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Supplementary figures

Dmp1 physically interacts with p53 and positively regulates p53's stability, nuclear localization, and function

Donna P. Frazier, Robert D. Kendig, Fumitake Kai, Dejan Maglic, Takayuki Sugiyama, Rachel L. Morgan, Elizabeth A. Fry, Sarah J. Lagedrost, Guangchao Sui, and Kazushi Inoue

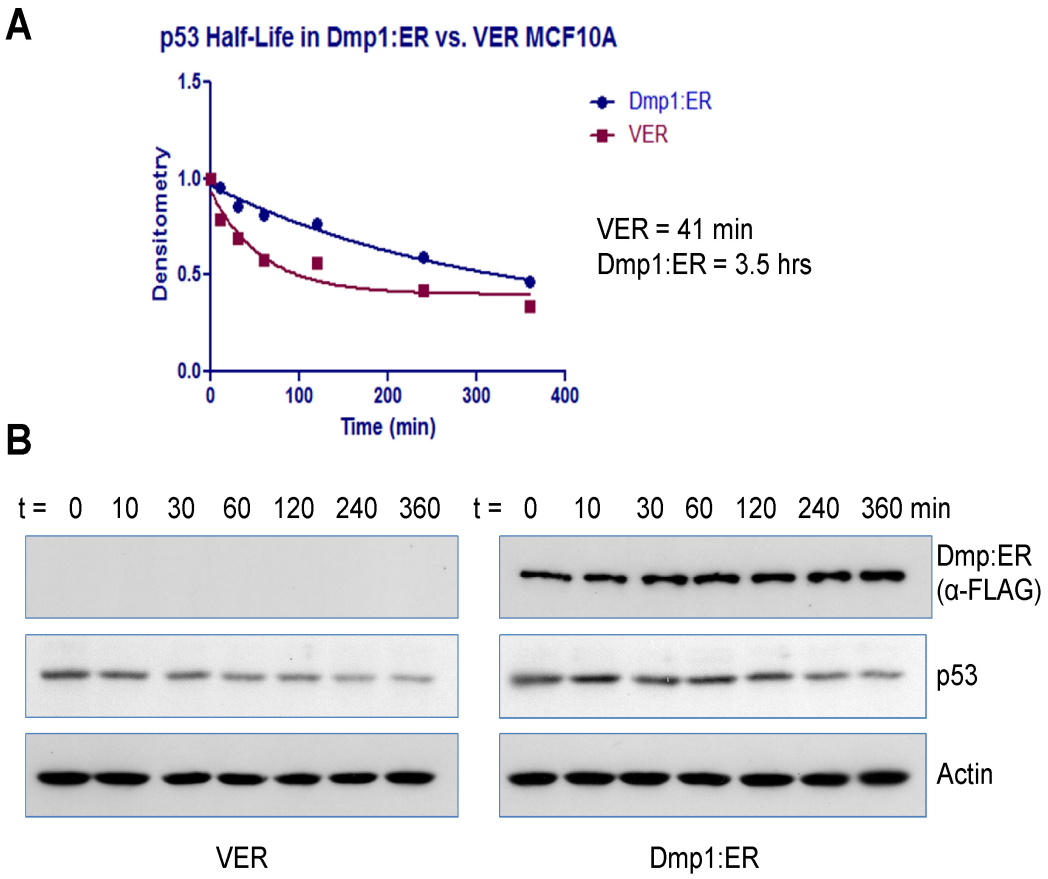
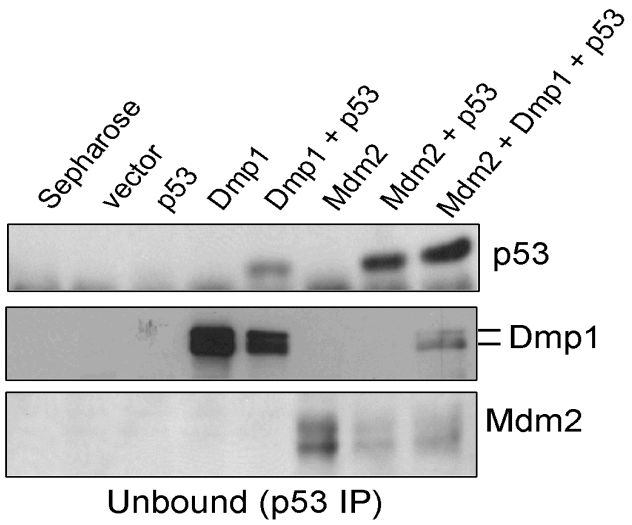


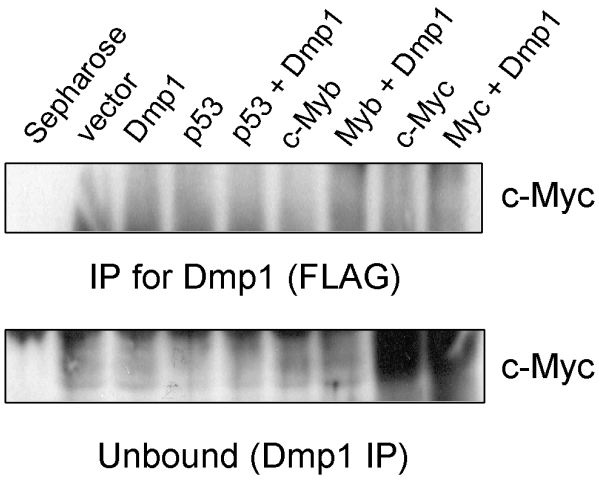
Figure S1. Endogenous p53 protein is stabilized in *ARF*-null MCF10A cells by activated Dmp1:ER.

Dmp1:ER or vector:ER virus-infected MCF10A cells (*p53* wild-type, *ARF*-null) were treated with 2μM 4-hydoxytamoxifen for 24 hours**,** and the p53 protein half-life was measured by treating cells with cycloheximide to stop novel protein synthesis. The p53 protein half-life was prolonged from 41 minutes to 3.5 hours in the presence of Dmp1:ER.

A


Sepharose
vector
p53
Dmp1
Dmp1 + p53
Mdm2
Mdm2 + p53
Mdm2 + Dmp1 + p53
p53
Dmp1
Mdm2
Unbound (p53 IP)

B


Sepharose
vector
Dmp1
p53
p53 + Dmp1
c-Myb
Myb + Dmp1
c-Myc
Myc + Dmp1
c-Myc
IP for Dmp1 (FLAG)
c-Myc
Unbound (Dmp1 IP)

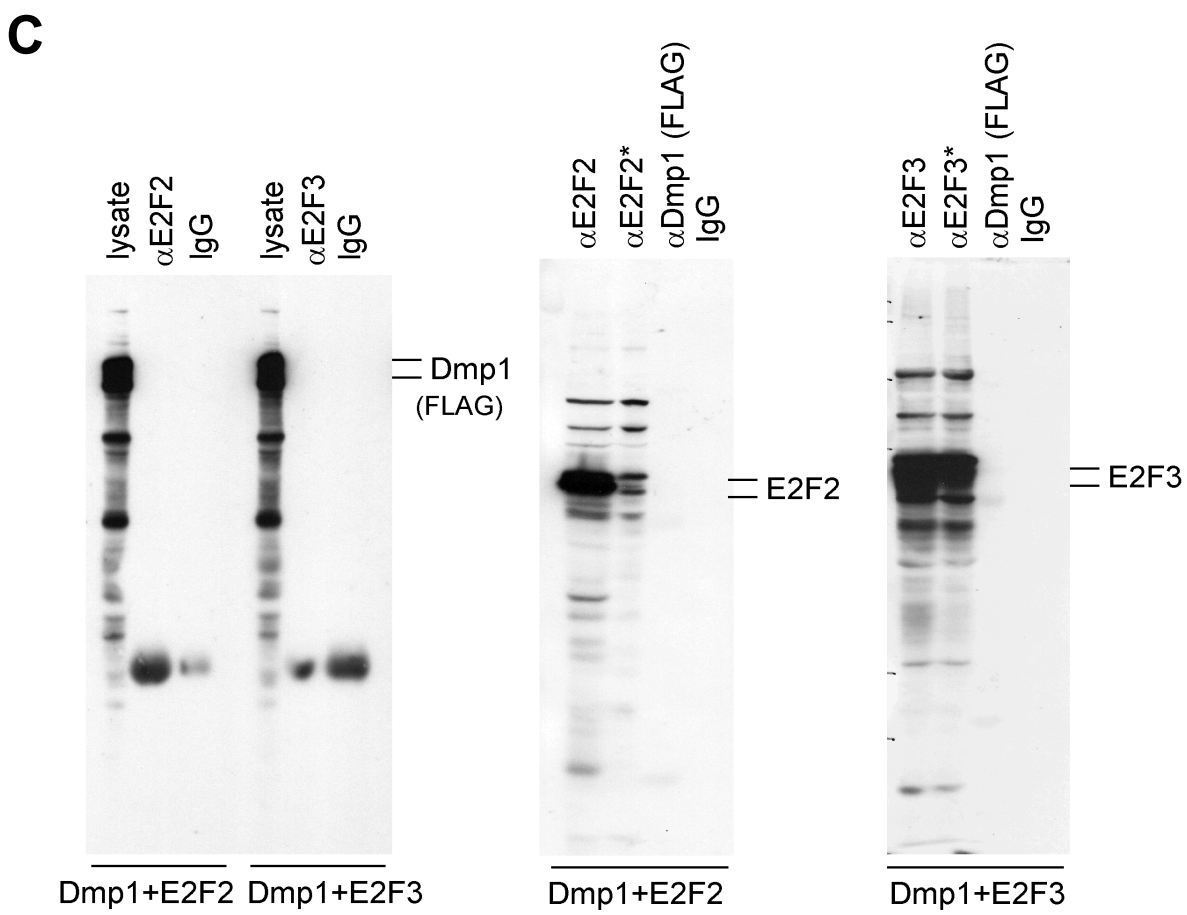


Figure S2. Dmp1 does not bind to c-Myb, c-Myc, E2F2 or E2F3.

(**A**) Detection of p53, Dmp1, Mdm2 proteins in the unbound fractions of cell lysates used in Fig. 1*C*. Cleared cell lysates from 3T3 cells co-expressing FLAG-Dmp1, HA-p53, and/or Mdm2 were immunoprecipitated with antibodies to p53 (HA). The unbound fraction was analyzed by Western blotting. Approximately 50% of Dmp1 or Mdm2 proteins had been recruited to the p53 complex.

(**B**) Dmp1 does not bind to c-Myc. pCMV-c-Myc and pFLEX1-Dmp1 (Inoue and Sherr, 1998) were co-transfected onto NIH 3T3 cells and their physical interaction was studied by IP-Western blotting.

(**C**) Dmp1 does not bind to E2F2 or E2F3. FLAG-Dmp1 and E2F2 or E2F3 were transiently co-expressed in 293T cells and cleared lysates were analyzed by IP-Western blotting. (Left) FLAG-Dmp1 was not found in the immunoprecipitates of E2F2 or E2F3. Likewise, E2F2 (middle) or E2F3 (right) was not found in the immunoprecipitates of FLAG-Dmp1. Asterisk shows the immunoprecipitation of lysates from 293T cells transfected with pFLEX1-Dmp1 only.

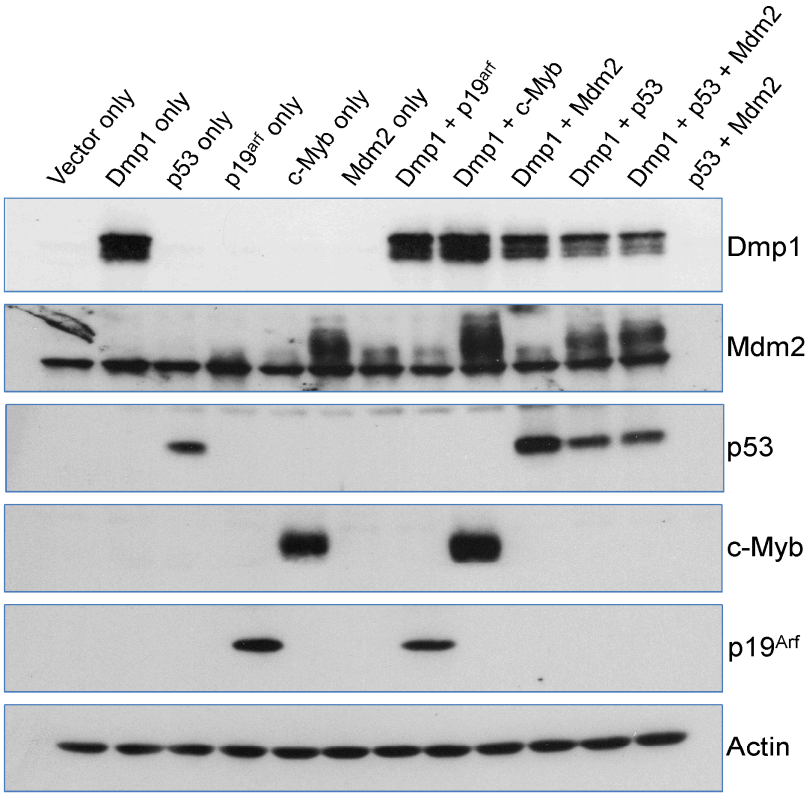


Figure S3. Protein expression in cell lysates used in Figure 1. NIH 3T3 cells were transiently transfected with each expression vector (for Dmp1, Mdm2, po53, c-Myb, and/or $p19^{Arf}$) and expression of each protein was analyzed by Western blotting with specific antibody.

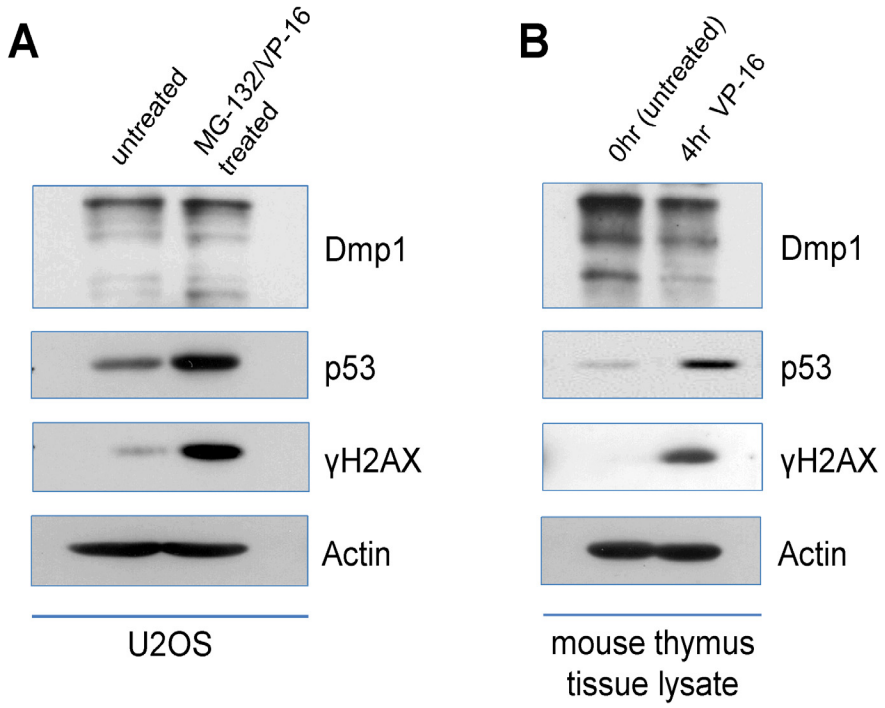
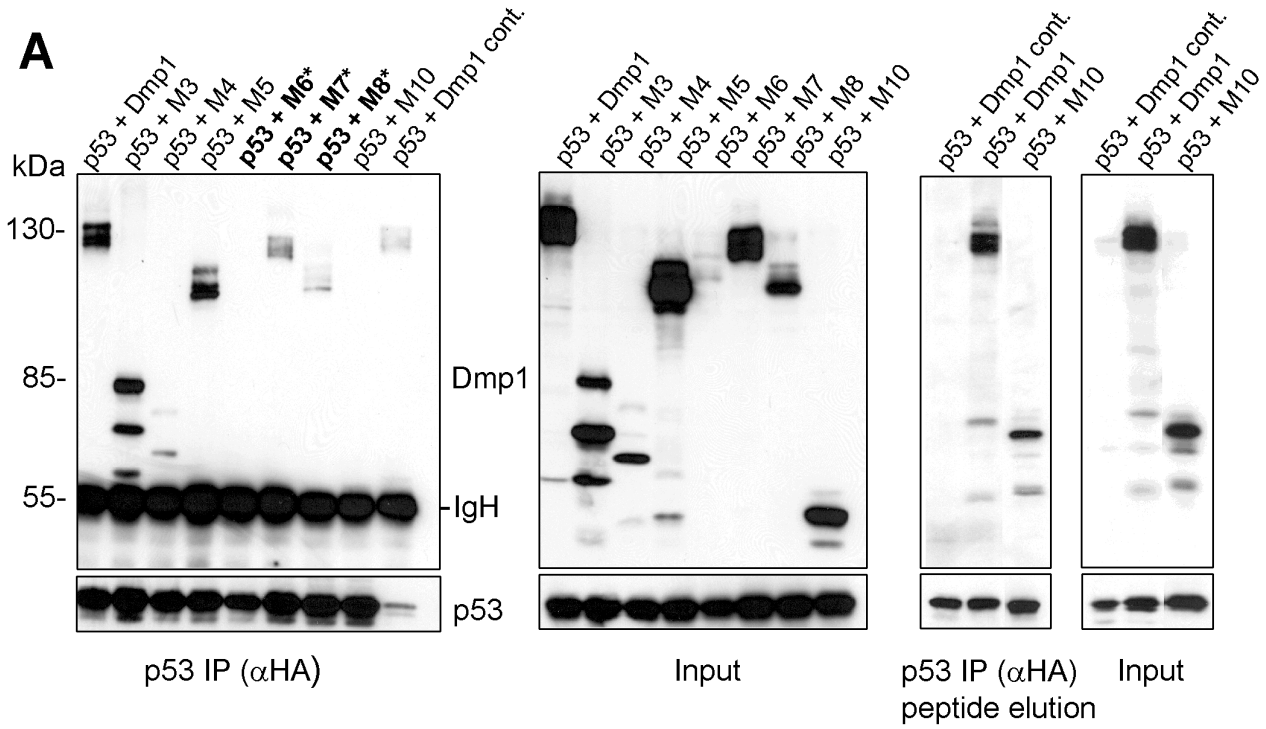
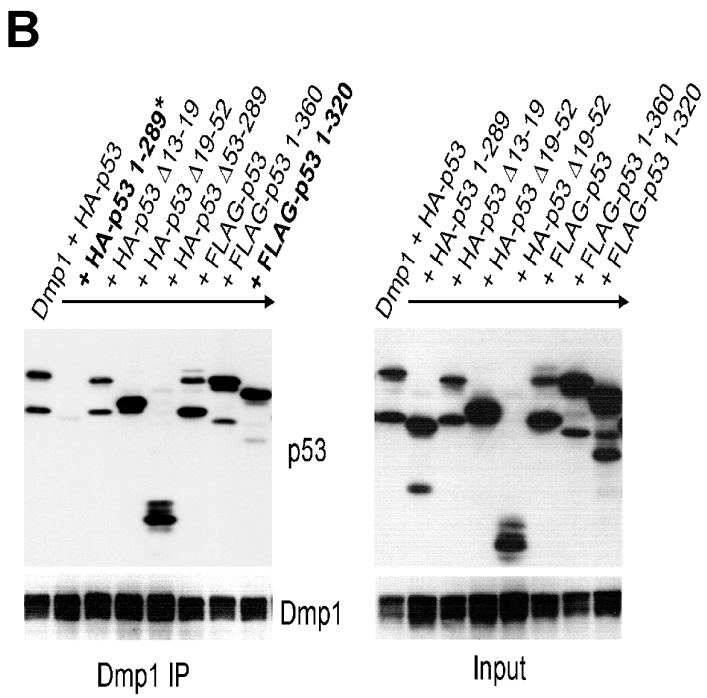


Figure S4. DNA damage response of U2OS cells and mouse thyme used in this study.

(A) U2OS cells were treated with 2 μM etoposide (VP-16) and 25 μM MG-132 for 6 hours for endogenous Dmp1-p53 binding assay for Figure 1*C*. Significant increase of γH2A was observed in VP-16 treated cells.

(B) Mice were tail injected with 0.6 mg of etoposide (per kg of mouse weight) and the thymus was harvested 4hrs after injection, and endogenous Dmp1-p53 binding was studied.


A
kDa
130-
85-
55-
p53 + Dmp1
p53 + M3
p53 + M4
p53 + M5
p53 + M6*
p53 + M7*
p53 + M8*
p53 + M10
p53 + Dmp1 cont.
Dmp1
IgH
p53
p53 IP (αHA)
Input
p53 + Dmp1 cont.
p53 + Dmp1
p53 + M10
p53 IP (αHA)
peptide elution
Input


B
Dmp1 + HA-p53
+ HA-p53 1-289*
+ HA-p53 Δ13-19
+ HA-p53 Δ19-52
+ HA-p53 Δ53-289
+ FLAG-p53
+ FLAG-p53 1-360
+ FLAG-p53 1-320
p53
Dmp1
Dmp1 IP
Input

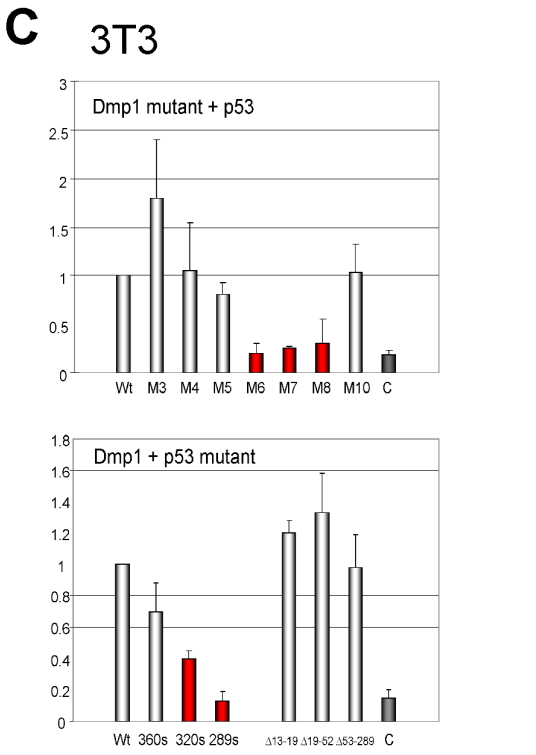

C
3T3
Dmp1 mutant + p53
Wt M3 M4 M5 M6 M7 M8 M10 C
Dmp1 + p53 mutant
Wt 360s 320s 289s
Δ13-19 Δ19-52 Δ53-289 C

Figure S5. Mapping of the p53-binding domain on Dmp1 and Dmp1-binding domain on p53 in NIH 3T3 cells.

(**A**) Mapping of p53-binding domain on Dmp1. HA-p53 and Flag-Dmp1 or mutant expression vectors were transiently transfected onto NIH 3T3 cells, immunoprecipitated with HA beads, and probed with Dmp1 antibody, RAD (29). The input panels show the level of expression of each Dmp1 mutant and p53. Dmp1-p53 binding was significantly impaired in M6, M7, and M8 mutants (shown in bold asterixis) (20).

(**B**) Mapping of Dmp1-binding domain on p53. NIH 3T3 cells were co-transfected with FLAG-Dmp1 and HA-p53 wild-type or mutant expression vectors, and the lysates were immunoprecipitated with the Dmp1 antibody RAD. The filter was probed with p53 antibody (sc-6243). Dmp1–p53 binding was significantly impaired in p53 (1-289) and p53 (1-320) mutants (bold asterixis).

(**C**) Summary of densitometric analyses from 3 independent assays in NIH 3T3 cells. Top: Dmp1 M6, M7, and M8 mutants did not interact with p53. Bottom: the p53 289stop mutant did not interact with Dmp1 while 320stop showed partial interaction. C: background signals from control immunoprecipitation with IgG. Error bars indicate SD.

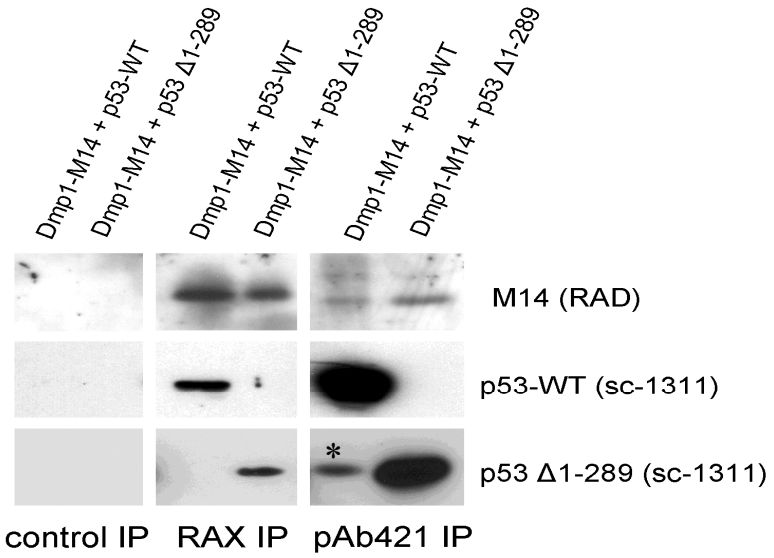


Figure S6. Direct binding of bacterially purified minimal region of Dmp1 (M14) and p53 (Δ1-289).

His-tagged minimal-binding region of Dmp1 for p53 (M14: 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 with 4μg of each protein by IP-Western blotting. Dmp1 M14 bound directly to wild-type p53 and p53Δ1-289. * : background signal from wild-type p53.

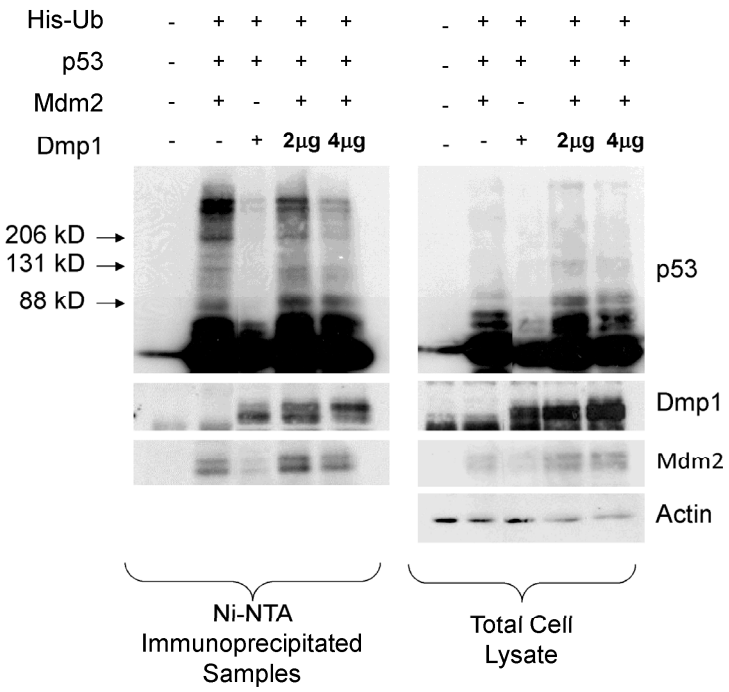


Figure S7. p53 ubiquitination assay without proteasome inhibitor.

H1299 cells were transfected with the indicated cDNAs and treated with the proteasome inhibitor MG-132 to stabilize ubiquitinated proteins. Ubiquitinated proteins were immunoprecipitated with Ni-NTA resin. Proteins were analyzed by Western blotting. Dmp1 inhibited Mdm2-mediated polyubiquitination of p53 in dose-dependent fashion. The dose of DNA represents the amount of DNA used for transfection per 10mL of cell culture.

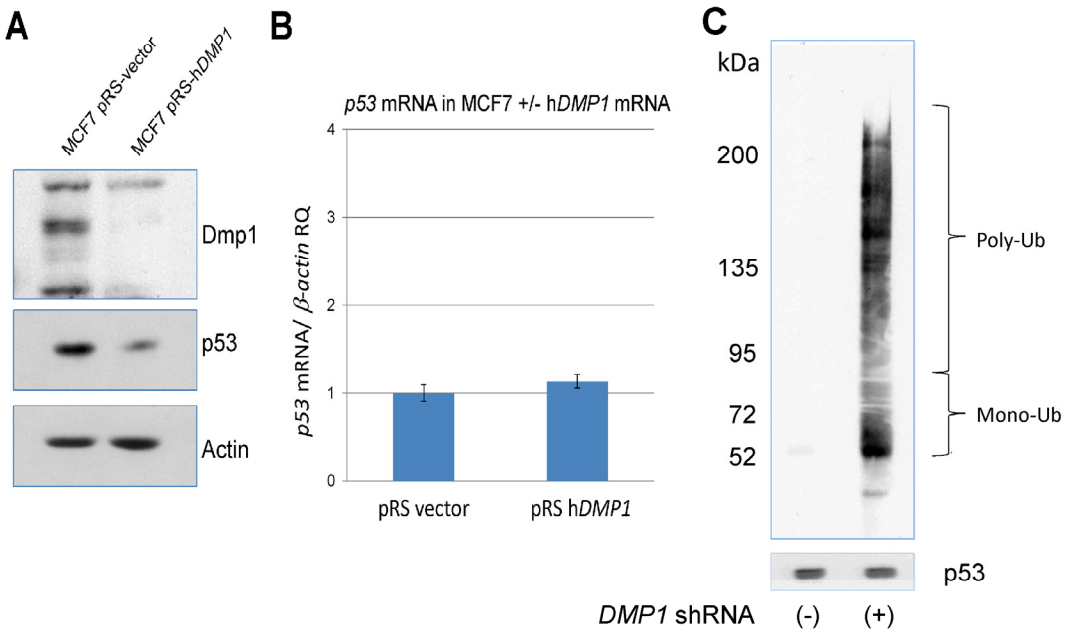


Figure S8. Endogenous *DMP1* knockdown decreases p53 protein levels. **(A)** *ARF*-null, *p53* wild-type MCF7 cells were infected with pRetroSuper vector that knockdown h*DMP1* (23) and the expression of DMP1 and p53 proteins were studied. **(B)** Depletion of h*DMP1* did not affect endogenous *p53* mRNA level suggesting that the p53 downregulation occurred at the protein level. **(C)** Depletion of h*DMP1* increases polyubiquitination of p53. Lysates from MCF7 cells with or without *DMP1* shRNA (80% knockdown at RNA level) were immunuprecipitated with antibody to p53 and filter was probed for ubiquitinated proteins.

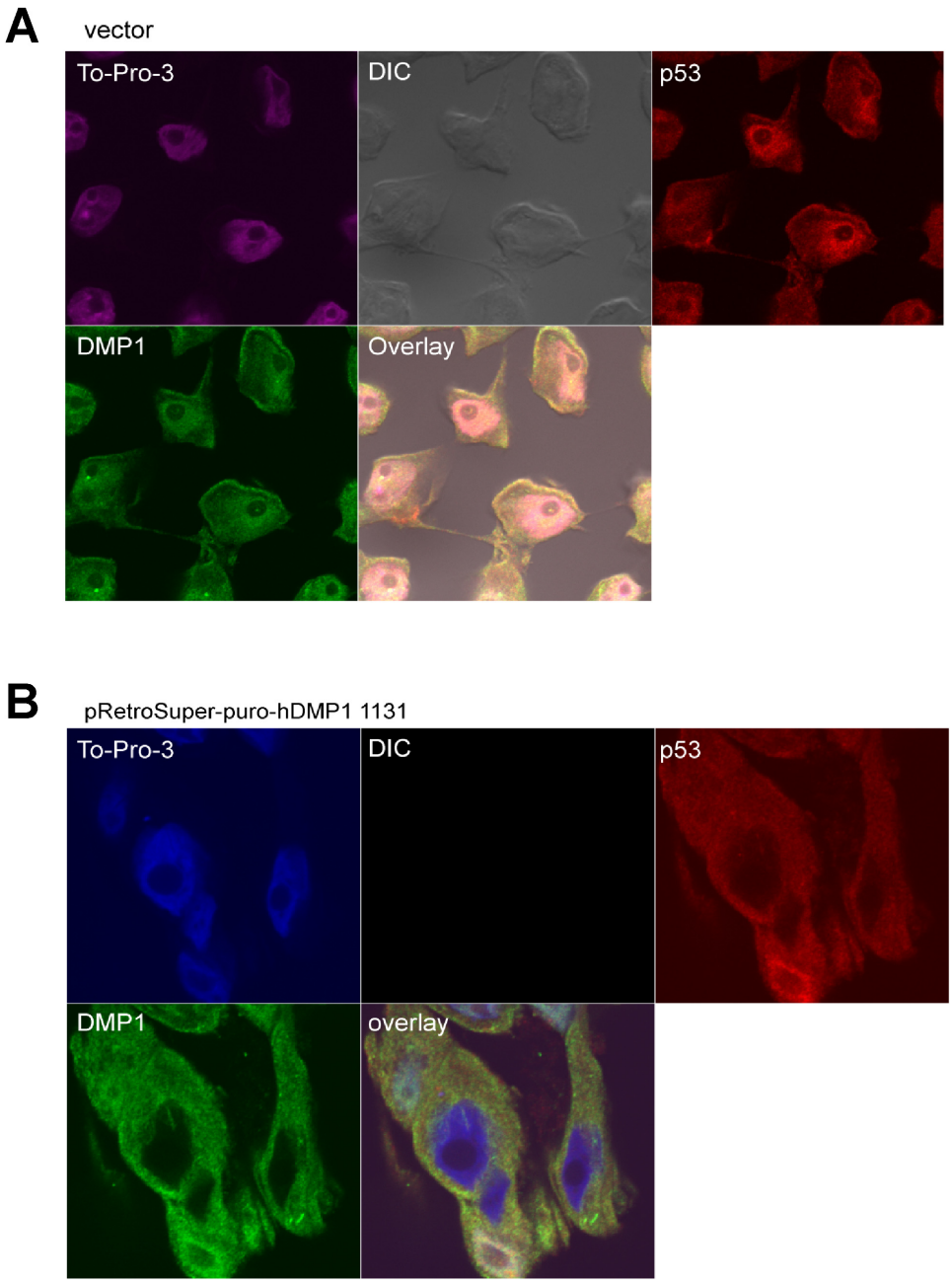


Figure S9. p53 nuclear localization is lost upon *DMP1* ablation by shRNA.

MCF7 cells (*ARF*-null) were transduced with empty vector **(A)** or pRetroSuper-puro virus encoding shRNA for *DMP1* **(B)** and selected with 2 μg/mL puromycin for 7 days. Nuclear exclusion of DMP1, indicative of protein knock-down, directly correlated with nuclear exclusion of p53. In cells expressing vector only, p53 and DMP1 are co-localized in the nucleus.

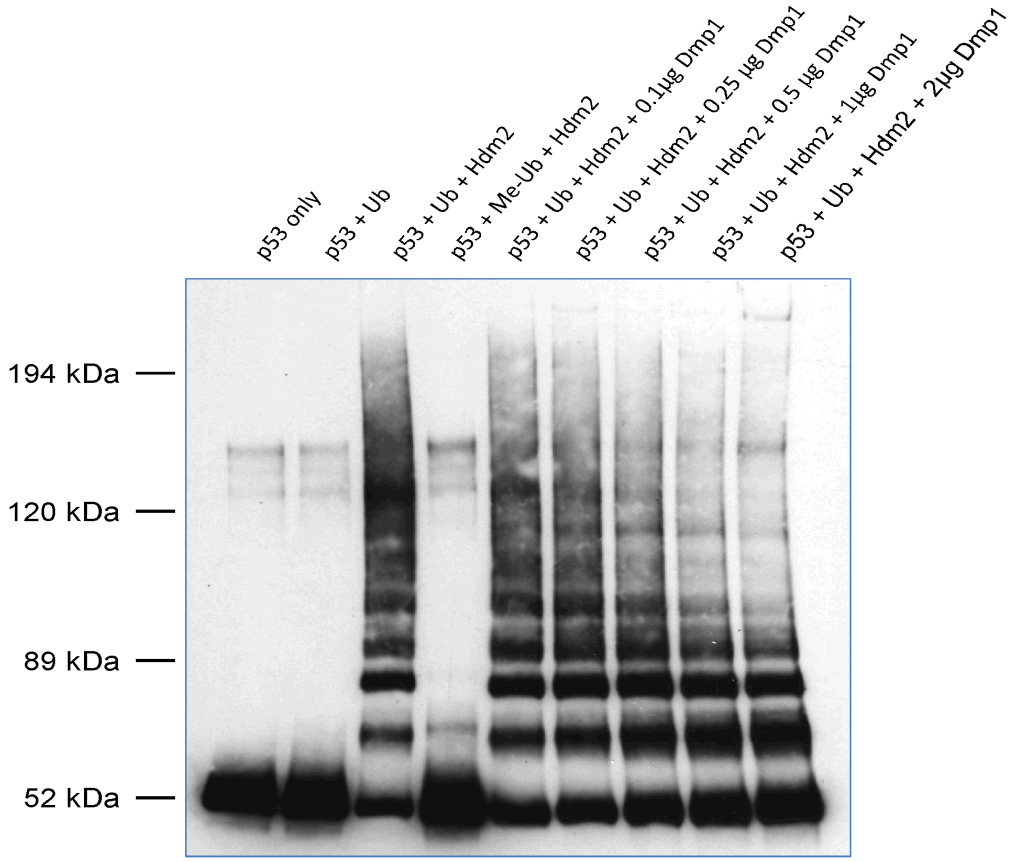


Figure S10. Dose-dependent inhibition of Hdm2-mediated p53 ubiquitination by Dmp1.

In vitro ubiquitination assay was performed in the presence of purified p53, purified Hdm2 (E3), purified E2 (ubch5a), purified E1, and ATP using either Ub (poly- and mono-ubiquitination) or Me-Ub (mono-ubiquitination only) with increasing amount of Dmp1. Dmp1 inhibits Hdm2-mediated p53 ubiquitination in dose-dependent fashion.

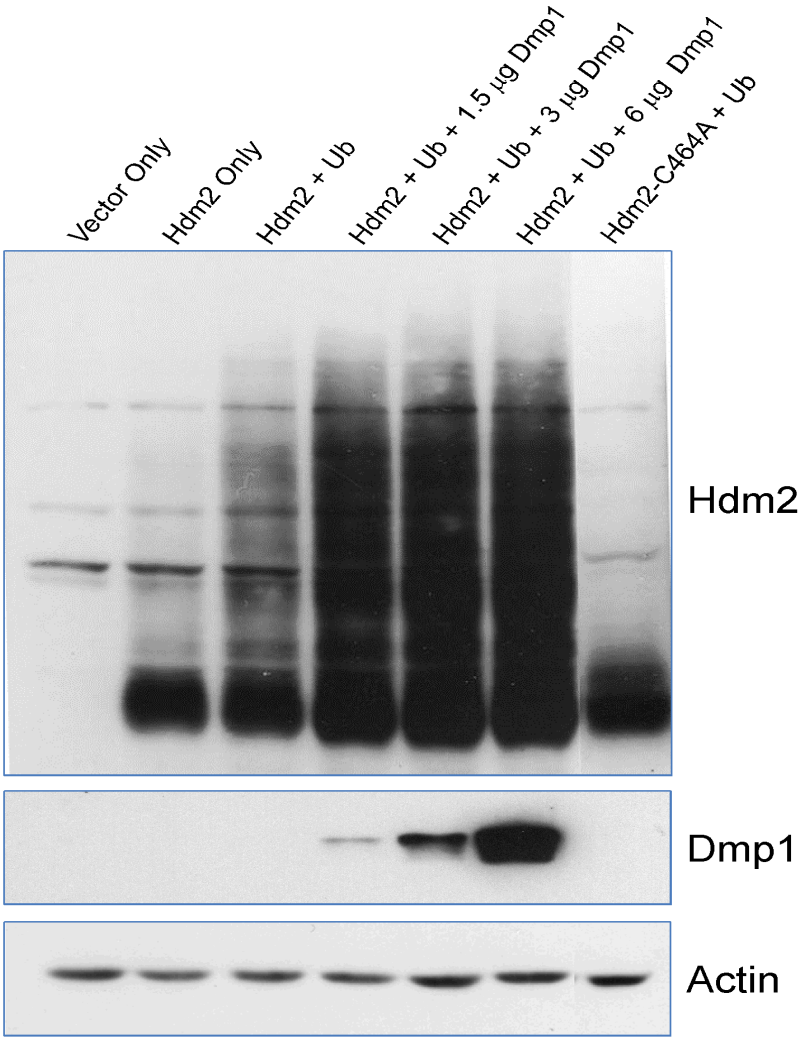


Figure S11. Dmp1 does not inhibit block Hdm2 E3 auto-ubiquitination. Autoubiquitination assay was conducted with recombinant Hdm2 in the presence or absence of Dmp1. Hdm2 C464A is a Hdm2 ring finger mutant that does not auto-ubiquitinate by itself. The results show that Dmp1 did not influence the Hdm2 auto-ubiquitination reaction even when 4 times molar excess of Dmp1 was added.

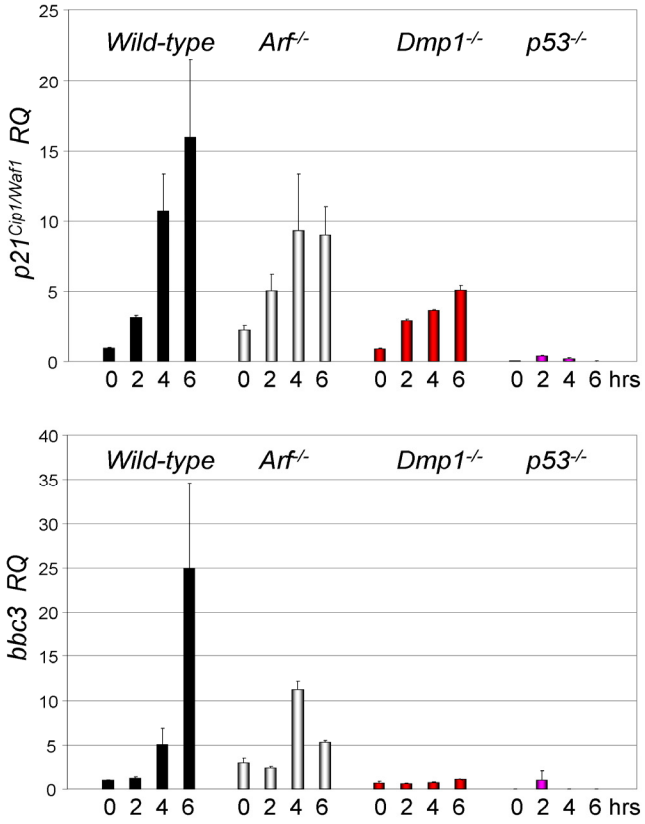


Figure S12. Real-time PCR analyses for physical interaction between Dmp1 and p53 *in vivo*.

The p53 target gene expression in mouse lungs injected with doxorubicin. Mice of each genotype were tail-injected with 0.6 mg doxorubicin/30 g mouse and lungs were harvested at 2 hr intervals.

(A) Real-time PCR analysis of p*21*^{Cip1} in mouse lungs. Error bars indicate SEM.

(B) Real-time PCR analysis of *bbc3* in mouse lungs. Both *p21*^{Cip1} and *bbc3* mRNA inductions were more significantly compromised in *Dmp1*$^{-/-}$ lungs than in *Arf*$^{-/-}$ lungs, suggesting *Arf*-independent mechanism of p53 regulation by Dmp1. Error bars indicate SEM.