Mouse Double Minute 2 Associates with Chromatin in the Presence of p53 and Is Released to Facilitate Activation of Transcription

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Abstract

The tumor suppressor p53 is a potent transcription factor of which the ability to mediate transcription is inhibited through an interaction with the oncoprotein mouse double minute 2 (Mdm2). The present study has tested the hypothesis that Mdm2 inhibits the p53 response in normally growing cells by binding to chromatin-associated p53. Using chromatin immunoprecipitation, we show that Mdm2 localizes with p53 at its responsive elements on the *waf1* and *mdm2* genes in human cell lines expressing p53, but not in cell lines lacking p53 expression, indicating that Mdm2 is recruited to regions of DNA in a p53-dependent manner. Interestingly, our results show a decrease of Mdm2 protein associated with p53-responsive elements on the *waf1* and *mdm2* genes when p53-induced transcription is activated either by DNA damage or through controlled overexpression of p53. Rapid activation of p53 transcriptional activity before increasing p53 protein levels was observed with addition of either small-molecule inhibitors to disrupt the p53-Mdm2 interaction or small interfering RNA to *mdm2*. These findings indicate Mdm2 transiently localizes with p53 at responsive elements and suggest that latent p53 results from the recruitment of Mdm2 to chromatin.

Introduction

In response to genotoxic stress, the tumor suppressor p53 conducts numerous pathways such as cell cycle arrest, apoptosis, and DNA repair, all averting cellular transformation. Acting principally as a transcription factor, p53 binds to DNA as a tetramer, localizing to specific p53-responsive elements on genes, the protein products of which mediate the p53 pathway (1). In the absence of stress, p53 levels remain low due in part to the association of p53 with its negative regulator, mouse double minute 2 (Mdm2; refs. 2, 3). *Mdm2* is a p53 target gene (4), the protein product of which regulates p53 activity by multiple mechanisms. Mdm2 protein binds to a region on the NH2 terminus of p53 and inhibits the transcriptional activity of p53 by blocking the ability of p53 to associate with transcriptional machinery (5, 6). In addition, Mdm2 acts as an E3 ubiquitin ligase targeting p53 for degradation in proteasomes (7, 8). However, hypomorphic *mdm2* mice that express reduced levels of Mdm2 have enhanced p53 activity without increased p53 protein levels, showing that targeted degradation of p53 by Mdm2 protein is not the only way Mdm2 inactivates p53 (9). Our recent results in cancer cells containing a single nucleotide polymorphism at position 309 in the *mdm2* gene promoter have shown a mechanism for inactivation of p53 that results from a chromatin-associated p53-Mdm2 complex rather than degradation of p53 protein (10). This inactivation of p53 in human cancer cells may be an enhanced form of inactivation that occurs as a basic regulatory mechanism in normal cells.

In the presence of cellular stress (e.g., DNA damage), stress kinase pathways signal to and phosphorylate p53 on numerous sites. Phosphorylation of p53 on amino acids within the Mdm2 binding site (e.g., serine 15, threonine 18, and serine 20) disrupt the Mdm2-p53 complex formation (11–13) and promote the transcriptional activity of p53. Recent studies have begun to delineate how Mdm2 mediates transcription of its target genes by determining which cooperative factors colocalize with p53 at its responsive elements. The regions encompassing the p53 binding sites on target genes, such as *mdm2* and *gadd45*, have been found to be nucleosome-free and poised for transactivation (14–16). Studies using chromatin immunoprecipitation have found that p53 bound to its responsive elements before gene activation. Furthermore, increasing levels of p53 localize to these specific DNA binding sites following activation by stress (17, 18). A DNA damage signal, however, is not required for p53 to associate with DNA (19). Using chromatin immunoprecipitation, overexpressed p53 has been shown to associate with p53-responsive elements on target genes in the absence of DNA damage (20, 21). Chromatin immunoprecipitation has shown that p53 promotes transcription by colocaling with numerous components of the transcription initiation complex (18), the histone acetyltransferases GCN5 and p300 (20), and the histone acetyltransferase complex scaffolding protein TRRAP (22). It is not known, however, whether p53, bound to the responsive elements of its target genes, is associated with Mdm2 before the initiation of transcription and/or if this association is relieved on p53 target genes during transcription initiation. Recently, Minsky and Oren (23) have shown that overexpressed oncogenic Mdm2 directs histone ubiquitination modifications by binding to chromatin in a complex with p53, and in this way may repress p53-activated transcription during oncogenic transformation. We have examined the role of Mdm2 in chromatin-mediated regulation of p53-dependent *mdm2* and *waf1* transcription when Mdm2 is not overexpressed. We found that normally expressed Mdm2 was capable of chromatin association in the presence of p53. Our study indicates for the first time that Mdm2 expressed at normal levels in growing cells associates with p53-responsive elements in the presence of chromatin-associated p53 protein. A mathematical model proposed by Lev Bar-Or et al. (24) predicts oscillations in p53 and Mdm2 protein levels due to the feedback between these two proteins, which has been confirmed in cells treated with ionizing radiation. In this study, we observed another type of oscillation involving the release of Mdm2 from stable DNA-bound p53, which allows for activation of p53 transcriptional activity. Rapid
activation of p53 transcriptional activity after the addition of either Nutlins, to disrupt the p53-Mdm2 complex (25), or small interfering RNA (siRNA) to mdm2 suggested that p53 is poised on the chromatin waiting to be released from its inhibitor. Interestingly, Mdm2 can cycle off chromatin sites during the activation of p53 target gene transcription after DNA damage treatment. Taken together, our data suggest that the dissociation of Mdm2 from p53-responsive genes is important in promoting p53 function and, therefore, constitutive Mdm2-p53 chromatin interaction could lead to tumor promotion.

**Materials and Methods**

**Reagents.** Etoposide and doxycycline were purchased from Sigma (St. Louis, MO); fetal bovine serum (FBS), gentamycin (G418), and the penicillin-streptomycin solution were purchased from Gemini. McCoy’s 5A and RPMI 1640 were purchased from Mediatech (Herndon, VA). Hygromycin B and Nutlins were purchased from Calbiochem (San Diego, CA) and the siRNA to mdm2 was from Dharmacon (Lafayette, CO).

**Cell culture.** ML-1 (a generous gift from M. Kastan, Hematology-Oncology, St. Jude Children’s Research Hospital, Memphis, TN) and K562 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 containing 10% FBS and 2,500 units of penicillin-streptomycin. DLD-1 cells (American Type Culture Collection, Manassas, VA) were grown in McCoy’s 5A medium containing 10% FBS and 2,500 units of penicillin-streptomycin. DLD-1 cells were grown in McCoy’s 5A medium containing 10% FBS and 2,500 units of penicillin-streptomycin whereas D-A2 cells required additional 0.4 mg/ ml G418, 20 mg/ml doxycycline, and 0.25 mg/ml Hygromycin B (both lines were a generous gift from B. Vogelstein, Oncology Center, Johns Hopkins School of Medicine, Baltimore, MD). All cells were incubated with 5% CO2 (37°C).

**Protein extract preparation.** Protein extracts were prepared as described by Dinigam et al. (26) with additional 0.1 mL/mg trichostatin A (Sigma) and 1% phosphate inhibitor cocktail 1 (Sigma) to the lysins buffers.

**Western blot analysis.** Samples were electrophoresed (10% SDS-PAGE), electrotransferred to nitrocellulose (Amersham, Piscataway, NJ), probed with p53-specific AB-6 or anti-phosphoserine-15 p53 (Oncogene Research, San Diego, CA), or Mdm2-specific antibodies D-7 or SMP-14 (Santa Cruz Biotechnology, Santa Cruz, CA), or with antiactin (Sigma), and visualized by chemiluminescence.

**Quantitative reverse transcription-PCR.** RNA was isolated using QiA shredder columns and the RNAeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of RNA were used for cDNA synthesis using the high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The primer probes for gapdh (Applied Biosystems Pre-developed Assay Reagents) and waf1/cip1 and mdm2 (exons 5 and 6; Applied Biosystems, Celera Discovery Systems Assays on Demand) were used for TaqMan PCR using the Applied Biosystems 5700 Sequence Detection System (Perkin-Elmer Life Sciences, Wellesley, MA) as follows: one cycle, 2 minutes (50°C), one cycle, 10 minutes (94°C), and 40 cycles, 15 seconds (94°C) and 1 minute (60°C).

**Chromatin immunoprecipitation.** Boiled 37% formaldehyde was diluted to an 11% solution in solution 1 [0.1 mol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L HEPES (pH 8.0)], added to cells to a 1% solution, and incubated for 30 minutes (37°C). Glycine was added to 0.125 mol/L and incubated for 5 minutes (room temperature). The cells were washed twice with cold PBS, spun down at 1,850 rpm for 7 minutes (4°C), resuspended in 5 mL of 100 mmol/L Tris-Cl (pH 9.4) with 20 mmol/L DTT, incubated for 15 minutes (30°C), and spun down at 2,000 rpm for 5 minutes (4°C). The pellet was resuspended in 2 mL of solution 2 [0.25% Triton X-100, 0.5% IGE-PAL (Sigma), 10 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris-Cl (pH 8.0), 1 mol/L phenylmethylsulfonyl fluoride (PMSF)] incubated for 10 minutes (ice), spun down, resuspended in 2 mL of solution 3 [0.2 mol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris-Cl (pH 8.0), 1 mol/L PMSF], sonicated 10 times for 10 seconds (ice), and spun down at 14,000 rpm for 10 minutes (4°C). Radioimmunoprecipitation assay buffer 10× (1% Triton X-100, 1% deoxycholate, 1.4 mol/L NaCl, 1% SDS) was added to each supernatant to make each 1×; 0.2 mL was designated “input.” Antibody, 2 µg of AB-6, D-7, 10 µg of 482, or anti-phosphoserine-15 p53 (1:200) was added to each immunoprecipitant and rocked overnight (4°C). Fifty microliters of Protein A/G Plus beads (Santa Cruz) were added, rocked for 2.5 hours (4°C), and spun down at 3,400 rpm for 2.5 minutes (4°C). The beads were washed once in wash 1 [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), 150 mmol/L NaCl], wash 2 [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), 500 mmol/L NaCl], wash 3 [0.25 mol/L LiCl, 1% IGE-PAL, 1% Deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-Cl (pH 8.1)], and twice in Tris-EDTA buffer [10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA (pH 8.0)], rocked each time for 10 minutes (4°C) and spun down. Then 0.1 mL of elution buffer (1% SDS, 0.1 mol/L NaHCO3) was added to immunoprecipitated and input samples and incubated overnight (65°C). The resulting DNA was isolated using the QIA quick PCR Purification Kit (Qiagen).

**PCR and quantitative analysis of chromatin immunoprecipitation samples.** Five microliters of the total 50-µL volume of isolated DNA were used in each 25-µL PCR reaction [50% TaqMan Universal PCR Mix (Applied Biosystems), 0.3 pmol/µL primers] with the quantitative PCR conditions for the number of cycles warranted. Radioactive dATP was included in some reactions to increase the signal. The primer sequences were as follows: mdm2 forward, 5’-CGGGAGTTCAGGGTAAAGGT-3’; reverse, 5’-AGCAAGTTCGTTCTACTG-3’; waf1/cip1 forward, 5’-GTGGCCTCTATGGGCTTTCTG-3’; reverse, 5’-CTGAAAACGGGACCCAA-3’. The PCR reaction was electrophoresed by 8% PAGE. Quantitative PCR using chromatin immunoprecipitation samples showed fold change in the specific binding normalized to gapdh and mock immunoprecipitation values. The primer probes used for waf1 chromatin immunoprecipitation were as previously described by Kaaser and Igo (21).

**siRNA.** Mdm2 expression was lowered using mdm2 siRNA (SmartPool, a mixture of four different mdm2 siRNAs; Dharmacon). siRNA, 200 pmol, was transfected into cells at 70% to 80% confluence using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Control siRNA (SmartPool, Dharmacon) has no known target in mammalian genomes.

**Results**

p53 target genes are up-regulated by high levels of p53 protein in the presence or absence of DNA damage. The relationship between Mdm2 and p53 in the regulation of cell growth has been examined in numerous studies, but how these two antagonistic proteins interrelate on chromatin has only begun to be examined. We have studied the localization of p53 and Mdm2 on the p53-responsive elements of two p53 downstream target genes, mdm2 and waf1. The interaction of Mdm2 with p53 protein on chromatin during the process of gene activation has not been examined. To examine the interaction of these two proteins when high levels of p53 were expressed in the absence of stress, we used the p53-inducible cell line D-A2. p53 induction after removal of doxycycline (−DOX) for 6 hours results in an induction of both mdm2 and waf1 transcripts (27). Etoposide treatment has been shown to induce global up-regulation of p53 targets, including waf1 and mdm2, in cells that express wild-type p53 (28). Using quantitative reverse transcription-PCR (RT-PCR), we confirmed that high levels of p53 in D-A2 cells after removal of doxycycline (−DOX) for 6 hours resulted in up-regulated mdm2 and waf1 both in the absence or presence of DNA damage (Fig. 1A). We were able to significantly activate the early transcriptional activity of the p53 (in the absence of increasing the p53 protein level) in the D-A2 cells by subjecting them to 0.5 mol/L etoposide treatment for 24 hours before doxycycline withdrawal (Fig. 1B, +DOX and −DOX, 1.5-, 3-, and 6-hour samples; Fig. 1C and D for protein). This increased

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activation of target genes indicated that the etoposide treatment influenced the process.

The levels of p53 protein in the D-A2 cells under the different conditions were analyzed by Western blot analysis using p53-specific antibodies (Fig. 1C and D, p53). No p53 was detected in the parental DLD-1 cell line (Fig. 1C, lane 1). High exposures of D-A2 (+DOX) cell extract Western blots revealed low-level p53 expression in the absence of doxcycline withdrawal (data not shown) and great p53 induction after 6 hours of doxcycline withdrawal (Fig. 1C, compare lanes 2 and 3). Consistent with previous work in our lab showing that DNA damage treatment does not change the level of p53 expressed by the tetracycline-regulated constructs in TR9-7 cells (16), no significant change in induced p53 protein level was detected in the presence of etoposide for 4 or 24 hours, respectively (Fig. 1C, lanes 3-6, and Fig. 1D, lanes 4 and 5). We examined by Western blot with a p53 phosphospecific antibody if etoposide treatment induced p53 phosphorylation at serine 15 (Fig. 1C, middle). Phosphorylation of p53 protein at serine 15 activates p53 (29-31) and is indicative of the convergence of stress responsive pathways on p53 (29, 32). Serine 15 phosphorylated p53 was observed in D-A2 cells grown in the absence of doxcycline (−DOX), potentially due to the stress of the overexpression system (Fig. 1C, lane 3). Slightly higher levels of phosphoserine-15 p53 were observed when the cells were treated with etoposide for 4 hours (Fig. 1C, lanes 4-6). When pretreated with etoposide for 24 hours before doxcycline withdrawal (−DOX), the cells gave an increase in activation of p53 target genes without or with doxcycline withdrawal (Fig. 1B). In −DOX samples that received 24-hour etoposide treatment before doxcycline withdrawal, no increase in p53 protein or Mdm2 protein resulted from the added DNA damage (Fig. 1D, lanes 4 and 5). Importantly, the transcriptional activity of p53 was increased by the 24-hour etoposide pretreatment in the presence or absence of doxcycline beginning from early time points (Fig. 1B), suggesting that the change in p53 activity was due to increased p53 transcriptional activity and not the amount of p53 protein. Activation of p53 transcription in etoposide-treated D-A2 cells +DOX (Fig. 1B) was explained by observing p53 and Mdm2 proteins in overexposed Western blots (data not shown). We will therefore discuss +DOX D-A2 cells from here on as low-level p53-expressing cells and the isogeneic cell line DLD-1 will be compared for no wild-type p53 activity.

Mdm2 requires p53 to localize on chromatin and dissociates after DNA damage. Chromatin-associated p53 has been detected at the waf1 and mdm2 response elements but increased levels of p53 protein do not always result in increased p53 chromatin association or increased target gene activation (17, 21). Due to the fact that p53 protein levels do not show a correlation with the p53 chromatin association, we predicted that other mechanisms, in addition to the control of p53 protein level, might be involved in the regulation of p53 target gene activation. The Mdm2 protein is known to associate with and inhibit p53 transactivation function (7, 8). We hypothesized that latent/inactive p53 resulted from Mdm2 protein associated with chromatin-bound p53 protein. Increased p53 activity was observed in D-A2 cells when they were treated for 24 hours with etoposide. We examined if a release of Mdm2 from chromatin was associated with this increased p53 activity. Using chromatin immunoprecipitation, we found less Mdm2 protein localized on the chromatin of the p53-responsive elements in D-A2 cells pretreated with etoposide before the removal of doxcycline (Fig. 2A and B). The amount of immunoprecipitated waf1 DNA resulting with Mdm2- or p53-specific antibodies was quantified by real-time PCR (Fig. 2A), p53 protein associated with waf1 chromatin in D-A2 cells without and with etoposide treatment (Fig. 2A, p53 ChIP histogram and gel). Strikingly, the association of Mdm2 protein was significantly

Figure 1. p53 target genes are up-regulated by high levels of p53 protein in the presence and absence of DNA damage. A, the levels of mdm2 and waf1/cip1 transcripts were determined by quantitative PCR carried out on cDNA synthesized from D-A2 cells grown in the presence (+DOX) or absence (−DOX) of doxcycline for 6 hours. Where indicated, samples were treated with etoposide (ETOP) for 4 hours following 2 hours of growth in the absence of doxcycline. Samples were normalized using TaqMan probes for gapdh. The fold induction was calculated over the D-A2 (+DOX) sample. Representative of two independent experiments. B, the levels of mdm2 and waf1/cip1 transcripts were determined by quantitative PCR carried out on cDNA synthesized from D-A2 cells treated with etoposide for 24 hours before the absence of doxcycline (−DOX) for 1.5, 3, or 6 hours as indicated. C, nuclear extracts were isolated from DLD-1 cells (lane 1), D-A2 cells (+DOX, lane 2), D-A2 (−DOX, lane 3), or D-A2 treated with increasing dosages of etoposide (−DOX, lanes 4-6). One hundred micrograms of the resulting nuclear extract were electrophoresed by 10% SDS-PAGE and subjected to immunoblotting using pAb1801 (top) or an anti–phosphoserine-15 p53 antibody (middle). To show equal loading between lanes, these samples were also immunoblotted for actin (bottom). D, nuclear extracts were isolated from D-A2 cells treated with 5 μmol/L etoposide or 10 μmol/L Nutlin for 24 hours as indicated (+DOX or with subsequent −DOX for 6 hours).
decreased by the etoposide treatment (Fig. 2A, Mdm2 ChIP histogram and gel). The association of Mdm2 with waf1 chromatin required p53 as no p53 or Mdm2 localized to the chromatin in DLD-1 cells lacking wild-type p53 (Fig. 2A, histogram, and Fig. 2C). In D-A2 cells, etoposide treatment also provoked the release of Mdm2 protein from the mdm2 gene whereas p53 protein remained associated (Fig. 2B, lanes 3 and 4 and lanes 7 and 8). In the DLD-1 cells without p53, no association of p53 or Mdm2 was observed on either the waf1 or mdm2 gene above the bead background (Fig. 2A-C). Taken together, these data show that the Mdm2 protein associates with the chromatin of p53-responsive elements in the presence of p53 and can be released by etoposide treatment.

The ability of etoposide treatment to induce the association of phosphorylated p53 with chromatin was examined using phosphospecific p53 antibodies. Decreased quantities of chromatin were immunoprecipitated with phosphoserine-15 p53-specific antibodies from D-A2 cells that had not been treated with etoposide (Fig. 2D, compare lane 5 to lane 6 in mdm2 gene panel and waf1 gene panel), indicating that before etoposide treatment, the chromatin-associated p53 (identified by p53-specific antibodies) was not phosphorylated at serine 15 (Fig. 2D, compare lanes 3 and 4 to lanes 5 and 6). Thus, there is a correlation between chromatin-bound phosphorylated p53 and the release of Mdm2 from the chromatin.

Mdm2 dissociates from chromatin during p53-activated transcription. We found Mdm2 and p53 colocalized on the waf1 and mdm2 genes in D-A2 cells that were not treated with etoposide. Again, it is important to stress that we have determined D-A2 cells +DOX to be low-level p53-expressing cells and p53 and Mdm2 protein levels are increased substantially after doxycycline removal (Fig. 1D, compare lanes 1 and 4). We analyzed the dynamics of p53-Mdm2 associations on chromatin in D-A2 cells when doxycycline was withdrawn in the absence of etoposide treatment. Less Mdm2 protein was detected on the waf1 and mdm2 genes at 0.5, 1.5, and 3 hours during p53-dependent transcription (Fig. 2D, compare lane 5 to lane 6 in mdm2 gene panel and waf1 gene panel), indicating that before etoposide treatment, the chromatin-associated p53 (identified by p53-specific antibodies) was not phosphorylated at serine 15 (Fig. 2D, compare lanes 3 and 4 to lanes 5 and 6). Thus, there is a correlation between chromatin-bound phosphorylated p53 and the release of Mdm2 from the chromatin.

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transactivation after doxycycline withdrawal (Fig. 3A, lanes 6-9). However, Mdm2 protein was reproducibly seen to reassociate with the chromatin at later time points (Fig. 3A, lanes 17 and 18). When a low level of p53 was present before doxycycline withdrawal, antibodies to p53 and Mdm2 proteins immunoprecipitated modest quantities of chromatin from both mdm2 and waf1 genes (Fig. 3A, lanes 2 and 3). Interestingly, p53 association with the chromatin increased slightly whereas Mdm2-specific antibodies reproducibly precipitated less DNA as early as 30 minutes following the induction of p53, indicating a reduction in Mdm2 protein associated with the mdm2 and waf1/p53-responsive elements during increased initiation of transcription (Fig. 3A). The amount of DNA immunoprecipitated with p53-specific antibodies increased slightly over time, confirming that p53 association with these regulatory sites during the doxycycline withdrawal period was increased (Fig. 3A). After 6 or 9 hours of doxycycline withdrawal, Mdm2 protein association with the chromatin was observed (Fig. 3A, lanes 17 and 18). The presence of Mdm2 and p53 proteins on the responsive elements after 6 and 9 hours of doxycycline removal indicates that after a period of transcriptional activity, Mdm2 relocalizes with p53 on chromatin. The re-formation of this Mdm2-p53 complex may serve to initiate down-regulation of the p53 response after a short lag.

To address the possibility that the association of a p53-Mdm2 complex with chromatin might correspond with the inhibition of p53-mediated transactivation in low-level p53-expressing cells, as well as when p53 is induced, we examined early activation of p53-mediated transcription. For this, we employed the small-molecule inhibitors of the p53-Mdm2 interaction, called Nutlins, and examined their influence on the level of waf1 and mdm2 transcription in the presence of doxycycline and with 1.5, 3, and 6 hours of doxycycline withdrawal. Significantly, we saw that the addition of Nutlins to the growth medium activated p53 transcriptional activity at all time points of doxycycline withdrawal (Fig. 3B) whereas no difference in p53 or Mdm2 protein level was caused by the addition of Nutlins in the doxycycline withdrawal samples (Fig. 1D, compare lanes 4 and 6). Interestingly, the addition of Nutlins in the presence of doxycycline not only confirmed the leaky nature of the D-A2 cells but also showed that the lack of waf1 and mdm2 gene activation by low levels of p53
could be reversed by targeting the p53-Mdm2 interaction (Fig. 3B). Furthermore, siRNA to mdm2 also significantly activated waf1 transcription in D-A2 cells in the presence of doxycycline while blocking mdm2 (Fig. 3C). A dynamic equilibrium hypothesis is further supported by the fact that the level of p53-activated transcript increased after 3 and 6 hours of doxycycline withdrawal but decreased by 9 hours (following a lag after the reassociation of Mdm2 with the chromatin; Fig. 3D).

Mdm2 requires p53 to localize to chromatin in hematopoietic cells. We examined Mdm2 localization on chromatin in cells that have a well-documented wild-type p53 response after DNA damage to further investigate a dynamic equilibrium hypothesis. The myeloid leukemia cell line ML-1 has been used in numerous studies examining p53 activation following stress (33–36). The p53-null erythroid leukemia cell line K562 was used to examine the association of Mdm2 with chromatin in the absence of p53. Transcriptional activation of mdm2 and waf1 genes after etoposide treatment was analyzed by quantitative RT-PCR. Etoposide treatment of ML-1 cells promoted high levels of mdm2 transcripts; however, no significant induction of mdm2 was observed in K562 cells treated with etoposide (Fig. 4A). The profile of gene activation over time showed that mdm2 transcription was increased after 3 and 6 hours of etoposide treatment (Fig. 4A) whereas the Mdm2 protein level was not substantially increased (Fig. 4B, lanes 1-3). The p53 protein was stabilized after 3 and 6 hours of etoposide treatment (Fig. 4B, lanes 2 and 3). In addition, chromatin immunoprecipitation with p53-specific antibody detected chromatin-associated p53 at both 3- and 6-hour time points (Fig. 4C, lanes 3 and 4, p53) whereas Mdm2-specific antibodies were able to immunoprecipitate the p53-responsive element only from the 6-hour etoposide-treated sample (Fig. 4C, lane 4, Mdm2). There was no association of Mdm2 or p53 protein detected with the chromatin in K562 cells which lack p53 (Fig. 4D, lanes 2-4). Similar levels of chromatin were obtained from each input portion removed from the samples, indicating that the DNA levels before immunoprecipitation were normalized. The fact that Mdm2 protein did not associate with the chromatin until after 6 hours of etoposide treatment (Fig. 4C, lane 4) further supports the idea of a dynamic cycling of p53-Mdm2 complexes with chromatin. Such complexes would then result in the attenuation of transcription after a short lag, as activation remained high at the 6-hour time point but was reduced after 12 hours of etoposide treatment (Fig. 4A). These data support our findings in D-A2 cells showing that Mdm2 association with chromatin changes during the activation of p53 target genes.

**Discussion**

The interaction of p53 protein with chromatin has been shown by both chromatin immunoprecipitation and in vivo footprinting (15–18, 37). Whereas this p53-chromatin interaction has been determined and an association between p53 and Mdm2 has been well described (38, 39), the colocalization of these two proteins on chromatin continues to require investigation. To determine if Mdm2 protein was able to localize with p53 on chromatin, we examined two p53-responsive genes (mdm2 and waf1) in...
different cell lines under different conditions. Mdm2 localized to chromatin in a manner that suggested a mode for transiently attenuating p53 transcriptional activity. When considering that the Mdm2-p53 feedback loop is cyclical, we reasoned that repression of p53 function on chromatin should also be cyclical.

Mdm2 inhibits the ability of p53 to promote transcription (5, 6). However, there has been a latent versus active p53 paradox with regards to chromatin-associated p53 that has remained unexplained. The p53 protein has been shown to associate with chromatin in an inactive latent state. The coassociation of Mdm2 protein with p53 protein on chromatin is one way to explain this paradox of latent chromatin-associated p53. Our data indicate that Mdm2 protein can localize with p53 protein at responsive elements before the activation of transcription. Following activation of the p53 protein, we found that Mdm2 disassociated from the chromatin and then returned at later time points, most likely reforming an inhibitory complex. We observed similar phenomena on p53 target genes in the p53-inducible cell line D-A2 and the wild-type p53–containing cell line ML-1. Although the proposed Mdm2-mediated chromatin repression model probably does not account for all the inhibition of p53 activity noted under different conditions, it can explain the latent p53 paradox (see Fig. 5).

Importantly, we found Mdm2 protein bound to chromatin when p53 protein was chromatin associated. No Mdm2 protein was detected on the p53-responsive elements of either mdm2 or waf1 genes in the cell lines DLD1 or K562, which lack wild-type p53. Minsky and Oren (23) have shown the association of a p53-Mdm2 complex with chromatin in a cell line overexpressing Mdm2 and that Mdm2 chromatin colocalization requires p53. Our data extend this observation to include cells that do not overexpress Mdm2 but have p53-Mdm2 chromatin complexes. The localization of Mdm2 protein with p53 protein on the p53 binding sites of mdm2 and waf1 genes correlated with down-regulation of the level of transcription evident from these loci. However, the transcriptional activity at these loci increased when the cells were treated with etoposide, which correlated with the release of Mdm2 from the chromatin. Additionally, we also saw levels of mdm2 and waf1 transcription increase for a short period after doxycycline removal. Association of the Mdm2 protein with the chromatin on the mdm2 and waf1 genes in the D-A2 cell line preceded decreased p53-dependent transcription. Mdm2 association with p53 protein has been shown to repress p53 transcriptional activity (6, 38, 40) and early dissociation of Mdm2 from p53 may be a critical event in promoting the assembly of a transcriptional complex that exhibits the highest level of activity.

Although many reports have stated phosphorylation of p53 interrupts the interaction of p53 with Mdm2 (11, 41), others have argued that this is not always the case (42, 43). In our study, we found that Mdm2 protein localized on chromatin when p53 phosphorylated at serine 15 also interacted with chromatin. The possibility exists that some p53 molecules in the tetrameric p53 are phosphorylated at various sites, whereas others are not, or that mixed populations of cells exist and are indistinguishable in our assay. In our hypothetical model depicted in Fig. 5, we propose that two of the four molecules of latent p53 bound to DNA are associated with Mdm2 protein before activation (Fig. 5, 1). The transcriptional repression mediated by Mdm2 protein is relieved by the phosphorylation of p53 during activation (Fig. 5, 2) but, over time, Mdm2 is again able to associate with dephosphorylated subunits of the tetramer (Fig. 5, 3). Furthermore, Mdm2 protein cannot associate with the region encompassing the p53-responsive element without p53 bound to this site (Fig. 5, 4).

In our present study, we observed another facet of the intriguing interplay between p53 protein and its antagonistic inhibitor protein Mdm2 on chromatin. Whether Mdm2 serves to inhibit the transcriptional activity of p53 on chromatin or also targets p53 for ubiquitination on DNA has yet to be determined. Current studies suggest that Mdm2 inhibits p53 function by multiple mechanisms using both ubiquitination and transcriptional repression. Recent studies using chromatin immunoprecipitation by Szak et al. (17) that acetylated p53 can be detected on p53-responsive elements following DNA damage. In addition, acetylation of p53 at its COOH-terminal lysine residues following DNA damage has been shown to stabilize p53 by preventing ubiquitination by Mdm2 at these sites (44–46). For chromatin-bound p53 to be ubiquitinated by Mdm2, deacetylases would have to be recruited to these enhancer elements. The histone deacetylases HDAC1 and Sir2α have been shown to associate with p53 and repress transcription (47–49). Additionally, HDAC1 has recently been found to associate with Mdm2 (49). It would be interesting to examine whether Mdm2 protein and HDAC1 colocalize with p53 protein on the p53 binding sites and if the presence of this complex (if it exists on chromatin) correlates with decreased levels of transcription.
of acetylated p53 as well as lower levels of p53-dependent transcription. These data may then begin to shed useful insight into the kinetics of p53 ubiquitination and transcriptional repression by Mdm2 and whether these events occur on both “free” and “chromatin-bound” p53 populations in the nucleus. Minsky and Oren (23) found overexpressed oncopgenic Mdm2 protein asso-ciated with chromatin from the waf1 p53-responsive element and monoubiquitinated the histone subunit H2B. Ubiqui-tination of histone proteins has been shown to play a role in gene silencing (50). The Mdm2-p53 feedback loop may make use of p53 targeting Mdm2 protein to ubiquitinate histones as an additional transcription regulation component. The mechanism by which Mdm2 mediates its repression of p53-dependent transcription may be multifaceted and involves not only the recruitment of histone deacetylases but also its ability to ubiqui-tinate histone protein subunits at enhancer sites.

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Mouse Double Minute 2 Associates with Chromatin in the Presence of p53 and Is Released to Facilitate Activation of Transcription

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