Supplemental Data
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Acetylation Is Indispensable for p53 Activation

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Supplementary Experimental Procedures

Antibodies

The polyclonal antibody specific for the acetylated p53 at K164 (anti-Ack164-p53) was produced in collaboration with Bethyl Laboratories, Inc. Rabbits were immunized with the acetylated human p53 peptide (160-MAIY$_{AC}$KQSQH-168) where $_{AC}$K represents the acetylated K164. Antisera from the immunized rabbits were first depleted with the unacetylated peptide (160-MAIYKQSQH-168) and then affinity- purified by use of the acetylated p53 peptide. Anti-p21 (sc-397), anti-Tip60 (N-17, sc-5725), anti-Tip60 (K-17, sc-5727), anti-Pig3 (C-20, sc-16327), anti-p53 (DO-1, sc-126), anti-p53 (1801), anti-Mdm2 (SMP14, monoclonal), and anti-p53 (FL, sc-6243), were purchased from Santa Cruz biotechnology, Inc.; anti-phospho-p53 (Ser-15) (#9284) from Cell Signaling Technology; anti-HA (A190-108A), anti-Pirh2 (BL588), and anti-Mdmx (A300-287A) from Bethyl Laboratories, Inc.; anti-Bax (06-499) and anti-AcK320-p53 (06-915) from Upstate; anti-actin (A1978) from Sigma; anti-Puma (PC-686) and anti-p53 (P$_{Ab}$421) (OP03L, Ab-1) from CALBIOCHEM. Anti-acetylated-p53 (CT) was produced in our laboratory (Luo et al., 2000) and anti-AcK120-p53 was described in (Tang et al., 2006). Anti-Tip60 (CLHF) was a gift from Dr. Chiara Gorrini and Dr. Bruno Amati. The rabbit polyclonal anti-Mdm2 antibody was raised against the GST-human Mdm2 fusion protein.

Plasmids

The plasmids expressing various p53 mutants were derived from pCin4-Flag-p53 by mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instuctions. Mutations at the specific sites were confirmed by DNA sequencing. To construct pTRE2hyg-Flag-p53 and pTRE2hyg-Flag-p53-8KR, the Flag-p53 fragments from pCin4-Flag-p53 were subcloned into the pTRE2hyg vector (a gift from Dr. Privses).

Protein purification and Mass spectrometry

To purify the acetylated p53 protein for mass spectrometric analysis, H1299 cells were co-transfected with CMV-Flag-p53 and CMV-CBP-HA, cultured for 18 hours, and then treated with 1 μM TSA + 5 mM Nicotinamide for 6 hours. Cells were harvested and lysed in the Flag-lysis buffer (50 mM Tris-HCl pH 7.9, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, and fresh proteinase inhibitor cocktail (Sigma)) plus 2 μM TSA and 10 mM Nicotinamide , and the cell extracts were

immunoprecipitated with the anti-Flag monoclonal antibody-conjugated M2 agarose beads (Sigma). The Flag peptide eluted material was resolved by SDS-PAGE on a 4-20% Tris-Glycine gradient gel (Invitrogen). The p53 bands were excised and subject to mass spectrometric analysis.

PCR primers

Primers for ChIP-PCR amplification were:
p21: 5' CTTTCACCATTCCCCTACCC 3' and 5' AATAGCCACCAGCCTCTTCT 3';
Mdm2: 5' GGATTGGGCCGGTTCAGTGG 3' and 5' GCGTCCGTGCCCACAGGTC 3';
pig3: 5' GATCCCAGGACTGCGTTTTGCC 3' and 5' GGGAACGAGACCCAACCTCTTG 3'; pih2: 5' CCAGAGCCTAACCACAGACAAGC 3' and 5' ACATTCCTTTCCTAAACTCGGTGG 3'.
Primers for amplification of DNA fragment containing the p53 binding site from the p21 promoter were: 5' TGCTGCCTGCTTCCCAGGAACA 3' and 5' CCATCCCCTTCCTCCCTGAAA 3'.

GST Pulldown

The ^{35}S-methionine-labeled in vitro translated proteins or purified proteins as indicated were incubated with 5 µl GST beads bound with 1 µg of the GST protein or GST fusion proteins in binding buffer BC100 (20 mM Tris pH7.9, 100 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.5% Triton X-100, and fresh proteinase inhibitor cocktail) at 4^{o}C for 2 hours with gentle rotation. The GST beads were washed 4 times with binding buffer and eluted with 40 µl binding buffer plus 20 mM reduced glutathione for 2 hours with gentle rotation. Half of the eluted materials were resolved on an 8% SDS-PAGE gel and the presence of the labeled proteins was detected by autoradiography or western blot.

Ablation of Mdm2 and Mdmx by siRNA

Ablation of the endogenous Mdm2 and Mdmx proteins was achieved by transfection of Tet-off-p53-8KR cells with a mixture of siRNA duplexes for Mdm2 (ON-TARGETplus SMARTpool, L-003279-00-0010) and Mdmx (ON-TARGETplus SMARTpool, L-006536-00-0010) using Lipofectamine2000 according to the manufacture's protocol. Additional Mdm2 siRNA (J-003279-12) and Mdmx siRNA (J-006536-09) were used to confirm the absence of off-target effect. A mixture of non-specific siRNA duplexes (ON-TARGETplus siCONTROL Non-TARGET Pool, D-001810-10-20) was used as a specific siRNA control. All siRNAs were purchased from Dharmacon.

Adenoviral Infection and Apoptosis

Production of adenoviruses expressing GFP (control), p53 or p53-8KR, infection of H1299 cells, and analysis of apoptosis were carried out as previously described (Tang et al., 2006).

In Vitro Acetylation Assay

In vitro protein acetylation assays were carried out essentially as previously described (Tang et al., 2006) with some modifications. The Flag-p300, CBP-HA, and Flag-p53 mutant proteins were purified from the transfected 293 cells to homogeneity under stringent conditions (500 mM NaCl + 1% Triton X-100). Twenty microliter reactions contained 50 mM Tris (pH 7.9), 10% glycerol, 1 mM DTT, 10 mM sodium butyrate, 20 μM of acetyl-CoA, 200 ng of purified Flag-p53-8KR or Flag-p53-K120R/6KR, and 10 ng of CBP-HA or Flag-p300, and incubated at 30°C for 1 hr. The reaction mixture was then subject to electrophoresis on SDS-PAGE gels, followed immunoblot.

Immunoprecipitation and Immunoblot

To immunoprecipitate the ectopically expressed Flag-p53 proteins, cells were lysed in the Flag-lysis buffer (50 mM Tris-HCl [pH 7.9], 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% sarkosyl, 10% glycerol, and fresh proteinase inhibitor cocktail). The whole-cell extracts were immunoprecipitated with the anti-Flag monoclonal antibody-conjugated M2 agarose beads (Sigma). The Flag peptide elutes were resolved by SDS-PAGE and detected by antibodies as indicated.

To detect acetylation of the endogenous p53 proteins at K164, HCT116 cells were lysed in the Flag lysis buffer. Two milligrams of the cleared total cell extracts was incubated with 2 μg of the anti-p53 (FL) antibody overnight with gentle rotation. Fifteen microliters of protein A agarose beads was then added and incubated for an additional 2 hr. Beads were washed five times with the Flag-lysis buffer, and the bound materials were eluted in the SDS sample buffer with boiling. The eluted materials were resolved on SDS-PAGE gel and detected by the anti-AcK164-p53 antibody.

To detect acetylation of the endogenous p53 proteins at K120, K164, and C-terminus in response to DNA damage, U2OS cells were lysed in the Flag lysis buffer. Two milligrams of the cleared total cell extracts were incubated with 2 μg of the site specific acetylation antibodies overnight with gentle rotation. Fifteen microliters of protein A/G agarose beads were then added and incubated for an additional 2 hr. Beads were washed five times with the Flag-lysis buffer, and the bound materials were eluted in the SDS sample buffer with boiling. The eluted materials were resolved on SDS-PAGE gel and detected by monoclonal antibody against p53 (DO1).

Supplementary Figures with Figure Legends

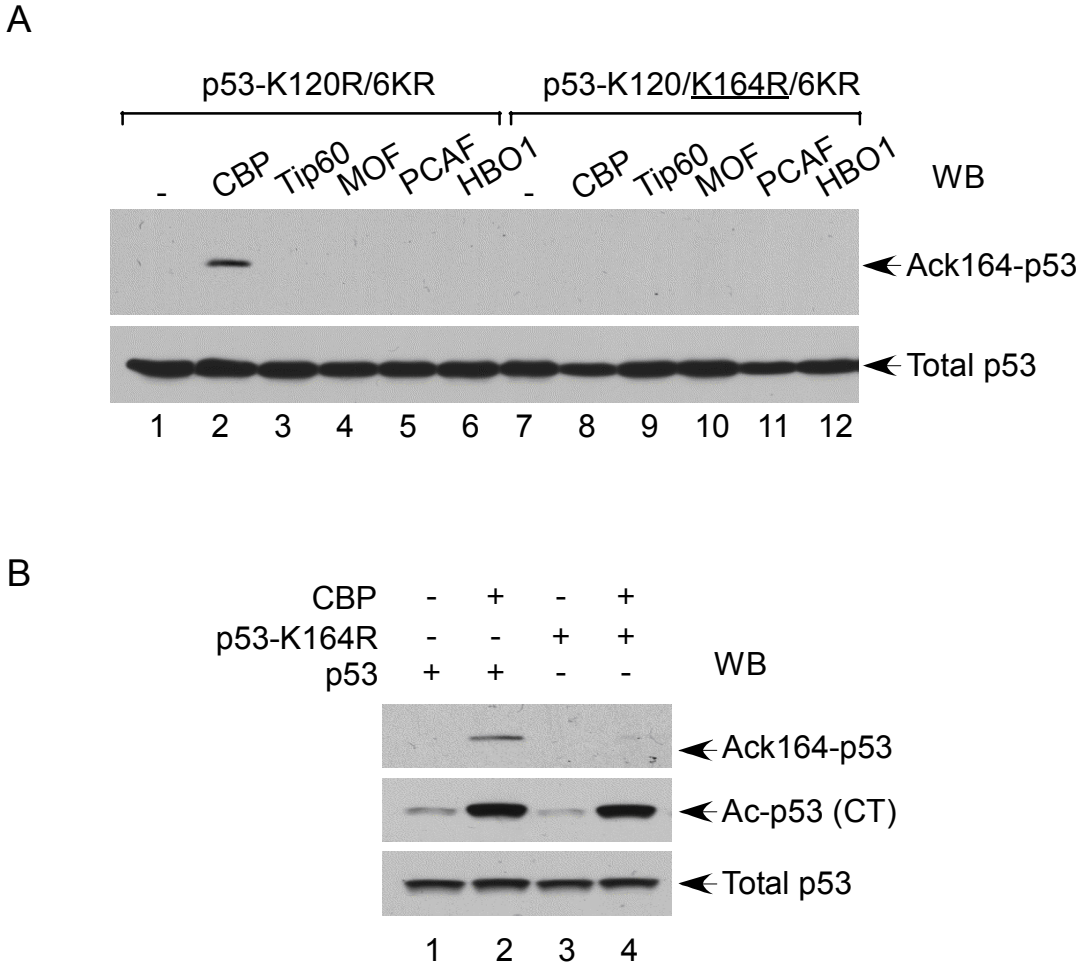


Figure S1. A. K164 is specifically acetylated by CBP in vivo. H1299 cells were transfected with the plasmids expressing the Flag-p53 mutants and different acetyltransferases as indicated. The p53 proteins immunoprecipitated by M2 beads were analyzed by Western blot using the anti-AcK164-p53 and anti-p53 (1801) antibodies. B. In vivo acetylation of p53 at K164 by CBP. H1299 cells were transfected with the plasmid DNA expressing Flag-p53 (WT or K164R mutant) with or without CBP. As in A, the M2 immunoprecipitated materials were analyzed by Western blot using the anti- AcK164-p53, anti-Ac-p53 (CT), and anti-p53 (1801) antibodies, respectively.

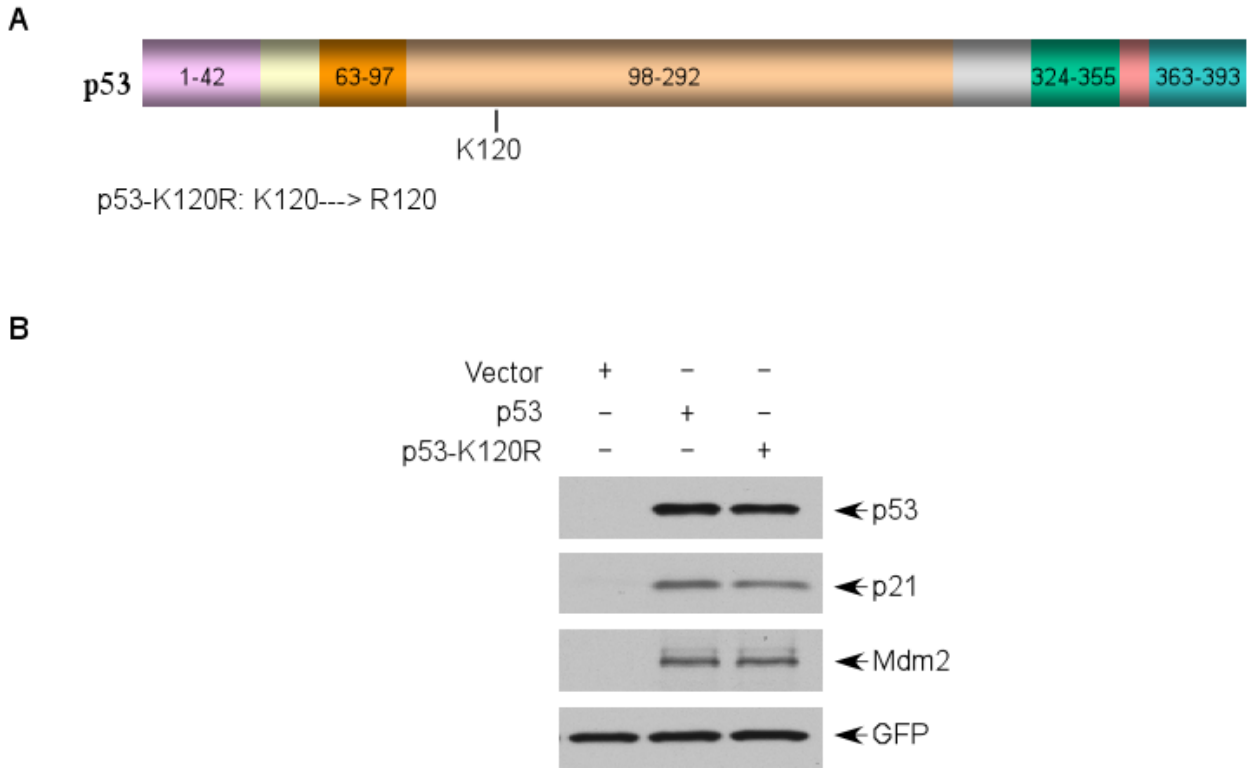


Figure S2. Transcriptional activity of p53-K120R mutant

A. Scheme of p53 mutant with lysine-120 replaced by arginine (p53-K120R).

B. Transcriptional activity of p53-K120R mutant in H1299 cells. The total cell extracts from H1299 cells transfected with control vector, CMV-p53, or CMV-p53-K120R were subjected to Western blot using antibodies against p53 (DO-1), p21, and Mdm2. GFP as used as a control for transfection efficiency.

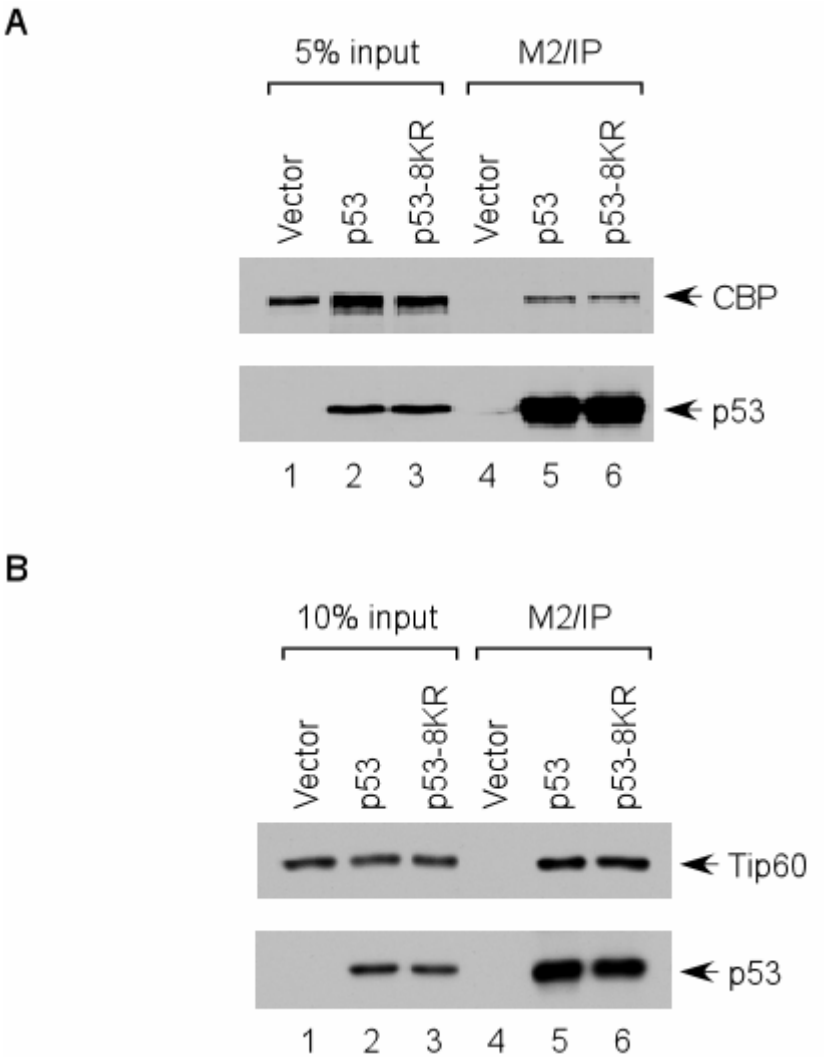


Figure S3. In vivo interaction of p53 or p53-8KR mutant with transcriptional co-activators

H1299 cells were co-transfected with CMV-CBP-HA (A) or CMV-Tip60 (B) abd with control vector (lanes 1 and 4), or plasmid DNA expressing either Flag-p53 (lanes 2 and 5) or Flag-p53-8KR (lanes 3 and 6). The total cell extracts and the immunoprecipitates by M2 beads were fractionationed and analyzed by Western blot using antibodies against p53 (DO1), HA, and Tip60.

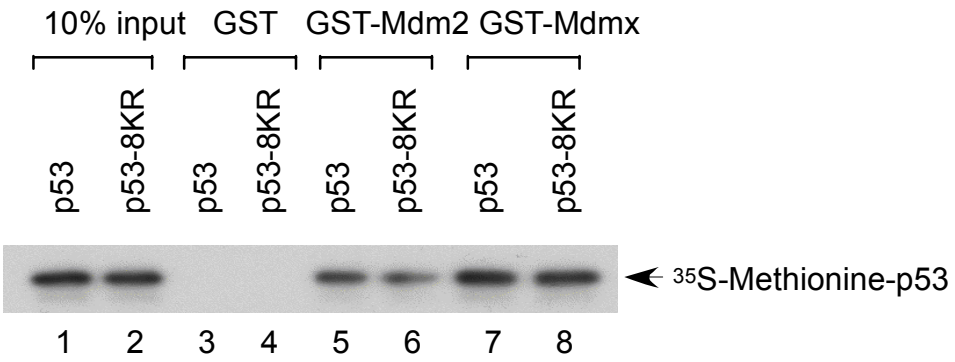


Figure S4. In vitro binding of p53 or p53-8KR to Mdm2 or Mdmx

In vitro translated 35S-methionine labeled p53 or p53-8KR protein was used in the pulldown assay with GST (lanes 3 and 4), GST-Mdm2 (lanes 5 and 6), or GST-Mdmx (lanes 7 and 8). The glutathione elutes and 10% input materials (lanes 1 and 2) were resolved on an SDS-PAGE gel and visualized by autoradiography.

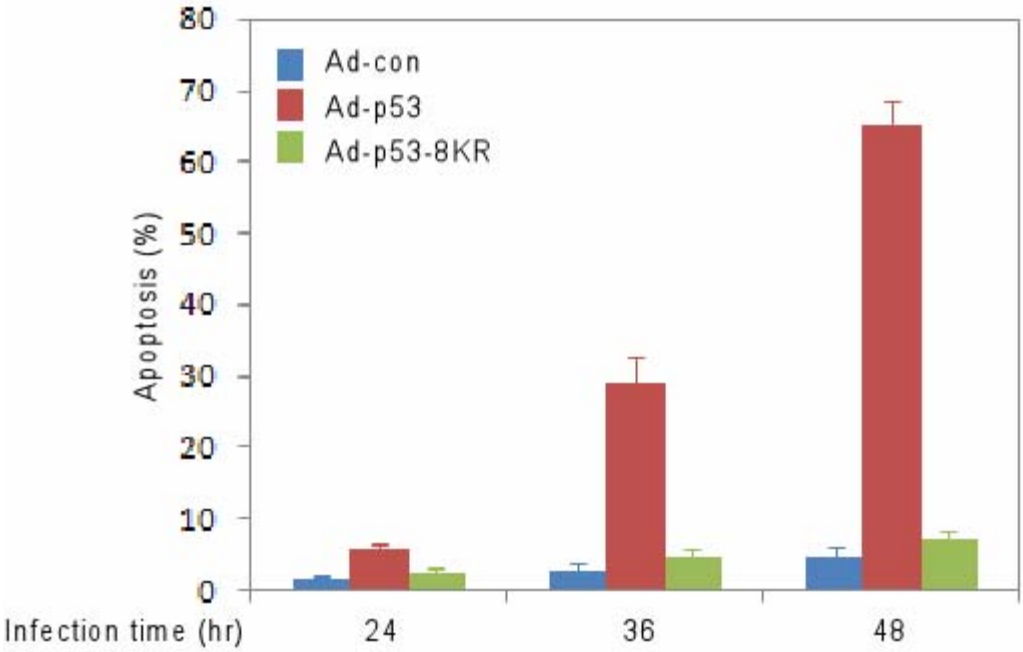


Figure S5. Apoptosis induced by adenovirus-mediated expression of p53 and p53-8KR

H1299 cells infected for 24, 36, or 48 hours by control adenovirus or adenoviruses expressing either p53 or p53-8KR mutant were collected, washed in PBS, fixed in cold methanol, stained with PI, and analyzed by flow cytometry. Apoptosis was quantitated by counting the number of cells with sub-G1 DNA content. The data presented as mean ± SD represent three independent experiments.

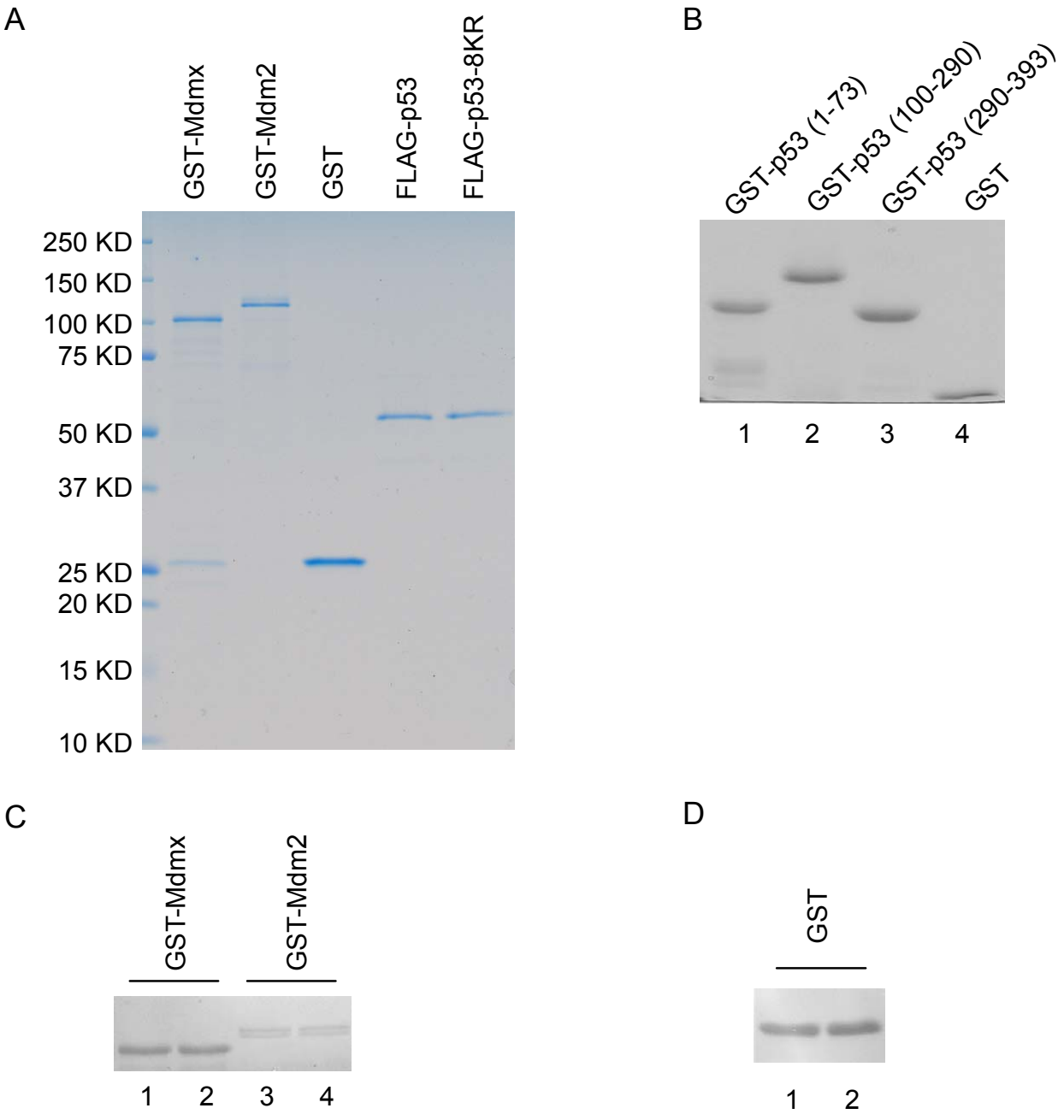


Figure S6. Coomassie blue staining of the purified recombinant proteins resolved on a 4-20% gradient SDS-PAGE gel. A. The recombinant proteins were used in gel-shift assay. B-D. Shown are the proteins in GST pulldown experiments as in Figure 5 D and 5E.

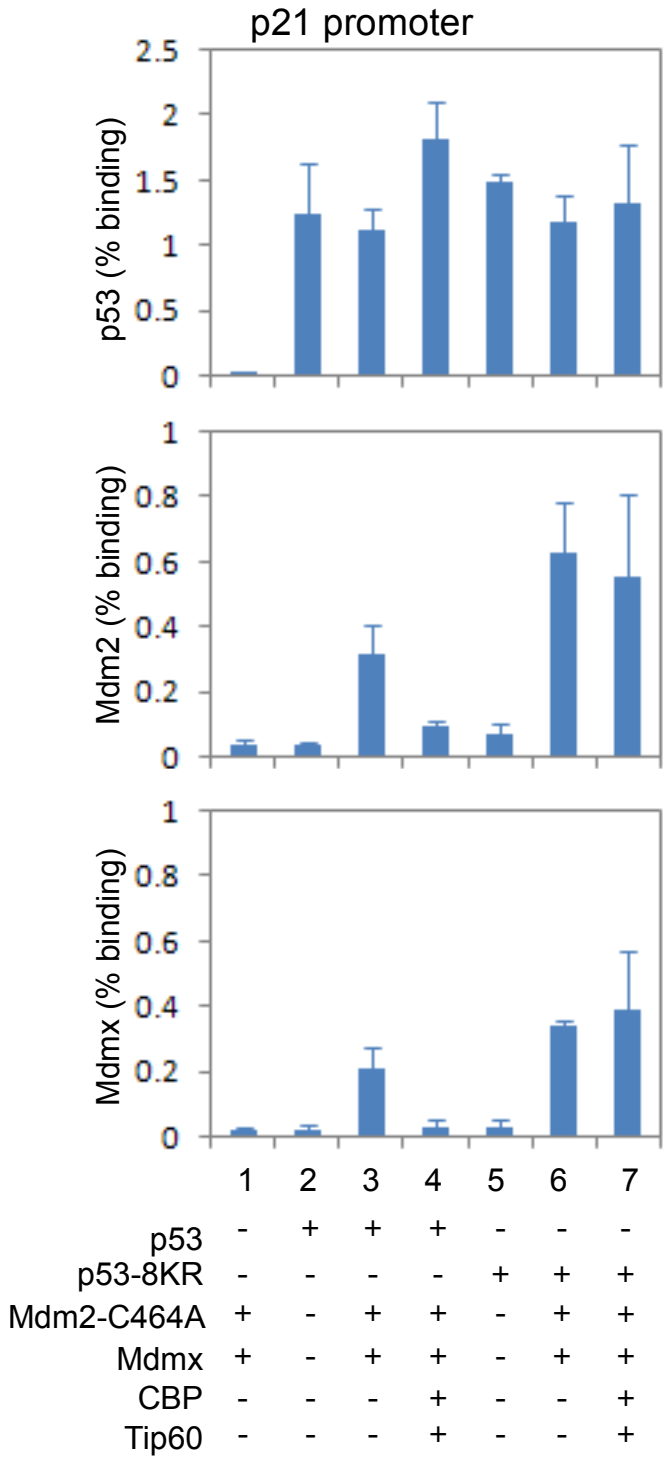


Figure S7. Real-time PCR to quantitate the recruitment of p53, Mdm2, and Mdmx to the p21 promoter, as shown in Figure 4C top panel. The data presented as mean ± SD represent three independent experiments.

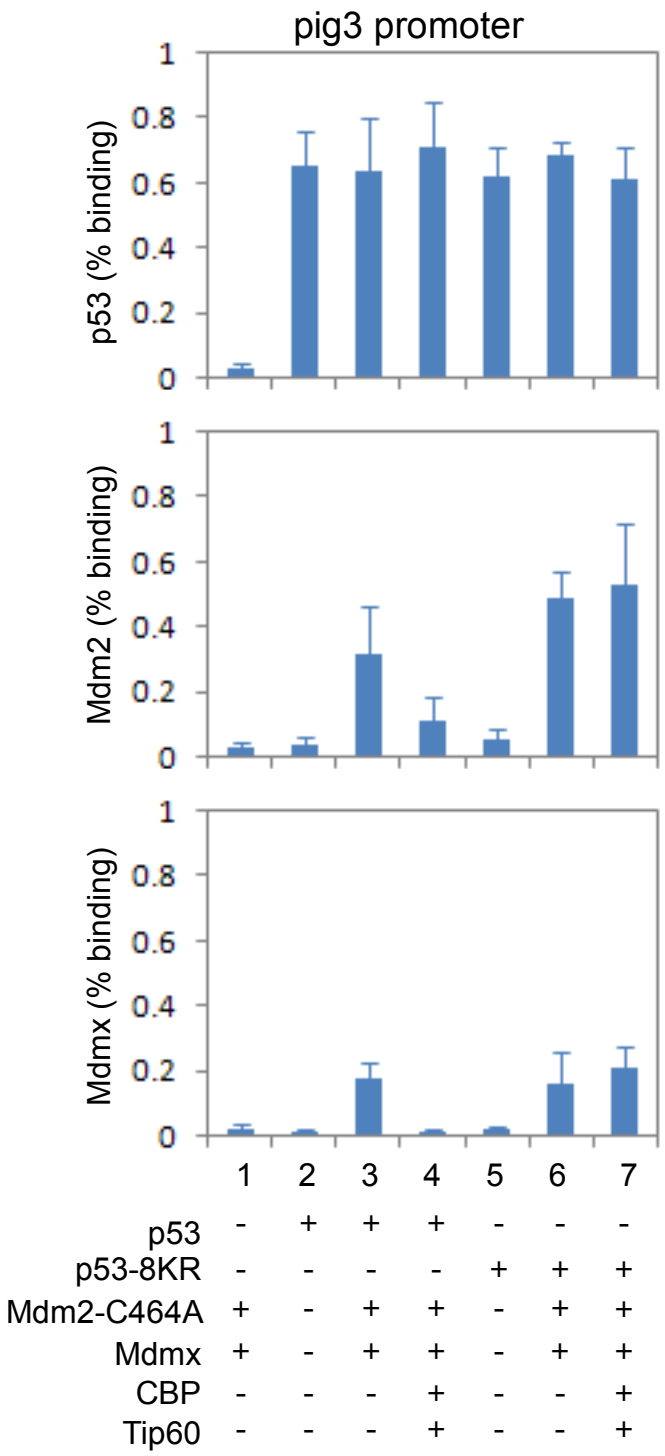


Figure S8. Real-time PCR to quantitate the recruitment of p53, Mdm2, and Mdmx to the pig3 promoter, as shown in Figure 4C middle panel. The data presented as mean ± SD represent three independent experiments.

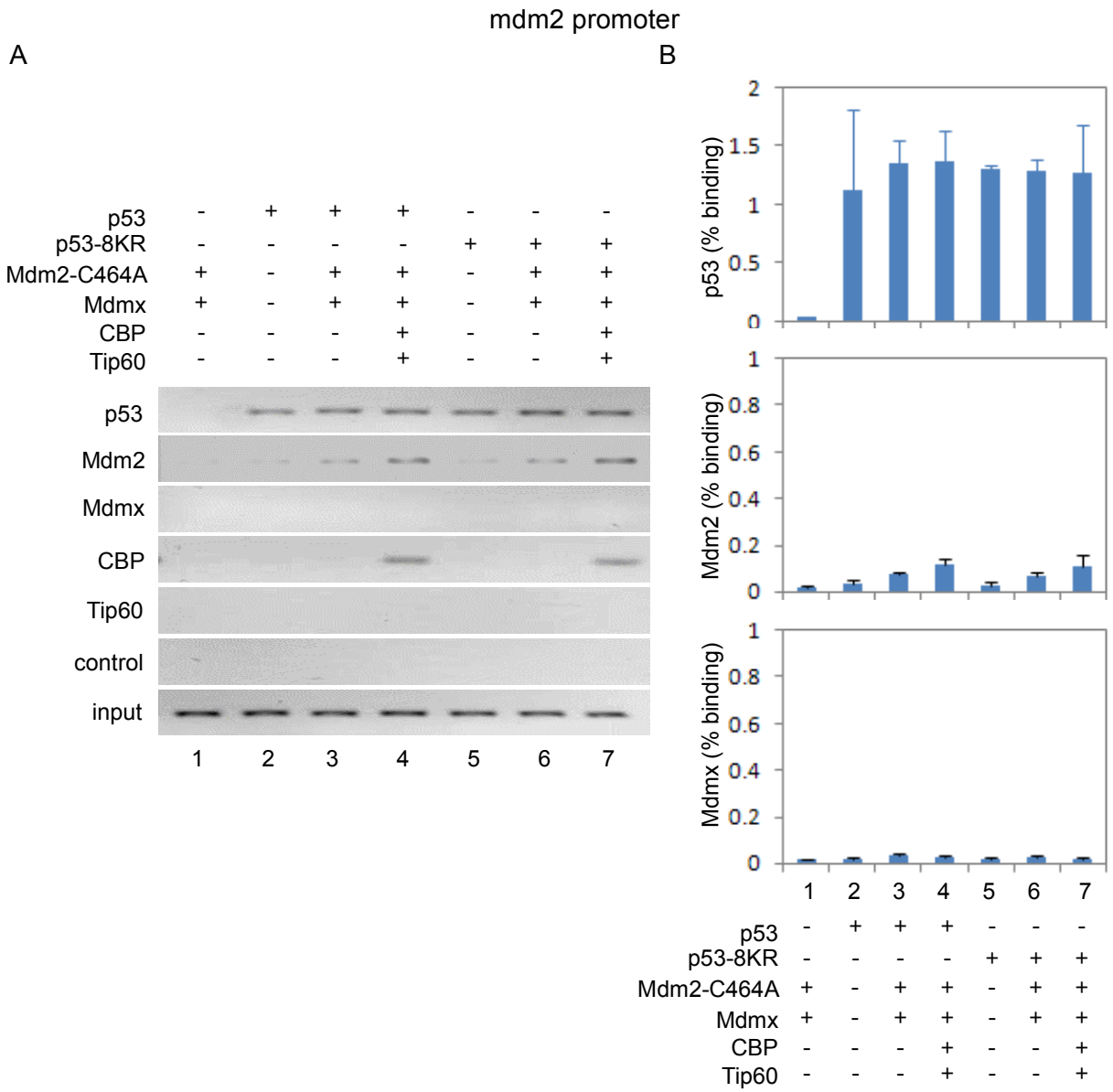


Figure S9. A. Recruitment of Mdm2 to the mdm2 promoter. Compared with Figure 4C bottom panel, PCR reactions using DNA samples from anti-Mdm2 immunoprecipitations were performed for 3 more cycles. B. Real-time PCR to quantitate the recruitment of p53, Mdm2, and Mdmx to the mdm2 promoter, as shown in Figure 4C. The data presented as mean ± SD represent three independent experiments.

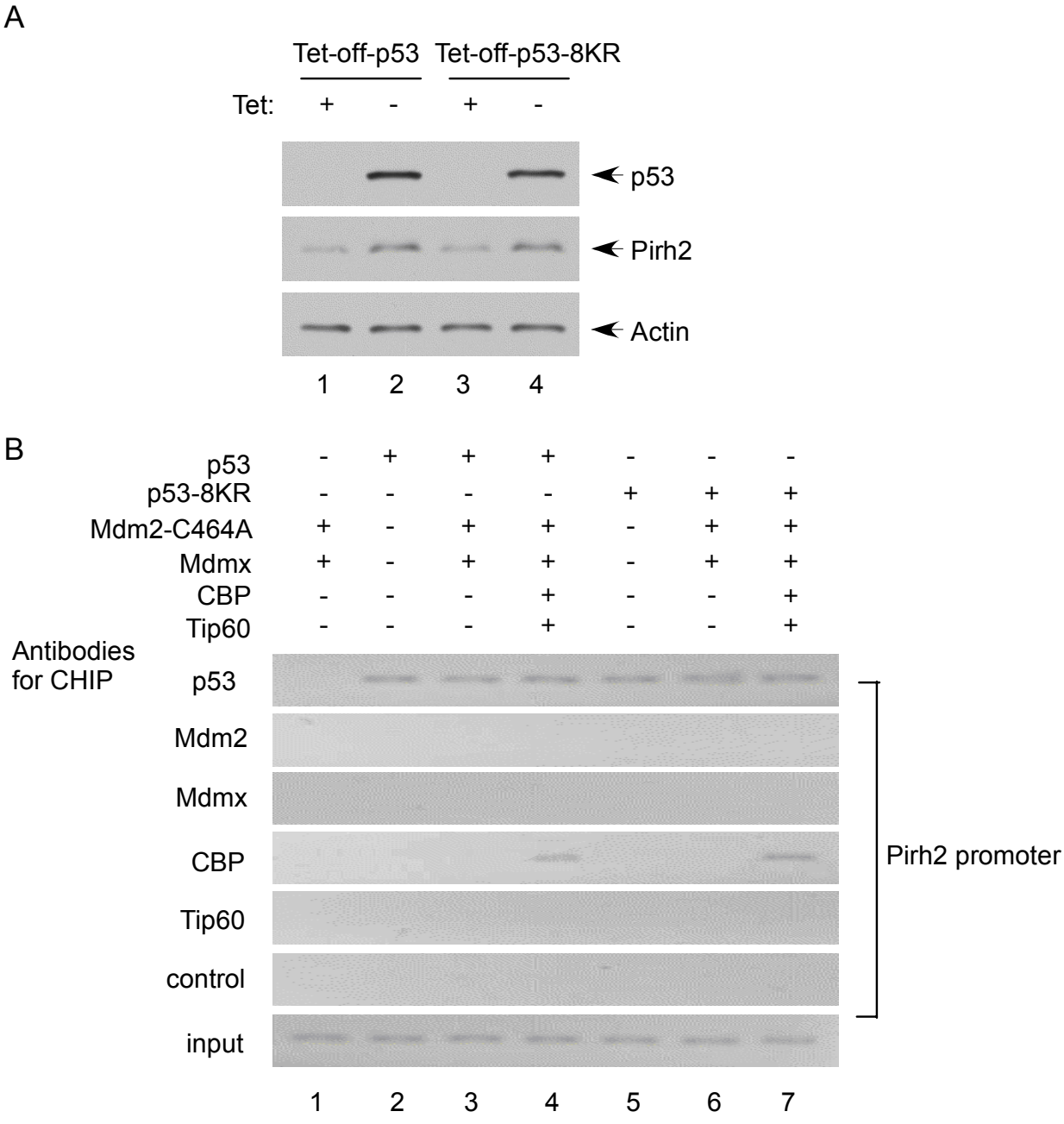


Figure S10. A. Tet-off-p53 and Tet-off-p53-8KR cells were induced for 0 and 3 days. The total cell extracts were analyzed by Western blot using antibodies against p53 (DO-1), Pirh2, and actin. B. Recruitment of p53, Mdm2, Mdmx, CBP, and Tip60 to the pirh2 promoter was analyzed as in Figure 4C.

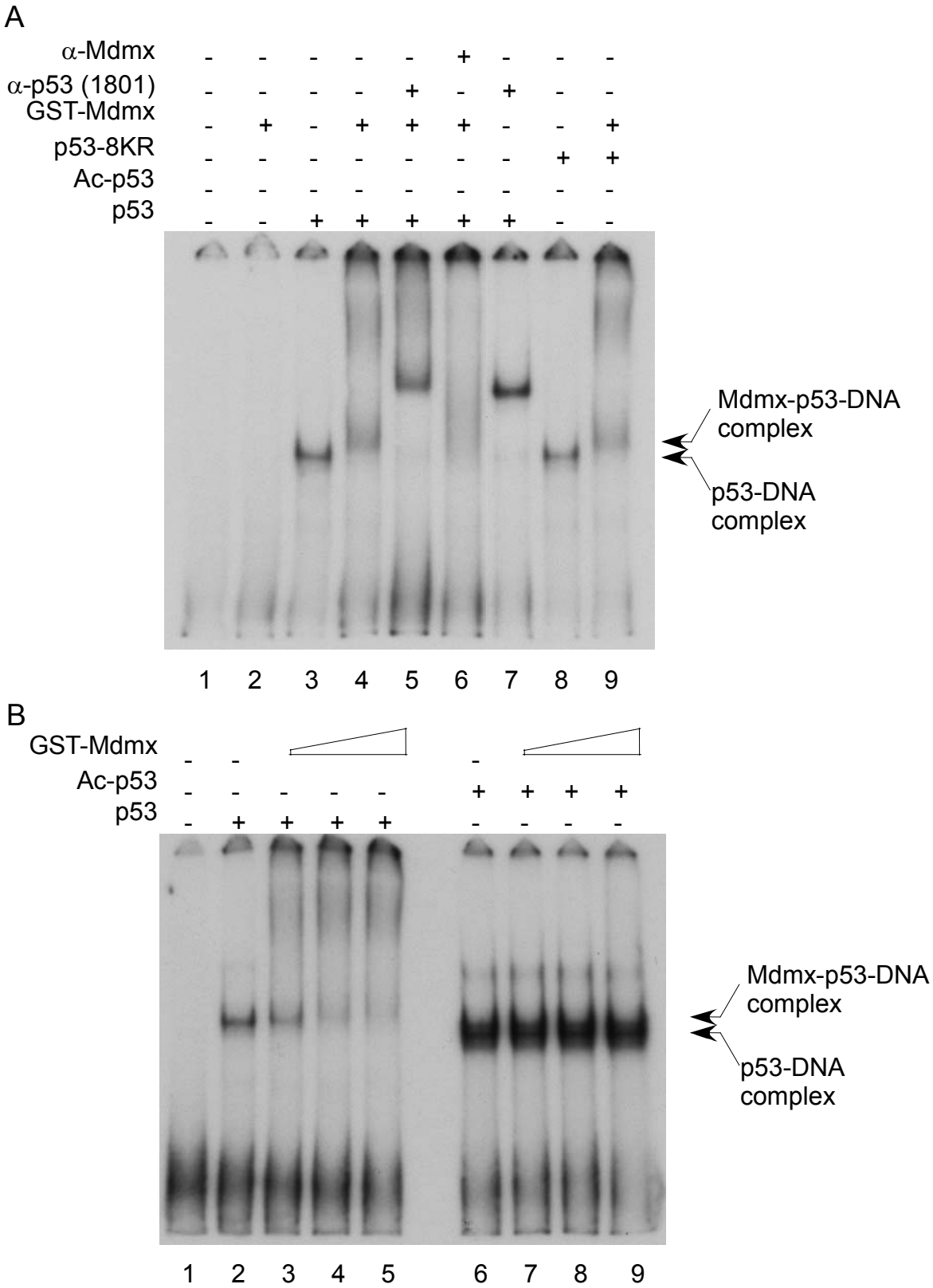
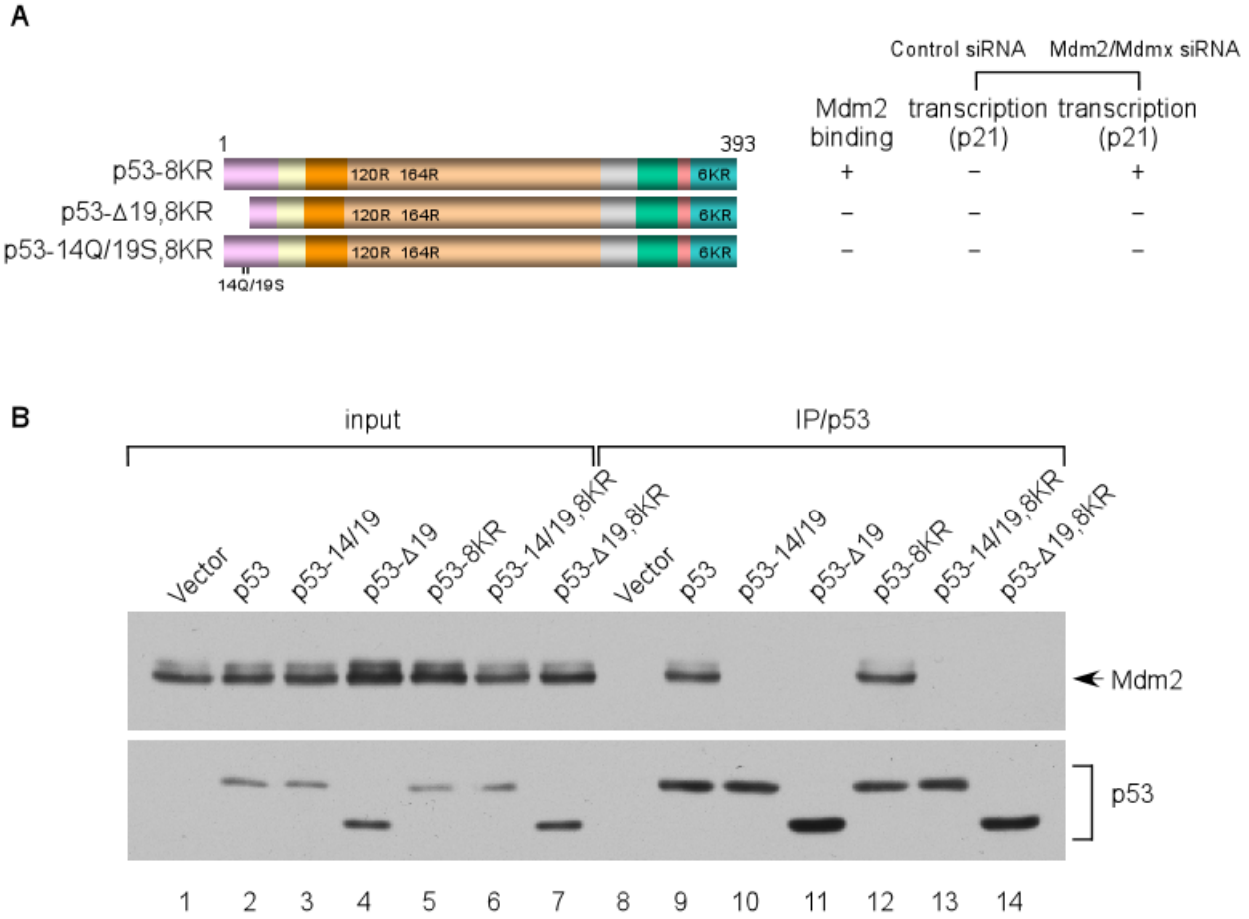


Figure S11. Acetylation of p53 abrogates the p53-Mdmx-DNA complex formation. In vitro formed DNA-p53-Mdmx complex assayed by gel-shift as in Figure 5B and 5C.



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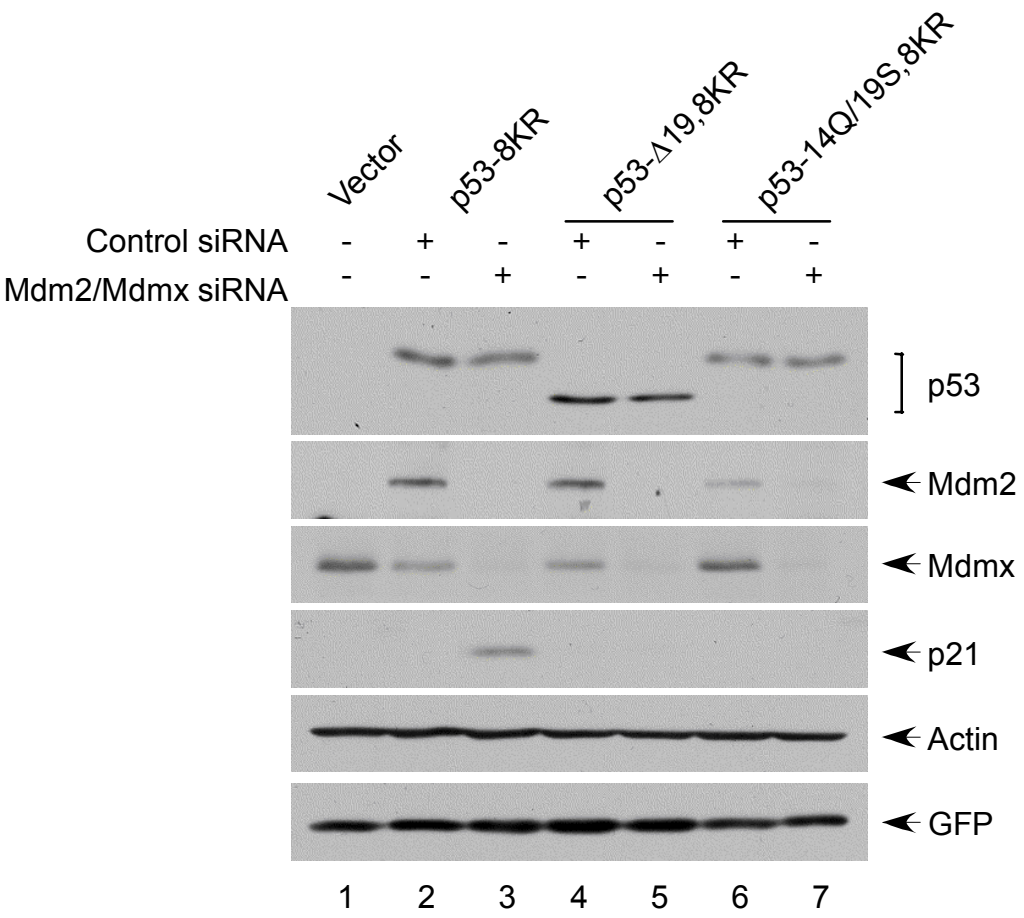

Figure S12. Transcriptional activities of the Mdm2-binding deficient p53 mutants.

A. Scheme of p53-8KR, p53-D19,8KR, and p53-14Q/19S,8KR mutants. The Mdm2 binding and transcriptional (p21) activities were summarized on the right panel. "+" indicates the presence of activity and "-" the absence of activity.
B. Binding of p53 mutants to Mdm2 in cells. H1299 cells were transfected with CMV-Mdm2 and with or without the plasmid DNA expressing Flag-p53 or various Flag-p53 mutants. The total cell extracts and the immunoprecipitates by M2 beads were fractionationed and analyzed by Western blot using antibodies against p53 (FL) and Mdm2.
C. After treated with Mdm2 and Mdmx siRNAs for 2 days, H1299 cells were co-transfected with p21 luciferase construct and with the indicated plasmids. Luciferase activities were measure using the Dual Luciferase Reporter Assay System Kit from Promega according the manufacturer's protocol. The data presented as mean ± SD represent three independent experiments.
D. As in C, cells were transfected with plasmids expressing p53 mutants and GFP, as a transfection control. Cell extracts were assayed for p53, Mdm2, Mdmx, p21, actin, and GFP.