Different Oligomeric States of the Tumor Suppressor p53 Show Identical Binding Behavior towards the S100β Homodimer

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Supporting Information

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Materials and Methods

Protein Expression and Purification

Expression and purification of full-length human p53 (wild-type, L344A, L344P) were performed as previously described\(^\text{[1]}\).

The sequence of full-length human S100\(\beta\) was synthesized as codon-optimized gene for recombinant expression in Escherichia coli by GeneArt. For cloning, the full-length human S100\(\beta\) sequence with N-terminal (His)\(_4\) tag and thrombin cleavage site were incorporated into the pET28(+) plasmid with cleavage sites Xhol and Ndel. Escherichia coli BL21(DE3) cells were transformed with the ligated plasmid\(^\text{[2]}\). Cells were induced at OD\(_{600}\)=1.2 with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and harvested after overnight incubation at 37°C. Protein was purified (ÄKTA pure, GE Healthcare) by immobilized metal-ion affinity chromatography (IMAC) (1 mL HisTrap FF column, GE Healthcare) in 50 mM 2-[4-(2-hydroxyethyl)-piperazine-1-yl]ethanesulfonic acid (HEPES), 300 mM NaCl, 2.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 0-500 mM imidazole at pH=8.0 followed by size exclusion chromatography (SEC) (HiLoad\(^\text{TM}\) 16/600 Superdex\(^\text{TM}\) 200pg, Cytiva) in 50 mM 2-[4-(2-hydroxyethyl)-piperazine-1-yl]ethanesulfonic acid (HEPES), 300 mM NaCl, 2.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 10% v/v glycerol at pH=7.2. Recombinant, tag-free human S100\(\beta\) protein was obtained from ProSpec (PRO-2312).

All chemicals were obtained from Sigma at the highest purity if not stated otherwise. Milli-Q-water was used for all reactions.

Covalent Modification of Proteins

N-hydroxysuccinimide (NHS) esters, such as disuccinimidyl dibutyric urea (DSBU, CF Plus Chemicals), mainly react with the primary amines of lysines and the N-termini of proteins. Reactions with amino acids that contain hydroxy groups, such as serines, threonines, and tyrosines, are also observed. A recent comparative community-wide cross-linking mass spectrometry (XL-MS) study\(^\text{[3]}\) reported that serines, threonines, and tyrosines account for ~30% of NHS ester reaction sites. In human p53, here are 21 primary amine groups and 69 hydroxy groups there are 90 potential reactive sites of p53. The same calculation for S100\(\beta\) with 8 primary amine groups and 9 hydroxy groups results in 17 potential reactive sites. Conclusively, the molar concentration of nucleophilic residues in solution is \([\text{p53}] \times \text{p53}_{\text{reactive sites}} + [S100\beta] \times \text{S100\beta}_{\text{reactive sites}} = 6 \, \mu\text{M} \times 42 + 6 \, \mu\text{M} \times 11 = 318 \, \mu\text{M}. Thus, the molar ratio of DSBU over the nucleophilic residues in solution is 120 \, \mu\text{M} / 318 \, \mu\text{M} = 0.38, corresponding to ~ 0.4 equivalents of the cross-linker DSBU.

All covalent modification experiments were performed at 4°C in buffer (50 mM HEPES, 300 mM NaCl, 2.5 mM TCEP, and 10% glycerol, pH 7.2). Each variant of p53 (wild-type, L344A, and L344P) was used at a final concentration of 6 \, \mu\text{M}, S100\(\beta\) was used at a final concentration of 6 \, \mu\text{M}, and DSBU with a final concentration of 0.12 mM (20-fold molar excess of p53). 1 mM of calcium chloride was added to calcium-containing DSBU-modified samples, while for calcium-depleted DSBU-modified samples 1 mM of the chelating agent EGTA was added. Quenching of covalent-linking reactions was performed by adding 20 mM of ammonium bicarbonate after 60 minutes. Buffer exchange was conducted immediately after the addition of ammonium bicarbonate.
**Buffer Exchange**

Prior to ESI-MS experiments, buffer exchange to 500 mM ammonium acetate (pH 6.8) was performed. Buffer exchange of the cross-linked samples (L344P and L344A variants of p53) was performed with Amicon Ultra centrifugal filter units (molecular weight cut-off 30 kDa, Merck Millipore). For the cross-linked samples of wild-type p53, an online buffer exchange (OBE) procedure was performed. The sample was loaded via the autosampler on the Ultimate 3000 RSLC nano-HPLC system (Thermo Fischer Scientific). The sample was then desalted and buffer exchanged with a self-packed polyacrylamide P6 column (BioRad).

**ESI-MS**

All ESI-MS experiments were performed with a High-Mass Q-TOF II instrument (Micromass/MS Vision). Experiments were measured with following instrumental settings: Capillary voltage, 2 – 2.2 kV (for online buffer-exchanged samples), 1.2 – 1.4 kV (for manually buffer-exchanged samples with Amicon filters); sample cone voltage, 60-80 V; collision energy, 30-100 V; source pressure, 1.0 x 10^{-1} mbar; collision cell pressure, 1.0 x 10^{-2} mbar – 1.2 x 10^{-2} mbar. The transmission range was set between m/z 1000-8000. For collision-induced dissociation tandem mass spectrometry (CID-MS/MS), the collision energy was set to 120 – 140 V.

**Cross-linking of Proteins**

All cross-linking reactions were performed in three replicates and analyzed separately.

**DSBU cross-linking**

A solution containing 6 μM wild-type p53 or L344A or L344P variants of p53 and 6 μM S100β in buffer (50 mM HEPES, 1 mM CaCl$_2$, 300 mM NaCl, and 2.5 mM TCEP, pH 7.2) was cross-linked at 4°C for 1 h with DSBU at 100-fold molar excess. DSBU was dissolved in neat dimethyl sulfoxide (DMSO) at a concentration of 0.6 mM, immediately before adding it to the protein solution. The reaction was quenched by adding ammonium bicarbonate to a final concentration of 20 mM.

**EDC/sulfo-NHS cross-linking**

A solution containing 6 μM wild-type p53 or L344A or L344P variants of p53 and 6 μM S100β in buffer (50 mM HEPES, 1 mM CaCl$_2$, 300 mM NaCl, and 2.5 mM TCEP, pH 7.2) was cross-linked at 4°C for 2 h with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo-N-hydroxysuccinimide (sulfo-NHS) (both Thermo Fisher) at 500-fold molar excess. EDC was dissolved in neat DMSO at a concentration of 3 mM, immediately before adding it to the protein solution. Sulfo-NHS was dissolved in a buffer containing 50 mM HEPES, 1 mM CaCl$_2$, 300 mM NaCl, and 2.5 mM TCEP, pH 7.2) at a concentration of 3 mM. The reaction was quenched by adding ammonium bicarbonate to a final concentration of 20 mM.

**Sulfo-SDA crosslinking**

A solution containing 6 μM wild-type p53 or L344A or L344P variants of p53 and 6 μM S100β in buffer (50 mM HEPES, 1 mM CaCl$_2$, 300 mM NaCl, and 2.5 mM TCEP, pH 7.2) was cross-linked at 4°C for 2 hrs with EDC and sulfo-NHS at 500-fold molar excess. Sulfosuccinimidyl 4,4'-azipentaonate
(sulfo-SDA) was dissolved in DMSO at a concentration of 0.6 mM, immediately before adding it to the protein solution. After the reaction with EDS/sulfo-NHS the sample was irradiated for 15 s at 365 nm (Panasonic Aicure UJ30/35 in a home-built irradiation chamber. The reaction was quenched by adding ammonium bicarbonate to a final concentration of 20 mM.

**Enzymatic Digestion**

All cross-linked samples were reduced to a volume of 2-3 µl in a SpeedVac concentrator. 25 µl of 8 M urea in 400 mM ammonium bicarbonate was added to each sample. After 5 min of sonication, samples were incubated with dithiothreitol (DTT, 6.4 mM) for 30 min at 56°C and iodoacetamide (12.5 mM) for 20 min at room temperature in the dark. The alkylation reaction was quenched by adding 9 mM of DTT. After diluting the samples to 1 M urea with water, they were incubated overnight at 37°C with AspN (NEB) at an 1:20 (w/w) enzyme-substrate ratio and digested with trypsin (Promega) at 1:20 (w/w) for 4 h at 37 °C. All proteolytic digestions were stopped by adding 10% (v/v) TFA.

**Nano-HPLC-MS/MS**

Digested samples were analyzed by nano-HPLC-MS/MS on an UltiMate 3000 RSLC nano-HPLC system (Thermo Fisher Scientific) coupled to a timsTOF Pro mass spectrometer equipped with CaptiveSpray source (Bruker Daltonik). Peptides were trapped on a C18 column (precolumn Acclaim PepMap 100, 300 µm × 5 mm, 5 µm, 100 Å) (Thermo Fisher Scientific) and separated on a μPAC 50-cm column (PharmaFluidics). After trapping, peptides were eluted by a linear 90-min gradient from 3% (v/v) to 40% (v/v) ACN. For elution, a flow gradient was employed ranging from 900 to 600 nl/min in 15 min, followed by a constant flow rate of 600 nl/min. The column was washed at a flow rate of 600 nl/min with the following gradient: 40% (v/v) to 85% (v/v) ACN (5 min), 85% (v/v) ACN (5 min), 85% (v/v) to 3% (v/v) ACN (5 min). All separations were performed at room temperature.

For the settings of the timsTOF Pro mass spectrometer, the following parameters were adapted, starting with the parallel accumulation serial fragmentation (PASEF) method for standard proteomics. The values for mobility-dependent collision energy ramping were set to 95 eV at an inverted reduced mobility (1/k₀) of 1.6 V s/cm² and 23 eV at 0.73 V s/cm². Collision energies were linearly interpolated between these two 1/k₀ values and kept constant above or below. No merging of TIMS scans was performed. Target intensity per individual PASEF precursor was set to 20 000. The scan range was set between 0.6 and 1.6 V s/cm² with a ramp time of 166 ms. 14 PASEF MS/MS scans were triggered per cycle (2.57 s) with a maximum of seven precursors per mobilogram. Precursor ions in an m/z range between 100 and 1700 with charge states ≥2+ and ≤8+ were selected for fragmentation. Active exclusion was enabled for 0.4 min (mass width 0.015 Th, 1/k₀ width 0.015 V s/cm²).

**Data Analysis**

Identification of cross-links was performed with MeroX 2.0.1.7. The following settings were applied: Proteolytic cleavage: C-terminal at Lys and Arg for (3 missed cleavages were allowed) and N-terminal at Asp and Glu (3 missed cleavages were allowed); peptide lengths of 5 to 30 amino acids; modifications: alkylation of Cys by iodoacetamide (fixed), oxidation of Met (variable); cross-linker specificity: Lys, Ser, Thr, Tyr, N-terminus (DSBU), Lys, N-terminus, Asp, Glu, C-terminus (EDC/sulfo-NHS); Lys, N-terminus, Asp, Glu, C-terminus (sulfo-SDA); search algorithm: RISEUP mode with up to three missing ions mode with minimum peptide score of 0; precursor mass accuracy: 10 ppm.
fragment ion mass accuracy: 20 ppm; signal-to-noise ratio > 1.5; precursor mass correction enabled; 10% intensity as prescore cut-off; 1% false discovery rate (FDR) cut-off, and minimum score cut-off: 50. Mass recalibration was performed for all files.

MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the project accession PXD029914, username: reviewer_pxd030001@ebi.ac.uk; password: OJIKK9rp.

Mapping of cross-links was visualized with xiNET\textsuperscript{[4]}

**Surface Plasmon Resonance (SPR)**

All SPR measurements were acquired with MP-SPR Navi 200 OTSO instrument (BioNavis). A CMDP sensorchip (Xantac) was used to perform experiments to investigate the protein-protein binding interactions between p53 (tetrameric wild-type, dimeric variant L344A, monomeric variant L344P) and S100\(\beta\). All buffers were degassed and filtered prior to SPR experiments. Pre-concentration experiments were performed in order to test the optimal immobilization pH (3.6, 3.9, 4.2) of 10 mM sodium acetate, where a pH value of 3.9 was found to be the most optimal.

The running buffer for ligand immobilization and binding experiments consisted of 50 mM HEPES, 300 mM NaCl, 2.5 mM TCEP, and 1 mM of CaCl\(_2\) (pH 7.2). For protein immobilization, 5 \(\mu\)M of S100\(\beta\) was diluted with 10 mM sodium acetate buffer (pH 3.9). The buffer for immobilizing S100\(\beta\) was 10 mM sodium acetate (pH 3.9). The amine coupling chemistry was applied for immobilization by using EDC/NHS to activate the CMDP surface and 1 M ethanolamine to deactivate non-reacted NHS-esters on the sensor surface.

Full-length p53 (wild-type, L344A, L344P) was injected separately at the following concentrations: 0.5, 1, 3, 5, 7 \(\mu\)M (wild-type p53), 0.8, 1, 4, 5, 7, 8 \(\mu\)M (L344A), 0.2, 0.5, 0.8, 3, 5, 8 \(\mu\)M (L344P). The running buffer consisted of 50 mM HEPES, 300 mM NaCl, 2.5 mM TCEP, and 1 mM calcium chloride. Prior to injection of each concentration, a regeneration buffer with 10 mM NaOH and 10 mM EGTA was used to remove bound analyte. Data processing was performed with the software TraceDrawer (BioNavis) where the on/off association (\(K_a\))/dissociation (\(K_d\)) constants were used to calculate the dissociation constant (\(K_D\)).
Amino Acid Sequences

Amino acid sequence of human p53

Wild-Type (tetrameric p53 variant)

MEEQPSDSPV EPPLSQETFS DLWKLPPENN VLSPLPSQAM DDLMLSPDDI
EQQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
KTYQGYSYGFRLGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
PPPGRTRVARAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
LRLVEYLDNRNTFRHSVVVPY EPPEVGSRCT TIHYNYMCNS SCMGGMNRRP
ILTIIITLEDSSGNLLGRNSFEVRCACPCGR DRRTEEENLRKKGEPHHELP
PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FREANEALEL
KDAQAGKEPG GSRAHSSHLK SKGQSTSRH KKLMFKTEGP DSD

L344A (dimeric p53 variant)

MEEQPSDSPV EPPLSQETFS DLWKLPPENN VLSPLPSQAM DDLMLSPDDI
EQQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
KTYQGYSYGFRLGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
PPPGRTRVARAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
LRLVEYLDNRNTFRHSVVVPY EPPEVGSRCT TIHYNYMCNS SCMGGMNRRP
ILTIIITLEDSSGNLLGRNSFEVRCACPCGR DRRTEEENLRKKGEPHHELP
PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FREANEALEL
KDAQAGKEPG GSRAHSSHLK SKGQSTSRH KKLMFKTEGP DSD

L344P (monomeric p53 variant)

MEEQPSDSPV EPPLSQETFS DLWKLPPENN VLSPLPSQAM DDLMLSPDDI
EQQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
KTYQGYSYGFRLGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
PPPGRTRVARAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
LRLVEYLDNRNTFRHSVVVPY EPPEVGSRCT TIHYNYMCNS SCMGGMNRRP
ILTIIITLEDSSGNLLGRNSFEVRCACPCGR DRRTEEENLRKKGEPHHELP
Amino acid sequence of human S100β

The N-terminal GSH tag is highlighted in red.

**GSHMSELEKA MVALIDVFHQ YSGREGDKHK LKKSELKELI NNELSHFLEE IKEQEVVDKV METLDNDGDG ECDFQEFMAF VAMVTTACHE FFEHE**
Additional Figures and Tables

SDS-PAGE of S100β purification

Figure S1. SDS-PAGE of the S100β protein purification
SDS-PAGE of p53 purification

**Wild-Type**

![SDS-PAGE Image](image)

**Figure S2.** SDS-PAGE of the p53 (Wild-Type, tetrameric) protein purification
**Figure S3.** SDS-PAGE of the p53 (L344A, dimeric variant) protein purification
Figure S4. SDS-PAGE of the p53 (L344P, monomeric variant) protein purification
Figure S5. ESI-MS of wild-type p53 and S100β without DSBU modification.
Wild-Type (tetrameric)

\[
m/z
\]

\[
\begin{align*}
+32 & \quad 6260.024 & \quad (200288 \text{ Da}) \\
+31 & \quad 6461.591 & \quad (200278 \text{ Da}) \\
+30 & \quad 6659.522 & \quad (199755 \text{ Da}) \\
+29 & \quad 6881.975 & \quad (199548 \text{ Da}) \\
\end{align*}
\]

\~200 kDa

(One p53 tetramer + one S100\beta dimer)

**Figure S6.** ESI-MS spectra show complex formation of S100\beta and wild-type tetrameric p53. Peak assignments of calcium-added and -depleted samples are presented.
**L344A (dimeric)**

| $m/z$ | (111 kDa) |
|-------|------------|
| +23   | 4829.25    | (111049 Da) |
| +22   | 5049.26    | (111061 Da) |
| +21   | 5289.60    | (111061 Da) |
| +20   | 5562.60    | (111232 Da) |

(one p53 dimer + one S100B dimer)

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**Figure S7.** ESI-MS spectra show complex formation of S100β and dimeric p53 L344A variant. Peak assignments of calcium-added and -depleted samples are presented.
Figure S8. ESI-MS spectra show complex formation of S100β and monomeric p53 L344P variant. Peak assignments of calcium-added and -depleted samples are presented.
Figure S9. SDS-PAGE (4-20% gradient gel) of XL experiments of S100β and p53 with DSBU; (a) tetrameric wild-type, (b) dimeric L344A, and (c) monomeric L344P p53.
Figure S10. SDS-PAGE (4-20% gradient gel) for XL experiments of S100β and p53 with sulfo-SDA; (a) tetrameric wild-type, (b) dimeric L344A, and (c) monomeric L344P p53.
**Figure S11.** SDS-PAGE (4-20% gradient gel) for cross-linking experiments of S100β and p53 with EDC/NHS; (a) tetrameric wild-type, (b) dimeric L344A, and (c) monomeric L344P p53.
Figure S12. Mapping of DSBU cross-linked samples of S100β and (a) tetrameric wild-type, (b) dimeric L344A, and (c) monomeric L344P p53. Cross-links are visualized with xiNET[^4].
Figure S13. Mapping of sulfo-SDA cross-linked samples of S100β and tetrameric wild-type p53. Cross-links are visualized with xiNET[4].
**Figure S14.** Mapping of EDC/NHS cross-linked samples of S100β and (a) tetrameric wild-type, (b) dimeric L344A, and (c) monomeric L344P p53. Cross-links are visualized with xiNET\[^4\].
Figure S15. Selected MS/MS spectra and cross-linking assignment of DSBU with MeroX.
Figure S16. Selected MS/MS spectra and cross-linking assignment of Sulfo-SDA with MeroX.
Figure S17. Selected MS/MS spectra and cross-linking assignment of EDC/NHS with MeroX.
**Figure S18.** SDS-PAGE (4-20% gradient gel) for cross-linking experiments of recombinant, tag-free S100β and tetrameric wild-type p53 with DSBU.

|        | DSBU | 3 replicates |
|--------|------|--------------|
| WT p53 | +    | + + + + + + + |
| S100β  | +    | + + + + + + + |
| CaCl₂  |      | + + + + + + + |
| EGTA   |      | + + + + + + + |

- 250 kDa
- 130 kDa
- 100 kDa
- 70 kDa
- 55 kDa
- 35 kDa
- 25 kDa
- 15 kDa
- 10 kDa
Figure S19. Mapping of DSBU cross-links of recombinant, tag-free S100β and tetrameric wild-type p53. Cross-links are visualized with xiNET\(^4\).
Figure S20. Selected MS/MS spectra and cross-linking assignment of recombinant, tag-free S100β and tetrameric wild-type p53 with DSBU with MeroX.
**Figure S21.** Instrumental setup of the online buffer exchange (OBE) at load and inject position\textsuperscript{[5]}. 
Figure S22. Surface plasmon resonance (SPR) experiments of p53 variants binding to immobilized S100β. (a) Wild-type, tetrameric; (b) L344A dimeric; (c) L344P monomeric p53. Dashed lines represent curve fittings of the one-to-one model. The $K_D$ value of wild-type p53 and S100β is 41.2 ± 0.01 μM. For dimeric p53 (L344A variant) the $K_D$ value is 60.6 ± 0.3 μM and for the monomeric p53 (L344P variant) the $K_D$ value is 40.3 ± 0.6 μM.
Table S1. Binding affinities ($K_D$) of reported p53 peptides/S100β and other protein binding partners.

| Interaction | Binding affinity ($K_D$) (µM) | Method                      |
|-------------|--------------------------------|-----------------------------|
| p53 (aa. 293-393)/S100β | 0.25 ± 0.05[^6]                   |                             |
| p53 (aa. 325-339)/S100β | 172 ± 4                                         | Fluorescence Anisotropy |
| p53 (aa. 325-355)/S100β | 112 ± 7                                          |                             |
| p53 (aa. 340-351)/S100β | 302 ± 7                                         |                             |
| p53 (aa. 367-393)/S100β | 102 ± 3                                          |                             |
| p53 (aa. 305-322)/S100β | 570 ± 50                                        | Analytical Ultracentrifugation |
| p53 (aa. 325-339)/S100β | 180 ± 20                                       |                             |
| p53 (aa. 325-355)/S100β | 77 ± 11                                        |                             |
| p53 (aa. 340-351)/S100β | 260 ± 20                                       |                             |
| p53 (aa. 367-393)/S100β | 82 ± 6                                         |                             |
| p53 (aa. 379-393)/14-3-3σ | 25.7[^7]                                       | Isothermal Calorimetry     |
| p53 (aa. 379-393)/14-3-3σ | 34.4                                           | Surface Plasmon Resonance  |
| p53-K382acetylated (aa. 372-389)/Sir21m | 4.3 ± 0.5[^8]                          | Isothermal Calorimetry     |
Table S2. Theoretical and experimental masses of p53 and complexes where differences are contributed by different numbers of DSBU modifications.

| Species                        | Theoretical Mass (kDa) | Experimental Mass (kDa) |
|-------------------------------|------------------------|------------------------|
| S100β monomer                 | 10.994                 | 11.062                 |
| S100β dimer                   | 21.988                 | 22.010                 |
| Tetrameric Wild-Type p53/S100β complex | 196.948               | 199.967               |
| Tetrameric Wild-Type p53      | 174.960               | 177.432               |
| Dimeric L344A p53/S100β complex | 109.468              | 111.100               |
| Dimeric L344A p53 variant     | 87.480                | 88.215                 |
| Monomeric L344P p53/S100β complex | 65.728               | 66.430                 |
| Monomeric L344P p53 variant   | 43.740                | 44.063                 |
Table S3. Summary of cross-links identified between p53 and S100β with cross-linker DSBU.

| Wild-Type tetrameric | Peptide 1 (p53) | From | To | Peptide 2 (S100β) | From | To | Site1 | Site2 |
|----------------------|-----------------|------|----|--------------------|------|----|-------|-------|
| KGQSTSR             | 374             | 380  | GSHMSELEK | 0       | 9    | K374  | N-terminus |

| L344A dimeric | Peptide 1 (p53) | From | To | Peptide 2 (S100β) | From | To | Site1 | Site2 |
|---------------|-----------------|------|----|--------------------|------|----|-------|-------|
| KGQSTSR     | 374             | 380  | GSHMSELEK | 0       | 9    | K374  | N-terminus |
| DAQAGKEPGGSR | 353             | 364  | GSHMSELEK | 0       | 9    | K358  | S5    |
| SKKGQSTSR   | 372             | 380  | HKLK   | 29      | 32   | S379  | K30   |

| L344P monomeric | Peptide 1 (p53) | From | To | Peptide 2 (S100β) | From | To | Site1 | Site2 |
|-----------------|-----------------|------|----|--------------------|------|----|-------|-------|
| DAQAGKEPGGSR   | 353             | 364  | GSHMSELEK | 0       | 9    | K358  | N-terminus |
| KGQSTSR        | 374             | 380  | GSHMSELEK | 0       | 9    | K374  | N-terminus |
**Table S4.** Summary of the cross-links identified of p53 and S100β with cross-linker sulfo-SDA.

| Wild-Type                | Peptide 1 (p53) | From | To | Peptide 2 (S100β) | From | To | Site1 | Site2 |
|--------------------------|-----------------|------|----|-------------------|------|----|-------|-------|
| EMFRELNEALEIK            | 340             | 352  |    | ELSHFLEEIK        | 43   | 52 | E344  | S45   |
**Table S5.** Summary of the cross-links identified of p53 and S100β with cross-linker EDC/sulfo-NHS.

### Wild-Type tetrameric

| Peptide 1 (p53) | From | To  | Peptide 2 (S100β) | From | To  | Site1       | Site2         |
|----------------|------|-----|-------------------|------|-----|-------------|---------------|
| DIEQWFTE       | 50   | 57  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D50           |
| DPGPDEAPR      | 58   | 66  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D62           |
| TEGPDSD        | 388  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D392          |
| TEGPDSD        | 388  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D394          |

### L344A dimeric

| Peptide 1 (p53) | From | To  | Peptide 2 (S100β) | From | To  | Site1       | Site2         |
|----------------|------|-----|-------------------|------|-----|-------------|---------------|
| DIEQWFTE       | 50   | 57  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D50           |
| DPGPDEAPR      | 58   | 66  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D62           |
| TEGPDSD        | 388  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D392          |
| TEGPDSD        | 388  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D394          |

### L344P monomeric

| Peptide 1 (p53) | From | To  | Peptide 2 (S100β) | From | To  | Site1       | Site2         |
|----------------|------|-----|-------------------|------|-----|-------------|---------------|
| DDLMLSPD       | 42   | 49  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D49           |
| DDIEQWFTE      | 49   | 57  | {GSHMS}           | 0    | 5   | N-terminus  | D49           |
| DIEQWFTE       | 50   | 57  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D50           |
| DPGPDEAPR      | 58   | 66  | {GSHMSELEK}       | 0    | 9   | N-terminus  | D62           |
| LMKTEGPDS      | 384  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D392          |
| TEGPDSD        | 388  | 394 | {GSHMSELEK}       | 0    | 9   | N-terminus  | D394          |
**Table S6.** Summary of the cross-links identified of p53 and recombinant, tag-free S100β with cross-linker DSBU.

| Wild-Type | Peptide 1 (p53) | From | To  | Peptide 2 (S100β) | From | To  | Site1 | Site2      |
|-----------|----------------|------|-----|-------------------|------|-----|-------|-----------|
|           | DAQAGKEPGGSR   | 353  | 364 | {MSELEK}          | 0    | 6   | K358  | N-terminus|
|           | AHSSHLKSK      | 365  | 373 | {MSELEK}          | 0    | 6   | K371  | N-terminus|
|           | KGQSTSR        | 374  | 380 | {MSELEK}          | 0    | 6   | T378  | N-terminus|
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