for 15 min in phosphate-buffered saline–Tween 20 before addition of horseradish peroxidase-conjugated anti-mouse (1:2,500) or anti-rabbit (1:5,000) secondary antibodies (Amersham Pharmacia Biotech) for 1 h at room temperature. Membranes were washed as before and signal was detected by Western Lightning chemiluminescence reagent (Perkin Elmer).

Immunoprecipitation. Cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were lysed in SLIP buffer plus bovine serum albumin (0.5 mg/ml) in the presence of protease inhibitors and *N*-ethylmaleimide (10 mM) for 10 min on ice. Cell lysates were then centrifuged at 20,000 × *g* and protein concentration in the supernatant was determined using Bradford reagent (Bio-Rad).

Four milligrams of cellular lysate was precleared by incubating with protein G Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C followed by brief centrifugation. Precleared supernatants were then incubated with either 2 μg of anti-MDM2 SMP14 antibody or 2 μg of anti-ubiquitin P4D1 antibody or with the same amount of the isotype control antibody Leu-16 against CD20 for 1 h at 4°C. Following this, the lysates were incubated with protein G Sepharose beads for 2 h at 4°C; the bead pellets were washed in SLIP buffer and resuspended in 1× protein sample buffer prior to analysis by Western blotting.

In vivo ubiquitination assay. H1299 cells were cotransfected with either MDM2 and p53 (6:1 plasmid mass ratio) or with MTBP, MDM2, and p53 (20:6:1 ratio) expression plasmids using GeneJuice reagent as described above. Forty-eight hours after transfection cells were harvested. Samples were analyzed by immunoprecipitation and/or Western blotting as indicated.

RNA extraction and Northern analysis. Total cellular RNA was extracted using RNA-Bee (Tel-Test) according to the manufacturer's instructions. Ten micrograms of total RNA was separated on a 1.2% agarose denaturing gel and transferred to a Hybond XL membrane (Amersham Pharmacia Biotech). Partial length probes for p53 (608 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (233 bp), and *lacZ* (500 bp) were generated by PCR using p53: pCEP4, U2OS cell line cDNA, and pFB-Neo-*lacZ* (Stratagene), respectively, as templates. Primers used to generate fragments were the following: p53, 5′-GGT TTC CGT CTG GGC TTC TT-3′ and 5′-TTG GGC AGT GCT CGC TTA GT-3′; GAPDH, 5′-TGC CGT CTA GAA AAA CCT GC-3′ and 5′-ACC CTG TTG CTG TAG CCA AA-3′; and *lacZ*, 5′-CTC TGG CTC ACA GTA CGC GTA A-3′ and 5′-CCA TCA ATC CGG TAG CTT TTC CG-3′. Primers were supplied by MWG. Probes were labeled with [α-^{32}P]-dCTP using the Megaprime DNA labeling system (Amersham Pharmacia Biotech).

Luciferase reporter assay. For reporter assays, cells were cotransfected with 7 μg per 10-cm dish (nominal) of a p53-responsive luciferase reporter construct pp53-TA-luc (Mercury Pathway Profiling Systems; Clontech).

Cells were lysed and luciferase activity measured 8 s after addition of sample to substrate using a luciferase assay kit (Stratagene) essentially according to the manufacturer's instructions with an integration period of 20 s in a TD 20/20 luminometer (Turner Design).

RESULTS

We originally identified MTBP as an MDM2 binding protein in a yeast two-hybrid screen and later confirmed the interaction in vitro and in vivo (3). We were interested in the following question: what might the effect be of MTBP binding upon MDM2 function? Numerous studies of MDM2 function have determined that MDM2 is essential for the regulation of p53 (22, 37). Although MDM2 has several effects upon p53 that contribute to its critical role as regulator of the "guardian of the genome," one of the most important effects of MDM2 upon p53 is mediated through its ability to target p53 for degradation (18, 25, 35). We therefore investigated the effect of MTBP on the steady-state levels of MDM2 and p53 in H1299 cells transfected with expression vectors for MTBP, MDM2, and p53 as indicated in Fig. 1A. As expected, addition of MDM2 resulted in a reduction in the steady-state level of p53. Interestingly, addition of MTBP augmented this reduction, and moreover raised the steady-state level of MDM2. We wanted to see whether this effect occurred in other cell types, and since the two most common cancers of the developed

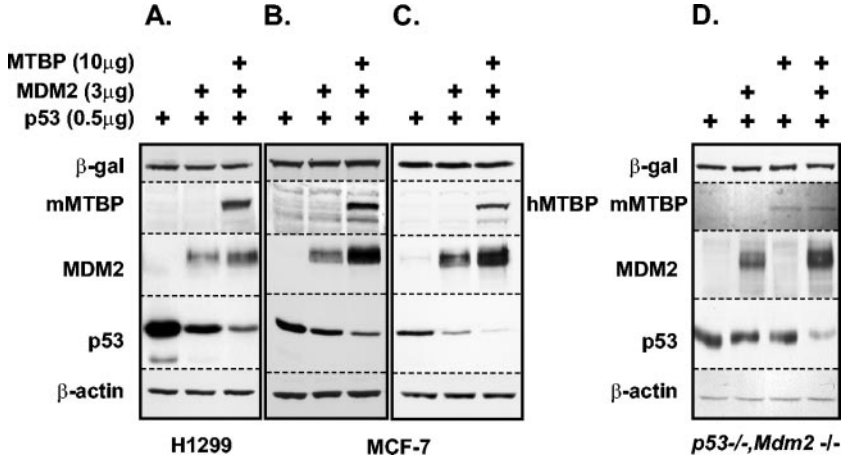

FIG. 1. hMTBP and mMTBP promote stabilization of MDM2 with the consequent destabilization of p53. Cells were transfected with the indicated amount of each plasmid. Total cell lysates were analyzed by Western blotting with the indicated antibodies. Note that in panels A, B, and C murine and human MTBP are detected with a polyclonal anti-MTBP serum, antisera 1, while in D the C-terminally HA-tagged mMTBP is detected with an anti-HA monoclonal antibody. Cells are (A) H1299, (B and C) MCF-7, and (D) double-null ($p53^{-/-}$ $MDM2^{-/-}$) MEFs.

world are lung (represented by H1299s, a non-small cell lung carcinoma cell line) and breast, we examined the effect of MTBP in the MCF-7 breast adenocarcinoma cell line. As shown in Fig. 1B and C, both mMTBP and hMTBP promote an increase in the steady-state level of MDM2 with a concomitant decrease in the level of p53 in MCF-7 cells. It is noteworthy that H1299s express $p14^{ARF}$ whereas MCF-7 cells do not (31, 33). Thus the effect that we have observed is not dependent upon *ARF* status. To determine whether the effect of MTBP upon p53 was dependent upon MDM2, we performed similar experiments in *p53*/*Mdm2*-null mouse embryo fibroblasts (double-null MEFs). As shown in Fig. 1D, under conditions in which neither MDM2 (there being insufficient MDM2 to elicit a substantial effect) nor MTBP alone have any detectable effect upon p53 levels, addition of MTBP downregulates p53 in an MDM2-dependent manner. Thus we conclude that MDM2 is necessary for MTBP-enhanced downregulation of p53.

We found that titration of MDM2 was necessary to establish the suboptimal conditions for MDM2-mediated degradation of p53, essential for the study of MTBP's MDM2-dependent effects. We therefore developed a set of standard conditions in which p53 and MTBP input levels are constant but MDM2 is titrated as illustrated by Fig. 2A. By comparing lanes 1 and 5 we can see that even solely in the presence of endogenous MDM2 transfection of MTBP decreases the level of p53. This format also makes the effect of MTBP upon both MDM2 and p53 levels more apparent: compare lanes 2 and 6, 3 and 7, or 4 and 8.

Since the effect of MTBP upon p53 is dependent upon MDM2, we wanted to investigate whether this was mediated by proteasomal degradation. For these experiments cells were transfected as before and the proteasome inhibitor MG132 was added 3 h before harvesting. Figure 2B shows that addition of MG132 substantially rescues p53 from the effects of MDM2 (compare with Fig. 2A) in either the presence or absence of MTBP. It is important to appreciate that addition of MG132 occurs after MTBP has already increased the steady-state level of MDM2. Thus, addition of MG132 would be expected to

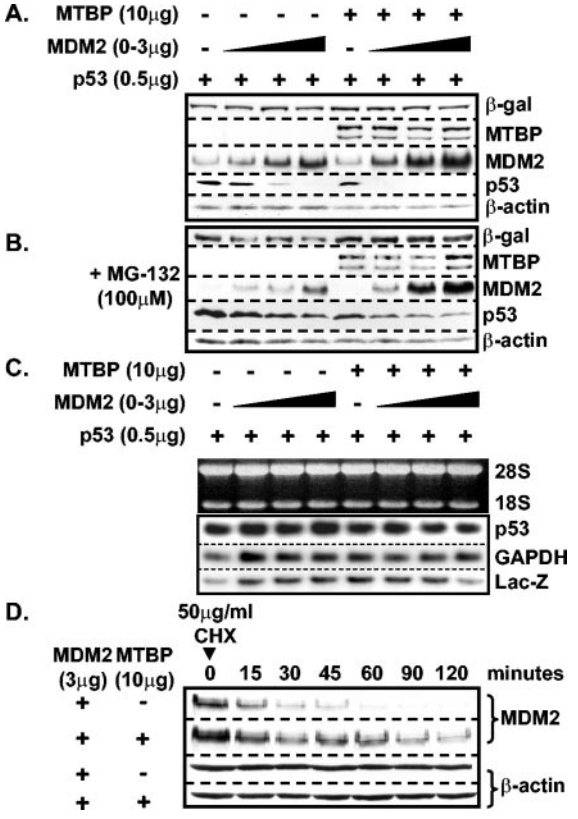

FIG. 2. MTBP promotes p53 degradation via a proteasome-dependent pathway. (A) H1299 cells were transfected with the indicated plasmids for 24 h. Three hours prior to harvest, cells were treated with dimethyl sulfoxide. Cell lysates were then prepared and analyzed by Western blotting. Panel B is as in panel A, but cells were treated with the proteasome inhibitor, MG132 (100 μM), 3 h prior to harvest. (C) H1299 cells were transfected as in panel A. After 24 h, total cellular RNA was extracted and subjected to Northern analysis using the indicated probes. The top panel shows an ethidium bromide-stained, agarose denaturing gel, loaded with 10 μg of total cellular RNA from each transfection condition. (D) H1299 cells transfected as indicated were treated with 50-μg/ml cycloheximide (CHX) and incubated for the times indicated. Cell lysates were then prepared and analyzed by Western blotting with the indicated antibodies.

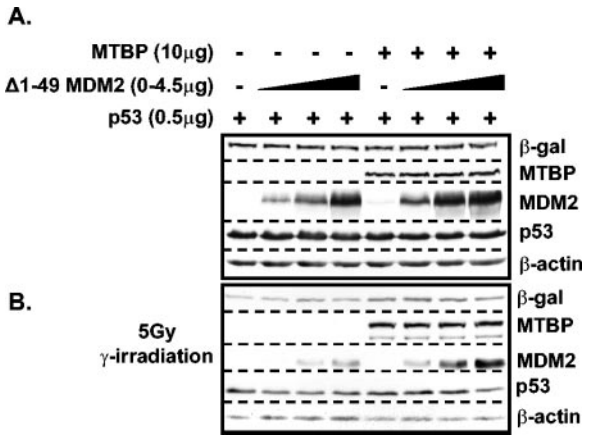

FIG. 3. Binding of MDM2 to p53 is necessary for MTBP to promote degradation of p53. (A) H1299 cells were transfected with the indicated plasmids for 24 h and cell lysates analyzed by Western blotting as indicated. (B) H1299 cells were transfected as indicated for 24 h. Six hours prior to harvest, cells were subjected to 5-Gy γ-radiation and cell lysates analyzed by Western blotting as indicated.

stabilize MDM2 to a higher level in the presence, compared to the absence, of MTBP (because there are more molecules of MDM2 at the time of adding MG132 in the presence of MTBP) and this is what we observe. We have also examined these cells to determine whether MTBP alters the mRNA levels of *p53* or *MDM2* and have found that it does not, as illustrated in Fig. 2C. Clearly if MTBP is increasing the steady-state level of MDM2 in a proteasome-dependent manner we would expect that MTBP would increase the half-life of MDM2. Figure 2D shows that, in the presence of an inhibitor of de novo protein synthesis (cycloheximide), this is indeed the case. We conclude that the effect of MTBP upon MDM2 and p53 is regulated at the level of protein turnover and this is substantially mediated by proteasomes.

Although we thought it unlikely, we considered the possibility that the MDM2-dependent effect of MTBP upon p53 levels does not necessarily require binding of MDM2 to p53. We therefore created a mutant of human MDM2 lacking the first 49 amino acids that has been previously shown not to bind to p53 (15) but which retains the ability to bind to MTBP (3). As shown in Fig. 3A, Δ1–49 MDM2 has no effect upon the level of p53 in the presence or absence of MTBP. Note that this mutant form of MDM2 is still stabilized by MTBP. To further investigate this question, we next examined whether MTBP altered p53 steady-state levels under conditions in which the interaction between MDM2 and p53 is blocked, i.e., in the presence of ionizing radiation. As illustrated in Fig. 3B, we observed that p53 steady-state levels were not affected by the addition of increasing amounts of MDM2, with or without transfection of MTBP, when cells were exposed to 5 Gy of γ-irradiation. Interestingly, we still observed an increase in MDM2 steady-state levels under these conditions. MTBP retains the ability to bind to MDM2 under these conditions (data not shown) and so we conclude that the interaction between MTBP and MDM2 is not inhibited by ionizing radiation.

Since we had consistently observed that MDM2 levels rose in the presence of MTBP, we wanted to determine whether this might be due to inhibition of MDM2 autoubiquitination by MTBP. To examine this we immunoprecipitated ubiquitinated proteins essentially as described previously (2), in the presence and absence of transfected MTBP, and used Western blot analysis to measure the electrophoretic patterns of MDM2 and p53 in the presence of an inhibitor of deubiquitination (*N*-ethylmaleimide). Figure 4A shows the typical effect of MTBP expression upon MDM2 and p53 steady-state levels. It is striking that even though there is considerably less p53 present in MTBP transfected cells, when ubiquitinated proteins are immunoprecipitated (Fig. 4B), there is an increase in the level of slower-migrating forms of p53 protein (ubiquitinated) in the presence of MTBP. We therefore conclude that MTBP increases the ubiquitination of p53 and in so doing promotes p53 degradation. We have noted in this experiment and in similar

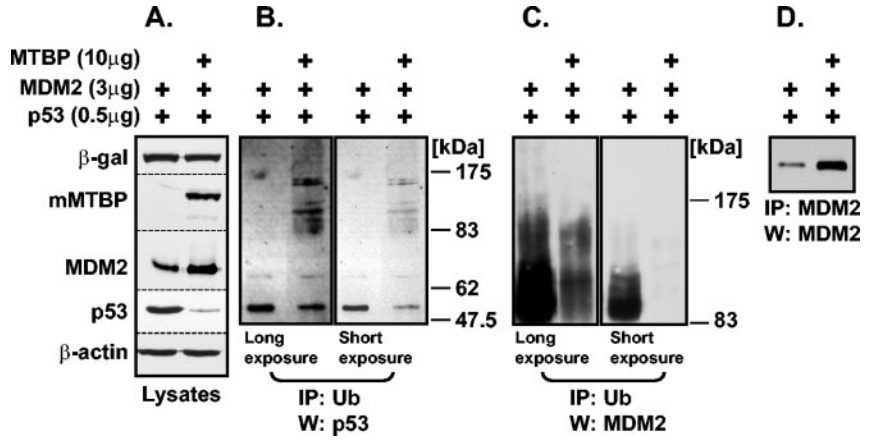

FIG. 4. MTBP induces an increase in the amount of ubiquitinated p53 and a decrease in the ubiquitination of MDM2. (A) H1299 cells were cotransfected as indicated. Total cell lysates were analyzed by Western blotting as indicated. (B) H1299 cells were cotransfected as in panel A. Cellular extracts were immunoprecipitated (IP) with antiubiquitin P4D1 or isotype control antibody (data not shown) followed by Western analysis (W) with an anti-p53 antibody (Ab2433). (C) H1299 cells were cotransfected as in panel A, immunoprecipitated as in panel B, and Western blotted with an anti-MDM2 antibody (Ab-1). (D) Cell extracts prepared as for panels B and C were immunoprecipitated with anti-MDM2 SMP-14 followed by Western blotting with an anti-MDM2 antibody (Ab-1).

experiments in other cells that there is an approximately 50-kDa, p53 immunoreactive band detected, although we do not know the identity of this band.

We have also examined the effect of MTBP upon MDM2 ubiquitination, and as shown in Fig. 4C, this is dramatically reduced in the presence of MTBP. Moreover, comparing Fig. 4A, C, and D, it is clear that the normal primary electrophoretic form of MDM2 (ca. 90 kDa) is markedly reduced and possibly even absent from the ubiquitin immunoprecipitation in the presence of MTBP but is substantially increased in both the lysate and when an anti-MDM2 antibody is used for the immunoprecipitation. Thus in the presence of MTBP, this primary form of MDM2 is not bound to ubiquitin and ubiquitination of MDM2 is greatly reduced.

If MTBP stabilizes MDM2 by inhibiting the autoubiquitination reaction, then a mutant of MDM2 that lacks ubiquitin ligase activity should be neither stabilized by MTBP nor should it promote MTBP-mediated enhancement of p53 down-regulation. Figure 5A shows that in H1299 cells the RING finger mutant (Cys464Ala) of MDM2 (49) is neither stabilized nor stimulated to degrade p53 by the addition of MTBP, in spite of the fact that this mutant still binds to MTBP (Fig. 5C). We conclude that MTBP acts to stabilize MDM2 by inhibiting the autoubiquitination reaction without inhibiting the ability of MDM2 to act as an E3 ligase for p53. We also conclude that the ability of MTBP to stimulate MDM2-mediated down-regulation of p53 depends upon the ubiquitin ligase activity of MDM2 encoded by the RING finger domain.

Having detected an effect of MTBP upon the ubiquitination and steady-state levels of p53, we wanted to determine whether this translated into a reduction in p53 transcriptional activity. In Fig. 6, by using an experimental design similar to that described in Fig. 2A, we have measured the level of reporter gene expression from a p53-dependent luciferase construct. Addition of MTBP alone results in a twofold reduction in p53 transcriptional activity (compare lanes 2 and 6). We have concluded from a number of observations, including the data shown in Fig. 1D, that this is the result of MTBP stabilizing endogenous MDM2 (as can also be seen in Fig. 5A, compare lanes 1 and 5, and is emphasized in Fig. 5B) in these cells. Notwithstanding this, by comparing in Fig. 6, lanes 3 with 7 in particular (5.2-fold reduction with MTBP) but also lanes 4 and 8 (2.2-fold) or 5 and 9 (4.3-fold), we can see that addition of MTBP leads to a reduction in the steady-state protein level of p53 with a concomitant reduction in p53 transcriptional activity.

To examine the physiological contribution of MTBP to MDM2 activity we used siRNA. As shown in Fig. 7A, siRNA for MTBP down-regulates transfected MTBP. This demonstrates that even supraphysiological levels of MTBP are effectively reduced by this siRNA. By using anti-MTBP serum (antisera 1) we are able to detect, albeit weakly, endogenous MTBP in a range of cells. Note that the identity of the specific MTBP band has been confirmed in multiple systems including siRNA, peptide competition, matrix-assisted laser desorption ionization mass spectrometry, and transfection experiments (data not shown). In Fig. 7B we show that the endogenous MTBP signal in MCF-7 cells is abolished by siRNA for MTBP. Under the conditions used, siRNA for MTBP also induces a significant reduction (2.3-fold) in endogenous MDM2 with a concomitant increase in the steady-state level of endogenous p53 (1.8-fold). To determine the significance of this effect we next compared the effect of siRNA for MTBP with siRNA for MDM2 and also a scrambled control siRNA for MTBP, as shown in Fig. 7C, followed by measurement of p53-mediated transcriptional activity under the same conditions. As shown in Fig. 7D, siRNA for MTBP induces an increase in p53 transcriptional activity that is smaller but comparable to that elicited by down-regulating MDM2 with siRNA. Thus, we conclude that under normal growth conditions MTBP significantly contributes to MDM2/p53 homeostasis in these cells.

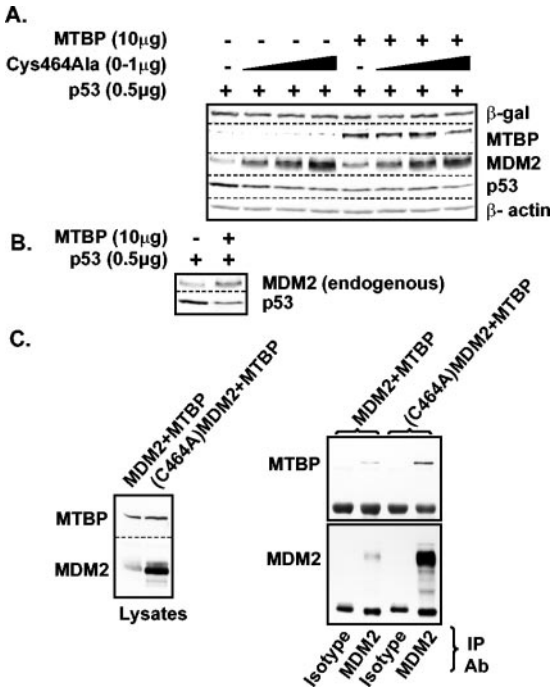

FIG. 5. The RING finger domain of MDM2 is necessary for MTBP to promote degradation of p53. (A) H1299 cells were transfected with the indicated plasmids for 24 h and cell lysates analyzed by Western blotting. (B) Elements of lanes 1 and 5 from panel A juxtaposed to facilitate visualization of the effect of MTBP upon endogenous MDM2 and concomitantly upon transfected p53. (C) H1299 cells were cotransfected with either wild-type human MDM2 and MTBP (1:1 plasmid mass ratio) or with the RING finger mutant of MDM2 (C464A) and MTBP (1:1 ratio) as indicated. The panel on the left shows the steady-state protein levels in lysates from these cells analyzed by Western blotting as indicated. The panel on the right shows Western blot analysis of immunoprecipitations (IP) performed on the same lysates. Cellular extracts were immunoprecipitated with either anti-MDM2 SMP14 antibody or an isotype control as indicated followed by immunoblotting with anti-HA antibody to detect MTBP or with anti-MDM2 antibody Ab-1 as indicated.

We have shown that in unstressed cells MTBP is a cofactor for MDM2-mediated regulation of p53. What happens when cells are exposed to p53-activating stresses? We had already seen that γ-irradiation resulted in MTBP-mediated stabilization of MDM2 in the absence of any effect upon p53 (Fig. 3B). There is an interesting difference between the regulation of the steady-state level of MDM2 following exposure to ionizing and UV radiation. Following ionizing irradiation, MDM2 levels remain constant whereas after exposure to UV MDM2 is down-regulated (41). Exposure of cells to 40-J/m^2 UV-irradi-

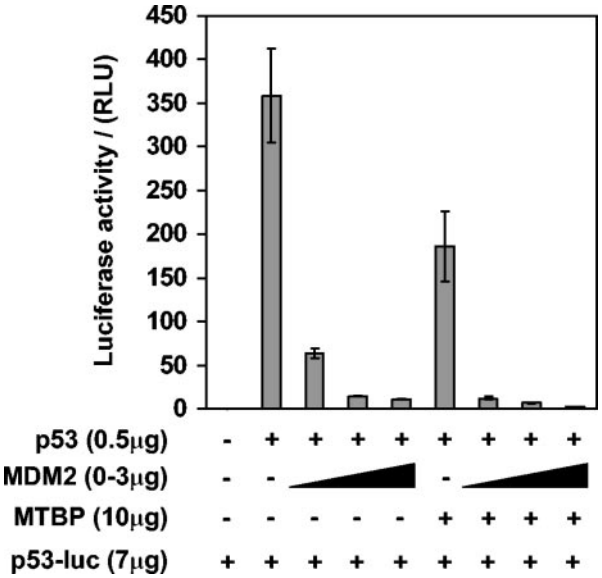

FIG. 6. MTBP promotes a reduction in p53 transcriptional activity. H1299 cells were transfected with the indicated plasmids for 24 h. Cells were then lysed and luciferase activity measured as described in Materials and Methods. Results are representative of three independent experiments. Data are shown as mean ± standard error of the mean. RLU, relative light units.

ation led to a reduction in the steady-state level of the MTBP protein (as shown in Fig. 8A). This is due to a reduction in the half-life of the protein from >2 h to ca. 60 min as Fig. 8B shows. We conclude that MTBP augments the ubiquitin ligase activity of MDM2 in unstressed cells but that in response to UV (but not γ)-irradiation, both MTBP and MDM2 are destabilized as part of the cellular response to UV-induced DNA damage.

DISCUSSION

Our results show that the MDM2 binding protein MTBP alters the ubiquitin ligase activity of MDM2 in vivo, such that it is stimulated with respect to p53 but inhibited with respect to MDM2. In support of this we have shown that in transient transfection experiments both human and murine MTBP have a similar effect, and this occurs in tumor cell lines of different origin (lung, H1299 and breast, MCF-7) and also in immortalized mouse embryo fibroblasts. The effect of ectopic expression of MTBP is entirely dependent upon the presence of MDM2 and is independent of the status of a known inhibitor of MDM2 ubiquitin ligase activity, $p14^{ARF}$, since H1299 cells possess wild-type ARF but MCF-7 cells have deletions of the *ARF* gene (31, 33). By using siRNA we also show that endogenous MTBP contributes significantly to MDM2/p53 homeostasis in unstressed cells and to the regulation of p53

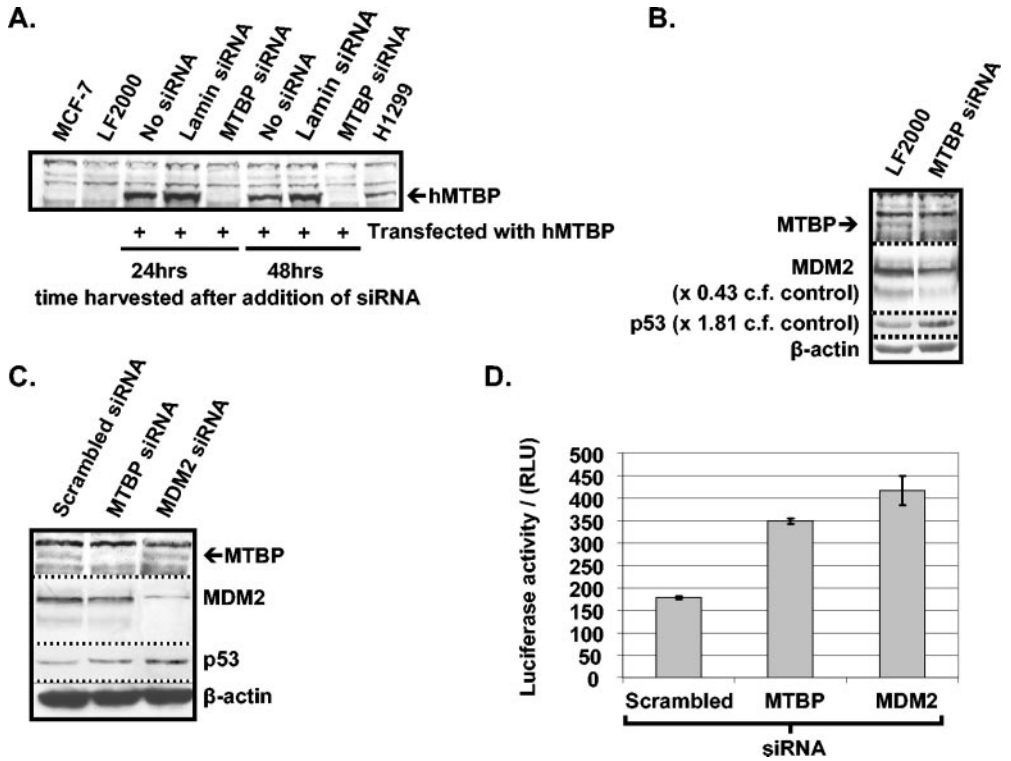

FIG. 7. Endogenous MTBP regulates MDM2/p53 homeostasis. (A) MCF-7 cells were transfected with a plasmid that expresses hMTBP as indicated and also with siRNA for the indicated times or treated with the transfection reagent alone (LF2000). MCF-7 and H1299 indicate untreated cells. Lamin siRNA was used in this experiment as a negative control. Lysates from these cells were analyzed by Western blotting with an anti-MTBP serum (antisera 1) to determine the steady-state level of MTBP protein. (B and C) MCF-7 cells were transfected with the indicated siRNA and harvested 24 h later. Lysates from these cells were analyzed by Western blotting as indicated. Densitometry in panel B was performed using KODAK 1D version 3.5 software. (D) MCF-7 cells were transfected with the indicated siRNA, and after 24 h, cells were retransfected with pp53-TA-luc plasmid. Cells were lysed and luciferase activity measured as described in Materials and Methods. Results are representative of three independent experiments. Data are shown as mean ± standard error of the mean. RLU, relative light units.

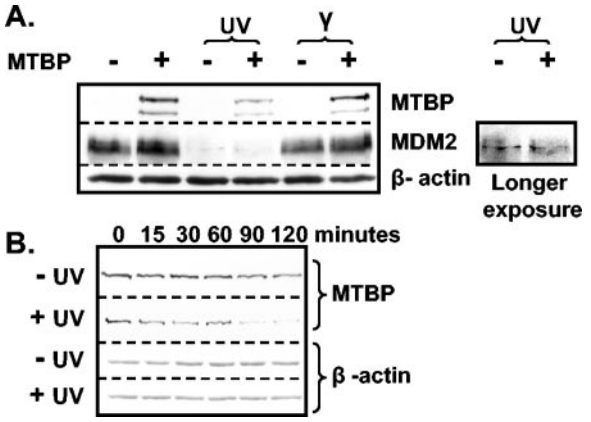

FIG. 8. MTBP stabilizes endogenous MDM2 in unstressed cells and is destabilized following exposure of H1299 cells to UV-irradiation. (A) H1299 cells were transfected with MTBP (10 μg) or empty pCEP vector control for 24 h. Cells were then either left untreated prior to harvest or exposed to UV- (40 J/m^2) or γ (5 Gy)-irradiation 6 h prior to harvesting. Cell lysates were then analyzed by Western blotting. On the right a longer exposure of the MDM2 track is shown from cells exposed to UV and transfected as indicated. (B) H1299 cells were transfected with MTBP (10 μg) for 24 h. Two hours prior to harvest, cells were either left untreated or exposed to UV-irradiation (40 J/m^2) and then all cells were treated immediately with an inhibitor of de novo protein synthesis (cycloheximide, 50 μg/ml). Cells were then harvested at the indicated times following addition of drug, and cell lysates were analyzed by Western blotting as indicated.

activity in these cells. Finally, we show that MTBP is destabilized as part of the cellular response to UV- but not γ-irradiation.

Our initial observation was that the steady-state levels of MDM2 rose when MTBP was transfected into cells. Simultaneously we noted a reduction in steady-state p53 levels. There are several important questions that we need to address. Firstly, how does MTBP differentially alter the steady-state levels of these two critical molecules in cells? Secondly, what is the physiological significance of this effect? Thirdly, can these observations be reconciled with our earlier findings that ectopic expression of MTBP inhibits cell growth by inducing G_1 arrest in a p53-independent, MDM2-sensitive manner (3)?

Regarding the first question, we have shown that by inhibiting proteasomal degradation with MG132 (Fig. 2B; we have also seen the same effect with MG115 and lactacystin) we protect p53 from degradation by MDM2 in the presence and absence of MTBP. This suggests that whatever the mechanism of action of MTBP, a major component depends upon proteasome activity. We and others have found that it is not possible to fully rescue p53 from degradation using proteasome inhibitors (48), whereas MDM2 is very efficiently rescued using any one of a range of proteasome inhibitors. This may suggest that p53 degradation is not exclusively proteasomal or at least that, when proteasomes are inhibited, other pathways or proteolytic enzymes such as calpain compensate by inactivating p53 (26). Presumably this is not the case for MDM2. Alternatively, the p53 in these cells may be partially stabilized as a result of low levels of cellular stress and this idea may be supported by our luciferase assay results on essentially unstressed cells.

If proteasomes are required for the effect of MTBP then what is the role of MDM2 in this? Clearly, as shown in Fig. 1D the effect of MTBP upon p53 depends on MDM2. The simplest explanation for this is that MDM2 is necessary because the effect of MTBP depends upon the ubiquitin ligase activity of MDM2. In support of this we found, in experiments such as those shown in Fig. 5A, that a RING finger mutant of MDM2 that lacks ubiquitin ligase activity is not stabilized by MTBP nor does it display any capacity to degrade p53 in the presence of MTBP. Interestingly, as shown in Fig. 4B and C we have seen that MTBP inhibits the autoubiquitination reaction of MDM2 while increasing ubiquitination of p53. For these experiments we have used two different detection antibodies for MDM2 and p53 and have obtained similar results with each (data not shown). This observation cannot be explained simply by MTBP inhibiting MDM2 E3 ligase activity, so how does MTBP promote this effect? One possibility is that MTBP physically protects sites of MDM2 ubiquitination while leaving the E3 ligase activity intact. An alternative possibility is that MTBP binding to MDM2 alters the rate of transfer of ubiquitin such that there is a relative increase in the *trans* reaction compared to the *cis*. Future in vitro experiments will be required to try to resolve this question.

In addition to our own studies there is recent circumstantial evidence which supports our identification of MTBP as an important regulator of MDM2 ubiquitin ligase activity. We previously showed that MTBP binds to a region bounded by amino acids 167 to 304 (3) encompassing the central acidic region of MDM2. Two recent studies have found that this region of MDM2 is essential, though not sufficient, for MDM2 to transfer ubiquitin to p53 in vivo (23, 34). We have observed low levels of MTBP in several cell lines including the H1299 and U2-OS lines used in the above studies (unpublished data) and therefore this low-level endogenous MTBP may provide the necessary but not sufficient effect described. Thus, MTBP may provide an explanation for the effects observed by these authors.

The second major question we wanted to address was that of the physiological significance of MTBP regulation of MDM2 and p53 levels. As mentioned above, others have seen that the region of MDM2 to which MTBP binds is critically involved in the efficient transfer of ubiquitin to p53, for as-yet-unidentified reasons, and this could reflect the effect of MTBP. To investigate the question of physiological relevance we examined the effect of down-regulation of endogenous MTBP with siRNA. As we showed in Fig. 7, this results in a reduction in the steady-state level of MDM2 with a concomitant increase in the p53 steady-state level which, not surprisingly, also results in a 1.8-fold increase in p53-dependent transcription. Given that down-regulation of MDM2 with siRNA leads to an approximately 2.3-fold increase in p53 activity, we conclude that MTBP contributes significantly to the regulation of MDM2/p53 homeostasis in unstressed cells. We also wanted to examine what happens in response to a physiological insult such as irradiation. Whereas γ-radiation efficiently protected p53 from MDM2, in the presence and absence of MTBP, without having any effect upon the level of either of these proteins, UV-radiation induced a substantial reduction in the level of MTBP (Fig. 8A) and also, as expected at this early time point, of MDM2 (41). Figure 8B shows that in response to UV-irradiation the half-life of MTBP is reduced, and this accords well with our observed reduction in steady-state levels. It is impor-

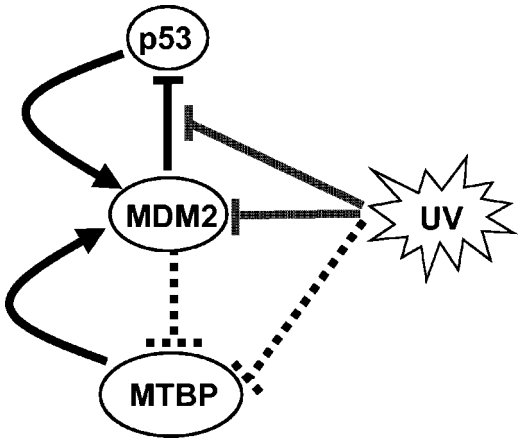

FIG. 9. A proposed model of the relationship between MDM2, p53, and MTBP. Solid arrows are based upon experiments with physiological levels of MTBP and dashed arrows are based upon studies involving ectopic expression.

tant to point out that if MTBP still stabilized MDM2 under these conditions, then we would expect that the reduced steady-state levels of MDM2 protein would still reflect the prestress ratios, but as shown in the longer exposure for Fig. 8A, lanes 3 and 4, this is apparently not the case.

The data presented here show that MTBP can act as a significant cofactor for efficient MDM2-mediated degradation of p53 in unstressed cells. Thus we arrive at a model that integrates MTBP into the MDM2-p53 pathway as illustrated in Fig. 9. Similar to the interaction of p53 with MDM2, there may exist a feedback loop between MDM2 and MTBP. We have previously found that high-level expression of MTBP induces growth arrest in a p53-independent manner (3). However, MDM2-mediated ubiquitination of p53 is stimulated by MTBP. It is possible that ectopic expression of MTBP leads to supraphysiological stimulation of MDM2 (we have not yet tested whether MTBP can induce cell growth arrest in *MDM2*$^{-/-}$ cells), leading to the degradation of additional targets, and clearly this possibility requires investigation. Indeed, transfection of the MDM2 cDNA inhibits cell proliferation in many cell lines (4). A recent study found that overexpression of truncated forms of MDM2 could also inhibit proliferation in primary MEFs and that the RING finger domain was both necessary and sufficient for this effect (7). Might MTBP induce growth arrest by altering MDM2 activity, similar to the effect of transfection of truncated forms of the gene? This possibility may be increasingly attractive given that MTBP has differential effects upon the *cis* and *trans* ubiquitination activities described here. To address this possibility we are currently testing whether MDM2, or parts thereof, is necessary for the growth inhibitory effect of MTBP in *MDM2*$^{-/-}$ MEFs. Notwithstanding this, it would be hard to reconcile this hypothesis with the fact that ectopic expression of MDM2 can overcome the growth inhibition induced by MTBP (at least in H1299 and U2OS cells) (3). We have not observed anything that would indicate that MTBP is targeted for degradation by MDM2. Therefore the ability of MDM2 to eliminate the growth inhibitory activity of MTBP most likely requires an alternative explanation. MTBP is a protein of ca. 900 amino acids in both humans and mice. There is little homology to any other known mammalian gene with the only identified similarity to the yeast *BOI* genes being located in the carboxy terminus. This same region is necessary for interaction of MTBP with MDM2. It is quite possible therefore that other domains of MTBP mediate a growth inhibitory activity when expressed supraphysiologically. This effect would have to retain sensitivity to MDM2, and one way to envisage this dominance of MDM2 might be through regulation of MTBP subcellular distribution, as occurs with some other MDM2 interacting proteins (14). Our studies to date have utilized different ratios of MTBP and MDM2 plasmids; for MTBP stabilization of MDM2, MTBP is in excess by plasmid weight, whereas for MDM2 elimination of MTBP-mediated growth arrest the converse is true. Whatever the mechanism, we have identified several additional cell lines that are also sensitive to MTBP-induced growth arrest (data not shown) and so far this effect of MTBP is a general one. One of the obvious concerns with all of these experiments is the use of supraphysiological expression of MTBP. The experiments presented here using siRNA to regulate the levels of endogenous MTBP or MDM2 greatly increase our confidence in the physiological relevance of the pathway that we have discovered linking MTBP with MDM2/p53 homeostasis.

The p53-MDM2 autoregulatory pathway is becoming more and more complex. In addition to well-studied molecules that impinge on the pathway such as ARF and MDMX, new members are still being discovered such as COP1 (9) and Pirh2 (29). We now add a previously described MDM2 binding protein MTBP to this mix in a previously unsuspected role as a cofactor for MDM2.

It is vitally important for a metazoan organism to tightly and precisely regulate the activity of p53 to prevent inappropriate activation leading to cellular, or more catastrophically, organismal death. To achieve this tight regulation it is necessary to maintain the correct balance between p53 and MDM2, the levels of which vary considerably from tissue to tissue. MDM2 is constitutively expressed at a wide range of levels with the highest being found in the testis and ovaries (10). Interestingly, these same tissues also express the highest levels of MTBP mRNA (3) and protein (our unpublished results). Coincidentally, the substantially overlapping patterns of high expression of MTBP and MDM2 occur in tissues in which there are high levels of proliferation, and it is perhaps not surprising that these same tissues are (according to the current ICRP 1990) also the most radio sensitive in the body. In these cells there may be a greater need to ensure that p53 is not inadvertently activated and by expressing MTBP these cells increase the effect of MDM2 and thus, presumably, reduce this risk. The difference in regulation of MDM2 and MTBP stability in response to exposure to UV- and γ-radiation is intriguing. Clearly, this difference must reflect upstream differences in the response to these forms of cellular stress and it will be interesting to further investigate whether there is a connection between MTBP stability and activation of, for example, ATR by UV. MDM2/p53 homeostasis must be maintained for mammalian viability. By inhibiting the *cis* and promoting the *trans* reaction of the MDM2 E3 ubiquitin ligase, the role that we have discovered for MTBP may well provide a function that is essential for life.

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