Supporting information

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Supplemental figures

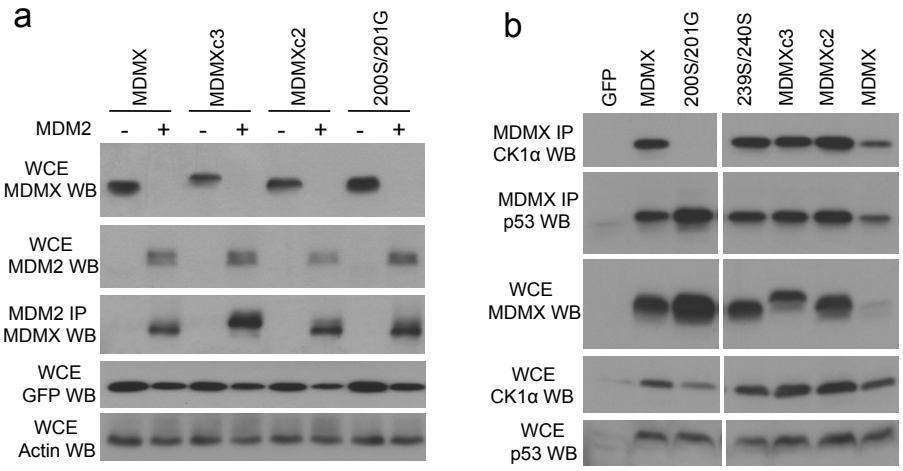


Figure S1. MDMXc3 has normal binding to MDM2, p53, and CK1α as MDMX (a) H1299 cells were cotransfected with MDMXc3 and MDM2 for 48 hours. MDMXc3 degradation by MDM2 was determined by western blot. MDMXc3 binding to MDM2 was determined by MDM2 IP-MDMX western blot. MDMXc2 is identical to MDMXc3 except not containing the PreScission cleavage site and epitope insert at residue 429. (b) H1299 cells were cotransfected with MDMXc3, p53 and CK1α. MDMX-CK1α and MDMX-p53 binding were determined by IP-western blot.

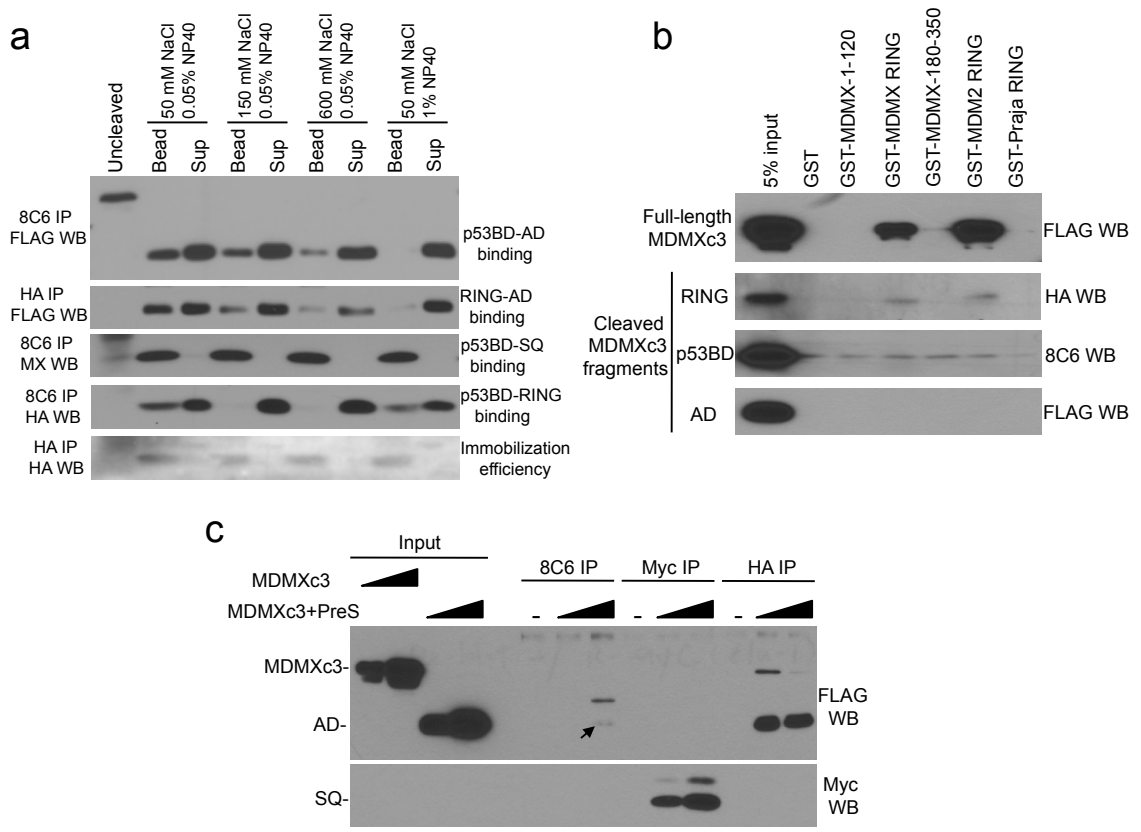


Figure S2. Characterization of MDMX intra-molecular interactions. (a) MDMXc3 was immobilized using 8C6 or HA antibody, cleaved with PreScission under the indicated buffer conditions for 20 min, and analyzed for the release of different fragments by western blot. (b) MDMXc3 in H1299 lysate was cleaved with PreScission and incubated with glutathione beads loaded with GST proteins. The capture of different MDMX fragments were determined by western blot. (c) Different amounts of MDMXc3 in H1299 lysate was cleaved with PreScission for 20 min, fragments were immunoprecipitated using indicated antibodies for 16 h. The coprecipitation of AD fragment with p53BD and RING was detected by FLAG western blot. Myc blot confirmed the presence of SQ fragment and absence of AD coprecipitation.

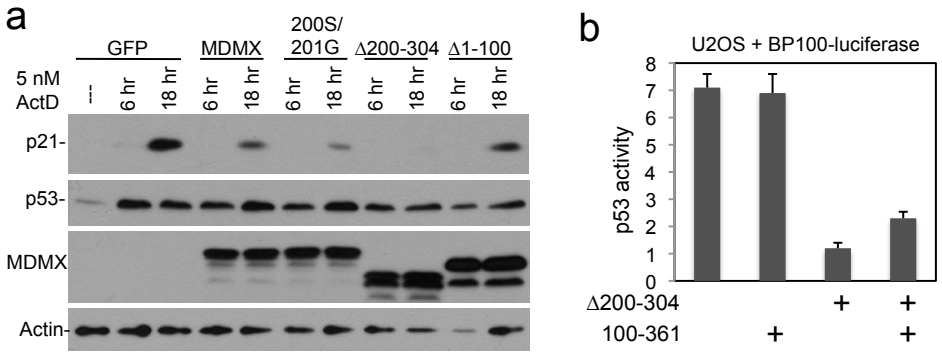
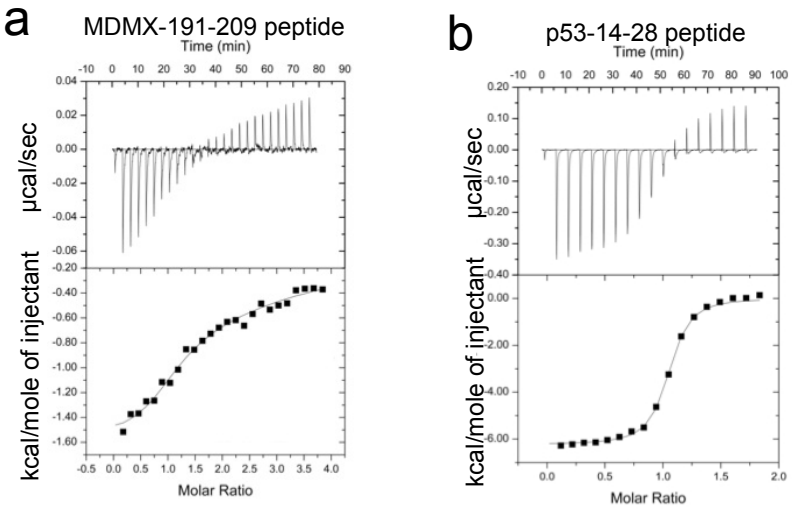


Figure S3. Regulation of p53 activity by MDMX mutants. (a) U2OS cells were transiently transfected with MDMX mutants, treated with 5 nM actinomycin D for 6 or 18 hours, and analyzed for p21 by western blot. (b) U2OS cells were cotransfected with BP100-luciferase and MDMX. Endogenous p53 activity was measured by luciferase assay and normalized to cotransfected CMV-lacZ.



c

Peptide	Binding model	Binding sites	ΔH (kcal/mol)	ΔS (cal/mol/deg)	K_D (μM)
MDMX-191-209	2 sequential binding sites	N.D.	(I) -1.7+/-0.03 (II) -5.2+/-0.4	(I) 18 (II) -2.4	(I) 8.3+/-0.1 (II) 340+/-40
p53-14-28	1 binding site	1.01+/-0.005	-6.2+/-0.04	7.1	0.5+/-0.05

Figure S4. *In vitro* binding of p53-mimetic peptide to MDMX N terminal domain. (a,b) MDMX-23-111 domain produced in *E. coli* was analyzed for binding to MDMX-191-

209 peptide and p53-14-28 peptide using isothermal titration calorimetry (ITC). (c) Dissociation constants (K_d), stoichiometry (N) and changes in enthalpy (ΔH) and entropy (ΔS) for peptide binding are summarized in the table.

Supplemental methods

GST pull down assay. Bacterial lysate expressing GST, GST-MDMX-1-120, and GST-p53 were applied to glutathione-agarose beads according to the manufacturer instruction (Pierce). The beads loaded with GST fusion proteins were incubated for 2 h at 4˚C with H1299 lysate transiently transfected with Myc-MDMX-100-361, MDMX mutants, or MDMXc3 digested with PreScission. The beads were washed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), boiled in Laemmli sample buffer and detected by western blot.

Isothermal titration calorimetry (ITC). MDMX-1-120 was cloned into pGEX-6P1, expressed as GST fusion proteins and purified from *E. coli*. The GST moiety was removed after cleavage with PreScission protease. The binding of MDMX-191-209 (EEWDVAGLPWWFLGNLRSN) peptide to the protein was analyzed with a MicroCal iTC200 titration calorimeter (GE Healthcare). The protein was exchanged into binding buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 0.5 mM DTT]. For the titration of the protein constructs, a total of 17 aliquots (2.01 µl each) of the peptide (600 µM) were injected into 200 µl of the protein solutions of MDMX-1-120 (69 µM) at 15˚C. The ITC cell mixture was constantly stirred at 1000 rpm and recorded for 300 seconds between injections at low feedback. The corrected heat values were fitted using a nonlinear least square curve-fitting algorithm (Microcal Origin 7.0) to obtain the stoichiometry (N), binding constant (Ka, Kd) and change in enthalpy of the enzyme-ligand interaction (ΔH). Biotin-p53 peptide control (Bio-Acp-LSQETFSDLWKLLPE) was synthesized by GenScript (Piscataway, NJ, USA).

Protein expression and NMR analysis. Uniformly ^{15}N-labeled samples of residues 23-111 from human MDMX were expressed using pGEX-6P-2 vector in BL21(DE3) cells grown in minimal media. Following purification using glutathione sepharose column, the GST tag was cleaved using HRV3C protease. Purified MDMX-23-111 were dialyzed into a buffer containing 50 mM Sodium Phosphate, 100 mM NaCl, 1 mM EDTA, 0.02%

Sodium Azide and 2 mM DTT. NMR experiments were carried out at 25°C on a Varian VNMRS 800 MHz spectrometer, utilizing a triple resonance pulse field Z-axis gradient cold probe. Sensitivity enhanced ^{1}H-^{15}N HSQC experiments were performed on uniformly ^{15}N-labeled samples of MDMX-23-111 in 80% H2O, 10% D_2O, and 10% DMSO PBS buffer, at pH 6.8. The sweep widths and complex points for the HSQC spectra were 9690 (t2) × 2500 (t1) Hz and 1024 (t2) × 128 (t1), respectively. All NMR spectra were processed with nmrPipe and analyzed in nmrView (38). Titrations were carried out by transferring equal volumes between two 600μl samples containing free MDMX-23-111 (0.1mM) or MDMX-23-111 (0.1mM) in the presence of a fivefold stoichiometric excess of either the WT peptide (MDMX-191-209: EEWDVAGLP**WW**FLGNLRSN), or the SG peptide (MDMX-191-209: EEWDVAGLP**SG**FLGNLRSN). Six transfers were made resulting in 14 HSQC spectra, which were then analyzed for chemical shift perturbations using the published assignments as a starting point.