SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Pull-down of SUMO proteins by the wild-type and mutant M-IR2 domains – The expression construct of the wild-type M-IR2 domain of RanBP2 encompassing residues 2686-2761 (1) was used to construct nine plasmids with the following mutations in the SIM region: K2708I, K2708L, K2708V, C2710S, C2710V, W2714I, W2714L, W2714V, and E2715K. The vectors were generated by the QuikChange site-directed mutagenesis kit (Stratagene) and all mutants were confirmed by DNA sequencing. The expression and purification of GST-SUMO1, GST-SUMO3 and the M-IR2 constructs was performed as described previously (1). The purified proteins were dissolved in 100 mM Sodium Phosphate, pH 6.0, for the pull-down assays. 96-well polystyrene plates (Costar) were coated with 0.1 mL of the ten M-IR2 domain proteins with a concentration of 5 mg/L overnight at 4ºC. Unoccupied sites were blocked by incubating with 5% BSA for 2 hours at room temperature. The plates were then washed with wash buffer (PBS and 1% BSA) followed by addition of 0.1 mL of purified GST-SUMO1 or GST-SUMO3 protein to each well at serial concentrations of 0, 2, 5 and 10 mg/L. After incubation for 1 hour at 37ºC to allow binding to the M-IR2 domain of RanBP2, the plates were washed three times with wash buffer and then treated with 0.1 mL/well of anti-SUMO1 mouse monoclonal IgG (Abgent, 1:1000) or anti-SUMO3 rabbit polyclonal IgG (Abcam, 1:1500). The antibodies were incubated for 1 hour at 37ºC and then washed three times with wash buffer. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Upstate, 1:2000) or HRP-conjugated goat anti-rabbit IgG (Bio-Rad, 1:3000) was applied at 0.1 mL/well to SUMO1 or SUMO3 containing wells, respectively. After incubation for 1 hour at 37ºC, the plates were washed three times. Then 0.1 mL/well of TMB peroxidase substrate solution (Thermo) was added and the reaction was stopped by addition of 1N phosphoric acid. The bound SUMO proteins were detected by measuring optical absorbance at 450 nm (Fig. S1).

NMR sample preparation and titration – SIM peptides were synthesized by the Peptide Synthesis Core Facility at City of Hope, purified by HPLC, and verified by mass spectrometry. SUMO samples used for NMR analysis were enriched with $^{15}N/^{2}H$ or $^{13}C/^{15}N/^{2}H$ isotopes using established protocols for expressing and purifying His-tagged proteins (2,3). All NMR samples contained 20 mM sodium phosphate buffer, pH 6.8, 0.03% sodium azide, 5 mM DTT, 90% H_2O/10%D_2O or 100% D_2O. All spectra were acquired at 298K on a Bruker 500MHz NMR spectrometer equipped with a room temperature probe and a Bruker 600MHz NMR spectrometer equipped with a cryogenic probe. Spectra were processed with Topspin 2.0 (Bruker Biospin, Inc.) and analyzed with Sparky 3 (Goddard T.D and Kneller D.G., Sparky 3, University of California).

SUMO-SIM NMR complex samples were prepared by monitoring the changes in SUMO1 or SUMO2 ^{15}N-^{1}H HSQC spectra upon incremental additions of SIM peptide until no more shifts occurred, which indicated protein saturation (Figs. S2-S4). The SIM titrant was dissolved in the same buffer as the SUMO protein and the concentration was calibrated against an internal standard, DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). Because of the M-IR2 peptides bound SUMO1 at NMR chemical shift exchange regimes ranging from fast to slow, the binding dissociation constant (K_d) (Table S1) was extracted by integrating lineshapes of the 1D ^{15}N slices of the 2D ^{15}N-^{1}H HSQC spectra of SUMO during titration and fitted to the Bloch-McConnell equations for 2-state chemical exchange (Fig. S4) (4). Chemical shift perturbation analyses used free versus SIM-saturated 2D ^{15}N-^{1}H HSQC NMR spectra of SUMO. The changes in the backbone amide chemical shifts for each residue were calculated as square root of ($25 \times \Delta\delta_{1H}{}^{2} + \Delta\delta_{15N}{}^{2}$).

Preparation of molecules for structure calculation by HADDOCK – The starting SUMO1 structure (residues 20-97) was extracted from the complex crystal structure of SUMO-RanGAP1-Ubc9-Nup358 (pdb 1Z5S) (5). Hydrogens were added and followed by minimization using Maestro (Schrödinger). The extended starting structure of the 13 amino acids of the M-IR2 SIM (DNEIEVIIVWEKK), which was fully flexible in HADDOCK calculations, was built using PyMOL (Schrödinger). HADDOCK calculations were carried out using all experimental intra-M-IR2 and intermolecular NOEs as unambiguous distance constraints.

Isothermal titration calorimetry (ITC) measurements – A Microcal (Amherst, MA) VP-ITC calorimeter was used to perform ITC measurements for the binding interaction of SUMO with SIM peptides. Titration was performed at 30°C with the peptides dissolved in the same buffer as SUMO (20 mM phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol). Peptide solutions (250-1000 μM in 10 μL increments) were injected at intervals of 180 sec and up to a total volume of 290 μL into a sample cell containing SUMO1 or SUMO2 (50 μM, 1.4 mL). The heat of dilution was subtracted for baseline correction and data were analyzed with Microcal ORIGIN software to extract binding thermodynamic parameters. K_d was obtained assuming a single binding site on SUMO as described before (2,3). Experiments were repeated at least three times.

SUPPLEMENTAL TABLES AND FIGURES

Table S1. Binding affinity from NMR linewidth and chemical shift perturbation analysis

M-IR2 SIM	[#]SEQUENCE	[*]SUMO1 K_d	[*]SUMO2 K_d
Wild-type	DNEKECIIVWEKK	Not Determined	Not Determined
C2710V	DNEKE**V**IIVWEKK	5.8 +/- 3.8 μM	18 +/- 13 μM
[‡]K2708I	DNE**I**E**V**IIVWEKK	1.8 +/- 1.2 μM	22 +/- 34 μM
W2714K	DNEKE**V**IIV**K**EKK	168 +/- 66 μM	> 1 mM
E2715K	DNEKE**V**IIVW**K**KK	81 +/- 18 μM	> 1 mM

[#]Mutation sites on the *wild-type* sequence are underlined. SIMs used for NMR studies have the C2710V mutation.
[*]Extracted from LineShapeKin as illustrated in Figure S4.
[‡]SUMO1-specific SIM used for structure determination.

Table S2. ITC derived thermodynamic parameters for the interaction of SUMO with SIM peptides derived from the PIASX SIM

PIASX SIM	SUMO1			SUMO2		
SEQUENCE	[†]K_d (μM)	ΔH (kcal/mole)	ΔS (cal/mol/K)	K_d (μM)	ΔH (kcal/mol)	ΔS (cal/mol/K)
KVDVIDLTIE	8.94±0.38	-2.13±0.02	16.06	5.07±0.51	-4.52±0.09	9.32
[§]K**G**DVIDLTIE		Not detected		7.46±0.28	-11.19±0.50	-13.5
K**Y**DVIDLTIE	7.41±-0.86	-20.25±2.43	-44.4	2.18±0.24	-6.46±0.12	4.23
KVD**Y**IDLTIE	5.49±0.91	-12.55±0.98	-17.3	1.36±0.14	-4.84±0.94	10.9
KVDV**Y**DLTIE	173±22	-10.43±147	-38.6	103±28.4	-16.8±42.5	-17.2
KVDVIDLTI**A**	158±80.4	-6.82±15.6	-5.46	59.9±29.3	-0.62±0.16	17.2

[†]Extracted from fitting ITC binding isotherms to a one-site model (Figure S7).
[§]SUMO2-specific SIM (V2G).

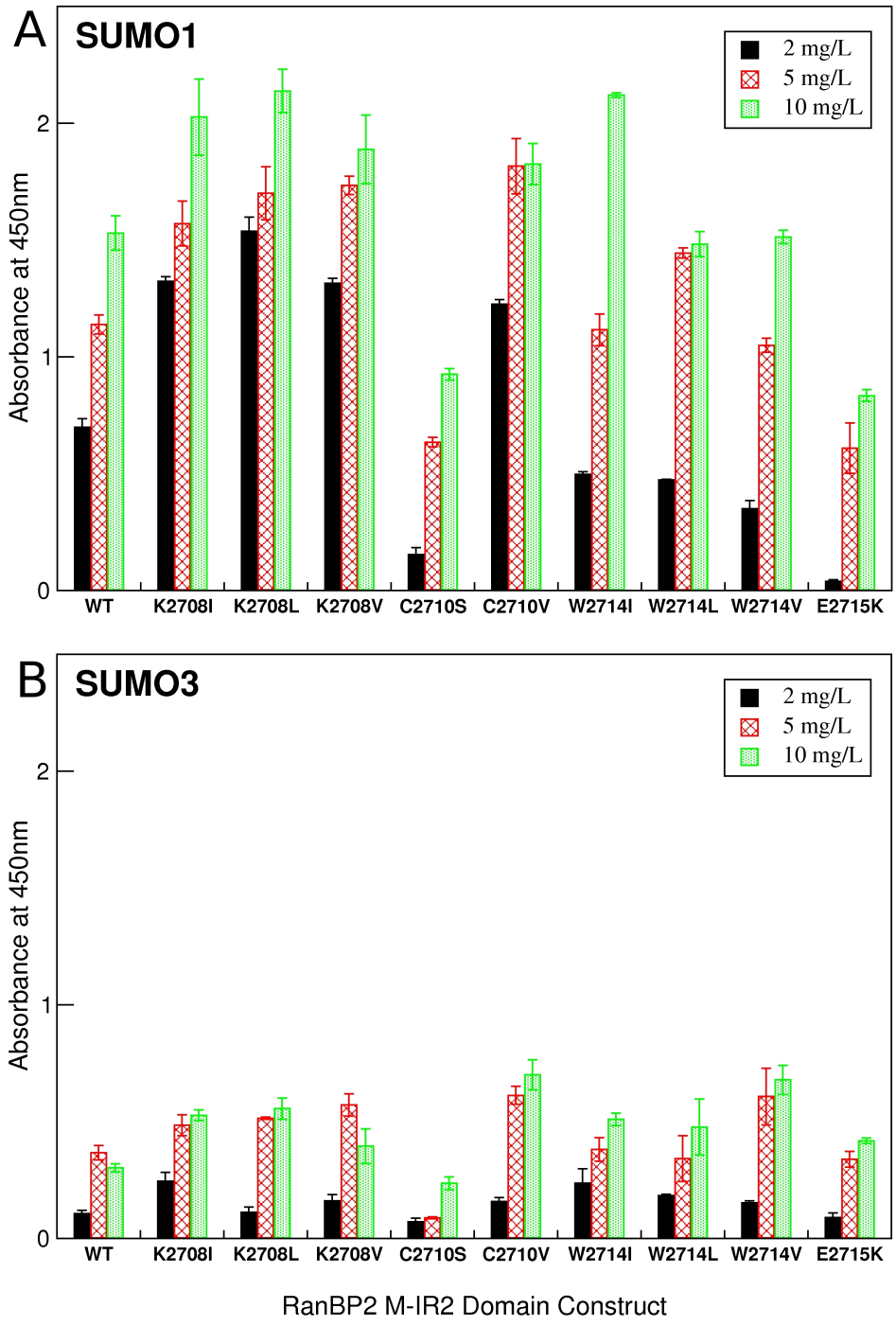

Figure S1. Validation of the peptide array results using M-IR2 domain constructs to pull down SUMO1 (A) or SUMO3 (B). The pull-down assays were carried out at three concentrations of GST-SUMO1 and GST-SUMO3, as indicated. The absorbance was averaged over triplicate data sets with higher values (taller bars) indicating tighter binding. Error bars represent the standard deviation.

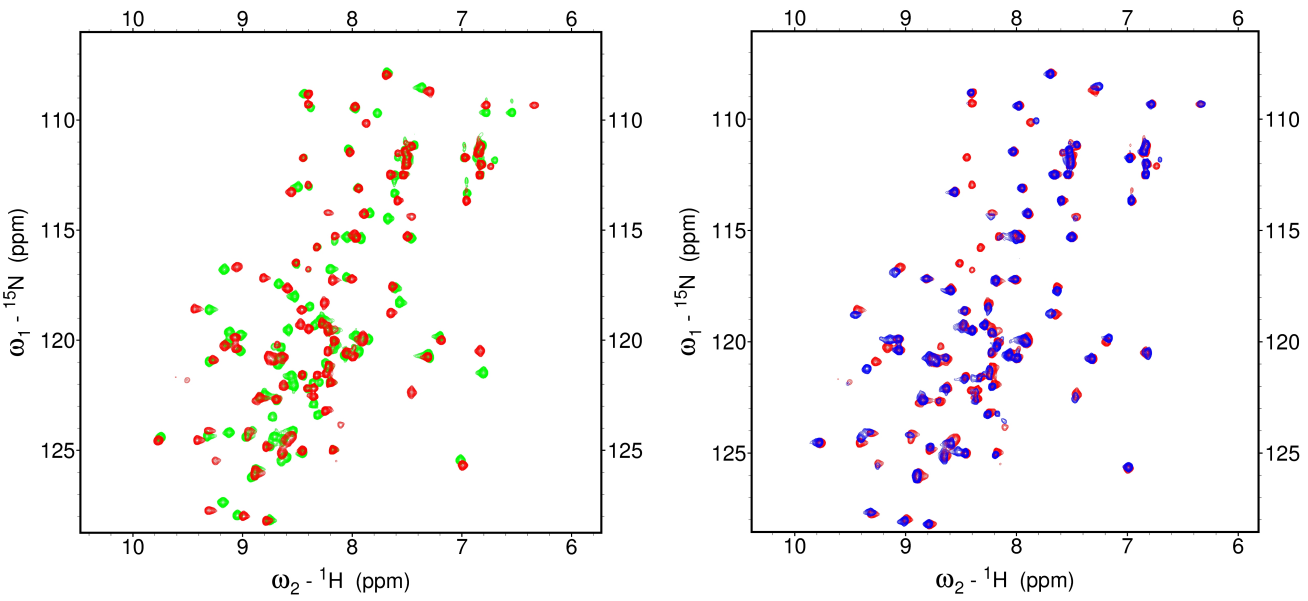

Figure S2. Superposition of 2D ^{15}N-^{1}H HSQC spectra of SUMO1, free (green) and bound to C2710V (red) or C2710V/K2708I (blue) M-IR2 SIM peptides.

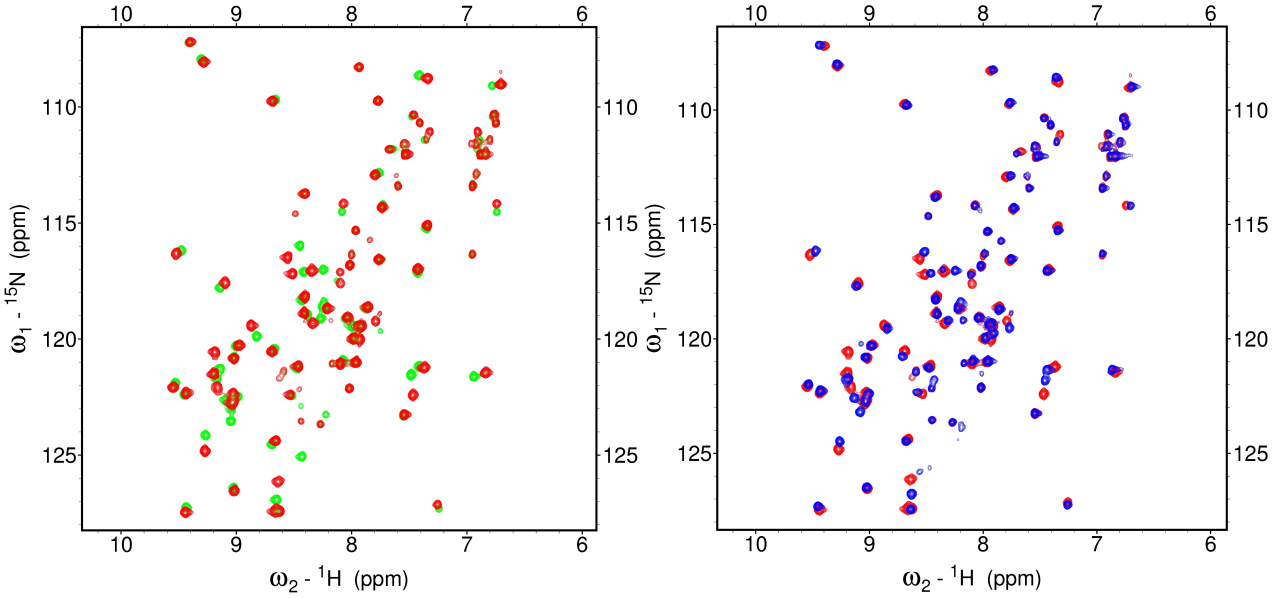

Figure S3. Superposition of the 2D ^{15}N-^{1}H HSQC spectra of SUMO2, free (green) and bound to C2710V (red) or C2710V/K2708I (blue) M-IR2 SIM peptides.

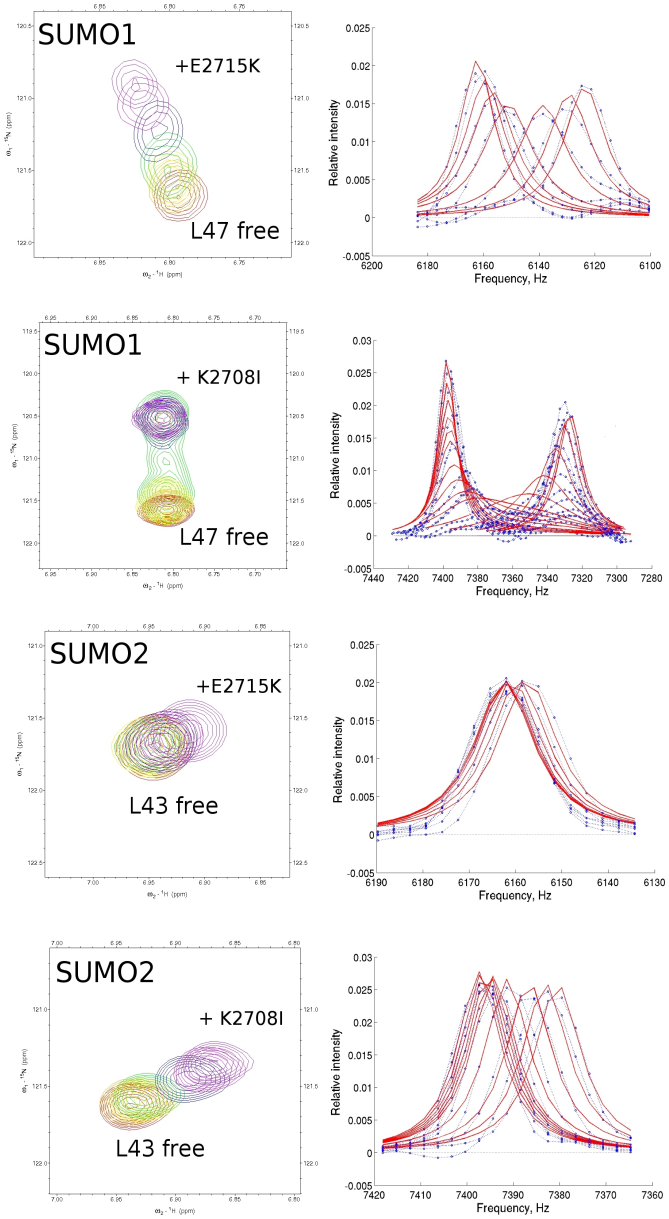

Figure S4. Representative NMR titration spectra and linewidth fits for calculating the M-IR2 SIM peptide binding affinities for SUMO1 or SUMO2. Overlays of the 2D ^{15}N-^{1}H HSQC spectra for SUMO complexed with peptide are shown on the left. The cross peaks of L47 of SUMO1 or the homologous L43 of SUMO2 are shown. Next to the ^{15}N-^{1}H HSQC spectra are the 1D ^{15}N slices (larmor frequency of 60 MHz) for each titration point shown in blue, and the linewidth fit shown in red. The fits were performed by LineShapeKin software and assumed a 2-state exchange binding process (free vs. bound).

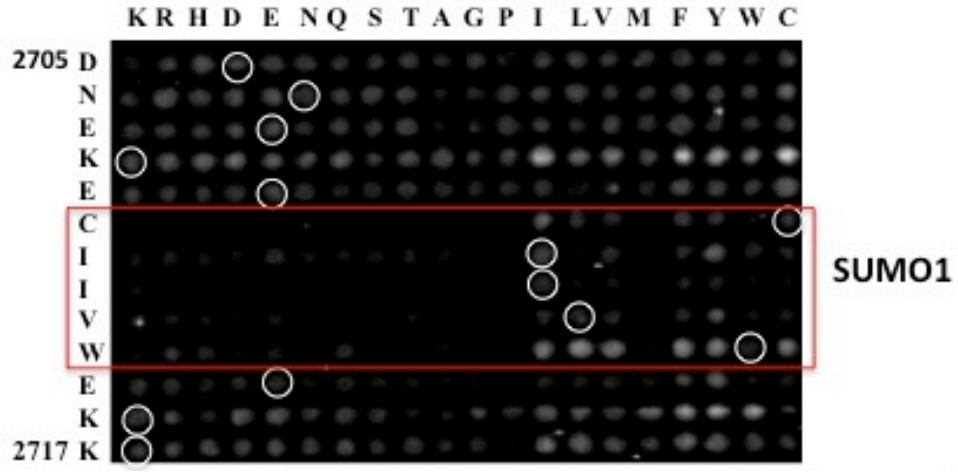

Figure S5. Peptide array based on the M-IR2 peptide. The SIM sequence is listed vertically with the N-terminus at the top, and the twenty amino acid substitutions are listed horizontally. The intensity of each spot relative to the wild-type SIM (circled spots) defines the binding affinity of the mutant to SUMO, with lighter spots indicating enhanced interaction. Segments of the SIM with strict sequence requirements are boxed.

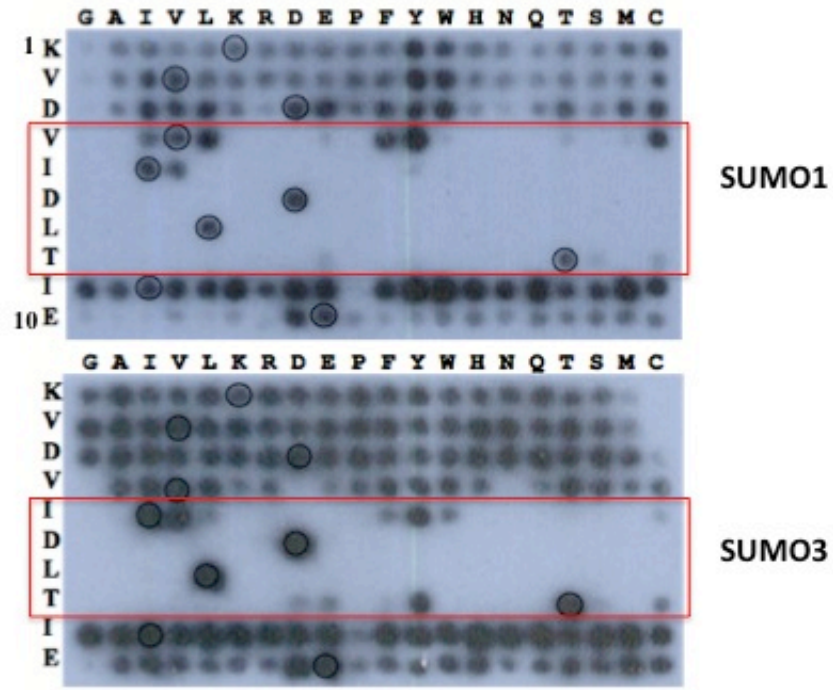

Figure S6. Peptide array based on the PIASX SIM for interaction with SUMO1 (upper panel) or SUMO3 (lower panel). The SIM sequence is listed vertically with the N-terminus at the top, and the twenty amino acid substitutions are listed horizontally. The intensity of each spot relative to the wild-type SIM (circled spots) defines the binding affinity of the mutant to SUMO, with darker spots indicating enhanced interaction. The boxed regions indicate segments on the SIM with strict sequence requirements.

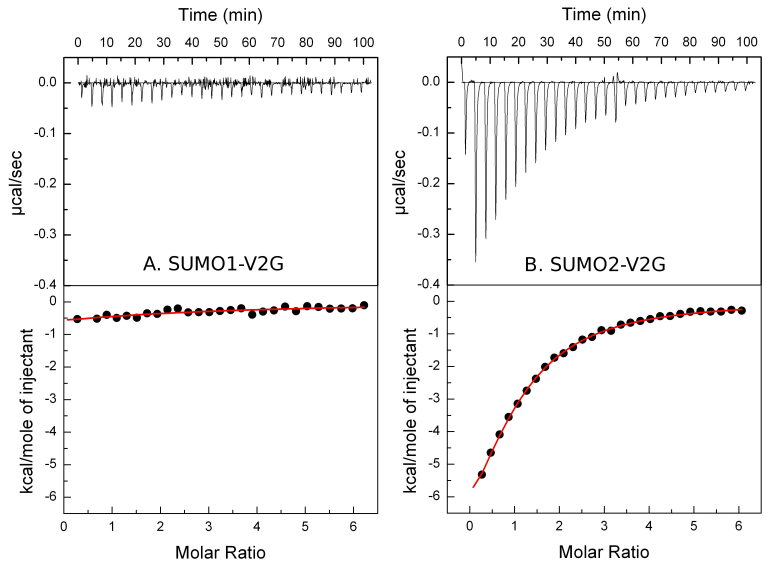

Figure S7. Representative ITC analyses to validate the peptide array based on the PIASX peptides. Shown are the interactions of SUMO1 (A) and SUMO2 (B) with the SUMO-2-specific V2G mutant peptide (K**G**DVIDLTIE). The top panel shows the measured heat that is released upon injecting the peptide to SUMO. The bottom panel shows curve fitting to extract K_d.

SUPPLEMENTAL REFERENCES

1. Tatham, M. H., Kim, S., Jaffray, E., Song, J., Chen, Y., and Hay, R. T. (2005) *Nat Struct Mol Biol* **12**(1), 67-74
2. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004) *Proceedings of the National Academy of Sciences of the United States of America* **101**(40), 14373-14378
3. Song, J., Zhang, Z., Hu, W., and Chen, Y. (2005) *J Biol Chem* **280**(48), 40122-40129
4. Kovrigin, E. L., and Loria, J. P. (2006) *Biochemistry* **45**(8), 2636-2647
5. Reverter, D., and Lima, C. D. (2005) *Nature* **435**(7042), 687-692