

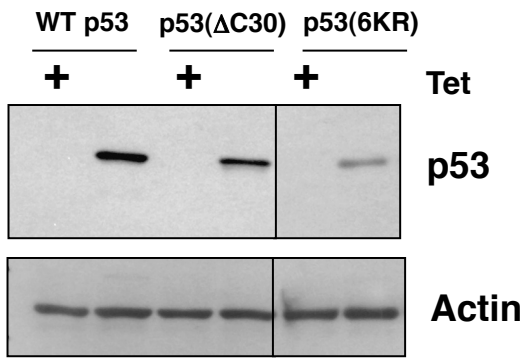
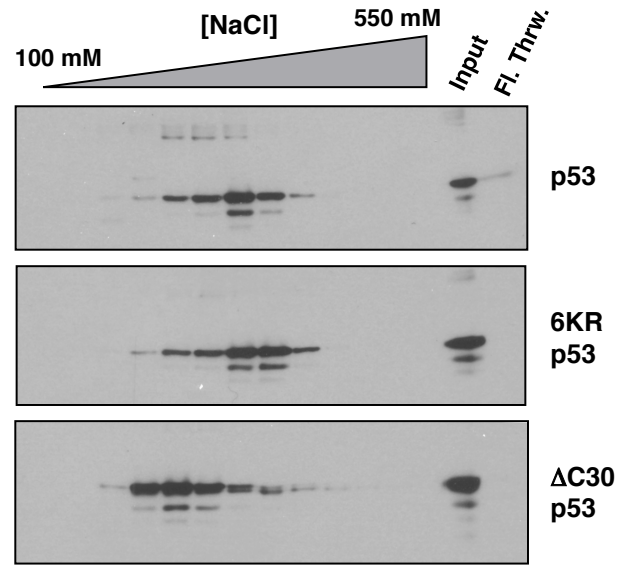
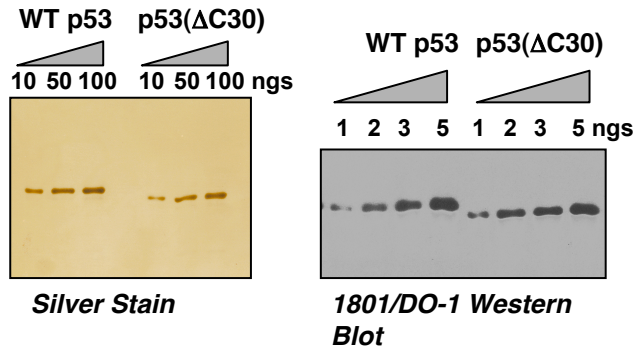
SUPPLEMENTARY INFORMATION

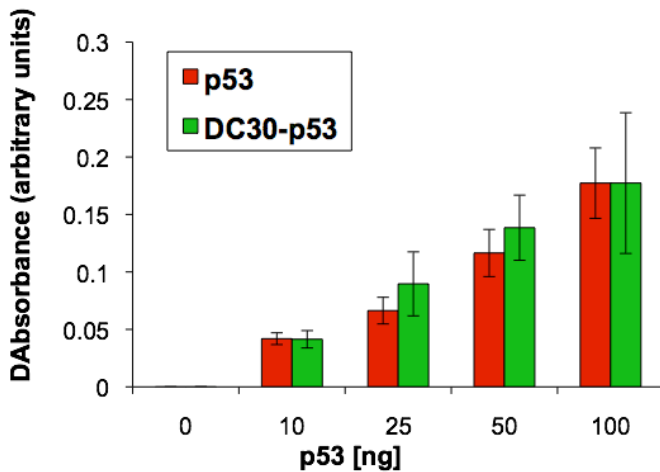
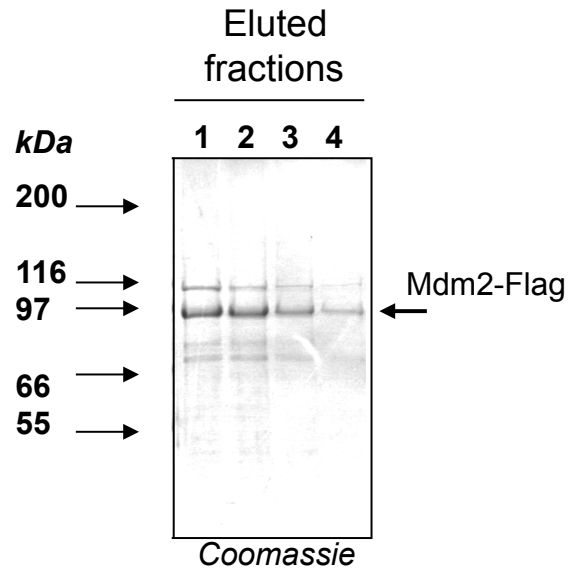
The C-terminus of p53 binds the N-terminal domain of MDM2

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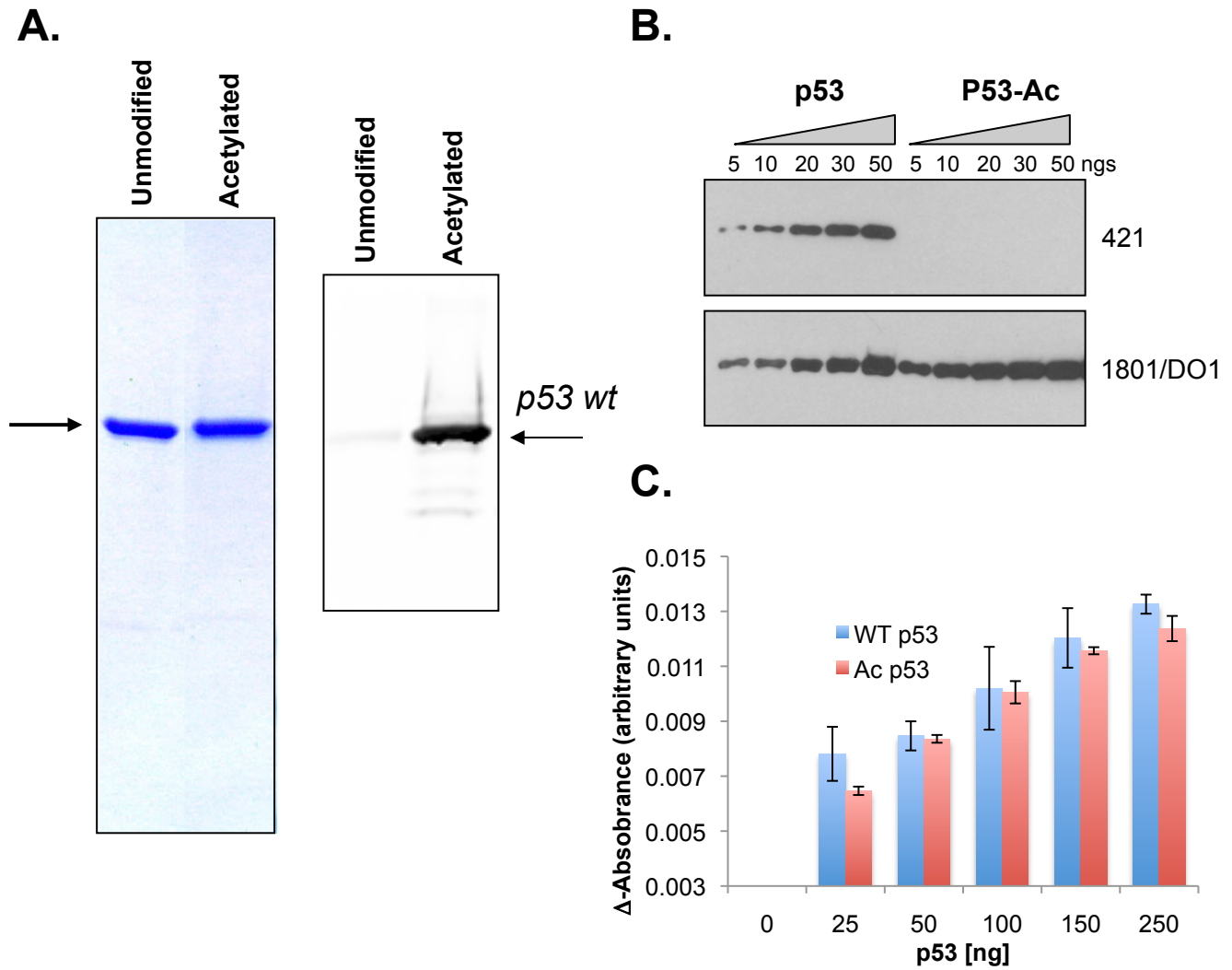
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A.**B.****C.**

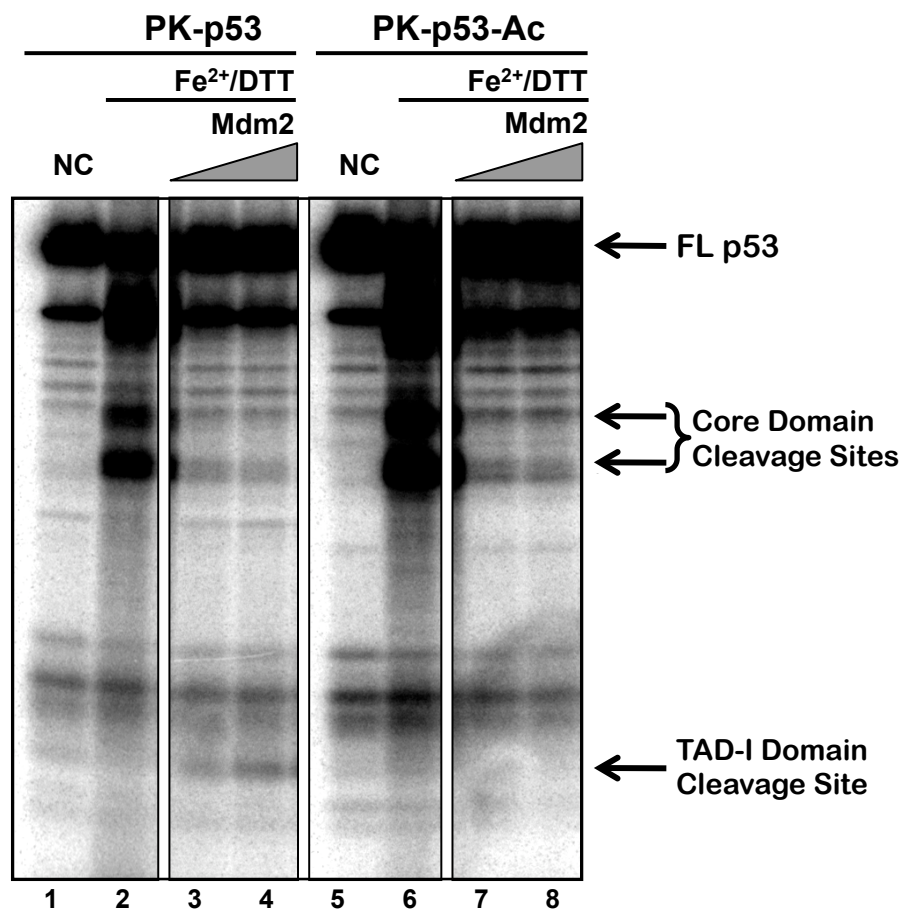
D.**E.****Supplementary Figure 1:****p53 and Mdm2 proteins used in EMSA and ELISA experiments.**

- A. Expression of wild type p53, p53(Δ C30) and p53(6KR) in H1299 cells. Soluble extracts of H1299 cells engineered to express tetracycline repressible full-length or C-terminally altered p53 were maintained in medium that contained tetracycline (+) or tetracycline was removed for 24 hours followed by cell lysis and extracts resolved by 10 % SDS-PAGE followed by immunoblotting for p53 and actin as indicated.
- B. Heparin elution profile of wild-type and C-terminally altered p53. H1299 cells expressing the indicated p53 variants as in A were placed in medium lacking tetracycline for 24 hours prior to lysis. Soluble whole cell extracts were separated by heparin column chromatography and p53 proteins were eluted by a NaCl gradient. Fractions from each p53 variant purification were visualized by immunoblotting with a mix of anti-p53 antibodies (PAb1801 and D0-1). Peak p53 fractions were used in the subsequent pulldown and EMSA experiments. Note that the lower salt elution profile of p53- Δ C30 is consistent with this protein missing the basic C-terminal nucleic acid binding region.
- C. Wild type p53 and p53(Δ C30) proteins. Left panel: silver-stained SDS-PAGE of bacterially expressed and purified 6xHis-p53 and p53(Δ C30) proteins. Total protein amounts are as indicated above the lanes. Right panel: Immunoblot of 6xHis-p53 and p53(Δ C30) using a mixture of anti p53 antibodies PAb1801-DO1. Total protein amounts are as indicated above the lanes.
- D. Normalization of wild type p53 and p53(Δ C30) in ELISA. ELISA experiments were performed as in Figure 1B. The wells were coated with 6xHis-p53 or p53(Δ C30) in PBS and the total p53 bound to the plate was detected with PAb1801.
- E. Baculovirus expressed and purified Flag-Mdm2. Flag-Mdm2 was purified from insect cells as described in Materials and Methods, and subjected to SDS-PAGE and Coomassie staining.



**Supplementary Figure 2:
Purification and normalization of unacetylated and acetylated p53 from SF9 cells.**

- A. Acetylated and unacetylated p53 proteins. Left panel: Coomassie blue-stained SDS-PAGE of HA-tagged baculovirus expressed and purified unacetylated HA-p53 or acetylated HA-p53 that was purified from insect cells that were co-infected with a baculovirus expressing p300 and subjected to 10% SDS PAGE. Right panel: Western blot of unacetylated and acetylated p53 detected by Pan-Acetyl antibody.
- B. Pab 421 cannot recognize p300-acetylated p53. Indicated amounts of unacetylated HA-p53 (p53) and acetylated-HA-p53 (p53-Ac) were resolved on 8% SDS-PAGE. The proteins were visualized by immunoblotting with Pab 421 (top panel) followed by stripping the membrane and reprobing it with a mixture of Pab 1801 and DO1 antibodies to detect total p53 levels.
- C. Normalization of p53 and Ac-p53 for ELISA. An ELISA was performed as in Figure 1B. Duplicate wells were coated with p53 or Ac-p53 and the total amount of protein in each well was detected by reactivity with Pab 1801.

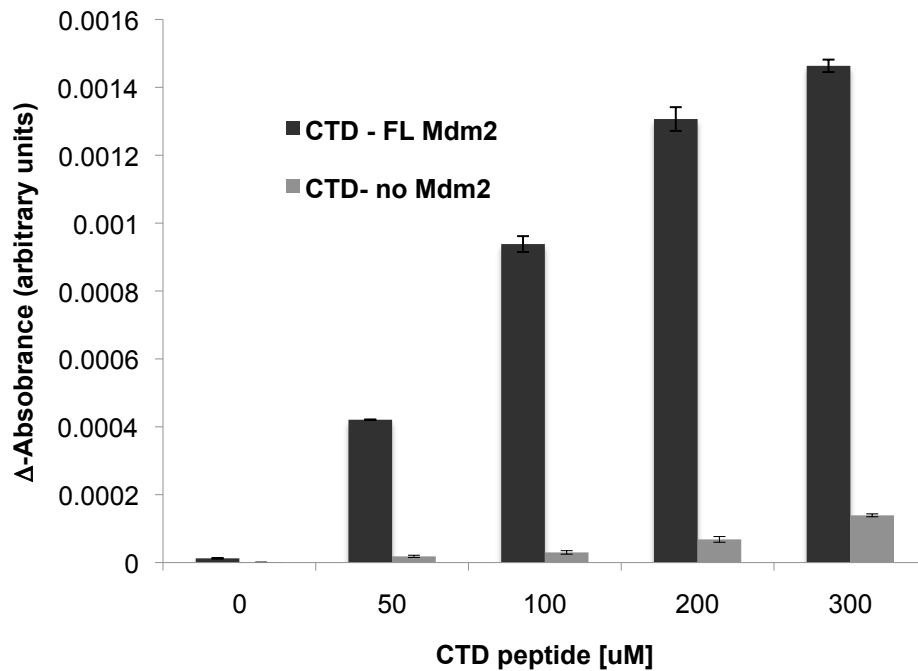


Supplementary Figure 3:
Fe²⁺ localized hydroxyl radical footprinting of acetylated p53 in the presence of Mdm2.

The unacetylated and p300 acetylated p53 proteins used in these assays were engineered with a PKA site at their N-termini and radiolabeled with [³²P] prior to the experiments and either treated or not (NC) with Fe²⁺-DTT. Substitution of Fe²⁺ for Zn²⁺ in the core domain of p53 produced a pattern of cleavage products mainly localized in the DNA-binding domain of p53. Migration of the products resulting from the cleavages of p53 DNA-binding domain and the N-terminal domain (TAD-I) is indicated. Reactions, containing indicated amounts of Flag-Mdm2 were performed in parallel. Following 10 min incubation at 20°C reactions were terminated by addition of 3x SDS-PAGE sample loading buffer. p53 cleavage products were separated by 13% SDS-PAGE and visualized by autoradiography. The size of the resulting cleavage products is dependent on the distance of the cleavage site from the N-terminus of p53.

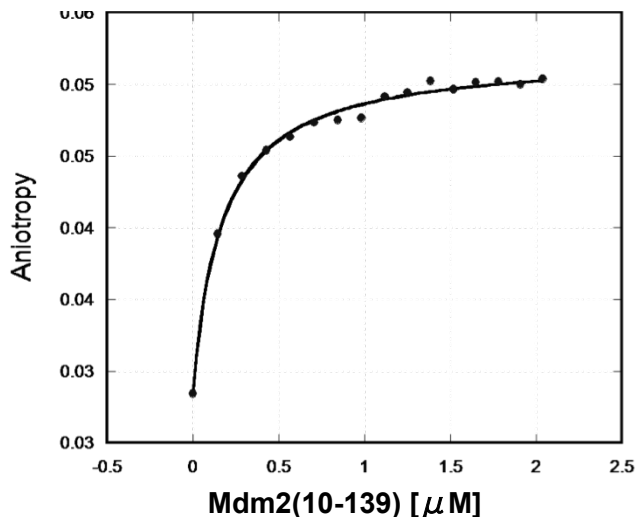
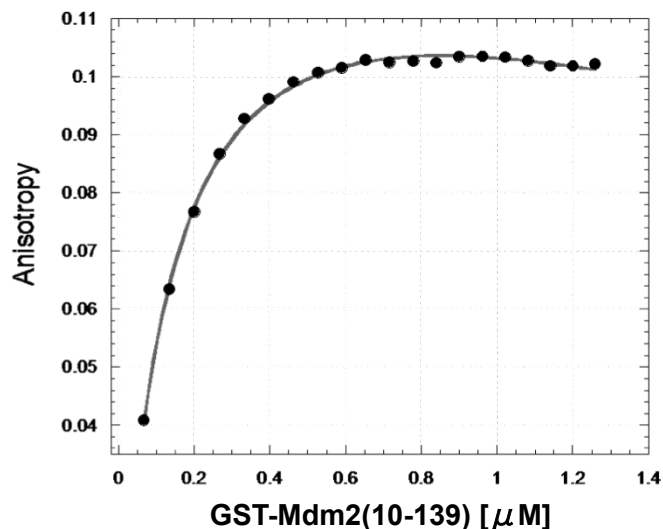
Note: Addition of Mdm2 into the reaction mixtures induced two significant changes in the p53-cleavage pattern. First, the presence of Mdm2 markedly reduced the cleavages within the core domain. This provides evidence that some of the contacts between p53 and Mdm2 remain intact upon p53 acetylation although it is also possible that Mdm2 may compete for the Fe²⁺ due to its possession of Zn²⁺ atoms. Second, addition of Mdm2 led to the appearance of a novel shorter fragment (lanes 3 and 4) whose site of cleavage was mapped to the TAD-I domain of p53. The induction of cleavage in the TAD-I domain is consistent with changes in the p53 N-terminus upon binding to Mdm2. Importantly, when we used acetylated p53 to bind Mdm2 we observed similar disappearance of the core domain cleavage sites but the cleavage product associated with the TAD-I domain was also absent (lanes 7 and 8).

This assay was an adaptation of a previously described protocol. For a more detailed explanation of the method refer to: Liptenko, O., Lee, J., Lomakin, I. & Borukhov, S. Transcript cleavage factors GreA and GreB act as transient catalytic components of RNA polymerase. *EMBO J* **22**, 6322-34 (2003).



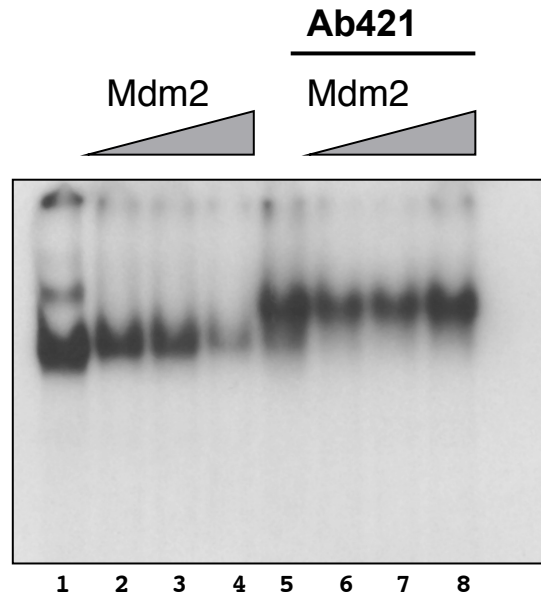
**Supplementary Figure 4:
Mdm2 binding to the p53-CTD.**

Full length Mdm2 binds to the C-terminal domain of p53 in ELISA. ELISA was performed as in Figure 2B. Wells were coated with 100 ng baculovirus-expressed full-length FLAG-Mdm2 overnight at 4°C. p53^{aa367-393} CTD peptide was added to wells at the indicated amounts and p53-CTD bound to Mdm2 was detected by reactivity with Pab 421.

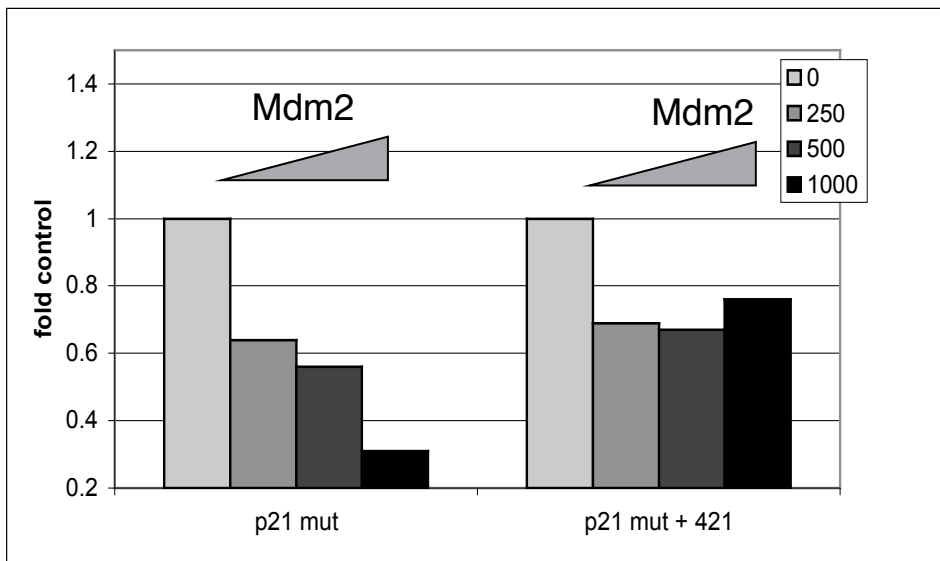
A.**B.****Supplementary Figure 5:****Binding of Mdm2(10-139) to the p53-TAD-I domain detected by fluorescence anisotropy.**

- A. Fluorescein-labeled p53^{aa1-42} peptide at 0.1 μM was placed in buffer containing 50 mM NaCl (IS = 50) in a cuvette and unlabeled Mdm2(10-139) protein [36 μM] was added at indicated concentrations at 1 min intervals and changes in fluorescence and anisotropy were recorded. The data were fit to a 1:1 binding model.
- B. Fluorescein-labeled p53^{aa1-42} peptide at 0.1 μM was placed (IS = 50) in a cuvette and unlabeled GST-Mdm2(10-139) protein [17 μM] was added at indicated concentrations at 1 min intervals and changes in fluorescence and anisotropy were recorded. The data were a fit to 1:1 binding model.

Calculated K_d was in the nM range in A and B, in agreement with previously published results in: Schon O. et al. Molecular mechanism of the interaction between Mdm2 and p53. *J. Mol Biol.* 2002 323(3):491-501.



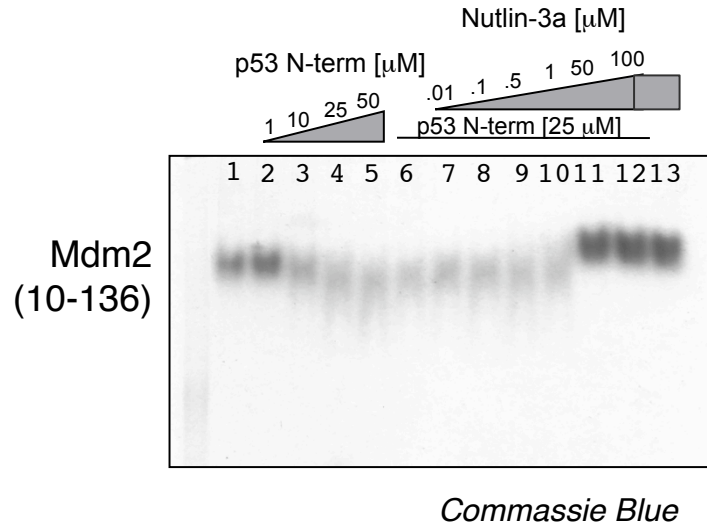
44bp-*p21* oligo
44bp-mut *p21* competitor (x30)



**Supplementary Figure 6:
PAb421 interferes with the ability of Mdm2 to block p53-DNA binding.**

Top panel: An EMSA experiment was performed as in Figure 2B. Reaction mixtures either lacked or contained 0.25, 0.5, or 1 μ g full-length baculovirus expressed and purified Flag-tagged Mdm2 (Mdm2). Reactions in lanes 5-8 also included 50 ng of purified Pab 421. Mixtures contained 30X molar excess of 44 bp oligonucleotides containing a mutated 5' -*p21* binding site as non-specific competitor.

Bottom panel: graphic representation of fold change in p53-DNA binding in the presence of Mdm2 and Pab 421.

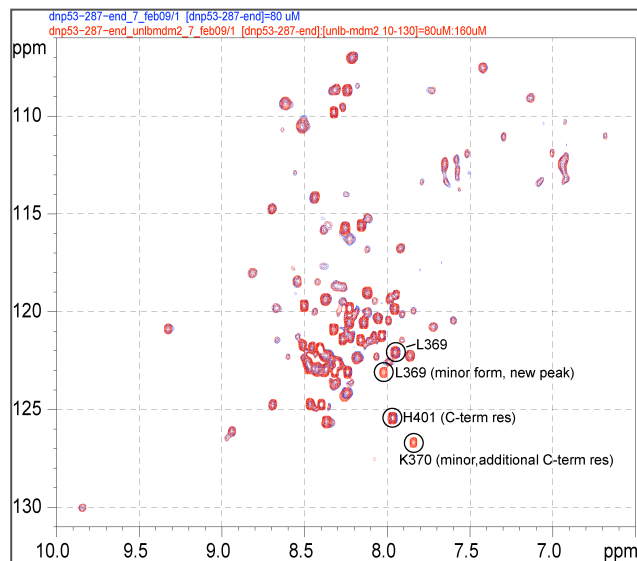


Supplementary Figure 7:

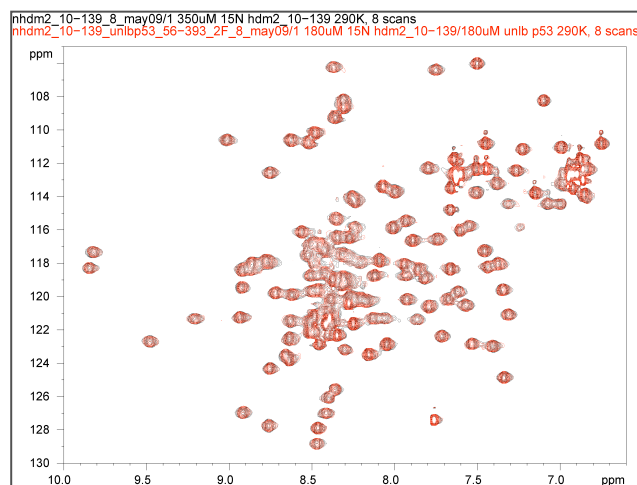
Nutlin completely disrupts the interaction of the N-terminus of p53 with the N-terminus of Mdm2.

Mdm2(10-139) ($2.5 \mu\text{g}$) was loaded onto a 4% acrylamide native gel either alone (lane 1) or in the presence of indicated concentrations of p53^{aa1-42} (lanes 2-5) or in the presence of p53^{aa1-42} ($25 \mu\text{M}$; lanes 6-12) with the indicated amounts of Nutlin (lanes 7-13). The gel was run for 2 hr at pH 8.5 after which Mdm2 migration was visualized by Coomassie staining.

A.



B.



**Supplementary Figure 8:
Binding of Mdm2(10-139) to the p53-CTD detected by NMR.**

- A. Superposition of ^1H - ^{15}N TROSY-HSQC spectra of U- ^{15}N , ^2H -labelled p53(257-393) in the absence (blue) and presence (red) of unlabeled Mdm2(10-139). Shifts assigned as L369 and K370 are results of proteolytic cleavage and not binding of Mdm2 and p53.
- B. Superposition of ^1H - ^{15}N TROSY-HSQC spectra of U- ^{15}N , ^2H -labelled Mdm2(10-139) in the absence (black) and presence (red) of unlabeled p53(56-393).

Protein purification and NMR experiments were performed as previously described in:
Ahn J et al. Insight into the Structural Basis of Pro- and Antiapoptotic p53 Modulation by ASPP Proteins *J. Biol. Chem.* 2009;284:13812-13822

SUPPLEMENTARY METHODS:

Cell lines, transfections and immunoblotting. *p53*^{-/-};*mdm2*^{-/-} (2KO) cells (a generous gift from G. Lozano, MD Anderson Cancer Center) and HCT116 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). H1299 cell lines with tetracycline (tet) regulatable wild-type p53 and p53(Δ C30) were previously described⁵⁷. The inducible cell line Tet-off-p53-6KR was established by transfection of H1299 cells with the plasmid DNA pTRE2hyg-p53-6KR (where lysine residues K370/372/373/381/382/386 were mutated to arginine) and selection in DMEM medium supplemented with 5 μ g ml⁻¹ doxycycline (Sigma), 0.2 mg ml⁻¹ G418 (EMD Biosciences), and 0.4 mg ml⁻¹ hygromycin B (Roche) for 3 weeks. Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's protocols. Cells were harvested 36 hours post transfection. Immunoblotting was performed as previously described²⁹. siRNA oligonucleotides targeting human Mdm2 were purchased from Invitrogen (Carlsbad, CA)⁵⁸. The negative control siRNA was the Qiagen All-stars duplex. siRNA transfections were performed using Dharmafect-1 (Dharmacon, Lafayette, CO) according to the manufacturer's instructions.

Protein purification and immuno-pulldown experiments. GST fusion proteins were expressed in BL21 (DE3) cells. After induction for 12 hrs at 16°C with 0.4 M IPTG, soluble proteins were extracted by sonication in lysis buffer (50 mM Tris at pH 7.0, 350 mM NaCl, 0.1% aprotinin, 1 mM DTT, 0.5 mM PMSF). The soluble protein fraction was incubated with glutathione-Sepharose beads (Pharmacia) at 4°C for 1 hr, and the bound protein was eluted with reduced glutathione. Purified GST-Mdm2 was subject

to thrombin cleavage at 4°C for 12 hr, at 1/100 thrombin/target ratio. Cleaved proteins were separated on another GST column in GST Elution Buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM DTT in 10% glycerol) and the flow-through fraction containing Mdm2 was collected. Purification of bacterially expressed 6 χ His-p53 and 6 χ His-p53(Δ C30) was previously described⁵⁹. Cloning, purification and characterization of p53(293-393) have been previously described⁶⁰. Soluble whole cell extracts from Tet-repressible p53, p53(Δ C30) and p53(6KR) stable cell lines grown without tet for 24 hr, were separated by heparin column chromatography. p53 proteins were eluted with a NaCl gradient in 20 mM Tris buffer (pH 7.6) containing 20 % glycerol and 0.1 mM EDTA. Fractions from each purification were visualized by immunoblotting with a mixture of PAb 1801/D0-1 antibodies. Peak p53 fractions were normalized for p53 content. For immuno-pulldown experiments 20 μ l of M2-Agarose (Sigma) beads were incubated with Flag-Mdm2 and heparin-purified p53 for 2 hr at 4°C. The complexes were isolated by 3 washes in PBS buffer followed by the addition of 3 χ SDS-PAGE buffer and immunoblotting. N-terminally Flag- and PKA-tagged wild-type p53 was affinity purified from Sf-9 cells infected with the p53 expressing baculovirus alone or co-infected with p300-expressing baculovirus (a generous gift from Prof. W.L. Kraus, Cornell University, Ithaca). N-terminally Flag-tagged full length Mdm2 was expressed from recombinant baculovirus infected Sf-9 cells and affinity purified on M2-agarose (Sigma) according to manufacturers protocol. All purified proteins were concentrated using Apollo concentrators, aliquoted, flash-frozen in liquid N₂ and stored at -80°C.