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Supplemental Information

Optimal Affinity Enhancement by a Conserved Flexible Linker Controls p53 Mimicry in MdmX

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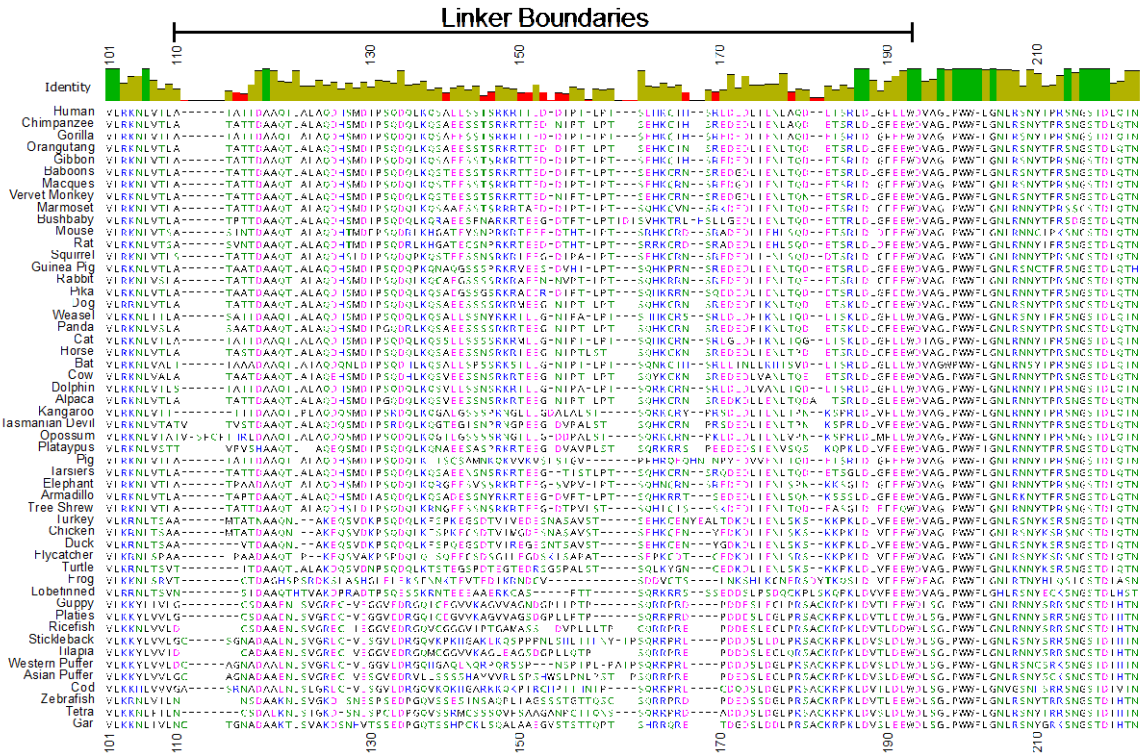


Figure S1: **Multiple protein sequence alignment of the MdmX linker from 52 MdmX homologues** including 10 primates, 24 other mammals, 4 birds, 1 amphibian, 1 reptile, and 12 fish. This alignment was used to generate the histogram in Figure 1.

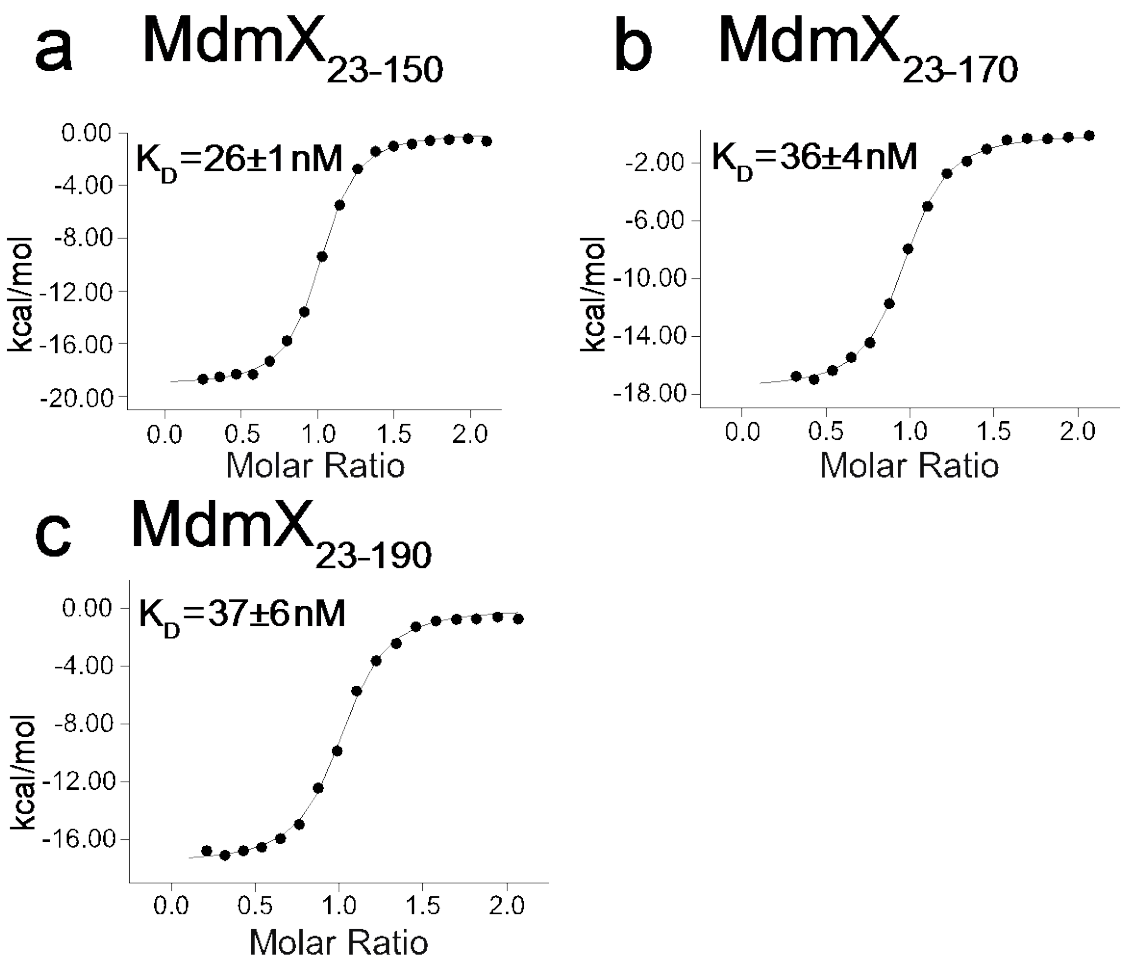


Figure S2: **p53TAD binding to fragments of MdmX with different lengths of the flexible linker.** Black circles show enthalpy per mole of injectant, measured using isothermal titration calorimetry, plotted as a function [p53TAD]/[MdmX]. Black lines show the fit to the data using a single site binding model. a. p53TAD binding to $MdmX_{23-150}$. b. p53TAD binding to $MdmX_{23-170}$. c. p53TAD binding to $MdmX_{23-190}$.

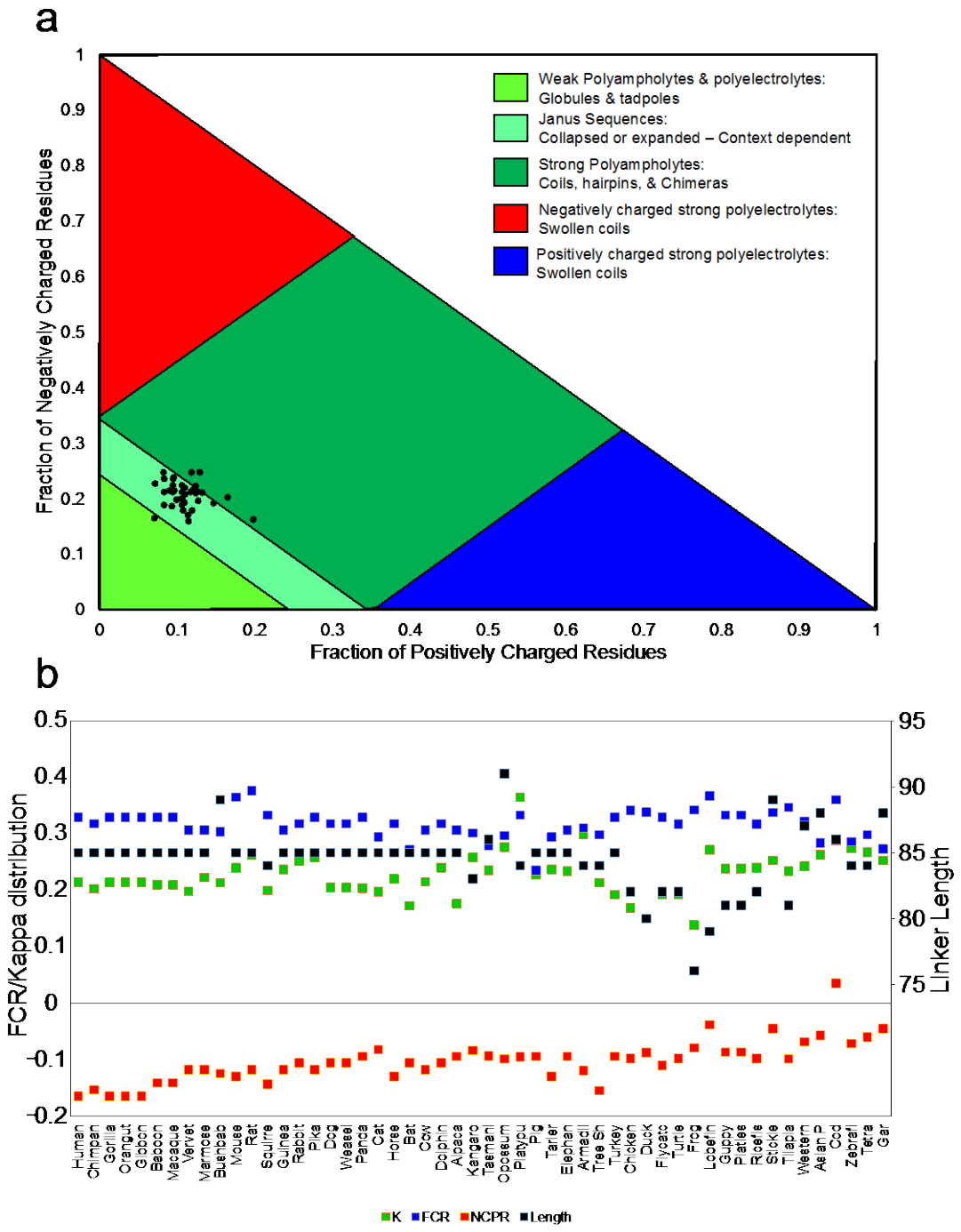


Figure S3: **CIDER analysis for MDMX linker sequences.** a. Diagram of states, the orthologues are primarily clustered within the Janus sequence zone of the plot. b. plot showing Kappa charge distribution value (K) in green, fraction of charged residues (FCR) in blue, net charge per residue (NCPR) in red, and the linker length in black for each of the MdmX orthologues. See reference 1.

Wormlike chain (WLC) modeling of the MdmX linker region

To model the behavior of the MdmX linker we used a previously described wormlike chain model that considers a polypeptide chain to behave as a continuous cylinder with a fixed but randomly directed radius of curvature (2,3). In this model, the end-to-end distribution depends only on two parameters, the persistence length *Lp* (length it takes for the motions to become uncorrelated) and the contour length *Lc* (total length of the chain). For our calculations, we used a persistence length *Lp* = 3Å which has been used previously for aminoacid chains [Zhou, 2004] and *Lc* = N_{res}*3.8 Å, where N_{res} is the number of residues in the linker (in this case 85), and 3.8 Å is the distance per chain element, in this case one amino acid.

P(r) calculations: The distribution function for the end-to-end distances can be written as

$$p(r) = 4\pi r^2 \left(\frac{3}{4\pi L_p L_c}\right)^{\frac{3}{2}} exp\left(\frac{-3r^2}{4L_p L_c}\right) \zeta\left(r, L_p, L_c\right) \quad [1]$$

Where the last term is defined as:

$$\zeta\left(r, L_p, L_c\right) = 1 - \left\{\frac{5L_p}{4L_c} - \frac{2r^2}{{L_c}^2} + \frac{33r^4}{80L_p{L_c}^3} + \frac{79{L_p}^2}{160{L_c}^2} + \frac{329L_p r^2}{120{L_c}^3} - \frac{6799r^4}{1600{L_c}^4} + \frac{3441r^6}{2800L_p{L_c}^5} - \frac{1089r^8}{12800{L_p}^2{L_c}^6}\right\} \quad [2]$$

C_{eff} calculations: The effective concentration in the bound state when the linker is restrained to a distance between binding sites r_o can be expressed as:

$$C_{eff} = \frac{p(r_o)}{4\pi r^2} \frac{10^{27} Å^3 l^{-1}}{L_o} \quad [3]$$

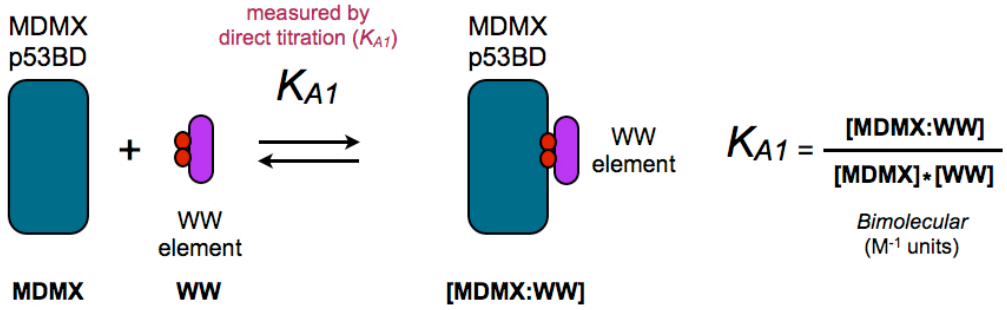
Where L_o is Avogadro's number, and C_{eff} is expressed in molar units. Multiplying Eq. [3] by 10^3 gives C_{eff} in millimolar units.

<u>Equilibrium scheme used for WLC modeling of binding affinity enhancement for the MdmX linker.</u>

We used the following equilibria to model the MdmX intramolecular interaction, and the MdmX-p53 interactions

I- Model for bimolecular interaction of WW with MdmX

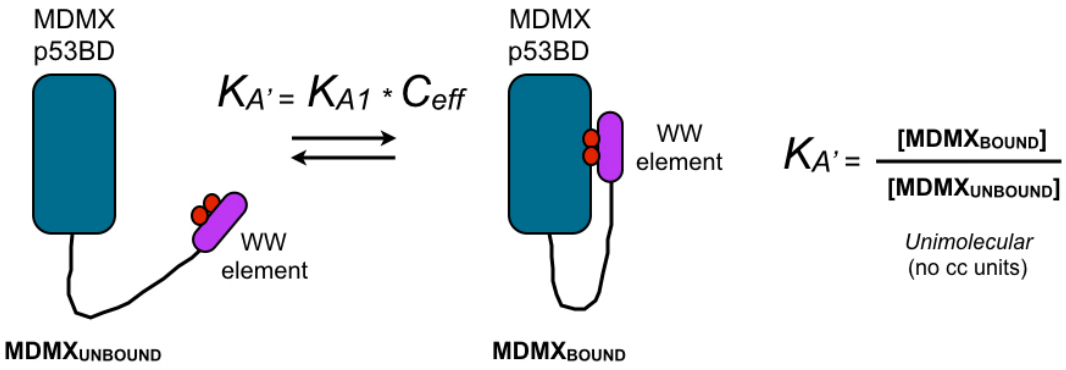
We define the first equilibrium as:



Where K_{A1} is the measured bimolecular association constant of the MdmX WW element to the MdmX p53 binding domain, in units of M^{-1}.

II- Model for intramolecular binding of WW to MdmX

We define the intramolecular MdmX equilibrium as:



And

$$K_{A'} = K_{A1} * Ceff \qquad [4]$$

Where $K_{A'}$ is the intra-molecular binding constant, and C_{eff} is the effective concentration, which was estimated from the WLC model (Eqn. [1]-[3]). $K_{A'}$ is unimolecular and therefore has no concentration units.

III- Calculating populations of $MdmX_{UNBOUND}$ and $MdmX_{BOUND}$ from $K_{A'}$

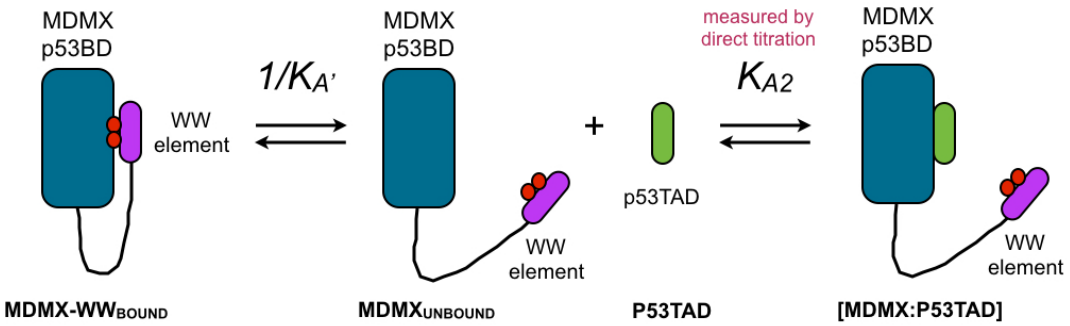
From the definition of $K_{A'}$ = [$MdmX_{BOUND}$]/[$MdmX_{UNBOUND}$] we can calculate the fractional population of the $MdmX_{BOUND}$ and $MdmX_{UNBOUND}$ conformers as:

$$f_{MdmXBOUND} = \frac{K_{A'}}{1+K_{A'}}; f_{MdmXUNBOUND} = \frac{1}{1+K_{A'}} \qquad [5]$$

Which does not depend on the concentration of MdmX.

IV- ***Modeling the competition between P53TAD and WW for binding to MdMX***

Taking into account the previous equilibria, the binding of p53TAD to the MdmX variant containing the intramolecular motif can be modeled as follows:



Where, based on measurements of the bimolecular association constant for binding of P53TAD to the MdmX domain without the intramolecular motif (K_{A2}), we can calculate the global equilibrium association constant for binding of p53TAD to the motif-containing MdmX construct as:

$$K_{A\ global} = \frac{1}{K_{A'}} * K_{A2} \qquad [6]$$

$$K_{D\ global} = K_{A'} * K_{D2} \qquad [7]$$

Note that K_D global = $K_{A'}$*K_{D2} and $K_{A'}$ = K_A*C_{eff}. This implies that higher values of C_{eff} lead to an increase in the global K_D value. This is equivalent to a decrease in the global binding affinity of p53TAD to MdmX. Therefore, higher values for C_{eff} lead to stronger MdmX intramolecular interactions and weaken P53TAD association to MdmX.

Supporting Material

Methods

Sequence Alignments

Mdmx alignments were carried out with the Geneious software suite version 10.0.7(4) using the ClustalW alignment algorithm (5) set to use the Blosum62 matrix with a gap open penalty of 12, extension penalty of 3, and 2 refinement iterations. The indicated residue numbers align with human mdmx.

Protein Expression and Purification

Mdmx cDNA constructs encoding residues 23-111, 23-150, 23-170, 23-190, and 23-210 were expressed using the pGEX-6p-2 vector transformed into BL21(DE3) cells and grown in minimal media. Overnight cultures were used to start cultures at OD600=0.04 and were grown to an OD600 of 0.6 and then induced with 1mM IPTG at 15 degrees Celsius for 16 hours. Pellets were re-suspended and lysed via French press in 50mM Tris, 300mM NaCl, 2.5mM EDTA, 1mM DTT, and 0.02% sodium azide pH 7.4 in the presence of ThermoScientific Pierce protease inhibitors (88665). GST tagged protein was then purified by passing the soluble fraction of lysate through a glutathione sepharose column (GE 17513201) and eluting with 10mM reduced glutathione. The GST tag was cleaved by incubation with the GST tagged HRV3C protease overnight at 4 Celsius, the glutathione was dialyzed away, and the cleaved tag was removed via a second glutathione sepharose column. The flowthrough is further purified via a superdex 75 SEC column (GE 28989333). The p53TAD construct encoding residues 1-73 in pET28A was prepared and purified as previously described (6).

Isothermal Titration Calorimetry

Mdmx and p53 polypeptides were co-dialyzed into 50mM NaPO4, 150mM NaCl, 1mM EDTA, 0.02% Sodium Azide, 8mM BME at pH 6.8. For mdmx constructs shorter than 23-210 3 replicate titrations were conducted with 2uM Mdmx in the cell and 20uM of p53 1-73 in the syringe using 15uL injections using a MicroCal-VP-ITC system at 25 celsius. For the 23-210 Mdmx construct 2 titrations were conducted using a MicroCal-ITC 200 system with 80uM Mdmx in the cell and 975uM of p53TAD 1-73 in the syringe using 2.05uL injections at 25 celsius. The corrected heat values were fit using Microcal origin software's (7.0) built in non-linear least square curve-fitting algorithm yielding the stoichiometry, enthalpy, and affinity constants reported.

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