Abstract

The transcription factor Dmp1 is a Ras/HER2-activated haplo-insufficient tumor suppressor that activates the Arf/p53 pathway of cell-cycle arrest. Recent evidence suggests that Dmp1 may activate p53 independently of Arf in certain cell types. Here, we report findings supporting this concept with the definition of an Arf-independent function for Dmp1 in tumor suppression. We found that Dmp1 and p53 can interact directly in mammalian cells via the carboxyl-terminus of p53 and the DNA-binding domain of Dmp1. Expression of Dmp1 antagonized ubiquitination of p53 by Mdm2 and promoted nuclear localization of p53. Dmp1–p53 binding significantly increased the level of p53, independent of the DNA-binding activity of Dmp1. Mechanistically, p53 target genes were activated synergistically by the coexpression of Dmp1 and p53 in p53−/−;Arf−/− cells, and genotoxic responses of these genes were hampered more dramatically in Dmp1−/− and p53−/− cells than in Arf−/− cells. Together, our findings identify a robust new mechanism of p53 activation mediated by direct physical interaction between Dmp1 and p53. Cancer Res; 72(7); 11. ©2012 AACR.

Introduction

Upon cellular stresses, such as DNA damage, oncogene activation, and hypoxia, the tumor suppressor p53 is activated and initiates a transcriptional program that induces DNA repair, cell-cycle arrest, apoptosis, or autophagy (1–3). This stress signaling plays critical roles in the prevention of tumor formation and, indeed, p53-null mice or p53 knock-in mice that express mutant p53 are highly prone to tumor development (4). A central regulator of the p53 pathway is the Mdm2 protein (Hdm2 in humans) that inhibits transcriptional activity, nuclear localization, and protein stability of p53 (5–7). Homozygous deletion of Mdm2 results in embryonic lethality at the blastocyst stage due to unrestricted apoptosis. Deletion of p53 abrogates this effect, indicating that the critical in vivo function of Mdm2 is the negative regulation of p53 activity (7). The Mdm2 (and also Hdm2) gene is regulated by p53 through direct binding of the protein to the p53-responsive elements located within the P2 promoter (7). Mutations in TP53 that disrupt p53 function occur in 50% of human cancers (8); the alteration of regulators for p53 is found in most of the remaining 50%. The Hdm2 gene is amplified in approximately 35% of human sarcomas and approximately 7% of all cancers without TP53 mutation, but the protein is overexpressed in 40% to 80% of late-stage metastatic cancers in the absence of gene amplification (7, 9).

The activity of Mdm2 is negatively regulated by p19Arf (p14ARF in humans) in response to oncogenic stress (10–13). p19Arf is an alternative reading frame gene product generated from the Ink4a/Arf locus, which also encodes the cyclin-dependent kinase inhibitor p16Ink4a. p19Arf directly binds to Mdm2, thereby stabilizing and activating p53. Arf induction by potentially harmful growth-promoting signals forces early-stage cancer cells to undergo p53-dependent and -independent cell-cycle arrest, apoptosis, or autophagy providing a powerful mode of tumor suppression (10–13). The Arf promoter monitors latent oncogenic signals in vivo, and thus Arf-null mice are also highly prone to spontaneous tumor development (14, 15). Arf is regulated at both transcriptional and protein levels (16–19). The Arf promoter is directly activated by E2Fs and Dmp1, whereas it is repressed by overexpression of nuclear proteins such as Bmi1, Twist, Tbx2/3, and Pokemon (16, 17).

Dmp1 (cyclin D–binding myb-like protein 1; Dmtf1) is a tumor suppressor that is deleted in approximately 40% of human non–small cell lung carcinomas (20–24). Mitogenic signals from oncogenic Ras and HER2/neu have been shown to activate the Dmp1 promoter, whereas physiologic mitogens as well as genotoxic stimuli mediated by NF-κB cause repression (25–28). It has been considered that the Dmp1 protein acts as a tumor suppressor by directly activating the Arf promoter, and thereby inducing Arf-, p53-dependent cell-cycle arrest (16, 21, 22). E2F-Myc, K-rasL14, and HER2/neu-driven tumor development was significantly accelerated in both Dmp1−/−
and Dmp1−/− mice with no significant differences in the survival between the 2 cohorts, suggesting that Dmp1 is haplo-insufficient for tumor suppression (22, 23, 28). In Eμ-Myc lymphomas, the combined frequencies of p53 mutation and Arf deletion in mice of Dmp1−/− or Dmp1−/− background were much lower than that in Dmp1−/− littermates, indicating that Dmp1 is a physiologic regulator of the Arf/p53 pathway in vivo (22). Of note, the frequency of p53 mutation (~40%) was significantly decreased in both Dmp1+/− and Dmp1−/− backgrounds (<10%), even in K-rasL4A lung cancer model, where the Ink4a/Arf involvement was rare (23). This suggests an Arf-independent mechanism of p53 regulation by Dmp1 in epithelial tissues.

In this study, we searched for binding partners for the Dmp1 protein to explain the Arf-independent function of Dmp1 in tumor suppression. We show that Dmp1 physically interacts with p53 to neutralize the activity of Hdm2 on the ubiquitination, nuclear localization, and transcription of p53. We also show that Dmp1 has Arf-independent mechanisms of p53 activation in vivo. We propose that the physical interaction between Dmp1 and p53 will synergize with Arf promoter activation by Dmp1 to enhance the activity of the p53 pathway.

Materials and Methods

Cell culture, retrovirus preparation, and infection

NIH 3T3, H1299, A549, and U2OS cells were cultured and transfected as described previously (16, 20). Passage 6 Arf−/−, p53−/− double-knockout mouse embryonic fibroblasts (MEF) were received from Drs. C. Sherr and M. Roussel. For preparation of retroviruses, human kidney 293T cells were transfected with a helper ecotropic retrovirus plasmid defective in psi-2 packaging sequences, together with pMSCV-IRESPuro vectors containing Dmp1, Dmp1 mutant, or p53 cDNA (16).

Detection of endogenous Dmp1–p53 interaction

Subconfluent U2OS cells were cotreated with 2 µmol/L etoposide (Toposar; Teva Pharmaceutical Industries Ltd.) and 25 µmol/L MG-132 for 6 hours, then harvested in EBC buffer. One milligram of total protein was immunoprecipitated with 4 µg of antibodies to Dmp1 [RAD to the full-length His-Dmp1 (ref. 29); RAX (ref. 26); or RAZ (ref. 24)], antibodies to p53 (sc-126 and sc-6243), or control immunoglobulins, followed by incubation with protein G-sepharose and extensive wash with lysis buffer (30). Sites of antibody bindings were visualized by sc-126 for p53 and RAD for Dmp1, followed by Trueblot secondary antibodies (Ebioscience). Endogenous Dmp1–p53 interaction was also studied with 1 mg of mouse thymus lysates injected with 0.6 mg/30 g etoposide.

In cell p53 ubiquitination assays

This assay was adapted from the work of Li and colleagues (31). H1299 cells were transfected with His-tagged ubiquitin, p53, Mdm2, and increasing amounts of Dmp1. Cells were treated with 25 µmol/L proteasome inhibitor MG-132 (Calbiochem) for 6 hours before harvesting to prevent degradation of ubiquitinated p53. Cells were lysed in EBC buffer without EDTA, sonicated, and cleared by centrifugation at 20,000 × g for 10 minutes. One milligram of cell lysate was incubated with 30 µL of 50% Ni-NTA agarose/EBC without EDTA slurry for 2 hours at 4°C with rotation. The samples were then centrifuged at 10,000 × g for 2 minutes at 4°C and the supernatant (unbound) lysate was removed and saved. The Ni-NTA resin was washed 4 times with 800 µL EBC without EDTA. His-tagged proteins were eluted from the Ni-NTA agarose by incubating in EBC buffer at 100°C for 5 minutes. The samples were then centrifuged at 10,000 × g for 2 minutes at 4°C and the supernatant (immunoprecipitated protein) was analyzed by Western blotting using antibodies for p53, Dmp1, Hdm2, and actin.

Induction of DNA damage response by doxorubicin injection in mice

Arf-null mice were obtained from National Cancer Institute Mouse Repository (Bethesda, MD). p53-null mice were purchased from the Jackson Laboratory. Six-week-old wild-type, Arf−/−, Dmp1−/−, and p53−/− mice (all C57BL/6) were intravenously injected with 0.6 mg doxorubicin/30 g mouse for 0, 2, and 4 hours, after which they were sacrificed, and thymi and lungs were harvested. Apoptosis was evaluated in vivo using antibodies to cleaved caspase-3 (Cell Signaling). For p53 target genes, p21Cip1/Waf1, bbc3 (puma), and puma, mRNA levels were quantitated by real-time PCR TaqMan assay (ABI 7500; refs. 26, 27). For quantification of p53 binding to p21Cip1/Waf1 and bbc3 promoters by real-time PCR, TaqMan assays were custom designed by ABI to detect target sequences with mouse β-actin genomic DNA as an internal control. All procedures involving mice were approved by Institutional Animal Care and Use Committee. In vitro protein binding assays, in vitro ubiquitination assay, immunofluorescence, immunohistochemical, luciferase, and electrophoretic mobility shift assays are explained in the Supplementary Materials and Methods.

Results

Dmp1 physically interacts with p53

Published studies indicate that Dmp1 regulates p53 levels in vivo as Dmp1−/− nulls have significantly decreased incidence of p53 mutation (22, 23). Thus, the half-life of p53 was studied by activating Dmp1 ER (estrogen receptor) in MCF10A cells (Arf-null) and found that it was extended from 41 minutes to 3.5 hours (Supplementary Fig. S1). To identify which key components of the Arf/Mdm2/p53 tumor suppressor pathway interact with Dmp1, the protein was transiently coexpressed with p53, Mdm2, p19Arf, or c-Myc in 3T3 cells (Fig. 1A and B; Supplementary Fig. S2A; the input protein levels are shown in Supplementary Fig. S3). Immunoprecipitation with Flag antibody showed that only p53 interacted with Dmp1 among these 4 proteins. Reciprocal immunoprecipitation with hemagglutinin antibody verified that Dmp1 was coimmunoprecipitated with p53 (Fig. 1B). Physical interaction of Mdm2−/− p53 was also confirmed by coimmunoprecipitation under the same experimental condition. Of note, the presence of Dmp1 did not interfere with Mdm2−/− p53 binding, suggesting that Dmp1 binds to p53 with a domain distinct from the Mdm2-binding region (Fig. 1B, last lane). In addition, Dmp1 did not
interact with c-Myc, E2F2, or E2F3, further showing the specificity of Dmp1–p53 binding (Supplementary Fig. S2B and S2C).

Next, the interaction of endogenous human DMP1 (hDMP1) with p53 was examined using U2OS cell lysates treated with the topoisomerase inhibitor etoposide. The cleared lysates were immunoprecipitated (IP) with antibodies to (A) Dmp1 (FLAG) or (B) p53 (HA) and analyzed by Western blotting. C, left, endogenous Dmp1–p53 interaction in U2OS cells treated with etoposide (Toposar). Etoposide/MG-132-treated lysates were immunoprecipitated with control GFP antibody (sc-9996), control IgGs, or antibodies to p53 (sc-126, sc-6243) or Dmp1 (RAD, RAX, RAZ), followed by blotting with Dmp1 or p53 antibodies. Right, endogenous Dmp1–p53 interaction in the mouse thymus injected with etoposide. Lysates were immunoprecipitated with control IgGs, or antibodies to p53 (sc-6243), or Dmp1 antibody (RAD) and analyzed by Western blotting. D, recombinant Flag-Dmp1, HA-p53, and Flag-Mdm2 proteins were purified from baculovirus-infected Sf9 cells. In vitro binding assays were conducted by using 4 μg of proteins followed by immunoprecipitation with p53 (sc-6243), Dmp1 (RAX), or control IgGs and analyzed by Western blotting.

Figure 1. Dmp1 coimmunoprecipitates with p53. A and B, NIH 3T3 cells were transfected with cDNA for Flag-Dmp1, HA-p53, c-Myb, p19Arf, and/or Mdm2 expression vectors. The cleared lysates were immunoprecipitated (IP) with antibodies to (A) Dmp1 (FLAG) or (B) p53 (HA) and analyzed by Western blotting. C, left, endogenous Dmp1–p53 interaction in U2OS cells treated with etoposide (Toposar). Etoposide/MG-132-treated lysates were immunoprecipitated with control GFP antibody (sc-9996), control IgGs, or antibodies to p53 (sc-126, sc-6243) or Dmp1 (RAD, RAX, RAZ), followed by blotting with Dmp1 or p53 antibodies. Right, endogenous Dmp1–p53 interaction in the mouse thymus injected with etoposide. Lysates were immunoprecipitated with control IgGs, or antibodies to p53 (sc-6243), or Dmp1 antibody (RAD), and analyzed by Western blotting. D, recombinant Flag-Dmp1, HA-p53, and Flag-Mdm2 proteins were purified from baculovirus-infected Sf9 cells. In vitro binding assays were conducted by using 4 μg of proteins followed by immunoprecipitation with p53 (sc-6243), Dmp1 (RAX), or control IgGs and analyzed by Western blotting.
identified in complex with HA-p53 in vitro. Reciprocal immunoprecipitation showed that HA-p53 (∼5% of input) was pulled down with an antibody to Dmp1 (Fig. 1D). We also confirmed direct interaction between Mdm2 and p53 but not Dmp1 and Mdm2 (Fig. 1D, middle). Together, our data show that Dmp1 physically interacts with p53 but not with p19Arf or Mdm2.

Mapping of p53-binding domain on Dmp1 and Dmp1-binding domain on p53

Mapping the p53-binding domain on Dmp1 was accomplished by coexpressing Dmp1 mutants with wild-type p53 in Sf9 and 3T3 cells. We found that the Dmp1 mutants M5, M6, M7, M8, and M9 (20) were unable to interact with p53, suggesting that amino acid residues 1 to 380 are vital to the interaction of Dmp1 with p53 (Fig. 2A and B). Specifically, the DNA-binding domain of Dmp1 (amino acids 87–380) is considered to play a major role in the Dmp1–p53 interaction as the M10 mutant is bound to p53 at the same level as wild-type. The p53 interaction domain on Dmp1 was mapped to amino acids 87 to 380 in NIH 3T3 cells (Supplementary Fig. S5A and S5C). Conversely, the Dmp1-binding domain was mapped to amino acid residues 290 to 360 of p53 (nuclear localization signal and tetramerization domain; Fig. 2C and D; Supplementary Fig. S5B and S5C), consistent with the finding that Dmp1 binding to p53 did not interfere with Mdm2–p53 interaction (Fig. 1B). For verification, the His-tagged minimal-binding region of Dmp1 for p53 (M14;
Figure 3. Dmp1 modulates p53 ubiquitination in vivo and in vitro. A, Dmp1 modulates p53 ubiquitination. H1299 cells were transfected with His-ubiquitin, p53, Hdm2, and increasing amounts of Dmp1. Cells were treated with the proteasome inhibitor MG-132 and ubiquitinated p53 was isolated with Ni-NTA resin and analyzed by Western blotting with the indicated antibodies. The numbers in the parentheses show densitometric values of polyubiquitinated p53. B, in cell p53 ubiquitination (monoubiq) assay using H1299 cells transfected with p14ARF shRNA. Polyubiquitination (polyubiq) of p53 by Hdm2 was significantly inhibited by Dmp1 (47% by 1/C2 Dmp1, 79% by 2/C2 Dmp1, and 98% by 4/C2 Dmp1) expression in H1299 cells with 90% downregulation of p14ARF by specific shRNA, indicating that the inhibitory effect of Dmp1 on p53 ubiquitination was independent of ARF. K.D., knockdown; MG, MG-132; RNAi, RNA interference. C, Dmp1 blocks p53 ubiquitination by Mdm2 in vitro. In vitro ubiquitination assays were conducted in the presence of purified p53, Mdm2 (E3), E2 (ubch5a), E1, and ATP by either ubiquitin (poly- and monoubiquitination) or methyl-ubiquitin (MeUb; monoubiquitination only). Dmp1-M6 and -M7 mutants that do not interact with p53 did not block poly- or monoubiquitination. *, endogenous Sf9 ubiquitination of p53. BSA, bovine serum albumin. D, both total and phosphorylated p53 levels are reduced in Dmp1/C0 thymus compared with wild-type. Total protein was isolated from the thymus of Dmp1-null and wild-type mice after intravenous injection with doxorubicin for 4 hours and resolved by SDS-PAGE. The numbers indicate relative expression levels of each protein determined by densitometric analyses of Western blotting data.
amino acids 87–224) and His-tagged, full-length, or carboxyl-terminal p53 (amino acids 290–393; Δ1–289) were purified from bacteria and protein–protein binding assays were conducted by immunoprecipitation–Western blotting (Supplementary Fig. S6). Our results show Dmp1-M14 bound directly to wild-type p53 and p53Δ1–289.

**Dmp1 antagonizes ubiquitination of p53 by Mdm2**

Mdm2 (or Hdm2) has been reported to inactivate p53 by (i) accelerating ubiquitin-mediated degradation of p53, (ii) inhibiting nuclear import of p53 to the nucleus, and/or by (iii) inhibiting transcriptional activity of p53 (5–7, 13). To assess the effect of Dmp1 expression on p53 ubiquitination by Hdm2, H1299 cells were coexpressed with His-tagged ubiquitin, p53, Hdm2, and increasing amounts of Dmp1. Ubiquitinated proteins were brought down with Ni-NTA resin, and samples were analyzed by Western blotting. Results show that Dmp1 overexpression decreased p53 polyubiquitination by Hdm2 in the presence or absence of proteasome inhibitor (Fig. 3A; Supplementary Fig. S7). H1299 cells transfected with short hairpin RNA (shRNA) to p14ARF also showed significant inhibition of p53 polyubiquitination by Dmp1, indicating that the effect was ARF-independent (Fig. 3B). To study the effects of endogenous Dmp1 on p53 stability, hDMP1 was knocked down by specific shRNA (23). Cells depleted for DMP1 exhibited more than 60% reduction in endogenous p53 protein levels compared with vector (Supplementary Fig. S8A), whereas p53 transcript levels remained unchanged (Supplementary Fig. S8B). Therefore, modulation of p53 levels by Dmp1 does not take place at the transcriptional level. Furthermore, endogenous p53 ubiquitination was drastically increased (Supplementary Fig. S8C) upon DMP1 knockdown. In vitro p53 ubiquitination reactions indicate that Hdm2 accelerated poly-ubiquitination of p53, whereas coexpression of Hdm2 and Dmp1 significantly inhibited Hdm2-mediated ubiquitination of p53 in dose-dependent fashion (Fig. 3C; Supplementary Fig. S10). Conversely, auto-ubiquitination of Hdm2 was not inhibited by Dmp1 (Supplementary Fig. S11) suggesting that the mode of action of Dmp1 involves its direct binding with p53 and does not function as a general/nonspecific inhibitor of the ubiquitin ligase activity of Hdm2. The M6 and M7 Dmp1 mutants that do not bind to p53 had little effect in p53 ubiquitination in vitro, indicating that direct physical interaction between Dmp1 and p53 is essential for Dmp1 to interfere with Hdm2 activity (Fig. 3C). To observe the effect of Dmp1 loss on p53 levels in vivo, 5-week-old Dmp1+/− and pure wild-type mice were treated by intravenous injection of doxorubicin for 4 hours. The animals were sacrificed, the thymus resected, and total protein was prepared for analysis by PAGE. Steady-state p53 levels in untreated animals were 50% lower in mice of Dmp1-null background than in wild-type. A 30% reduction in total p53 was observed in the thymus from Dmp1−/− mice treated with doxorubicin (Fig. 3D, fourth lane). Notably, the amount of phosphorylated p53 (pSer6, 15, 20) was further (60%–70%) reduced in Dmp1−/− thymus than in wild-type (Fig. 3D, first to third lanes), and the levels of p53 target gene products were proportionate to the levels of phosphorylated p53 (Fig. 3D, fifth and sixth lane). These data indicated that Dmp1 not only plays critical roles in...
setting basal p53 levels in tissues but also is involved in the activation of p53 in response to DNA damage.

**Dmp1 antagonizes nuclear export of p53 by Hdm2**

Next, we investigated whether Dmp1 altered the localization of p53. H1299 cells were cotransfected with GFP, p53, Hdm2, and/or Dmp1, probed for p53, and analyzed by confocal microscopy (Fig. 4A). As anticipated, Hdm2 expression resulted in an absolute reduction in p53 levels (Fig. 4A, second panel). This effect was rescued by concurrent expression of Dmp1 (Fig. 4A, third panel), whereas the M7 mutant was unable to reverse the negative effects of Hdm2 (Fig. 4A, fourth panel). Increased nuclear localization of p53 by Dmp1 was verified in endogenous proteins as MCF7 cells (ARF-null) depleted of hDMP1 by shRNA resulted in nuclear exclusion of p53, whereas in control cells, p53 was retained in the nucleus (Supplementary Fig. S9). Subcellular localization of p53 was also studied in A549 cells (ARF-null) coexpressed with p53-ZsGreen, Dmp1,
Synergism between Dmp1 and p53 on p53 target gene transcription in Arf-deficient cells

Next, p53 double-knockout cells were transiently coexpressed with wild-type p53 and Dmp1 (and its mutants) to study the consequences on p53 function. We saw significant accumulation of p53 in cells coexpressing p53 and Dmp1 wild-type or the M11 mutant that does not bind to DNA but not in those expressing p53 plus the M7 mutant that does not bind to p53 (Fig. 5A; ref. 20). The increase in p53 levels upon coexpression of Dmp1 correlated with synergistic activation of the p21- and bbc3-promoters in mouse thymus in response to doxorubicin (Dox) injection. We then tail-injected doxorubicin in wild-type and Dmp1-null mice, harvested the thymus at 0 and 4 hours posttreatment, and binding of p53 to target gene (p21 and bbc3) promoters was studied by chromatin immunoprecipitation, followed by real-time PCR. Significant binding of p53 to the p21 and bbc3 promoters was found in wild-type but not in Dmp1-null thymus, suggesting that Dmp1 is indispensable for p53 to bind to these promoters. Control IP, IP with protein G-sepharose only.

We then tail-injected doxorubicin in wild-type and Dmp1-null mice, harvested the thymus at 0 and 4 hours posttreatment, and binding of p53 to target gene (p21 and bbc3) promoters was studied by chromatin immunoprecipitation followed by real-time PCR (Fig. 5D). Significant binding of p53 (0.5% of input levels) to the p21 and bbc3 promoters was found in wild-type but not in Dmp1-null thymus, suggesting that Dmp1 is indispensable for p53 binding to these promoters in vivo. Together, our data indicate the following: (i) expression of Dmp1 interferes with the known activities of Hdm2 on the ubiquitination and nuclear localization of p53 in an Arf-independent fashion and (ii) p53 binding to target genes is Dmp1-dependent.

Figure 5. (Continued) D, Dmp1-dependent binding of p53 to the bbc3 and p21 promoters in mouse thymus in response to doxorubicin (Dox) injection. Wild-type and Dmp1-null mice (6-week-old) were tail-injected with 0.6 mg doxorubicin/30 g mouse and thymi were harvested at 0 and 4 hours. The binding of p53 to target gene (p21 and bbc3) promoters was studied by chromatin immunoprecipitation, followed by real-time PCR. Significant binding of p53 to the p21 and bbc3 promoters was found in wild-type but not in Dmp1-null thymus, suggesting that Dmp1 is indispensable for p53 to bind to these promoters. Control IP, IP with protein G-sepharose only.

Synergism between Dmp1 and p53 on p53 target gene transcription in Arf-deficient cells

Next, Arf, p53 double-knockout cells were transiently coexpressed with wild-type p53 and Dmp1 (and its mutants) to study the consequences on p53 function. We saw significant accumulation of p53 in cells coexpressing p53 and Dmp1 wild-type or the M11 mutant that does not bind to DNA but not in those expressing p53 plus the M7 mutant that does not bind to p53 (Fig. 5A; ref. 20). The increase in p53 levels upon coexpression of Dmp1 correlated with synergistic activation of the p21 promoter luciferase construct in Arfp53 double-knockout MEFs (Fig. 5B, left). The Dmp1-M11 mutant that does not bind to DNA (20) also synergized with p53 on the p21 promoter activation (Fig. 5B, right), suggesting that this synergistic effect between p53 and Dmp1 was independent of DNA binding of the latter. Because the M7 mutant did not contribute to the stability of p53, it had no effect on p53-mediated transactivation of the p21 promoter (Fig. 5B, right). The results of the p21 promoter reporter assay were further verified by stably coexpressing p53 and Dmp1 in Arf-/-; p53-/- MEFs and quantitatively endogenous p21 mRNA by real-time PCR (Fig. 5C). Synergism between p53 and Dmp1 was also shown by real-time PCR for bbc3 (puma) in Arf-/-; p53-/- MEFs, albeit to a lesser extent (Fig. 5C, right). Thus, synergistic activation of the p21 and bbc3 promoters by Dmp1 (wild-type and M11 mutant) and p53 was a consequence of increased p53 levels.

We then tail-injected doxorubicin in wild-type and Dmp1-null mice, harvested the thymus at 0 and 4 hours posttreatment, and binding of p53 to target gene (p21 and bbc3) promoters was studied by chromatin immunoprecipitation followed by real-time PCR (Fig. 5D). Significant binding of p53 (0.5% of input levels) to the p21 and bbc3 promoters was found in wild-type but not in Dmp1-null thymus, suggesting that Dmp1 is indispensable for p53 binding to these promoters in vivo. Together, our data indicate the following: (i) expression of Dmp1 interferes with the known activities of Hdm2 on the ubiquitination and nuclear localization of p53 in an Arf-independent fashion and (ii) p53 binding to target genes is Dmp1-dependent.
compromised in Dmp1<sup>−/−</sup> lungs than in Arf<sup>−/−</sup> lungs (Supplementary Fig. S12A and S12B), eliminating the possibility that this effect is cell type specific. Together, our data show Arf-independent activation of p53 by Dmp1 in response to genotoxic drug in vivo. The current model for the mechanism of regulation of p53 by Dmp1 is summarized in Fig. 6D.

Discussion

We have now shown that Dmp1 physically interacts with p53 and antagonizes all the known activities of Mdm2 (or Hdm2), that is, p53 ubiquitination, nucleus–cytoplasm shuttling, and transactivation of target genes. Dmp1 is a critical transcriptional activator for Arf, and p19<sup>Arf</sup> (or p14<sup>ARF</sup>) can, in turn, stabilize nucleoplasmic p53 by binding to Mdm2 and sequestering it in the nucleolus or by directly inhibiting the ubiquitin ligase activity of Mdm2 (6, 13). Moreover, Arf has also been shown to modulate the activity of other E3 ligases for Mdm2, such as ARF-BP1 (18). However, inhibition of ubiquitination and nuclear export of p53 by Dmp1 are considered to be Arf-independent as they were found in cells where p14<sup>ARF</sup> was knocked down or deleted. Synergistic activation of the p21<sup>Cip1/Waf1</sup> promoter by p53 and Dmp1 was also Arf-independent as it was found in p53;Arf-null cells. These Arf-independent mechanisms of p53 modulation by Dmp1 substantiate the decrease in frequency of p53 mutations in both Dmp1<sup>+/−</sup> and...
Dmp1−/− backgrounds, which we previously observed in the K-ras−4 lung cancer model, where p19ARF involvement is rare (23).

We have mapped the Dmp1-binding domain at the carboxy-terminus of p53, suggesting that Dmp1–p53 binding does not interfere with p53 binding to target genes or physical interaction between Mdm2 and p53. Conversely, the p53-binding domain was mapped to the DNA-binding domain of Dmp1, therefore binding of Dmp1 to p53 and DNA is considered to be mutually exclusive. Indeed, the Dmp1 mutant that does not bind to DNA retained the activity to stimulate the p21WAF1 promoter in a p53-dependent fashion. Among p53-binding partners reported so far, Dmp1 is unique in that it directly binds and activates the Arf promoter and at the same time physically interacts with p53 (32). Furthermore, most p53-interacting proteins that bind to the C-terminus of p53 (e.g., YB-1, YY1, or RelA) negatively regulate its function, whereas Dmp1 regulates p53 positively in terms of stabilization and nuclear localization (30, 32). Thus, Dmp1 is a critical antagonist of Mdm2 in regulating the activity of p53.

The current study showed an unexpected role of Dmp1 in apoptosis: Although it was induced to wild-type levels in Arf−/− tissues, the bbc3 gene was barely expressed doxorubicin in the thymi or lungs from Dmp1−/− or p53−/− mice. This indicates that the induction of bbc3 was dependent on Dmp1 and p53 but not Arf. Because both mouse and human bbc3 promoters lack Dmp1-binding motifs, it is speculated that Dmp1 physically interacts with p53 and regulates the bbc3 promoter. This model was supported by chromatin immunoprecipitation that showed binding of p53 to the bbc3 (and p21) promoter in wild-type thymus, but not in Dmp1−/− tissues (Fig. 5D).

Li and colleagues showed that low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote the polyubiquitination and nuclear degradation of p53 (31). Our results show that Dmp1 inhibits both monoubiquitination and polyubiquitination of p53, suggesting that Dmp1 can overcome Hdm2 antagonism at both high and low levels. Interestingly, in our localization studies, we found that p53 is degraded when it is coexpressed with Hdm2 in H1299 cells (p53-null, Arf wt) but is exported from the nucleus in A549 cells (p53 wt, Arf-null). This is likely due to the fact that A549 cells contain higher amounts of p53, reflecting higher endogenous expression, likely inducing Hdm2-medi- ated monoubiquitination and nuclear export of the protein. In both cases, Dmp1 stabilizes nuclear p53 providing clear evidence that Dmp1 can inhibit both p53 nuclear degradation and p53 nuclear export induced by Hdm2.

Augmentation of the activity of Dmp1 may be a useful target for future drug discovery for several reasons. First, Dmp1 initiates 2 independent mechanisms that act synergistically to neutralize the activity of Mdm2: one directly through the regulation of p53 stabilization, localization, and transactivation capabilities and the second indirectly through the regulation of the Arf promoter. In addition, Dmp1 is haplo-insufficient for tumor suppression; hence, tumors often retain one allele of Dmp1, making modulation of this intact gene more feasible.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D.P. Frazier, R.D. Kendig, D. Maglic, G. Sui, K. Inoue.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.P. Frazier, R.D. Kendig, F. Kai, R.L. Morgan, S.J. Lagedrost, K. Inoue.
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.P. Frazier, R.D. Kendig, T. Sugiyama, E.A. Fry, K. Inoue.

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Dmp1 Physically Interacts with p53 and Positively Regulates p53's Stability, Nuclear Localization, and Function


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