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Stabilization of the p53-DNA complex by the nuclear protein Dmp1α

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Abstract

We recently reported the existence of a physical interaction between the Myb-like transcription factor Dmp1 (Dmtf1) and p53 in which Dmp1 antagonized polyubiquitination of p53 by Mdm2 and promoted its nuclear localization. Dmp1 significantly stabilized p53-DNA complexes on promoters that contained p53-consensus sequences, which were either supershifted or disrupted with antibodies to Dmp1. Lysates from mice injected with doxorubicin showed that Dmp1 bound to *p21*^{Cip1}, *Bbc3*, and *Thbs1* gene regulatory regions in a p53-dependent fashion. Our data suggest that acceleration of DNA-binding of p53 by Dmp1 is a critical process for Dmp1 to increase the p53 function in *Arf*-deficient cells.

Keywords

Dmp1 (Dmtf1); p53; Mdm2; knockout; p21^{Cip1}; Bbc3; Thbs1; cancer; DNA binding; transcription; electrophoretic mobility shift assay (EMSA); chromatin immunoprecipitation

Introduction

Upon cellular stresses, such as DNA damage, oncogene activation, hypoxia, or nutrient deprivation, the tumor suppressor p53 is activated and initiates a transcriptional program in which a battery of genes that cause cell cycle arrest (e.g. *p21*^{Cip1}, *cyclin G*), apoptosis (*Bax, Bbc3, Noxa*), DNA repair (*PCNA*), or autophagy (*DRAM1*) are transactivated, all playing crucial roles in prevention of tumor formation (1–5). Indeed, both *p53*-null and gain-of-function knock-in mice that express mutant *p53* are highly prone to tumor development (6, 7; reviewed in 8). Human p53 consists of 393 amino acids with 5 proposed domains, i.e., transactivation domains 1 and 2; proline-rich domain; DNA-binding domain; tetramerization domain; and the C-terminal regulatory domain (9).

The central regulator of the p53 pathway is the Mdm2 protein (HDM2 in humans) that inhibits transcriptional activity, nuclear localization, and protein stability of p53 (10–13). Homozygous deletion of *Mdm2* results in embryonic lethality at the blastocyst stage due to apoptosis. Deletion of *p53* abrogates this effect, indicating the critical *in vivo* function of

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Declaration of interest

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Mdm2 is the negative regulation of p53 activity (10, 11, 13). 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 (10, 11, 13). Mutations in *TP53* that disrupt p53 function occur in 50% of human cancers (14, 15); the alteration of regulators for p53 is found in most of the human tumors with wild type p53. The *Hdm2* gene is amplified in ~35% of human sarcomas and ~7% of all cancers without *TP53* mutation, but the protein is overexpressed in 40–80% of late-stage metastatic cancers in the absence of gene amplification (14, 15), suggesting additional mechanisms.

The activity of Mdm2 is negatively regulated by $p19^{Arf}$ ($p14^{ARF}$ in humans) in response to oncogenic stress (16–18). $p19^{Arf}$ is an alternative reading frame gene product generated from the *Ink4a/Arf* locus which also encodes the cyclin-dependent kinase inhibitor $p16^{Ink4a}$. $p19^{Arf}$ directly binds to Mdm2, thereby stabilizing and activating p53. Arf is induced by all the reported oncogenic stresses triggered by mutant Ras, c-Myc, E2F1, or HER2 overexpression (16–19). The *Arf* promoter is directly activated by E2F1 or Dmp1 (20) while the protein is stabilized by c-Myc or nucleophosmin through abrogation of Ulf-mediated Arf ubiquitylation (21). Alternatively, the *Arf* promoter is repressed by overexpression of nuclear proteins such as Bmi1, Twist, Tbx2/3, and Pokemon (22). The *Arf* promoter is activated by latent oncogenic signals *in vivo*, thus *Arf*-null mice are highly prone to spontaneous tumor development (23, 24). This Arf induction forces early-stage cancer cells to undergo p53-dependent and -independent cell cycle arrest, apoptosis, and/or autophagy, providing a powerful mode of tumor suppression (16–18, 25). In p53 activation brought about by DNA damage, the ATM/CHK2 and ATR/CHK1 cascades are the two major signaling pathways driving the DNA damage response (DDR), a network of processes crucial for the preservation of genomic stability that act as a barrier against tumorigenesis and tumor progression (1–3, 26, 27). The p53 signaling activated by DDR is also mediated by JNK, NF-κB, and MEKK1 (28–31), which needs to be investigated in further detail.

Dmp1 (cyclin D binding myb-like protein 1; Dmtf1) is a tumor suppressor that is deleted in ~35% of human non-small cell lung cancer and 42% of breast cancer (32–38). Mitogenic signals from oncogenic Ras (39) and HER2/neu (19, 40) have been shown to activate the *Dmp1* promoter (39, 41) while physiological mitogens as well as genotoxic stimuli mediated by NF-κB cause repression (42). It has been theorized that the Dmp1 protein acts as a tumor suppressor by directly transactivating the *Arf* promoter, thereby inducing Arf-, p53-dependent cell cycle arrest (20, 33, 34, 43). *Eμ-Myc, K-ras*^{LA}, *HER2/neu,* and *cyclin D1*-driven tumor development was significantly accelerated in *Dmp1*-deficient mice (19, 34, 35, 44, 45). Of note, both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice showed acceleration of oncogene-induced tumor development with no significant differences in survival between the two cohorts, suggesting that Dmp1 is haplo-insufficient for tumor suppression (19, 34, 35; reviewed in 46). We recently reported that Dmp1 physically interacts with p53 to neutralize the known functions of Mdm2 (or HDM2), namely ubiquitination, nuclear-cytoplasmic transport, and suppression of transport (47), a very unique property among p53/Mdm2-binding transcription factors (48). The h*DMP1* locus encodes at least three splicing variants - h*DMP1α, β,* and *γ* with antagonizing functions (49–51, reviewed in 52). The h*DMP1α* gene corresponds to murine *Dmp1α* that positively regulates the $p19^{Arf}$-p53 pathway (761 amino acids [a.a.] in mice, 760 a.a. in humans). Conversely, the hDMP1β (272 a.a.) and γ

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(285 a.a.) isoforms lack the DNA-binding domain, and hDMP1β is dominant-negative over hDMP1α in *CD13* and *ARF* induction (49, 50). Our recent study showed that forced expression of hDMP1β stimulates cell proliferation in p53-independent fashion and induces aberrant growth of mammary glands and accelerates tumorigenesis (51).

Dmp1 does not directly bind to the $p21^{Cip1}$ and *Bbc3* promoters in response to DNA damage caused by DOX, yet Dmp1 plays an essential role in p53's response to stress signaling (47). Consistently, the induction of $p21^{Cip1}$ and *Bbc3* in mouse tissues following DOX injection (thymus, lung) was significantly impaired in *Dmp1*-deficient mice, but not in those deficient in *Arf*, indicating that Dmp1 must play a more important role than Arf in p53 activation in response to dsDNA breaks (47). In this study, we studied the effects of Dmp1 (Dmp1α) in p53's binding to target genes on genomic DNA using probes covering the p53-consensus sequences on the $p21^{Cip1}$ promoter and also that for general p53-binding (53).

Materials and Methods

Cell culture, retrovirus preparation, and infection

NIH 3T3, H1299, and A549 cells were cultured and transfected with Genejuice (EMD Millipore) as described previously (20, 32, 47).

Plasmid DNAs.—The expression vectors for mouse Dmp1α (32) and human p53 (47) have been described. For reporter assays with the mouse *p21* promoter, the 4kb construct was recovered from the pJFCATΔH-mp21-CAT1.9 plasmid DNA (from Dr. B. Vogelstein, ref. 54), which was then recloned into the pGL2-basic vector.

Electrophoretic Mobility-Shift Assay (EMSA).—The detailed procedures for EMSA have been described (32, 39, 55, 56). EMSA was conducted with either with recombinant proteins from Sf9 cells infected with baculoviruses (Figs. 1 and 2), or with *p53*-null H1299 cell lysates transfected with p53 or FLAG-Dmp1 expression vectors (Fig. 3). 5×10^5 cpm of [$\alpha-^{32}$P] labeled probe (~1ng) was used per lane. For competition assays, a 200 molar excess of unlabeled probe was pre-incubated with the recombinant protein or cell lysate before addition of ^{32}P-labelled DNA probe. For non-specific competition assays, annealed oligonucleotides covering the possible AML1-binding site on the murine *Dmp1* promoter (36 bps; ref. 39) were used.

For the Dmp1 binding site on the mouse *Arf* promoter [20], the sense 5'-TACCTCGTGGGGCGGATGCACAGAAGCAC-3' (the Dmp1-consensus sequence is underlined) and its reverse complementary strands were synthesized, annealed and end-labelled with T4 polynucleotide kinase with [$\gamma-^{32}$P] dATP. Recombinant FLAG-Dmp1 recognized both short (29 bps; Fig. 1B, left) and long (~300 bps; ref. 20) genomic DNA sequences on the *Arf* promoter. For EMSA with recombinant FLAG-Dmp1 in Fig. 1B, right, 300–400 base pair genomic DNAs on the mouse $p21^{Cip1}$ promoter construct (#1 [397 base pairs] and #2 [308 base pairs]; ref. 54) were PCR-amplified, purified from the agarose gel, and then end-labelled with [$\gamma-^{32}$P] dATP.

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For EMSA with recombinant FLAG-Dmp1 and/or p53 on the mouse *p21*^{Cip1} promoter p53 consensus #2 (Figs. 1A, 2A), the sense 5'-AGACTGGGCATGTCTGGGCA-3' and its anti-sense oligonucleotide DNAs were synthesized, annealed, and end-labelled (the p53 consensus sequences are underlined; ref. 54). For general p53-binding assay in Fig. 2B, EMSA was performed with oligonucleotide probes used in the previous study (53, 57–59) using oligonucleotide 5'-AGGCATGCCTAGGCATGCCT-3' and its complementary strand (named p53-g in this study; the two p53 consensus binding sites are underlined).

For EMSA with mammalian cell lysates (Fig. 3), *p53*-null H1299 cells were transfected with expression vectors for HA-p53 (pcDNA-HAp53; ref. 47) and/or FLAG-Dmp1 (pFLEX1-Dmp1; ref. 32). Nuclear lysates were prepared with the established protocol (60), and 2uL of lysate was incubated with the end-labeled oligonucleotide DNA obtained by annealing 5'-CATCAGGAACATGTCCCAACATGTTGGGCGTCGGCTGTCGGAGGAACATGTCCCAACATGTTGAGCTCT-3' containing two repeats of the p53 consensus sequence #1 on the human *p21*^{CIP1} promoter (the position of the consensus #1 is similar to that on the mouse promoter in Fig. 1A) and its anti-sense strand DNA (61). To verify the identity of the proteins in shifted complexes, reaction mixtures were incubated with control non-immune rabbit/mouse IgG, or with specific antibodies to p53 (PAb421, DO-1; refs. 47, 53, 62) or Dmp1 (RAF, RAJ, RAX, RAZ, RAD, and αFLAG). The RAX antibody was raised to Dmp1 amino acids 136–150 (41), RAF to Dmp1 amino acids 752–760 (55), RAZ to Dmp1 amino acids 741–755 (63), RAJ to the Myb-like repeats of Dmp1 (55), and RAD to the full-length His-tagged Dmp1 (47). The M2 monoclonal antibody to the FLAG epitope was purchased from Sigma-Aldrich (cat# F3165).

Chromatin Immunoprecipitation (ChIP).—ChIP was performed as described previously (19, 39, 42, 44, 64, 65) using thymi from DOX -injected mice. See http://genomecenter.ucdavis.edu/farnham/farnham/protocols/tissues.html for tissue ChIP. The antibodies were recovered by using Protein G-sepharose, and washed extensively (64). The immunoprecipitated DNA was recovered by digestion of the samples with proteinase K and RNase. ~250 base pair fragments covering each p53-consensus sequences on the mouse *p21*^{Cip1} (site #1) and *Bbc3* (site #1) promoters, and mouse *Thbs1* intron 7 (Fig. 1A) were amplified by PCR using 1 μCi of [α−^{32}P] dATP (GE Healthcare), separated in a 10 % non-denaturing polyacrylamide gel.

The sequences of primers used in ChIP PCR at Fig. 4A are as follows: p21^{Cip1}_1580SE 5'CCCTGT CCTTTTCTGGAAGTG-3', p21^{Cip1}_1975AS 5'-CTGGGGTCTCTGTCTCCATTC-3'; Bbc3_1201SE 5'-GGACCAAAATCATGGCTTCA-3'; Bbc3_1377AS 5'-TGGGGAGACC ACAGTTCAAA-3'. Thbs1_310SE 5'-GAAAGCCCTACTGGTCCATCC-3', Thbs1_560AS 5'-TGCACCATCACCACATTTCTC-3'.

Results

The Dmp1 protein is upregulated by genotoxic drugs

We recently searched for binding partners for the Dmp1 (Dmp1α) protein to explain the *Arf*-independent function of Dmp1 in tumor suppression, and found that Dmp1 physically bound

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to p53, but not to Mdm2, Arf, c-Myc, c-Myb, E2F2, or E2F3 (47). Arf-independent binding of Dmp1 to p53 was demonstrated in endogenous proteins in U2OS cells treated with the DNA-damaging agent dox as well as in thymi of mice 4 hrs after exposure to etoposide (47). We also observed endogenous Dmp1α-p53 interaction in both NIH 3T3 and A549 cells (both *ARF*-null, p53 wild type) treated with DOX (data not shown). DOX-induced Dmp1 played critical roles in p53 induction in response to these dsDNA breaks since 1) the levels of Dmp1α increased at 4hrs in response to DOX (47) or etoposide (Fig. S1), and 2) induction of the pro-apoptotic protein Bbc3 was dramatically impaired in both *Dmp1*-null and *p53*-null, but not in *Arf*-null cells (47).

Recombinant Dmp1 does not directly bind to the *p21* promoter

We showed that Dmp1α neutralized the activities of Mdm2 (or HDM2) in polyubiquitination and nuclear-to-cytoplasmic transport of p53, resulting in nuclear accumulation of p53 and increased nucleolar protein levels when assessed by confocal microscopy (Fig. S2; ref. 47). Both human and mouse $p21^{Cip1}$ and *Bbc3* genomic DNA lack DNA-binding consensus sequences for Dmp1, XXCG(G/T)ATG(T/C) (20, 32, 55, 56). However, we saw activation of the *p21* promoter in response to Dmp1 even in *Arf*-null, $p53^{WT}$ NIH 3T3 cells (~3 folds, data not shown), but not in *Arf;p53*-null cells (47), raising the possibility that this is a consequence of direct Dmp1-p53 interaction. Indeed, recombinant Dmp1 protein did not bind to the mouse $p21^{Cip1}$ promoter although it showed high affinity binding to the *Arf* promoter (Fig. 1B). Recombinant FLAG-Dmp1 did not bind to parts of the $p21^{Cip1}$ promoter other than #1 or #2 (both 300 −400 bps) as far as we studied with PCR-amplified genomic DNA fragments in EMSA (data not shown).

Recombinant Dmp1 stabilizes p53 binding to target genes

To explore the effect of Dmp1 on the DNA-binding of p53, synthetic oligonucleotides encoding the p53-binding site #2 on the $p21^{Cip1}$ promoter were used (Fig. 1A, top). This sequence was chosen because the sequence was common between mice and humans (54). Recombinant p53 protein prepared from Sf9 cells bound to the DNA, which was supershifted with monoclonal antibodies to p53 (PAb421, DO-1) (complex S, Fig. 2A; see Fig. S3 for a longer exposure). The presence of Dmp1 significantly increased the stability of the p53 complex A in comparison to the p53+BSA control (3 folds; Fig. 2A). The presence of Dmp1 within complex A was confirmed by supershift assays with 5 different antibodies to Dmp1: the antibodies RAX, RAZ, RAD, RAJ and αFLAG caused partial or total supershift of the complex A (Figs. 2A & S3, arrows).

We then repeated EMSA with the general p53-consensus probe (p53-g; ref. 53) that had been used where the activity of Arf was tested (Fig. 2B). Recombinant p53 did not bind to this sequence (see lanes p53#1, #2, p53 +control oligo, p53 +mouse IgG). However, the presence of p53 plus Dmp1 produced complexes B1 and B2 while p53 +DO-1 did not form any complexes. These two complexes were supershifted either with the antibody to p53 (DO-1, S) or Dmp1 (RAX or αFLAG, arrows), suggesting that they contained both p53 and Dmp1 (Fig. 2B). The complexes shown in white asterisks were supershifted bands of the complex B3 that consisted of specific Dmp1:p53-DNA and non-specific binding of Dmp1 to the probe indicated by a white pound symbol (see the Figure Legend).

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Dmp1 stabilizes p53 binding to target genes in cells

The effects of Dmp1 on p53's binding to DNA in cells was investigated by transiently transfecting H1299 cells with expression vectors for HA-p53 and/or FLAG-Dmp1 using human *p21*^{CIP1} promoter probe (site #1). Transfected p53 weakly bound to the probe, the complex of which was supershifted with DO-1 while Dmp1 aone did not bind (Fig. 3A). The p53-DNA complex was stabilized by Dmp1 although the presence of Dmp1 did not affect the mobility of the complex (p53+Dmp1) (Fig. 3B, 2nd lane). The p53-Dmp1 complex was supershifted with two different antibodies to p53 (DO-1 or PAb421, S2), which was not influenced by control antibodies rabbit IgG, mouse IgG, αGFP, and αSPC. The Dmp1:p53-PA421 complex was further supershifted with the DO-1 antibody (S1, see the lane of p53+Dmp1+PAb421+DO-1). Conversely, the complexes were completely disrupted by the Dmp1 antibodies RAD, RAX, RAZ, incompletely with RAF or RAJ, and slightly shifted with RAF, RAJ, or αFLAG (Fig. 3B).

Densitometric analyses of EMSA for Figs. 2 and 3 showed that Dmp1 increased p53 binding to target genes for 3 (Fig. 2A), 6 (Fig. 2B), and 3.5 folds (Fig. 3), respectively (Student t test, $p = 0.027$). In summary, Dmp1 stabilizes p53's binding to the DNA even though Dmp1 does not directly bind to the p53-consensus sequences.

Dmp1 binds to p53 target gene promoters in a p53-dependent fashion

Finally, we tested whether Dmp1 binds to the *p21*^{Cip1}, *Bbc3,* and *Thbs1* genomic regulatory regions in mouse thymus in response to DOX (Fig. 4A). These are genuine target genes for p53 in response to stress signaling (4, 5, 66, 67). Wild type and *p53*-null mice (6-week-old) were injected via tail vein with 0.6 mg DOX/30 g of a mouse, thymi were harvested at 0 and 4 hrs, and binding of Dmp1 to p53 target gene promoters (*p21, Bbc3*) or intron (*Thbs1*) was studied by chromatin immunoprecipitation. The primers used for amplification of p53-consensus sequences are shown in Fig. 1A (arrows). Significant binding of Dmp1 to the *p21*^{Cip1}, *Bbc3*, and *Thbs1* genomic regulatory regions were found in wild type, but not in *p53*-null thymus 4 hrs after DOX injection (Figs. 4A, S4) suggesting that the Dmp1-binding to these p53 target genes was indirectly mediated by p53. Of note, Dmp1 did not bind to these promoters before the drug injection (see 0 hr) suggesting that some kind of stress response is needed for the Dmp1-p53 interaction to happen on genomic DNA.

Discussion

The data shown in this study and our recent publication (47) indicate that Dmp1 (Dmp1α) physically interacts with p53, stabilizes p53's binding to its target genes, and increases the transcriptional activity of p53. Our EMSA data also show that Dmp1 increases *p21*^{Cip1} transcripts without directly binding to the *p21* promoter. Indirect regulation of p53's target genes by Dmp1 through physical interaction of Dmp1 and p53 will be applied to other genes as well since we saw increased binding of p53 to genomic DNA with the oligonucleotide probe that has different consensus sequences for p53 (53, 57–59). Stabilization of the p53-DNA complex through physical interaction with p53 has been reported in c-Abl (68–71), YB-1 (72–74), and HMG-1 (75, 76). In the case of c-Abl-p53 binding, c-Abl accelerated the DNA-binding of p53 to the ribosomal gene cluster (RGC) although it did not change

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mobility of the p53-DNA complex, which resulted in increased transactivation of the *p21* and *RGC* promoters by p53 (69). Similarly, YB-1 increased the DNA-binding of p53 to the $p21^{Cip1}$ promoter although it did not change the mobility of the p53-DNA complex on the promoter (72). Likewise, although HMG-1 stabilized the DNA-binding of p53 to the consensus sequence on the *GADD45* promoter, it did not affect the mobility of the p53-DNA complex nor did the antibody to HMG-1 supershift the p53-DNA complex (75). This increased DNA binding of p53 resulted in nearly 10-fold increase of p53's activity in transactivation. Since HMG-1 is capable of interacting with DNA in a non-sequence specific fashion and bend DNA, thereby providing pre-bent DNA to p53 (76), the molecular mechanism of increased p53-binding to target genes must be different between HMG-1 and the former two. In either case, the p53-binding protein does not have to stay on the p53-DNA complex for long once p53 binds to the target gene as a tetramer (77).

Although we did not see significant change in the mobility of the p53-DNA complex that was stabilized with Dmp1 (Dmp1α), partial supershift of the complex was observed with Dmp1 antibodies (RAX, RAZ, RAD, RAJ, and αFLAG) when EMSA was conducted using recombinant proteins (Fig. 2A). Regarding the EMSA employing a general oligonucleotide probe for p53, the DNA was recognized by Dmp1+p53 to form specific complexes which were supershifted with an antibody to p53 or Dmp1 (Fig. 2B). The Dmp1:p53-DNA complex development may be transient, i.e. Dmp1 leaves the complex after stabilization of p53's binding to the target gene, and addition of the Dmp1 antibody even destabilized the p53-DNA complex, dependent on the epitope on Dmp1 (Fig. 3B). This is apparently an Arf-independent process since these assays were conducted only with recombinant proteins.

Our data in Fig. 2A shows a greater abundance of p53 binding to the DNA as a result of the antibody (PAb421) binding to the C-terminus of p53 (62, 77). It appears that DNA-binding partners of p53 that enhance its interaction with DNA (c-Abl, YB-1, Dmp1) all bind to this region, thereby stabilizing the tetrameric conformation and resulting in a more stable p53-DNA complex.

We had very similar, but different data in EMSA using nuclear lysates from transfected cells. Dmp1 increased DNA binding of p53 to the *p21* promoter, which was supershifted with DO-1 or PAb421 (Fig. 3). In this case, the Dmp1:p53-DNA complex was totally disrupted with RAD, RAX or RAZ antibody in EMSA with cellular lysates (Fig. 3B). Conversely, addition of RAF, RAJ or αFLAG slightly changed the mobility of the Dmp1:p53-DNA complex in a similar fashion as the EMSA with recombinant proteins. We speculate that the presence of other nuclear proteins within the complex will explain the differential effects of Dmp1 antibodies on the Dmp1:p53-DNA complex.

Our results show that Dmp1 stabilizes p53's binding to the DNA even though Dmp1 does not directly recognize the p53-consensus sequences. These results are consistent with the increase of *p21* transcripts in *Arf;p53*-deficient cells by co-transfection of Dmp1 and p53 expression vectors, indicating that it is an Arf-independent mechanism of p53 activation (47). Consistently, significant binding of Dmp1 to the $p21^{Cip1}$, *Bbc3, Thbs1* promoters was found in wild type, but not in *p53*-null thymi, suggesting that Dmp1 recognizes p53 target genes only when p53 is present. Likewise, significant binding of p53 to the $p21^{Cip1}$ and

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Bbc3 promoters was found in wild type, but not in *Dmp1*-null thymi, and induction of both *p21* and *Bbc3* was significantly impaired in *Dmp1*-null, but not *Arf*-null mice when they were injected with DOX (47). Together, these data suggest that direct Dmp1-p53 interaction may play essential roles in cellular responses caused by stress signals caused by DNA damage response. Accumulating evidence suggests that Arf is a gateway of nearly all the oncogenic signals that initiate a p53 response; conversely, Dmp1 is selectively activated by oncogenic Ras, HER2, and cyclin D1 (19, 39, 44), but not with E2F1 or c-Myc (39).

The findings obtained from the current study are summarized in Fig. 4B. The *Dmp1* promoter is activated by oncogenic Ras or overexpression of HER2, which, in turn, activates the Arf-p53 pathway to induce cell cycle arrest or apoptosis to prevent the emergence of transformed cells. The promoter is also activated by TNFα (42) and dsDNA breaks caused by genotoxic drugs (47, this study). Dmp1α physically interact with p53 to accelerate its DNA binding to target genes, which has been shown by EMSA with supershift assays with different antibodies to Dmp1. Since not all Dmp1α molecules exist within the p53-DNA complex, it is hypothesized that Dmp1α dissociates from the complex after stabilization.

We have reported that Dmp1 antagonizes known negative functions of Mdm2 on p53, i.e. polyubiquitination, nuclear localization, and transcription (47). Here we showed that Dmp1 accelerates targeting DNA binding of p53 through physical interaction. It has been reported that Mdm2 inhibits target DNA binding of p53 through the acidic domain in ubiquitination-independent fashion (78). Other tumor suppressors that physically interact with p53 are ARF and PML (18, 79, 80). However, these molecules are different from Dmp1 in that they bind to p53/Mdm2 and translocate p53 to the specific nuclear structure -nucleoli (81) or nuclear bodies (82) respectively to protect p53 from the negative regulation by Mdm2 while Dmp1 does not have such activity. *PML* is a direct transcriptional target for p53 (83) while the *ARF* transcription is repressed by p53 (84). The role of p53 on *DMP1* transcription is currently under investigation; we have shown that the mouse *Dmp1* promoter lacks p53-consensus sequences (39). In summary, Dmp1 is a unique, naturally-occurring nuclear protein that antagonizes all the known functions of Mdm2 on p53 regulation through physical interaction, which can be activated in human cancers with wild type p53 for therapeutic purposes.

Recent studies indicate critical roles of aberrant RNA splicing in carcinogenesis (85–88). The human *DMP1* (*DMTF1*) locus encodes two other splice variants, DMP1β and DMP1γ. We recently reported that DMP1β accelerates G1-S progression and contributes mammary carcinogenesis *in vivo* (51). On the other hand, the biology of DMP1γ in cell proliferation, apoptosis, and/or tumorigenesis is currently unknown. Our study shows that DMP1β does not bind to p53 (data not shown); whether or not DMP1γ binds to p53 has not been studied. It is very likely that they DMP1γ does not affect the function of p53 because i) it lacks the 2nd and 3rd Myb-like domain required for the physical interaction with p53 (52), and ii) DMP1β accelerated the proliferation breast cancer cells independent of p53 (51). To date 40 splice variants have been reported from the *hDMP1* locus (89), but only two of them - aAug10 (hDMP1α) and bAUG10 (a variant that lacks the amino-terminal 88 amino acids) encode proteins that can bind to p53. Since the mRNA for the latter was reported only in the thymus at low levels, we expect that DMP1α is the only splice variant that physically bind

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and stabilize the DNA-binding of p53. Further studies will be conducted to study the effects of other *DMP1* splice variants on p53 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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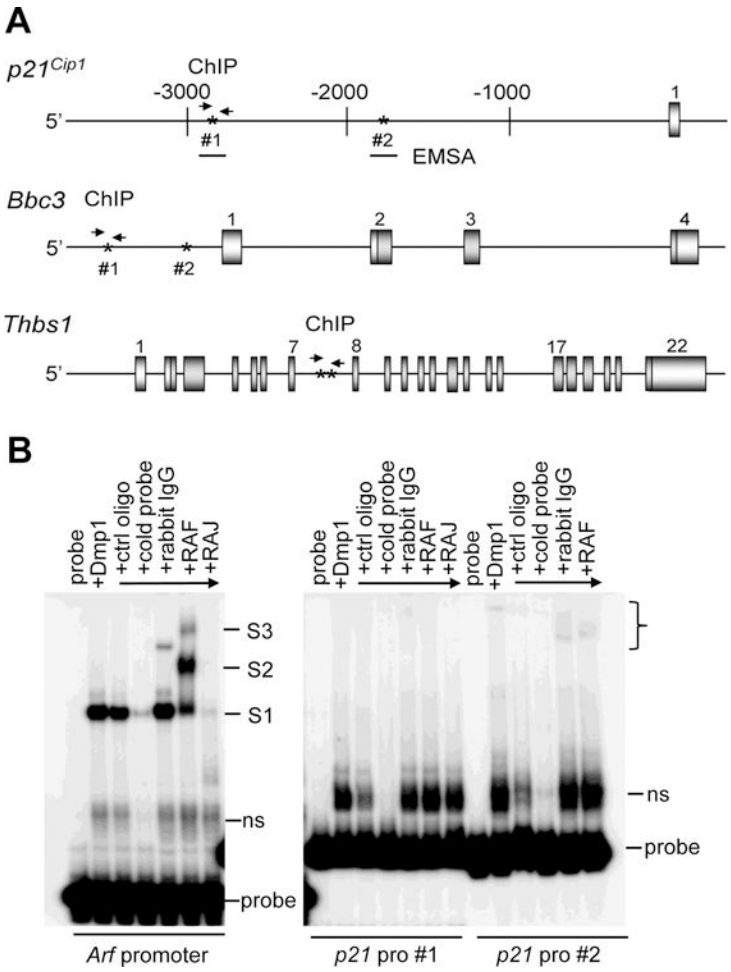

Figure 1. Genomic structures of the mouse *p21^{Cip1}*, *Bbc3*, and *Thbs1* loci and the locations of p53 consensus-sequences amplified in ChIP and EMSA.

A. The structure of the mouse *p21^{Cip1}*, *Bbc3,* and *Thbs1* promoters. Untranslated exons are shown in light silver while coding exons are shown in dark silver. The p53 consensus sequences are shown as asterisks. (top) The mouse *p21^{Cip1}* promoter has two (#1 and #2) p53-binding-consensus sequences at −1,800 and −2,800 bps from the transcription initiation site. The p53 consensus #1 was amplified in ChIP. The probe covering the mouse *p21^{Cip1}* promoter in EMSA (Figs. 2A & 3) are shown as thick bars. There is no Dmp1-binding consensus sequence on mouse or human *p21^{Cip1}* promoter. (middle) The structure of the mouse *Bbc* genomic locus. It has four exons. The p53 consensus #1 was amplified in ChIP. (low) The structure of the mouse *Thbs1* genomic locus. It consists of 22 exons. The p53 consensus sequences in intron 7 were amplified in ChIP. **B.** Recombinant Dmp1 protein binds to the *Arf* promoter, but not to the *p21^{Cip1}* promoter. (left) Recombinant FLAG-Dmp1 binds to the Dmp1-consensus sequence of the mouse *Arf* promoter (S1; refs. 20, 90), which was supershifted with the RAF antibody (to the C-terminus: S2 and S3). The complex S1 was disrupted by the RAJ antibody to the Myb-like repeats within the DNA-binding domain. (right) Genomic DNAs around the p53-binding sites (300–400 bps) were amplified from the plasmid DNA containing the mouse *p21^{Cip1}* promoter (54), end-labelled with [γ−^{32}P] ATP, and were mixed with recombinant FLAG-Dmp1 protein. The protein did not bind to either

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sequence (#1, #2) on the *p21*^{Cip1} promoter. ns indicates the non-specific (ns) complex that was not recognized by either RAF or RAJ antibodies. The higher bands indicated by blankets were also non-specific (ns) signals.

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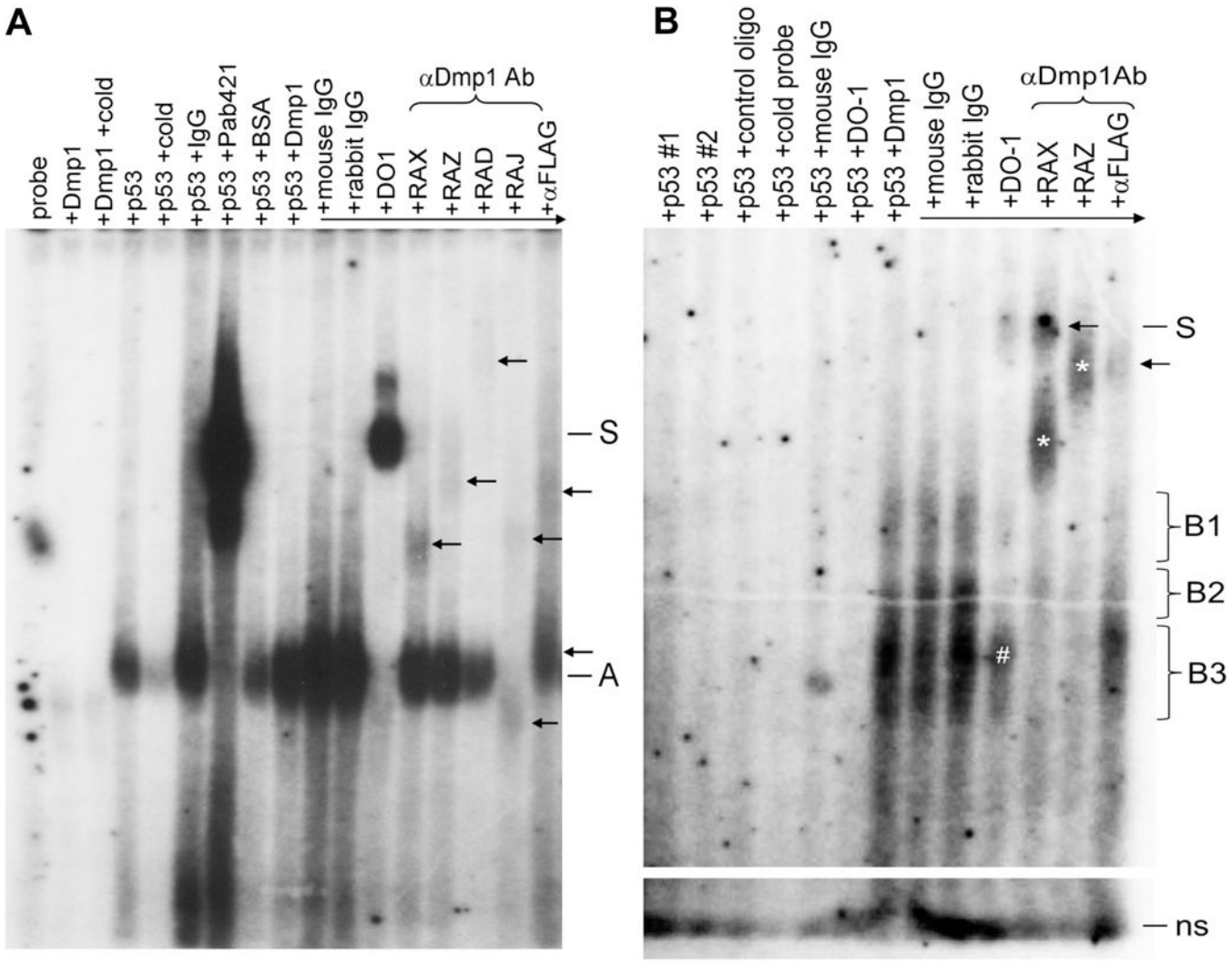

Figure 2. Dmp1 stabilizes p53 binding to the DNA and synergizes with p53 to activate p53 target genes *in vitro*.

A. EMSA was conducted with recombinant FLAG-Dmp1 and p53 proteins with ^{32}P-labeled probe covering the p53 consensus sequence #2 on the murine *p21*^{Cip1} promoter. Recombinant p53 bound to the *p21* promoter by itself (complex A), which was supershifted by the monoclonal antibody PAb421 or DO-1 (S). The presence of Dmp1 significantly increased the stability of the p53 complex A (p53+Dmp1, +mouse IgG or rabbit IgG) in comparison to the p53+BSA control. Although the presence of Dmp1 did not influence the mobility of the A complex, the presence of Dmp1 within complex was confirmed by supershift assays with 5 different antibodies to Dmp1 (arrows). Partially supershifted bands were found in the lanes of RAX, RAZ, and RAD. The complex A totally disappeared and shifted to faster and slower migrating complexes with RAJ that had been directed to the Myb-like repeats of Dmp1[55]. The mobility was decreased with αFLAG with production of partially shifted band. The epitope of each antibody is: RAX, amino acid (a.a.) 136–150 of Dmp1; RAZ: a.a. 741–755 of Dmp1; RAD: full-length His-Dmp1; anti-FLAG: monoclonal antibody to the FLAG tag. A longer exposure of the gel is shown in the Supplementary Fig.

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S3. **B**. EMSA was conducted with recombinant FLAG-Dmp1 and p53 proteins with ^{32}P-labeled probe containing the p53 consensus sequence published (p53-g, refs. 53, 57–59). Recombinant p53 protein did not bind to the probe by itself (lanes for p53#1, #2 [two different preparations]). However, the probe was recognized by Dmp1+p53 to form specific complexes B1 and B2, which were supershifted with either DO-1 (S), RAX, or αFLAG (arrows). The complex B3 is considered to be a mixture of specific Dmp1:p53-DNA complex and non-specific binding of FLAG-Dmp1 to the p53-g probe since the complex was decreased by DO-1 while B3 disappeared with simultaneous appearance of the supershifted band * with RAX or RAZ.

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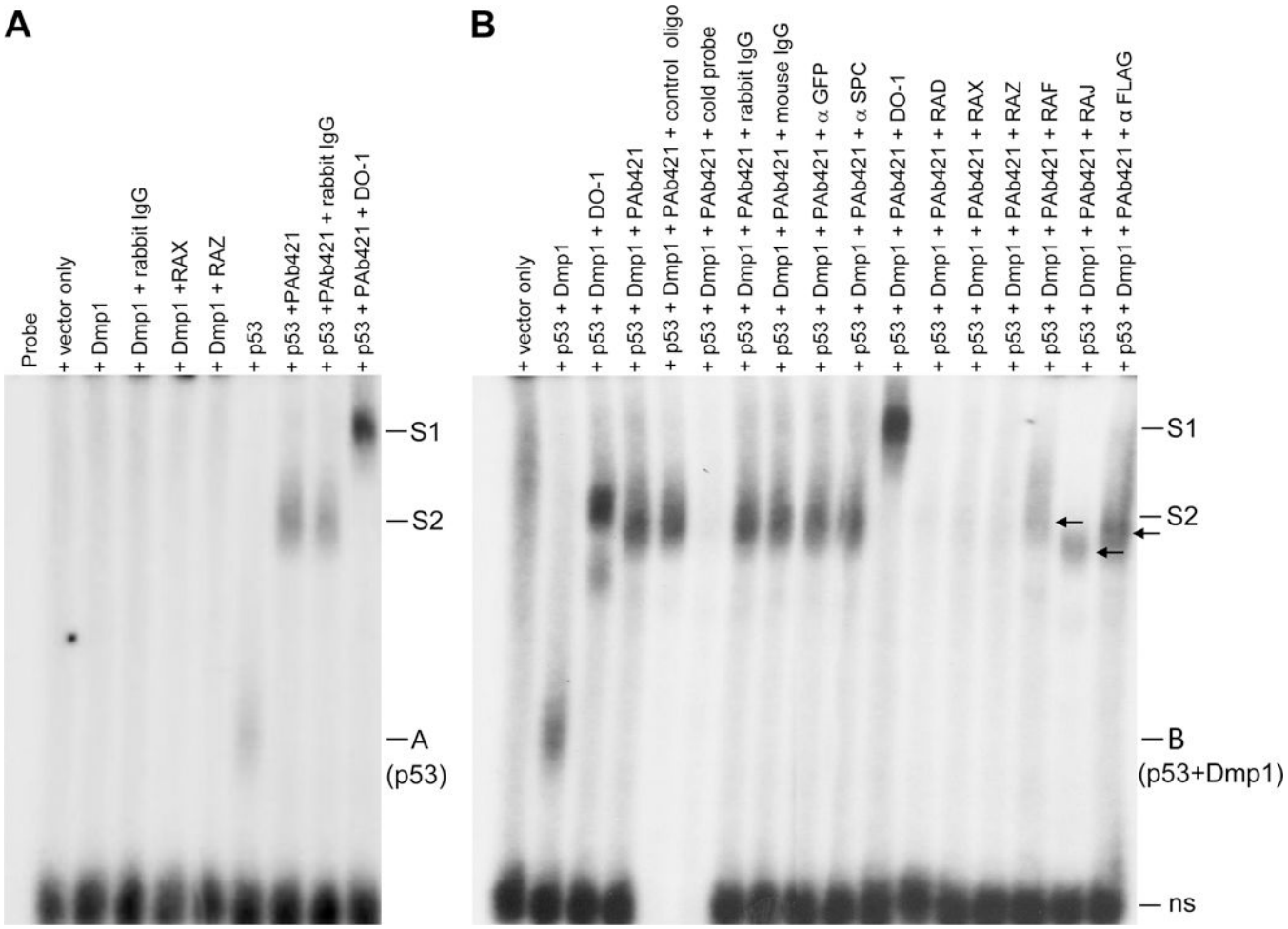

Figure 3. Dmp1 stabilizes p53 binding to the DNA and synergizes with p53 to activate p53 targets *in cells*.

p53-null H1299 cells were transiently transfected with either empty vector or expression vectors for Dmp1 and/or p53, nuclear lysates were prepared, and EMSA was conducted with the probe covering the human $p21^{CIP1}$ promoter, p53-binding site #1. **A**. EMSA with p53 only. Transfected p53 made a weak complex A, which was stabilized with an activating antibody PAb421 (S2), which was further supershifted with DO-1 (S1). **B**. EMSA with p53 plus Dmp1. The presence of Dmp1 stabilized the p53 complex A without changing its mobility, which was supershifted with either DO-1 or PAb421. The complex was not formed in the presence of x200 excess cold oligos, but was not influenced by control antibodies (rabbit/mouse IgG, αGFP [green fluorescent protein], αSPC [surfactant protein C]). The complex S2 was further supershifted with DO-1 (S1). The complex S2 completely disappeared with any one of antibodies to Dmp1 (RAD, RAX, RAZ), indicating that Dmp1 was in the complex. Other antibodies to Dmp1 (RAF, RAJ, and αFLAG) changed the mobility of the complex S2, again showing the presence of Dmp1 in the complex.

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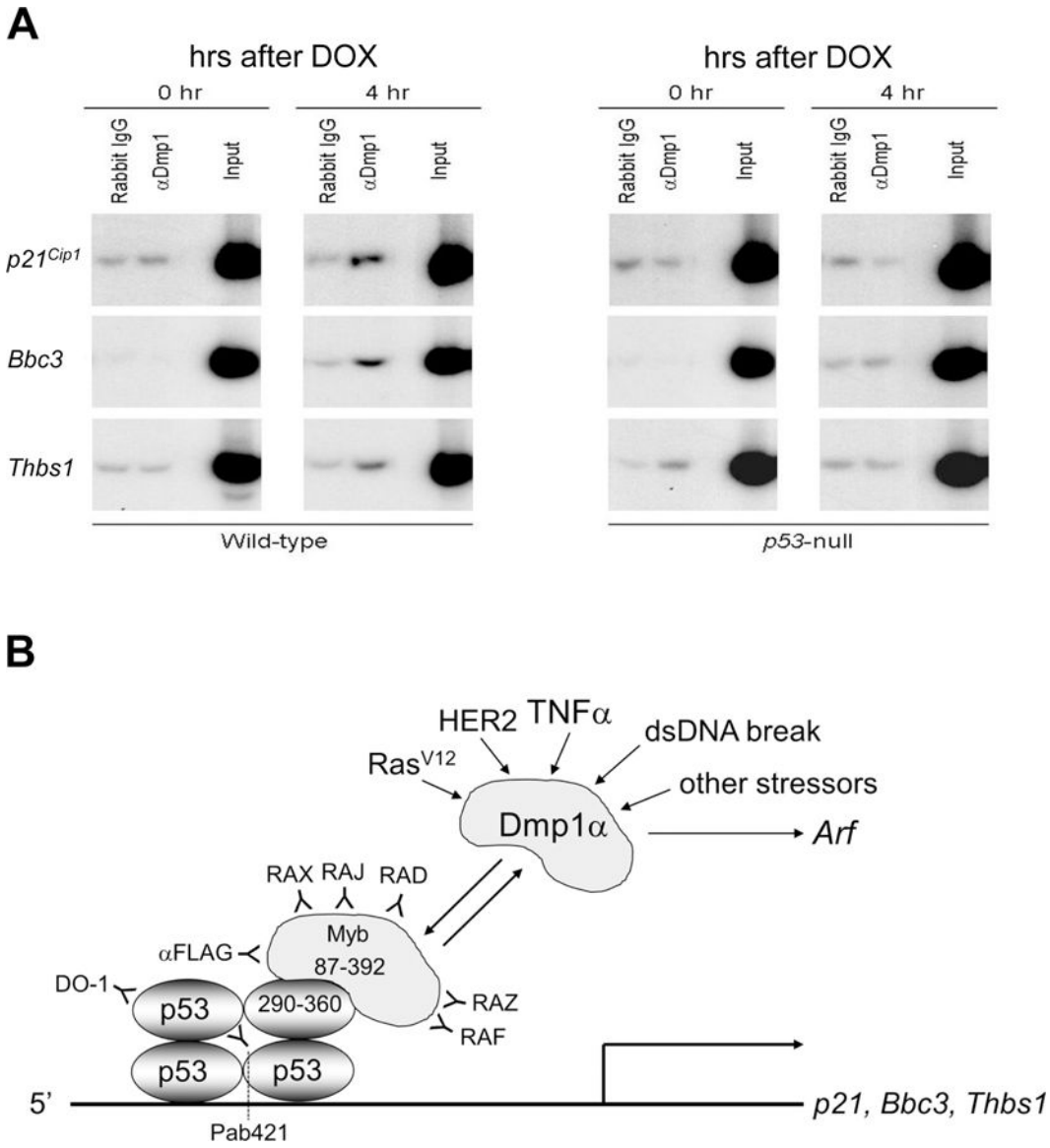

Figure 4. Chromatin immunoprecipitation of the thymus from wild type and *p53*-null mice treated with doxorubicin.

A. p53-dependent binding of Dmp1 to the *Bbc*, *p21*^{Cip1}, and *Thbs1* genomic loci in mouse thymus in response to DNA damage. Wild type and *p53*-null mice (6-week-old) were tail-injected with 0.6 mg DOX/30 g of a mouse, thymi were harvested at 0 and 4 hrs, and binding of Dmp1 to p53 target gene promoters were studied by chromatin immunoprecipitation. Significant binding of Dmp1 to the *Bbc*, *p21*^{Cip1}, and *Thbs1* promoters were found in wild type, but not in *p53*-null thymus, suggesting that Dmp1 binding to these p53 target genes was indirectly mediated by p53. The Dmp1 protein did not bind to the genomic DNA without Dmp1 or p53 consensus sequences (data not shown).

B. Schematic representation of the findings obtained in this study and previously published data[47]. The *Dmp1* promoter is activated by oncogenic Ras and overexpression of HER2, which subsequently activates the Arf-p53 pathway to induce cell cycle arrest or apoptosis to prevent the emergence of transformed cells. The promoter is also activated by TNFα and

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dsDNA breaks caused by DOX or etoposide. In *Arf*-deficient cells, Dmp1α (amino acids 87–392) directly binds to the p53 C-terminus (amino acids 290–360) and neutralizes all the known functions for Mdm2 on p53 (47) published data. This effect is mutually exclusive of DNA-binding of Dmp1α and thus is independent of Arf. In addition, Dmp1α-p53 binding accelerates DNA-binding of p53 to the target genes, which has been shown by EMSA with supershift assays with six different antibodies to Dmp1. See Materials and Methods about the epitope of each antibody.