Supporting Information

Sortase-Mediated Multi-Fragment Assemblies by Ligation Site Switching

J. Bierlmeier, M. Álvaro-Benito, M. Scheffler, K. Sturm, L. Rehkopf, C. Freund, D. Schwarzer*
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Experimental section

General methods

Standard amino acid (AA) derivatives for solid-phase peptide synthesis and coupling reagent HBTU were purchased from GLS (Shanghai, China). Fmoc-Cys(StBu)-OH and Fmoc-Adoa-OH were purchased from Bachem (Schwerte, Germany), and Fmoc-6-6Ahx-OH was obtained from Iris Biotech (Marktredwitz, Germany). Coupling reagents HATU and DIC were purchased from Merck Novabiochem (Darmstadt, Germany), and Tentagel RAM resin was purchased from Rapp Polymere (Tübingen, Germany). Other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), TCI (Eschborn, Germany), Bachem (Bubendorf, Switzerland) or Carbolution (St. Ingbert, Germany). Organic solvents were obtained from J. T. Baker (Deventer, Netherlands), VWR (Leuven, Belgium), Fisher Scientific (Loughborough, UK), Biosolve (Valkenswaard, Netherlands) and Th. Geyer (Renningen, Germany).

Peptide purifications were conducted on a Varian ProStar 210 HPLC system equipped with either a preparative Reprosil C18 column (5 μm, 100 Å, 20 x 250 mm, Dr. Maisch) and a flow rate of 13 mL/min in preparative scale or equipped with a Reprosil C18 column (5 μm, 100 Å, 10 x 250 mm, Dr. Maisch) at a flow rate of 4 mL/min in semipreparative scale. HPLC solvents A (0.1 % TFA in water) and B (80 % MeCN, 0.1 % TFA in water) were used for chromatography and samples were eluted with a gradient of 5 – 95 % solvent B over 50 min.

LC-ESI-MS analyses of peptides and ligation reactions were performed with an LC-MS2020 System (Shimadzu) equipped with a Kinetex C18 column (2.6 μm, 2.1 x 100 mm, phenomenex). HPLC solvents 0.1 % formic acid (FA) in water (solvent A) and 80 % ACN, 0.1 % FA in water (solvent B) were used as eluents with a gradient of 5 – 95 % solvent B over 10 min and a flow rate of 0.3 mL/min. The ESI-MS was operated in positive mode.
Solid-phase peptide synthesis (SPPS)

Peptides were synthesized by automated solid-phase peptide synthesis (SPPS) on a Syro I peptide synthesizer (MultiSynTech, Witten, Germany) applying the Fmoc / tBu strategy in 50 μmol scale on TentaGel HL RAM resin (capacity: 0.40 mmol/g). Amino acid side chains were protected as follows: Cys(Trt), Cys(StBu) Lys(Boc), Arg(Pbf), His(Trt), Trp(Boc), Thr(tBu), Ser(tBu), Tyr(tBu), Gln(Trt), Asn(Trt), Glu(OtBu).

Coupling reactions of standard amino acid building blocks (3 eq) were performed twice with coupling reagents DIC/Oxyma and HATU: DIC (3 eq) and Oxyma (3eq) for 30min, followed by a short washing step and coupling with HATU (N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide) (2.7 eq) as activator and NMM (N-methylmorpholine) as base for 40 min. The Fmoc group was removed by piperidine (20 % in DMF, 3 × 10 min). The resin was washed with DMF (3 ×), DCM (3 ×), DMF (3 ×) between each step.

Peptides were cleaved off the resin with 10 ml cleavage solution (TFA : phenol : triisopropylsilane : water, 85:5:5:5) under agitation for 3 h. After concentration under reduced pressure, cleaved peptides were precipitated in cold Et₂O (40 mL), centrifuged (4000 g, 10 min, −4 °C), dissolved in 50 % MeCN in water (v/v), and lyophilized. Peptides were purified by preparative HPLC and analyzed by LC-MS (see Supporting Figures S1, S3a, S5 and S8). Amino acid sequences of all peptides and ligation products are listed in Table S1. The exact, average and observed masses of all peptides and ligation products are listed in Supporting Table S2.
Sortase-mediated ligation
Ligation reactions were conducted in sortase reaction buffer (150 mM NaCl, 50 mM Tris, 5 mM CaCl$_2$, pH 7.5) at 25 °C. Concentration of sorting motif containing peptides and nucleophile substrates were adjusted to 2 mM and 1 mM, respectively. The reactions were initiated by adding Sortase A (Δ59, 76 μM) and stopped by adding H$_2$SO$_4$ (25 mM) or TFA (5 % v/v) in water. Ligation reactions without Cys(StBu) containing substrates were further supplied with 10 mM DTT. Progression of ligation reactions were monitored by LC-MS analysis and the reaction times were adjusted accordingly and varied between 1 and 96h (see below). The amounts of starting materials, isolated ligation products and HPLC-purification steps required for purifying the ligation products are listed in Supporting Table S3.

Duration of Ligation reactions

| Ligation product: | Total Ligation time: |
|------------------|---------------------|
| LP1              | 23 h                |
| LP3              | 17 h                |
| LP5              | 1 h                 |
| LP6              | 43.5 h              |
| LP7              | 43.5 h              |
| LP8              | 16.3 h              |
| LP9              | 96 h*               |
| LP10             | 16.3 h              |
| LP11             | 16.3 h              |
| LP12             | 42 h                |
| LP13             | 4.8 h*              |
| LP14             | 24 h                |

* A second aliquot of sortase A was added after the first half of the total ligation time.
Reduction of Cys(StBu)
The disulfide bond in Cys(StBu) containing peptides were reduced by treating the peptides with 10% (v/v) 2-mercaptoethanol in sortase reaction buffer for 1.5 – 2.5 h. Peptides were purified by preparative or semipreparative HPLC after completion of the reduction reaction.

Desulfurization of Cys(StBu) moieties to Ala
Alternatively, Cys(StBu) containing peptides were subjected to desulfurization reactions yielding an Ala moiety. To this end, 10 µL of a 5 mM Cysteine (side chain protected by tBu-SH or unprotected) containing peptide (solved in 20 % (v/v) ACN) was mixed with 10 µL 0.5 M TCEP/TEA, 0.5 µL 0.1 M VA-044 and 1 µL tert-Butylthiol. This mixture was incubated for 2 h followed by further addition of 0.5 µL 0.1 M VA-044. Reaction progress was monitored via LC-MS and stopped by adding 10 % TFA and subsequent HPLC purification.

Nvoc Deprotection
Nvoc containing peptides (5 mM) were dissolved in 25 mM H_2SO_4 in water and irradiated at 365 nm with a Blak-Ray® B-100AP UVP lamp (Ultra-Violet Products Ltd, Cambridge, UK). The reaction was monitored by LC-MS until starting materials were consumed and subsequently purified by HPLC. The duration of uncaging reaction varied between 60 and 150 minutes.
Recombinant expression of GST-BPTF and sortase A

Rosetta™(DE3)pLysS was transformed with plasmids encoding GST fusion of BPTF PHD and Bromodomain or Sortase A (59 - 206) of S. aureus. Cells were incubated overnight in 15 ml of LB media, containing ampicillin (100 mg/L) at 37 °C. Then 700 ml of fresh LB media with ampicillin (100 mg/L) were inoculated with 7 ml of overnight cultures and incubated at 37 °C until an optical density at 600 nm (OD$_{600}$) of 0.6 to 0.8 was reached. Media for GST-BPTF expression were further supplemented with 0.1 mM ZnCl$_2$. Expression was induced by adding 0.4 mM Isopropylthiogalactosid (IPTG) and cultured overnight at 20 °C. Cells were harvested by centrifugation (4000 g, 4 °C), pellets were suspended in a minimum of the lysis buffer (20 mM Tris, 0.1 % Triton X-100, pH 7.9). Cells were lysed with Emulsi Flex-C5 (max 1000 bar, 30 min, 4 °C) and the lysate was cleared by centrifugation (20,000 g, 30 min, 4 °C) followed by purification via IMAC or GSH-affinity chromatography.

IMAC purification

The cleared supernatant was incubated (rolling, 60 min, 4 °C) with Ni-NTA agarose beads (3 mL). The beads were washed with 40 mL of lysis buffer, followed by washing with 30 mL of IMAC wash buffer (20 mM Tris, 0.1 % Triton X-100, 30 mM imidazole, pH 7.9). Resin-bound proteins were eluted with 15 mL of IMAC elution buffer (20 mM Tris, 0.1 % Triton X-100, 300 mM imidazole, pH 7.9), fractions of 1 mL were collected and dialysed against pull-down buffer (20 mM HEPES, 100mM KCl, 20 % Glycerol, pH 7.9). The concentration of the fractions was determined by UV absorption on a nanodrop ($\varepsilon = 17,545$/mol/cm, $\text{MW} = 14.8$ kDa). Purity of samples was assessed by coomassie stained SDS-PAGE.

GSH-affinity chromatography

The cleared supernatant was incubated (rolling, 60-120 min, 4 °C) with 1.5 ml glutathione sepharose. The resin was washed with 45 ml GSH-binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH$_2$PO$_4$, pH 7.3), followed by elution of bound proteins with 15 ml of GSH-elution buffer (50 mM Tris, 10 mM reduced Glutathione, pH 8.0). Eluted fractions were further processed as described above.
**Gel Filtration**

Affinity purified proteins were subjected to a second purification step by gelfiltration on a Superdex75, HiLoad16/60, prep grade coloumn (GE Healthcare) using sortase reaction buffer. Purity of fractions was assessed by SDS-PAGE and pure fractions were pooled, concentrated and stored at -20 °C until usage.

**Immobilobilization of dual histone tail peptides**

The peptides were coupled to agarose matrix (*SulfoLink coupling resin*, Thermo Fisher Scientific) by incubating 300 µL of a 1 mM peptide solution in coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5) with 300 µL of resin suspension for 1h. The supernatant was discarded and the resin was subsequently treated with 50 mM 2-mercaptoethanol in coupling buffer in order to block excess binding sites of the matrix. Afterwards the resin was stored in ACN / H₂O (1 : 1) at -20 °C.

**Pull-Down reactions with PHD and Bromodomain of BPTF**

The resin-bound peptide probes were transferred into micro centrifuge filter units (Ultrafree-MC, Merck Millipore, Darmstadt, Germany) and washed three times with 200 µL pulldown buffer (20 mM HEPES, 100 mM KCl, 20 % glycerol, pH 7.9). The beads were incubated with 200 µL 500 nM GST-BPTF supplemented with 10 g/L BSA for 1 h at RT. Afterwards the beads were washed six times each with 500 µL pull-down washbuffer (20 mM HEPES, 300 mM KCl, 20 % glycerol, pH 7.9). The proteins were eluted by adding 20 µL 3-fold SDS sample buffer (150 mM Tris, 6 % SDS (w/v), 30 % Glycerin (v/v), 0.003 % bromophenoleblue (w/v), 300 mM DTT, pH 6.8) and heating for 10 min at 95 °C. After centrifugation the eluate was subjected to SDS-PAGE (Supporting Figure S16).
Immunological Assays

Cellular experiments: The K562 cell line expressing HLA-DR1 (DRA*0101-DRB1*0101), the HA1.7 and RD4 hybridomas were cultured in complete medium (RPMI supplemented with 10 % FCS, 5mM L-Gln, and 100 U/ml of penicilin and streptomycin) at 37 °C in the presence of 5 % CO2. For antigen binding and antigen presentation assays the FCS concentration was reduced to 2 %. For antigen binding assays 5x10^5 K562-DR1 cells were seeded on 48-well plates and incubated 16 h with the corresponding peptides. Serial dilutions of the antigens were prepared from a stock solution (10x the highest concentration in 100% DMSO). All subsequent dilutions were prepared in 10 % DMSO and a fixed volume of 1ul of the corresponding dilution was applied. After 16 h cells were washed, stained with L243-Alexa647 and measured by flow cytometry using a BD-CantoII flow cytometer. The generated FCS files were analyzed using FCS Express6. Antigen presentation assays were performed by detecting the secretion of IL-2 by the HA1.7 or RD4 hybridoma upon activation. Using 96-well plates, 10^5 cells of each hybridoma were cultured in the presence of 10^5 K562-DR1 cells for 36 h with titrated concentrations of antigenic peptides. After this time the cells were pelleted, supernatants collected and m-IL-2 measured by ELISA (Abcam). Control wells with the corresponding amount of DMSO but no antigenic peptide added were used. Additional experiments included the incubation of the two stated hybridomas with peptides in the absence of APCs. In this case, titrated concentrations of antigens were incubated under the same culture conditions as described above for a period of 54h.
Supporting Information

Supporting Information Figures

Supporting Information Figure S1: Peptide substrates of \textit{P4} characterization assay

\textbf{Supporting Figure S1:} LC-MS characterization of synthetic peptides used in \textit{P4} characterization assay. Detailed amino acid sequences and masses of used peptides are listed in supporting tables S1 and S2.
Supporting Information

Supporting Information Figure S2: four-fragment ligation scheme

Supporting Figure S2: Detailed ligation scheme of four-fragment assembly.
Supporting Information Figure S3: four-fragment ligation: 1st ligation step

Supporting Figure S3: LC-MS characterization of peptides and ligation products of the first ligation step and ligation site switching in the four-fragment assembly. a) Central fragment pep10 used in four-fragment assembly. The N- and C-terminal fragments correspond to peptides pep1 and pep9 (Fig. S1), respectively. b) Ligation reaction of pep9 and pep10. c) Switching of nucleophile activity by uncageing of Nvoc in LP1. The chromatogram was recorded after 1h of irradiation. d) Radical Desulfurization of LP1a in presence of reducing agent TCEP and radical inducing VA-044. The chromatogram was recorded after 200 min of reaction time. e) Isolated ligation product LP2 with activity switched nucleophile and sorting motif. Sections of chromatograms (b) and (e) are also shown in Figure 2.
Supporting Information Figure S4: four-fragment ligation: 2\textsuperscript{nd} & 3\textsuperscript{rd} ligation steps

Supporting Figure S4: LC-MS characterization of ligation products of the second and third ligation steps and ligation site switching in the four-fragment assembly. a) Ligation reaction of LP2 and pep10. b) Uncaged ligation product LP3a. The chromatogram was recorded after 150 min of reaction time. c) Isolated ligation product LP4, d) ligation reaction of LP4 and pep1 after 1h reaction time and e) Isolated ligation product LP5. Sections of chromatograms (a), (c), (d) and (e) are also shown in Figure 2. #: sortase A.
Supporting Information Figure S5: LC-MS characterization of peptides used in the assembly of dual-histone-tail probes. a) Peptides derived from the N-terminal tail of histone H4. b) Templating peptide pep13. c) Histone H3 tail derived peptides without and with a trimethylation mark at Lysine-4. Detailed amino acid sequences and masses of used peptides are listed in supporting tables S1 and S2.
Supporting Information

Supporting Figure S6: Assembly of dual-histone-tail probes with penta-acetylated H4 tails. a) Detailed ligation scheme of assembly reactions. b) LC and LC-MS analysis of ligation reactions and isolated ligation products. #: sortase A, * mass signals corresponding to unreacted pep11 with reduced StBu disulfide. The Nvoc group is marked with a red box, the reducable disulfide of the Cys(StBu) moiety is marked with a green box. LC tracs of ligation reactions are shown at 43h (pep11 + pep13) and 16h (LP6 + Pep14 and LP6 + Pep15). LC-MS analysis of isolated ligation products are shown in larger magnification in Figure S15a.
Supporting Figure S7: Assembly of dual-histone-tail probes with unmodified H4 tails. a) Detailed ligation scheme of assembly reactions. b) LC and LC-MS analysis of ligation reactions and isolated ligation products. #: sortase A. LC traces of ligation reactions are shown at 43h (pep12 + pep13) and 16h (LP7b + Pep14 and LP7b + Pep15). The Nvoc group is marked with a red box, the reducible disulfide of the Cys(StBu) moiety is marked with a green box. LC-MS analysis of isolated ligation products are shown in larger magnification in Figure S15b.
Supporting Information Figure S8: Dual-Histone-Tail Probe immobilization

Supporting Figure S8: Immobilization of dual-histone-tail probe peptides. This illustration shows the immobilization of LP8. Immobilization of LP9, LP10 and LP11 were conducted in the same way.
Supporting Information

Supporting Information Figure S9: Antigen starting materials

a) Antigen Peptide Monomer (pep16)  

b) Antigen central fragment (pep17)  

c) Antigen C-terminal fragment (pep18)  

d) Antigen N-terminal fragment (pep19)  

Supporting Figure S9: LC-MS characterization of peptides used in the assembly of dimeric and trimeric antigens. a) Antigen monomer. b) Central fragment used for the assembly of the trimeric antigen. c) C-terminal fragment for antigen dimer and trimer assembly. d) N-terminal fragment for antigen dimer and trimer assembly.
Supporting Information Figure S10: Ligation of antigen trimer

Supporting Figure S10: Assembly of trimeric antigen construct. The top rows illustrate the ligation scheme. The bottom row shows HPLC-traces and mass spectra of the ligation reactions and ligation products. LC-MS analysis of the isolated ligation product LP13 is shown in larger magnification in Figure S15c. All peptide building blocks were optimized for subsequent purification of ligation products by HPLC.
Supporting Information Figure S11: Ligation of antigen dimer

**Legend**
- **antigen**: -PKYVKQNTKLAT-
- **Adoa-Adoa-G-**
- **GG-Adoa-Ahx-Adoa-**
- **biotin-Adoa-Ahx-Adoa-**
- **5(6)-Carboxyfluorescein**

**Adoa**: 8-Amino-3,4Dioxoacatanoic acid
**Ahx**: Aminoheptanoic acid

Supporting Figure S11: Assembly of dimeric antigen construct. The top row illustrates the ligation scheme. The bottom row shows HPLC-traces and mass spectra of the ligation reactions and ligation products. LC-MS analysis of the isolated ligation product LP14 is shown in larger magnification in Figure S15c.
Supporting Information Figure S12: Antigenic peptide binding to cellular MHCII. Flow cytometry measurement of monomeric, dimeric and trimeric HA constructs at different concentrations to HLA-DRB1*01:01 expressing cell lines (K562). The measurement shows the ratio between the geometric mean fluorescence intensity of the peptide bound in each condition (FITC) relative to the HLA-DR (Alexa 647) expression levels. The bar chart represents the mean of n = 5 measurements and the error bars show the SD.
Supporting Figure S13: Scheme of TCR Stimulation Assay

**Supporting Figure S13:** Scheme of TCR stimulation assay. Antigen-presenting cells (APCs) used in this assay display MHCII complexes of the HLA-DRB1*01:01 (DR1) allotype on the cell surface. The antigen binding cleft accommodates antigen peptides derived from hemagglutinin (HA) covering amino acids 306-318. The APCs are supplied with antigen oligomers assembled by multi-fragment SML. The antigens are now presented on the DR1 MHCII complexes. Addition of hybridoma cells (HA1.7) expressing a TCR that recognizes the antigen presented on DR1 results in the formation of a ternary complex of TCR antigen peptide and DR1. The interaction of TCR with the DR1-HA complex induces results in activation of the TCR signaling pathway and release of Interleukin-2 (IL-2), which serves as assay readout.
Supporting Figure S14: All isolated ligation products

Supporting Figure S14: TCR stimulation assays. a) TCR expressing hybridoma cells are restricted to antigen-MHCII complexes as illustrated for HA1.7 cells, which are stimulated by APCs expressing DR1 in complex with HA antigen but not DR4 APCs with bound M. tuberculosis 85B (Mt85B) antigen. RD4 hybridoma T cells are restricted to DR4 APCs with bound Mt85B and are not stimulated by the DR1-HA complex. b) Activation profile of RD4 hybridoma (specific for DR4-Mt85B) determined by IL-2 secretion. K562-DR1 cells were incubated with the antigens, and then the hybridoma cells were added and co-cultured for approx. 36h. c) Activation profile of the two different hybridomas in the presence of different HA-constructs. The HA1.7 (DR1-HA restricted) and the RD4 (DR4-Mt85B) cells were cultured for 54h with the indicated amounts of antigen (HA-Monomer (Pep16), 2xHA-Dimer (LP14), 3xHA-Trimer (LP13), Monomer-Linker (Pep20) and Scrambled-HA (Pep21)) in the presence (filled symbols) or the absence (empty symbols) of K562-DR1 (APC). The T cell activation status was determined by measuring the IL-2 secreted into the cell culture medium after the indicated time via ELISA. (n=6 independent experiments).
Supporting Information Figure S15: All isolated ligation products

a) Isolated ligation products of divalent histone tail constructs with penta-acetylated H4

![LC-MS characterization of ligation products used in biochemical assays. a) Ligation products used for probing BPTF binding with divalent histone tail construct with penta-acetylated histone H4 tail. Sections of chromatograms and mass spectra are also shown in Figure S6. b) Ligation products used for probing BPTF binding with divalent histone tail construct with unmodified histone H4 tail. Sections of chromatograms and mass spectra are also shown in Figure S7. c) Ligation products of trimeric and dimeric antigen constructs. Sections of chromatograms and mass spectra of LP13 and LP14 are also shown in Figure S9 and S10, respectively.](image-url)
Supporting Figure S16: Triplicate of BPTF pulldown experiment with immobilized divalent antigen constructs LP8 (H3K4me3 & H4Kac5), LP9 (H3 & H4Kac5), LP10 (H3K4me3 & H4) and LP11 (H3 & H4). This figure shows the full-scale SDS-PAGE of the pull-down experiments stained with coomassie brilliant blue. The pull-down experiments are shown on the left side of the respective SDS-PAGE and the right sites shows the supernatant (1.5 µL) of the pull-down experiments. SDS-PAGE: 12 %, GST-BPTF 0.5 µM, BSA 10 mg/mL ft: flow through/ supernatant. A section of the SDS-PAGE of the top panel is shown in Figure 2.
Supporting Information Tables

**Supporting Information Table S1: Amino acid sequences of peptides and ligation products**

Table S1: Amino acid sequences of peptides and ligation products. Adoa: 8-Amino-3,4 Dioxoacatanoic acid, Ahx: 5(6)-Carboxyfluorescein, Bio: biotin, dBio: desthiobiotin, Dns: Dansyl; Nvc: Nvoc Photoprotection group, Nle: L-norleucine, Hle: L-homoleucine, AMT: L-2-amino-6-methylheptanoic, L-Neo: L-α-Neopentylglycine

| peptide   | amino acid sequence                                                                 |
|-----------|------------------------------------------------------------------------------------|
| Pep01     | Dns-GG-L-PKTGGRR-NH₂                                                              |
| Pep02     | Dns-GG-I-PKTGGRR-NH₂                                                              |
| Pep03     | Dns-GG-Nle-PKTGGRR-NH₂                                                             |
| Pep04     | Dns-GG-Hle-PKTGGRR-NH₂                                                             |
| Pep05     | Dns-GG-AMH-PKTGGRR-NH₂                                                             |
| Pep06     | Dns-GG-Neo-PKTGGRR-NH₂                                                             |
| Pep07     | Dns-GG-C(StBu)-PKTGGRR-NH₂                                                         |
| Pep08     | Dns-GG-C-PKTGGRR-NH₂                                                               |
| Pep09     | H₂N-GGGWW-NH₂                                                                      |
| Pep10     | Nvoc-GGSpKTGGRR-NH₂                                                                |
| Pep11     | Ac-SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLsPKTGGRR-NH₂                         |
| Pep12     | Ac-SGRGKGKKGLGGKGGAKRHRKVL-Cys(StBu)-PKTGGRR-NH₂                                   |
| Pep13     | H₂N-GGGK-[Nvoc-GGSGGEGGSGEGGSGEGSGE-K(dBio)]-NH₂                                  |
| Pep14     | H₂N-ARTK(me3)QTARKSTGGKAPRKQLATLPRTGGRR-NH₂                                      |
| Pep15     | H₂N-ARTKQTARKSTGGKAPRKQLATLPRTGGRR-NH₂                                            |
| Pep16     | Bio-GG-Adoa-Ahx-Adoa-PKVQNTTLKLAT-Adoa-Adoa-GK(FI)-NH₂                           |
| Pep17     | Nvoc-GG-Adoa-LLL-Adoa-PKYVKQNTTLKLAT-Adoa-Adoa-G-Cys(StBu)-PKTGGGRRR-NH₂         |
| Pep18     | GG-Ahx-Adoa-Adoa-PKYVKQNTTLKLAT-Adoa-Adoa-GK(FI)-NH₂                             |
| Pep19     | Bio-Ahx-Adoa-Adoa-PKYVKQNTTLKLAT-Adoa-LPKTGGFgw-NH₂                              |
| Pep20     | Fl-Ahx-Adoa-Adoa-PKYVKQNTTLKLAT-Adoa-LPKTGG-Ahx-Adoa-NH₂                         |
| Pep21     | Fl-Ahx-Adoa-Adoa-KLAPLTGYVTQN-Adoa-LPKTGG-Ahx-Adoa-NH₂                           |
| LP1       | Nvoc-GG-C(StBu)-PKTGGGWW-NH₂                                                       |
| LP2       | H₂N-GGAPKTGGGWW-NH₂                                                                |
| LP3       | Nvoc-GG-C(StBu)-PKTGGAPKTGGGWW-NH₂                                                |
| LP4       | H₂N-GGCPKTGGAPKTGGGWW-NH₂                                                          |
| LP5       | Dns-GGPLKTGGCPKTGGAPKTGGGWW-NH₂                                                    |
| LP6       | Ac-SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLsPKTGGGK-[Nvoc-GGSGGEGGSGEGGSGEGSGE-K(dBio)]-NH₂ |
| LP6b      | Ac-SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLCPKTGGGK-[GGSGGEGGSGEGGSGEGSGE-K(dBio)]-NH₂ |
| LP8       | Ac-SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLCPKTGGGK-[ARTK(me3)QTARKSTGGKAPRKQLATLPRTGGGSGEGGSGEGGSGEGSGE-K(dBio)]-NH₂ |
| LP9       | Ac-SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLCPKTGGGK-[ARTKQTARKSTGGKAPRKQLATLPRTGGGSGEGGSGEGGSGEGSGE-K(dBio)]-NH₂ |
| Name   | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| LP7    | Ac-SGRGKGGKGLGKGGAKRHRKVLsPKTGGGK-[Nvoc-GGGSGE-SGEGSGEGSGEGSGEGSGE-K(dBio)]-NH$_2$ |
| LP7b   | Ac-SGRGKGGKGLGKGGAKRHRKVLCPKTGGGK-[GGGSGE-SGEGSGEGSGEGSGEGSGE-K(dBio)]-NH$_2$ |
| LP10   | Ac-SGRGKGKGGKGLGKGGAKRHRKVLCPKTGGGK-[ARTK(me3)QTARKSTGGKAPRKQLATLPR-TGGSGE-SGEGSGEGSGEGSGEGGE-K(dBio)]-NH$_2$ |
| LP11   | Ac-SGRGKGKGGKGLGKGGAKRHRKVLCPKTGGGK-[ARTKQTARKSTGGKAPRKQLATLPR-TGGGSE-SGEGSGEGSGEGSGEGSGE-K(dBio)]-NH$_2$ |
| LP12   | Nvoc-GG-Adoa-LLL-Adoa-PKYVKQNTLKLAT-Adoa-Adoa-GCys(StBu)PKT-GG-Adoa-Adeo-PKYVKQNTLKLAT-Adoa-Adoa-GK(Fl)-NH$_2$ |
| LP12b  | H$_2$N-GG-Adoa-LLL-Adoa-PKYVKQNTLKLAT-Adoa-Adoa-GCPKT-GG-Ahx-Adoa-Adeo-PKYVKQNTLKLAT-Adoa-Adoa-GK(Fl)-NH$_2$ |
| LP13   | Bio-Ahx-Adoa-Adeo-PKYVKQNTLKLAT-Adeo-LPKT-GG-Adoa-LLL-Adoa-PKYVKQNTLKLAT-Adeo-Adoa-GCPKT-GG-Ahx-Adoa-Adeo-PKYVKQNTLKLAT-Adeo-Adoa-GK(Fl)-NH$_2$ |
| LP14   | Bio-Ahx-Adoa-Adeo-PKYVKQNTLKLAT-Adeo-LPKTG-Ahx-Adoa-Adeo-PKYVKQNTLKLAT-Adeo-Adoa-GK(Fl)-NH$_2$ |
### Supporting Information Table S2: Masses of peptides and ligation products

Table S2: chemical sum formula, monoisotopic mass, molecular mass (average) and observed mass of all peptides and ligation products. A + in the chemical sum formula indicates the presence of a quaternary ammonium cation (Pep14, LP8 and LP10).

| Peptide | chemical sum formula | monoisotopic mass | average mass | observed mass | SD of observed mass |
|---------|----------------------|-------------------|--------------|---------------|---------------------|
| Pep01   | C53H87N19O13S        | 1229.6451         | 1230.4590    | 1230.00       | 0.0000              |
| Pep02   | C53H87N19O13S        | 1229.6451         | 1230.4590    | 1229.97       | 0.1528              |
| Pep03   | C53H87N19O13S        | 1229.6451         | 1230.4590    | 1229.73       | 0.1768              |
| Pep04   | C54H89N19O13S        | 1243.6608         | 1244.4860    | 1243.95       | 0.1500              |
| Pep05   | C55H91N19O13S        | 1257.6764         | 1258.5130    | 1258.03       | 0.0354              |
| Pep06   | C54H89N19O13S        | 1243.6608         | 1244.4860    | 1243.83       | 0.2517              |
| Pep07   | C54H89N19O13S3       | 1307.6049         | 1308.6060    | 1307.88       | 0.3014              |
| Pep08   | C50H81N19O13S2       | 1219.5703         | 1220.4380    | 1219.83       | 0.2517              |
| Pep09   | C28H32N8O5           | 560.2496          | 560.6150     | 560.15        | 0.0000              |
| Pep10   | C143H252N54O38S2     | 3397.8888         | 3400.0490    | 3399.21       | 0.2720              |
| Pep11   | C133H242N54O33S2     | 3187.8360         | 3189.8640    | 3188.49       | 0.7486              |
| Pep12   | C104H160N34O51       | 2701.0982         | 2702.6110    | 2701.64       | 0.0382              |
| Pep13   | C138H254N55O38+      | 3289.9628         | 3291.8965    | 3290.86       | 0.6571              |
| Pep14   | C135H247N55O38       | 3246.9086         | 3248.8080    | 3247.39       | 0.6380              |
| Pep15   | C142H219N31O43S      | 3078.5624         | 3080.5480    | 3080.51       | 0.7075              |
| Pep16   | C173H298N50O53S2     | 3988.1602         | 3990.7040    | 3990.28       | 0.3135              |
| Pep17   | C132H205N29O41       | 2852.4848         | 2854.2540    | 2853.70       | 0.0866              |
| Pep18   | C161H252N38O41S      | 3405.8523         | 3408.0720    | 3407.80       | 0.2828              |
| LP1     | C65H86N16O18S2       | 1430.5747         | 1431.6060    | 1431.45       | 0.0707              |
| LP2     | C50H69N15O12         | 1071.5250         | 1072.1950    | 1071.59       | 0.3041              |
| LP3     | C86H123N23O25S2      | 1941.8502         | 1943.1860    | 1943.08       | 0.2474              |
| LP4     | C72H106N22O19S       | 1614.7725         | 1615.8350    | 1615.27       | 0.5798              |
| LP5     | C97H149N29O26S       | 2401.1460         | 2402.7810    | 2402.94       | 0.5940              |
| LP6     | C231H379N77O85S2     | 5655.7143         | 5659.1470    | 5659.25       | 2.3014              |
| LP6b    | C217H362N76O79S      | 5328.6366         | 5331.7960    | 5331.03       | 0.4382              |
| LP8     | C339H583N120O113S+   | 8175.3277         | 8180.1795    | 8177.60       | 1.0330              |
| LP9     | C336H576N120O108S    | 8132.2735         | 8137.0910    | 8135.25       | 0.7250              |
| LP7     | C221H369N77O80S2     | 5445.6614         | 5448.9620    | 5448.93       | 1.1026              |
| LP7b    | C207H352N76O74S      | 5118.5838         | 5212.6110    | 5120.07       | 0.5775              |
| LP10    | C329H573N120O108S+   | 7965.2749         | 7969.9945    | 7966.55       | 1.0330              |
| LP11    | C326H566N120O108S    | 7922.2207         | 7926.9060    | 7924.76       | 0.9770              |
| LP12    | C281H455N63O88S2     | 6184.2507         | 6188.2040    | 6188.90       | 0.3162              |
| LP12b   | C267H438N62O82S      | 5857.1730         | 5860.8530    | 5850.91       | 0.7829              |
| LP13    | C391H649N91O117S2    | 8555.7073         | 8561.1330    | 8561.80       | 1.4533              |
| LP14    | C256H416N58O76S      | 5551.0191         | 5554.5340    | 5553.96       | 0.5481              |
Supporting Information Table S3: Amounts of Peptides and Isolated Ligation Products

*Table S3*: amounts of the used starting materials and amount of the isolated ligation products.

| Educt 1 | Educt 2 | Isolated Ligation Product after all switching reactions | Total number of HPLC purification steps |
|---------|---------|-------------------------------------------------------|----------------------------------------|
| **4 Fragment Ligation** | | | |
| Pep9: 9.2 mg | Pep10: 0.8 mg | LP2: 0.9 mg | 2 |
| LP2: 0.9 mg | Pep10: 1.55 mg | LP4: 0.5 mg | 2 |
| LP4: 0.5 mg | Pep1: 0.5 mg | LP5: 0.2 mg | 1 |
| **Divalent Histone Tails** | | | |
| Pep11: 19.1 mg | Pep13: 19.8 mg | LP6b: 10.2 mg | 2 |
| LP6b: 4.8 mg | Pep14: 4.4 mg | LP8: 2.5 mg | 1 |
| LP6b: 4.8 mg | Pep15: 4.4 mg | LP9: 3.3 mg | 1 |
| Pep12: 16.2 mg | Pep13: 19.8 mg | LP7b: 10.6 mg | 2 |
| LP7b: 4.7 mg | Pep14: 4.4 mg | LP10: 2.6 mg | 1 |
| LP7b: 4.7 mg | Pep15: 4.4 mg | LP11: 3.3 mg | 1 |
| **Antigen** | | | |
| Pep17: 16.0 mg | Pep18: 11.4 mg | LP12b: 4.9 mg | 2 |
| LP12b: 2.4 mg | Pep19: 2.0 mg | LP13: 1.5 mg | 2 |
| Pep17: 8.0 mg | Pep19: 10.2 mg | LP14: 4.2 mg | 1 |