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Supplementary Information for

Mitochondrial protein interaction landscape of SS-31

Juan D. Chavez[a], Xiaoting Tang[a], Matthew D. Campbell[b], Gustavo Reyes[b], Philip A. Kramer[b], Rudy Stuppard[b] , Andrew Keller[a], Huiliang Zhang[c], Peter S. Rabinovitch[c], David J. Marcinek[b], and James E. Bruce[a*]

*To whom correspondence should be addressed. Email: jimbruce@uw.edu

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Supplementary Information Text

SI Material and Methods

Cross-linker synthesis

The protein interaction reporter (PIR) cross-linker amide-DP-NHP was synthesized by solid phase peptide synthesis using a CEM Liberty Lite peptide synthesizer. Amino acids were coupled to amide-Rink resin in the following order: Fmoc-Lys(Fmoc), Fmoc-Pro, Fmoc-Asp, succinic anhydride. The N-hydroxyphthalamide (NHP) ester of trifluoroacetic acid (TFA) was synthesized by dissolving 5.86 g of NHP in 20 mL of TFA anhydride in a 50 mL round bottom flask. The reaction was allowed to proceed for 1.5 h under a dry N_2 atmosphere with constant mixing via magnetic stir bar. After 1.5 h the mixture was dried under vacuum to obtain a white crystalline solid (TFA-NHP). The cross-linker (0.5 mmoles of peptide on resin) was esterified by reacting with a 12-fold molar excess of TFA-NHP in 10 mL of dry pyridine for 20 min at room temperature with constant mixing. The reaction mixture was transferred to a Bio-Rad poly prep column and the liquid was filtered away. The resin containing the esterified peptide was washed extensively with a total of 60 mL dimethyl formamide (DMF) followed by 60 mL of dichloromethane (DCM). The cross-linker was cleaved from the resin by incubation with 5 mL of 95% TFA, 5% DCM for 3 h at room temperature with constant mixing. The cross-linker was precipitated by adding the cleavage solution to ice cold diethyl ether at a ratio of 1:15 by volume. The cross-linker was pelleted by centrifugation at 3400 g for 30 min at 4^{o}C. The cross-linker pellet was washed by resuspending the pellet in 10 mL of fresh ice cold diethyl ether and repeating the centrifugation step. The cross-linker pellet was then dried by vacuum centrifugation and weighed. The cross-linker was dissolved in DMSO to a concentration of 270 mM, aliquoted and stored at -80^{o}C until used.

Cross-linked mitochondrial sample preparation

Cross-linked mitochondrial samples were lysed with 8 M urea in 0.1 M NH_4HCO_3. The sample was kept on ice and sonicated with a GE 130 ultrasonic processor using 5 cycles of 5 s pulses at an amplitude of 40. The total protein concentration was measured using the Pierce Coomassie Plus Bradfrod protein assay. Protein disulfide bonds were reduced with 5 mM TCEP for 30 min followed by alkylation of thiol groups with 10 mM iodoacetamide for 30 min. Samples were diluted 10-fold with 0.1 M NH_4HCO_3 and digested with a 1:200 ratio of trypsin to protein at 37^{o}C overnight. After digestion samples were acidified by adding trifluoroacetic acid (TFA) to a final concentration of 1%. Acidified samples were desalted using Waters C18 Sep Pak cartridges and associated vacuum manifold. Samples were passed through the cartridge at a flow rate of 1 mL/min, followed by 3 mL washes of water containing 0.1% TFA. Peptides were eluted from the Sep Pak cartridge with 1 mL of 80% acetonitrile, 20% water, 0.1% TFA. Desalted samples were concentrated by vacuum centrifugation using a Genevac EZ-2 system. Dried samples were reconstituted in 0.5 mL of 0.1 M NH_4HCO_3 and adjusted to pH 8 with 1.5 M NaOH. 100 uL of 50% UltraLink monomeric avidin slurry was added to the sample and incubated with constant mixing for 30 min at room temperature. The monomeric avidin resin was washed 5 times with 3 mL of 0.1 M NH_4HCO_3 followed by elution of bound peptides with 2 additions of 0.5 mL of 70% acetonitrile, 0.5% formic acid, each with 5 min incubation. The eluate fractions were pooled and concentrated by vacuum centrifugation. The biotin enriched sample was reconstituted in 30 uL of 0.1% formic acid in water and subjected to LC-MS analysis as described below.

LC-MS analysis of cross-linked peptide pairs

Samples were analyzed by LC-MS using two methods developed for analysis of cross-linked peptide pairs, namely ReACT [1] and Mango [2]. ReACT analysis was carried out on a Velos-FTICR mass spectrometer coupled with a Waters nanoAcquity UPLC. Peptides were fractionated by reversed-phase liquid chromatography by first loading the sample (5 µL injection) onto a trapping column (3 cm x 100 um i.d.) packed with Reprosil C8 beads (Dr. Maisch) using a flow rate of 2 µL/min of 98% solvent A (water, 0.1% formic acid) and 2% solvent B (acetonitrile, 0.1% formic acid). After trapping peptides were separated over an analytical column (60 cm x 75 um) maintained at 45^{o}C, packed with Reprosil C8 stationary phase using a flow rate of 300 nL/min and applying a binary gradient of 90% solvent A / 2% solvent B to 60% solvent A / 40% solvent B over 120 min followed by a wash cycle consisting of 20% solvent A / 80% solvent B for 20 min and a re-

equilibration period consisting of 98% solvent A / 2% solvent B for 20 min. Eluting peptides were ionized by electrospray ionization (ESI) by applying a voltage of 2.6 kV to a laser pulled spray tip at the end of the chromatography column. MS1 analysis (500-2000 m/z) was performed in the ICR cell with a resolving power setting of 50K at 400 m/z, and an automatic gain control (AGC) setting of 5E5. The most abundant precursor ion with a charge state ≥ 4 was selected for MS2 using an isolation window of 3 m/z and a normalized collision energy (NCE) of 25. Fragment ions were analyzed in the ICR cell using a resolving power setting of 12.5K at 400 m/z and an AGC setting of 2E5. MS2 spectra were searched in real-time for fragment ions that satisfy the expected PIR mass relationship (mass peptide 1 + mass peptide 2 + mass reporter = mass precursor) within a 20 ppm mass error tolerance. If satisfied the two released peptide ions were sequentially analyzed by MS3 in the Velos dual ion trap mass analyzer where they were isolated with a 3.0 m/z isolation window and fragmented with a NCE of 35 using an AGC setting of 5E4.
Samples were analyzed by Mango using a Thermo Q-exactive plus mass spectrometer coupled with a Thermo Easy nLC. 5 µL of sample was injected into the nLC system where it was loaded onto a trapping column (3 cm x 100 um i.d.) packed with Reprosil C8 beads (Dr. Maisch) using a flow rate of 2 µL/min of solvent A. After trapping peptides were separated over an analytical column (60 cm x 75 um) maintained at 45^{o}C, packed with Reprosil C8 stationary phase using a flow rate of 300 nL/min by applying the same binary gradient described for the ReACT analysis above. Eluting peptides were ionized by electrospray ionization (ESI) by applying a voltage of 2.6 kV to a laser pulled spray tip at the end of the chromatography column. MS1 analysis (400-2000 m/z) was performed in the orbitrap mass analyzer using a resolving power setting of 70K at 200 m/z and an ACG value of 1E6. This was followed by MS2 on the 5 most abundant precursor ions with charge states ≥ 4 using a 3 m/z isolation window, a resolving power setting of 70K at 200 m/z and an ACG value of 5E4.

Data analysis
LC-MS data files in .RAW format were converted to .mzXML format using the ReADW tool in the Trans Proteomic Pipeline software suite [3]. Comet [4] was used to search the .mzXML files against the Mitocarta 2 database [5] containing both forward and reverse protein sequences (2084 total sequences) along with the addition of the sequence for bSS-31 (Biotin-D-Arg-dimethyl Tyr-Lys-Phe-NH2). Due to the presence of non-canonical amino acids the bSS-31 sequence was entered using the following single letter amino acid sequence BJKZ. Within the Comet parameters file the mass of B (Biotin-D-Arg) was set to 382.17926 Da, the mass of J (dimethyl Tyr) was set to 191.094629 Da and the mass of Z (amidated Phe) was set to 146.084399 Da. Additional Comet parameters used for searching ReACT data included; a peptide mass tolerance of 20 ppm, allowance of -1/0/1/2/3 ^{13}C offsets, trypsin as the digesting enzyme considering only fully tryptic sequences with up to 5 missed cleavage sites. Oxidation of Met (15.9949 Da) was included as a variable modification while the cross-linker stump mass modification on Lys (197.032422 Da) was included as a required modification at any position within the peptide sequence except for the C-terminus, MS3 spectra were searched using a 1.0005 Da tolerance on the fragment ions with a bin offset of 0.4. Comet parameters used for Mango data were the same except that the Mango search parameter was set to 1, MS2 spectra were searched and a 0.02 Da tolerance with a 0.0 bin offset were used for fragment ions.

Validation of bSS-31 fragmentation spectrum
Ten µL of 10.6 mM bSS-31 in 18.2 MΩ H_2O was transferred to a 1.5 mL tube. 90 µL of 170 mM Na_2HPO_4 pH 8.0 was added. This was followed by addition of 1.11 µL of 270 mM DP-amide cross-linker stock solution in DMSO resulting in a 3 mM final cross-linker concentration. The reaction was carried out at room temperature for 1 h with shaking 1400 rpm. The sample was then acidified to 1 % TFA by volume. The sample was then desalted using a 50 mg size C18 Sep-Pak column. After loading the sample onto the Sep-Pak the peptides were washed with 3 additions of 1 mL H_2O/0.1% TFA. The sample was then eluted from the Sep-Pak column with 1 mL 50% ACN/ 2% CH_3COOH and analyzed by direct infusion MS with the Velos-FTICR mass spectrometer. SpectraST v 5.0 was used to search fragmentation spectra generated by ReACT against a spectral library of the cross-linker modified bSS31. Fragmentation spectra assigned as bSS-31 cross-links were required

to contain the accurate mass of the DP stump modified bSS-31 (Fig. S1C) and have a SpectraST assigned p-value of less than 0.1.

Ex Vivo Mitochondrial Respiration and H_2O_2 Production

In a parallel experiment, mitochondria were isolated from young (5-7 month-old) and old (36-37 month old) mouse hearts or gastrocnemius muscles as described above either in the presence or absence of 10 μM bSS-31. Approximately 100 μg mitochondrial homogenate was used in the 2 mL chamber of an Oxygraph 2K dual respirometer/fluorometer (Oroboros Instruments, Innsbruck, Austria) at 37°C and stirred gently during substrate and inhibitor titrations. Heart mitochondrial respiration and H_2O_2 production were measured simultaneously under the following conditions. State 4 was measured by adding 5 mM pyruvate, 2 mM malate, and 10 mM glutamate. State 3 was stimulated by adding 2.5 mM ADP (CI) followed by 10 mM succinate (CI+CII) then cytochrome C (6 mM). The rate of maximal uncoupled flux through the ETS was measured by adding 1 μM FCCP. Following FCCP, Antimycin A (2.5 uM) was added to block flux through complex III followed by TMPD and ascorbate (1mM and 4mM, respectively) to measure complex IV activity. The non-mitochondrial rate of oxygen consumption was subtracted from all measured functional parameters before reporting final values. In a separate experiment ADP sensitivity was measured in gastrocnemius muscles by measuring complex I stimulated respiration across a range of ADP conenctrations.H_2O_2 emission was measured in parallel with respiration using Amplex Red (10 mM) and HRP (0.1 U/mL).

In vivo ADP sensitivity assays

In order to assess in vivo ADP sensitivity we reanalyzed data from Siegel et al. [6]. Briefly, a short ischemic period was used to induce PCr breakdown. This rate of PCr breakdown is equal to the resting mitochondrial ATP production. The PCr recovery was measured over 6 min to determine a time constant of recovery (t_{PCr}) to yield ATPmax(= PCr_{rest}/ t_{PCr}). Free ADP concentration at rest and the start of recovery and ATP production at the start of recovery (ATP_{flux}= ΔPCr/ t_{PCr}) used to determine ADP sensitivity by fitting the [ADP] and ATPase rates at rest and initial recovery to Michaelis-Menton plots with a hill coefficient of 2.6 (**Fig. S3**)[6, 7].

In vivo superoxide production assay

Single ventricular myocytes were enzymatically isolated from mouse hearts as described previously[8, 9]. SS-31 (1 μM) or Biotin-SS31 (1 μM) were added to the cultured cardiomyocytes for 12hrs. The mitochondrial superoxide was quantified by the ratio of mitoSOX to mitoTrackerGreen using Leica SP8X confocal microscopy. MitoSOX Red (5 μM, excited at 540 nm with emission collected at > 560 nm) to mitoTrackerGreen (200 nM, excited at 488 nm and emission collected at 505-530 nm)[10].

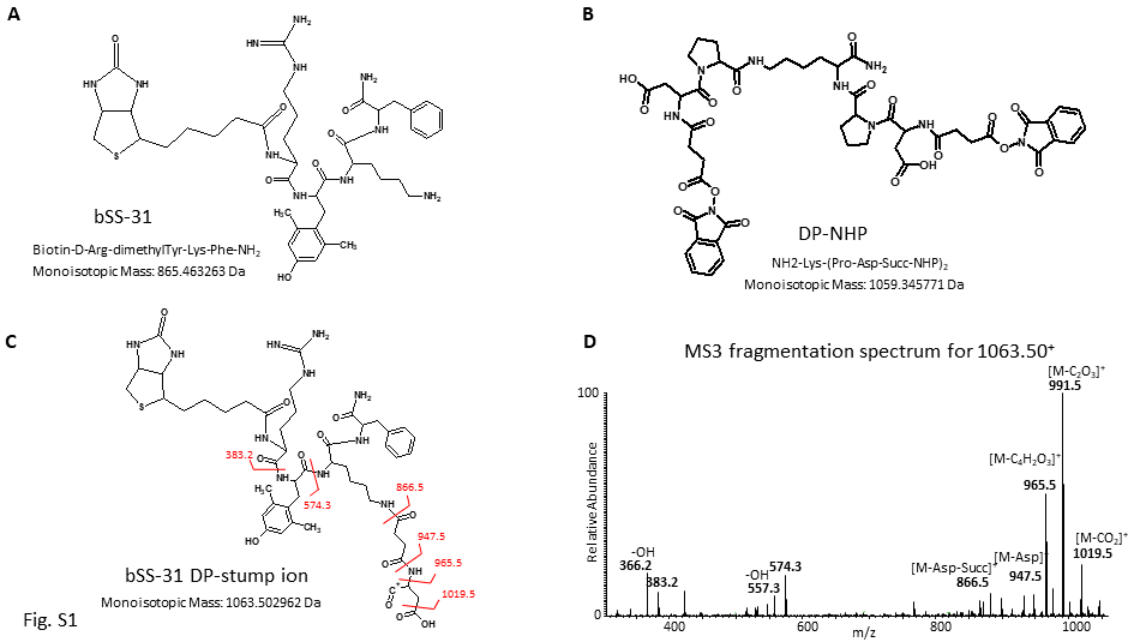
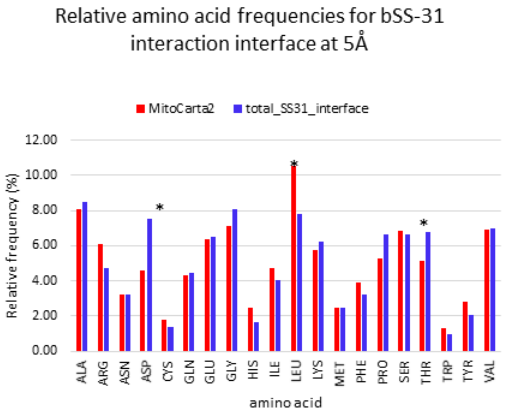


Fig. S1

Fig. S1. Structures of biotin SS-31 and PIR cross-linker DP-NHP. A) chemical structure of biotinylated SS-31. B) chemical structure of DP-NHP cross-linker. C) chemical structure for bSS-31 DP-stump ion which results from MS2 fragmentation of the PIR labile bonds of DP cross-linked bSS-31. Red lines with numbers indicate the bods which break to give rise to the major fragment ions observed in panel D. D) MS3 fragmentation pattern for bSS-31 DP stump ion with major fragment ions labeled. Bonds which fragment to give rise to these fragment ions are indicated in red in panel C. Ions at m/z 557.3 and m/z 366.2 result from loss of hydroxyl radical from fragments ions at m/z 574.3 and m/z 383.2 respectively.

A



B

COMPOSITION PROFILER

Ala	Not significant.	P-value=0.625319 (>0.050000)
Arg	Not significant.	P-value=0.130040 (>0.050000)
Asn	Not significant.	P-value=0.967645 (>0.050000)
Asp	↑Enriched.	P-value=0.000151 (≤0.050000)
Cys	Not significant.	P-value=0.452208 (>0.050000)
Gln	Not significant.	P-value=0.852845 (>0.050000)
Glu	Not significant.	P-value=0.837160 (>0.050000)
Gly	Not significant.	P-value=0.299125 (>0.050000)
His	Not significant.	P-value=0.158045 (>0.050000)
Ile	Not significant.	P-value=0.396237 (>0.050000)
Leu	↓Depleted.	P-value=0.018157 (≤0.050000)
Lys	Not significant.	P-value=0.514830 (>0.050000)
Met	Not significant.	P-value=0.947528 (>0.050000)
Phe	Not significant.	P-value=0.351050 (>0.050000)
Pro	Not significant.	P-value=0.091603 (>0.050000)
Ser	Not significant.	P-value=0.866523 (>0.050000)
Thr	↑Enriched.	P-value=0.040943 (≤0.050000)
Trp	Not significant.	P-value=0.411683 (>0.050000)
Tyr	Not significant.	P-value=0.244195 (>0.050000)
Val	Not significant.	P-value=0.508747 (>0.050000)

C

cross-linked residue	protein	biorep1	biorep2	biorep3	biorep4	control	# of Lys	probability
159	AATM_MOUSE						30	3.7037E-05
272	ADT1_MOUSE						23	0.003952569
33	ADT1_MOUSE						23	7.47178E-06
506	ATPA_MOUSE						31	0.001040583
506	ATPA_MOUSE						31	1.08281E-06
480	ATPB_MOUSE						23	0.001890359
644	ECHA_MOUSE						66	1.01325E-10
505	ECHA_MOUSE						66	1.53523E-12
360	IDHP_MOUSE						37	2.13175E-12
272	IDHP_MOUSE						37	5.7615E-14
180	IDHP_MOUSE						37	2.13175E-12
230	KCRS_MOUSE						28	4.55539E-05
74	NDUA4_MOUSE						8	0.000244141
582	ODO1_MOUSE						51	7.53858E-06
278	ODO2_MOUSE						26	0.038461538
199	QCR2_MOUSE						27	5.08053E-05
83	QCR6_MOUSE						7	0.002915452

D

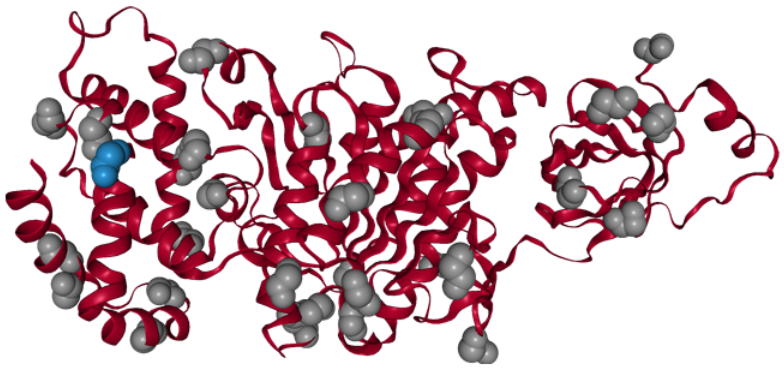


Fig. S2 (cont.)

Fig. S2. Amino acid composition of bSS-31 interaction interfaces – **A)** Bar plot indicating relative frequencies for the 20 amino acids in the MitoCarta2 database (red) and the amino acids comprising the bSS-31 interaction interface (residues within 5 angstroms of bSS-31 in docked models). Asterisks indicate a statistically significant difference ($p<0.05$) as calculated by the Composition Profiler tool. **B)** Output from Composition Profile comparing amino acid composition of the bSS-31 interaction interfaces to the MitoCarta2 database. **C)** Table of Lys residues cross-linked to bSS-31 indicating the number of biological replicates each was identified in (blue fill), as well as the probability that each residue would be linked to bSS-31 by random chance. **D)** Ribbon structure of ATPA indicating all Lys (31 total) as space filled residues. Grey residues were not found cross-linked to bSS-31 while K506 (blue) was the only residue cross-linked with bSS-31.

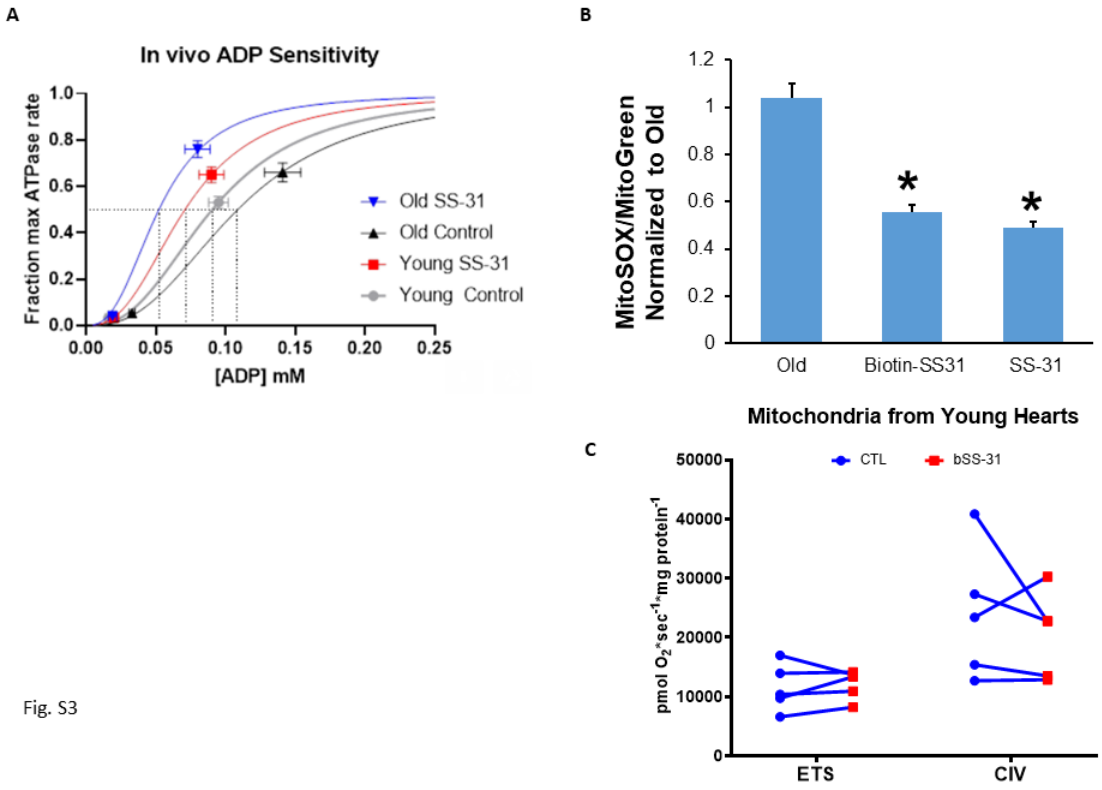


Fig. S3

Fig. S3. Effect of SS-31 on ADP sensitivity and superoxide production in vivo. **A)** Free ADP concentration at rest and the start of recovery and ATP production at the start of recovery (ATPflux= ΔPCr/ tPCr) used to determine ADP sensitivity by fitting the [ADP] and ATPase rates at rest and initial recovery to Michaelis-Menton plots with a hill coefficient of 2.6. **B)** Superoxide production by mitochondria from old cardiomyocytes treated with 1 μM bSS-31, SS-31 or vehicle control for 12h. Superoxide production is indicated the normalized relative fluorescence intensity of MitoSOX to MitoGreen was measured on cells (N=26-75 cells per group). * indicates statistical significance ($p < 0.01$). **C)** Maximum uncoupled respiration (ETS) in mitochondria isolated from young mouse hearts in the presence of bSS-31 (red) or vehicle control (blue) and complex IV activity (CIV) in the presence of bSS-31 (red) or vehicle control (blue).

Dataset S1 (separate file). List of identified bSS-31 cross-linked peptides

Video S1 (separate file). Cross-link directed docked models for bSS-31 ADT1 interaction in the c-state (PDB: 2c3e) and m-state (PDB: 6gci). ADT is shown as red ribbon structures with the cross-linked residues K33 and K272 displayed as yellow space filled residues. Electrostatic interactions between D232-K33 and E265-K272 stabilize the c-state of ADT and are not present in the m-state.

SI References

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