Electronic Supplementary Material (ESI)

An investigation into the effect of ribosomal protein S15 phosphorylation to its intermolecular interactions by using phosphomimetic mutant

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Materials and Methods

Materials: All chemicals were purchased from Sigma-Aldrich, Thermo Fisher Scientific and Bio-Rad unless otherwise stated. Synthetic DNA fragments (gBlocks) were purchased from Integrated DNA Technologies. Restriction enzymes were from New England Biolabs. *Escherichia coli* competent cells were from Agilent. The cloned expression vectors pGEX-4T-1-S18 and pGEX-4T-1-S18-Mut were purchased from GenScript. S15-S18 hybrid peptides were purchased from ChinaPeptides (Wujiang, Suzhou, China). The expression vector pNH-TrxT was a gift from Opher Gileadi (Addgene plasmid #26106).[1,2] The expression vector pGEX-2T was a gift from Ries Langley (The University of Auckland).

Molecular cloning, protein expression and purification of S15 and S18: Polyhistidine-thioredoxin-tagged WT/T136D S15 and S18, and GST-tagged WT/Mut S18 were produced following a method described previously.[3] Briefly, synthetic gene fragment (gBlocks; Integrated DNA Technologies) encoding WT/T136D S15 and WT S18 (Figures S14, S15 and S16) were cloned into the expression vector pNH-TrxT following the ligation independent cloning protocol described previously.[1,2] Plasmids encoding WT/Mut S18 (pGEX-4T-1, Figures S16 and S17) were purchased from GenScript. Resulting plasmids were used to transform *E. coli* BL21 (DE3). For protein expression, bacterial cells were grown in 2-YT media at 37 ºC until the optical density at 600 nm wavelength (OD_{600nm}) reached 0.5. Gene expression was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration) at 18 ºC overnight. Cells were lysed in buffer A containing 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 2 mM 2-mercaptoethanol, 10% glycerol with the addition of Halt Protease Inhibitor Cocktail (EDTA-Free, Thermo Fisher Scientific). Purification of polyhistidine-thioredoxin-tagged proteins were carried out using His GraviTrap affinity chromatography column (GE Healthcare). Polyhistidine-thioredoxin-tagged proteins were eluted with buffer A supplemented with 500 mM imidazole. Purification of GST-tagged

proteins were carried out using GSTrap HP column (GE Healthcare). GST-tagged proteins were eluted with buffer supplemented with 20 mM glutathione. Proteins were concentrated and dialysed in buffer A.

Production and purification of GST: pGEX-2T was obtained from Dr Ries Langley of Faculty of Medical and Health Sciences, The University of Auckland. The plasmid allows the expression of GST protein along with a thrombin cleavage site extension. For protein production, the plasmid was first transformed into *E. coli* BL21 (DE3), which were then grown in 2-YT media at 37 ºC until it reached an OD_{600} of around 0.6 to 0.8. Gene expression was then induced by adding 0.1 mM IPTG (final concentration) and incubated overnight at 18 ºC. The harvested cells were resuspended in phosphate buffer saline (PBS, pH 7.4) containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 with the addition of 10 mM dithiothreitol (DTT), Halt Protease Inhibitor Cocktail (EDTA-Free, Thermo Fisher Scientific), DNAseI (RNAse free, Thermo Fisher Scientific) and $MgCl_2$. After the cells were lysed by sonication, the filtered cell lysate was purified using a GSTrap HP affinity column (GE Healthcare) connected to an ÄKTA START protein purifier system. The protein was eluted using a buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 10% glycerol, 10 mM DTT and 20 mM glutathione (reduced). Purified GST was then concentrated using Amicon Ultra-15 10K centrifugal filter (Merck Millipore, Auckland, New Zealand) and stored at −80 ºC until use.

Ni-NTA Pulldown binding assays: 10 µM of polyhistidine-thioredoxin-tagged S15 or T136D S15 were mixed with 20 µM of GST-tagged S18 or GST-tagged Mut S18 or GST in 500 µL total volume of buffer A containing 5 mM imidazole. The mixture was incubated at room temperature for 10 min and at 4 ºC for 1 hour with gentle shaking. The mixture was loaded into a HisGraviTrap affinity column previously equilibrated with buffer A. The columns were extensively washed with a variant of buffer A containing 50 mM imidazole. Proteins were

eluted with a variant of buffer A containing 500 mM imidazole. Protein-protein interactions were evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Production and purification of ^{15}N labelled S18 tail: A codon optimised synthetic gene fragment encoding S18 tail (Figure S18) was cloned into the expression vector pNH-TrxT following a method described previously.[1,2] For the production of ^{15}N labelled polyhistidine-thioredoxin-tagged S18 tail, *E. coli* BL21 (DE3) cells were grown at 37 ºC in M9 medium containing ^{15}N ammonium chloride. When the OD_{600nm} reached 0.5, gene expression was induced by adding 0.1 mM IPTG at 18 ºC overnight. Cell lysis and protein purification were performed as per ribosomal protein S15. For the proteolytic cleavage of polyhistidine-tagged thioredoxin, eluted protein was incubated with tobacco etch virus (TEV) protease at 4ºC overnight. The sample was heated at 90 ºC for 20 minutes and centrifuged at 8000 g for 30 minutes. The supernatant, containing only polyhistidine-thioredoxin tag and S18 tail was further purified using high pressure liquid chromatography (HPLC). Reversed phase-HPLC (RP-HPLC) was performed on a GE Pharmacia ÄKTA purifier 10 system using a Phenomenex Luna 5 μm C18 100 Å (250 mm × 10 mm) column using 0.1% TFA in water as solvent A and 0.1% TFA and 0.9% water in 99% acetonitrile as solvent B at a flow rate of 5 mL per minute. The sample was eluted using a linear gradient of 20-80% of solvent B over 48 min with UV detection at both 214 nm and 280 nm. Polyhistidine-thioredoxin tag and S18 tail samples were separated and collected. Samples were flash frozen with liquid nitrogen, lyophilised overnight, and resuspended in H_2O or stored at -80 ºC. SDS-PAGE analysis confirmed the separation of S18 tail from polyhistidine-tagged thioredoxin. Samples were further characterised using matrix assisted laser desorption/ionisation/time-of-flight (MALDI/TOF) mass spectrometry.

After HPLC, 0.6 μL of each peak or each fraction of interest was mixed with 0.6 μL of α-cyano-4-hydroxycinnamic acid (MALDI matrix) and spotted onto the target MALDI plate. The mixed solution was then left air-dry for co-crystallisation. Samples were assessed by MALDI-

TOF / TOF mass spectrometer (Bruker UltrafleXtreme). The target peptides were best detected using linear positive mode and data were processed using flexanalysis (Figure S19). α-Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma Aldrich and prepared as follows: 10 mg of CHCA was dissolved in 50% acetonitrile in Type 1 water with 0.1% trifluoroacetic acid (TFA).

Isothermal Titration Calorimetry (ITC): MicroCal VP-ITC was used. Sample cell (1.5 mL volume) contained 15 μM polyhistidine-thioredoxin-tagged S18. The syringe contained 200 μM polyhistidine-thioredoxin-tagged WT/T136D S15. The titration experiment was carried out at 10 °C with a reference power of 8 μcal/sec with a total of 19 injections of 15 μL each. The interaction was exothermic with the amount of heat change decreasing with increasing titrant volume. All the proteins were in buffer A containing 500 mM imidazole and dialysis was avoided to prevent protein precipitation or aggregation. The proteins were degassed at 10 °C for 10 mins using a degassing station before the titration experiment.

Nuclear Magnetic Resonance (NMR) methods: NMR experiments were conducted at 600 MHz using a Bruker Avance spectrometer equipped with an inverse TCI cryoprobe. 3 mm MATCH NMR tubes (Bruker) containing 160 μL final sample volume or conventional 5 mm NMR tubes containing 500 μL sample volume were used. Pulse tip-angle calibration using the single-pulse nutation method was undertaken for each sample (Bruker *pulsecal* routine).[4] All experiments were conducted at 298 K.

Mass Spectrometry Protein Sequence Analysis of Gel Bands: Samples were analysed as described previously.[5,6]

Supplementary Figures

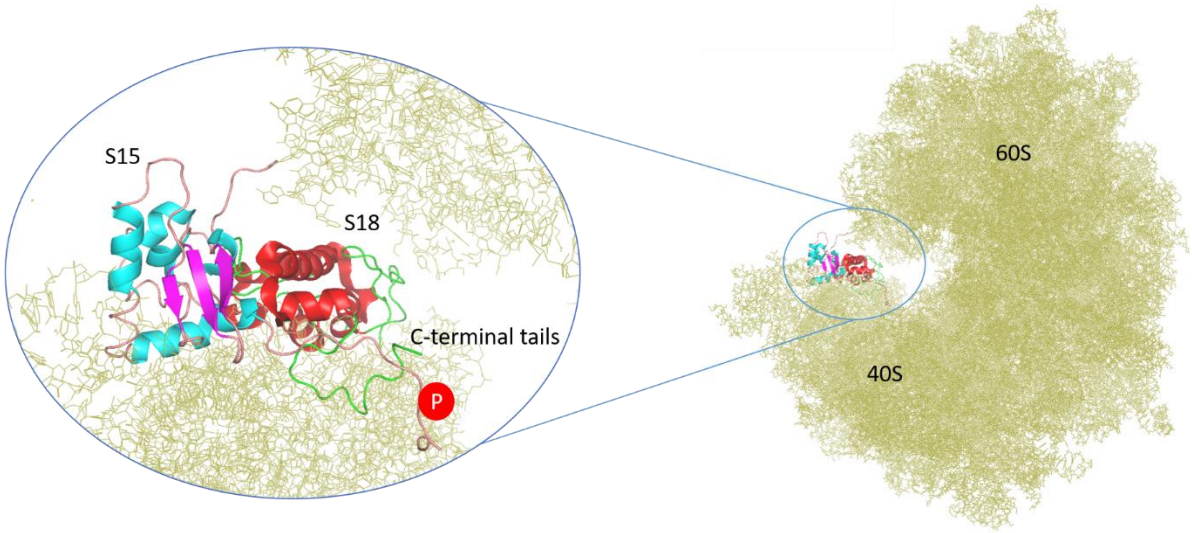

Figure S1: Cryo-EM structure of ribosomal protein S15 and S18 (extracted from PDB: 6EK0). The figure shows S15 α-helices (cyan), β-sheets (purple) and loops (pink), and S18 α-helices (red) and loops (green). The site of LRRK2-phosphorylation of S15 is indicated by a phosphate group (P) on S15 tail.

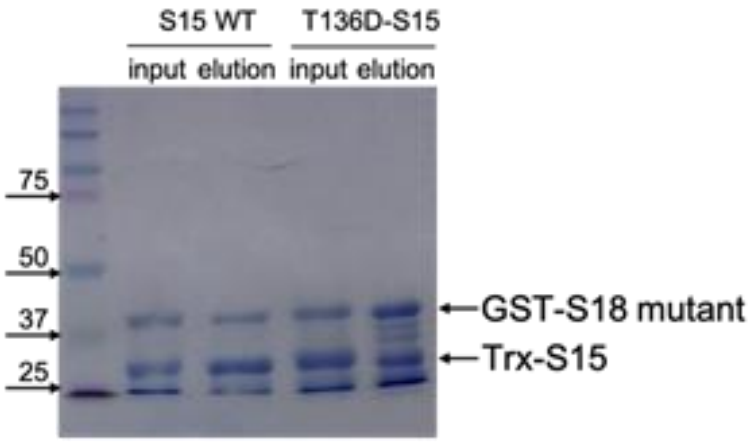

Figure S2: SDS-PAGE analysis of polyhistidine-tag affinity pulldown binding assay. The lanes that are shown as 'input' contain a mixture of bait proteins (N-terminal polyhistidine-thioredoxin-tagged WT or T136D S15) and prey proteins (N-terminal GST-tagged C-terminal deletion mutant of S18). Elution lanes show that the proteins co-eluted after the addition of elution buffer containing 500 mM imidazole. In order to aid visualisation, elution samples were concentrated before being loaded into the gel. Arrows on the right side of the gels indicate the expected positions of proteins: polyhistidine-thioredoxin-tagged S15 (~31 kDa) and GST-tagged C-terminal deletion mutant of S18 (~43 kDa).

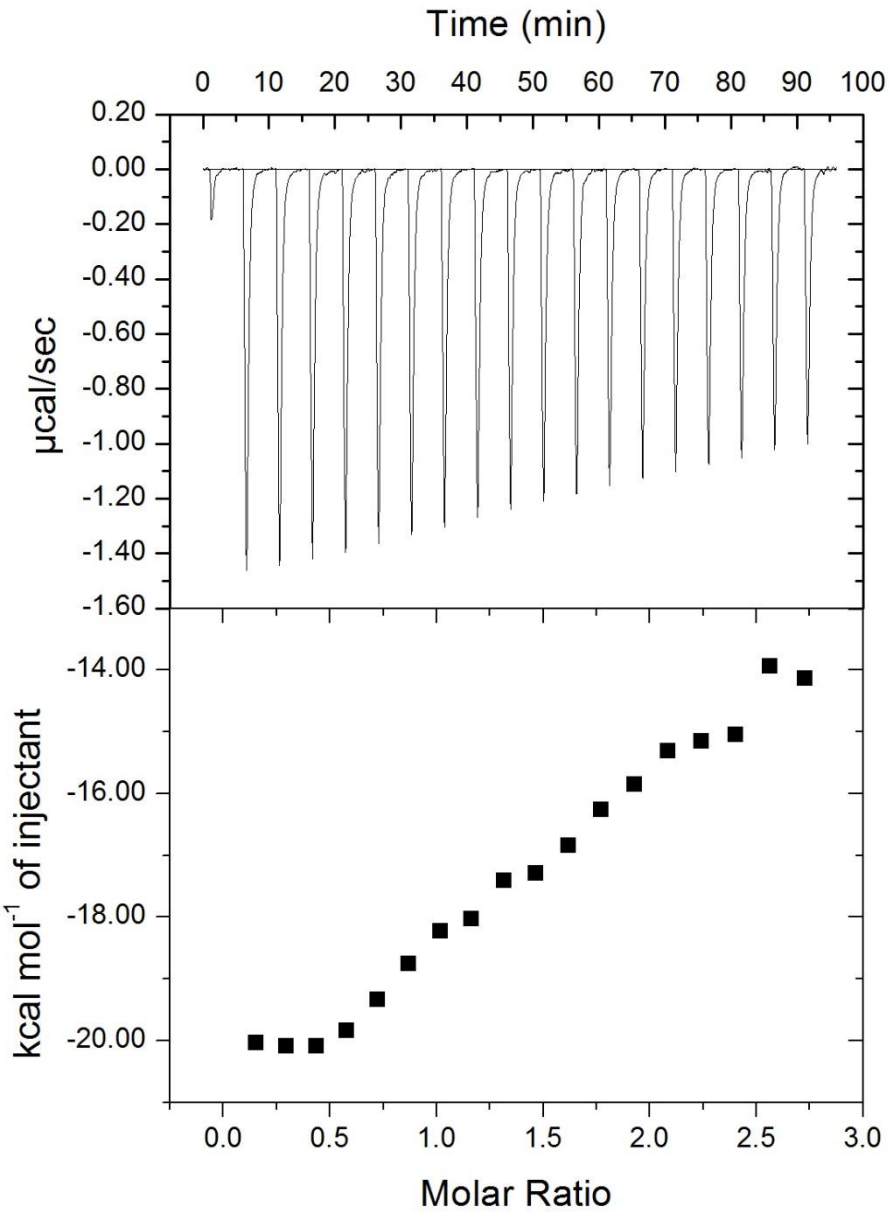

Figure S3: Titration of polyhistidine-thioredoxin-tagged WT S15 (200 µM stock solution; 15 µL per injection) to a sample cell containing 15 µM purified polyhistidine-thioredoxin-tagged WT S18 (1.5 mL sample cell volume).

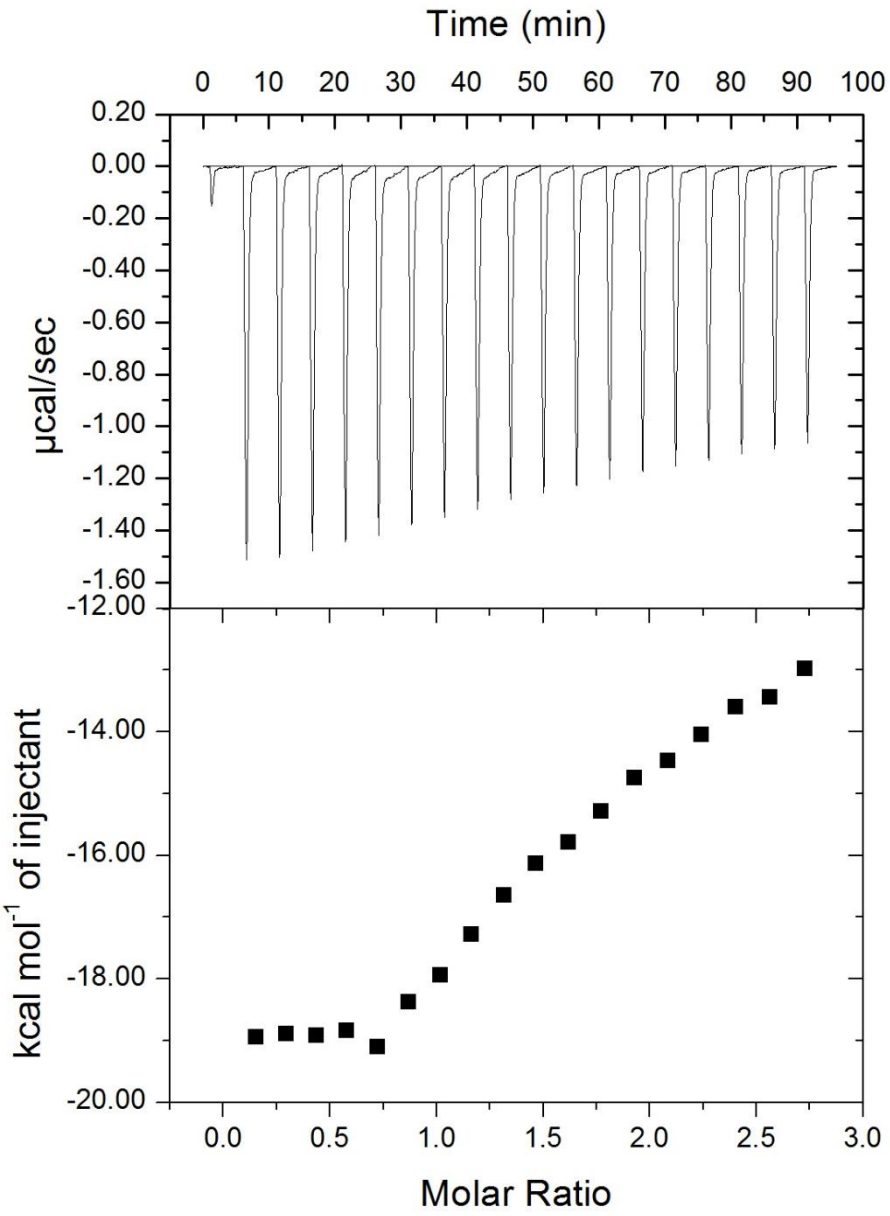

Figure S4: Titration of polyhistidine-thioredoxin-tagged T136D S15 (200 µM stock solution; 15 µL per injection) to a sample cell containing 15 µM purified polyhistidine-thioredoxin-tagged WT S18 (1.5 mL sample cell volume).

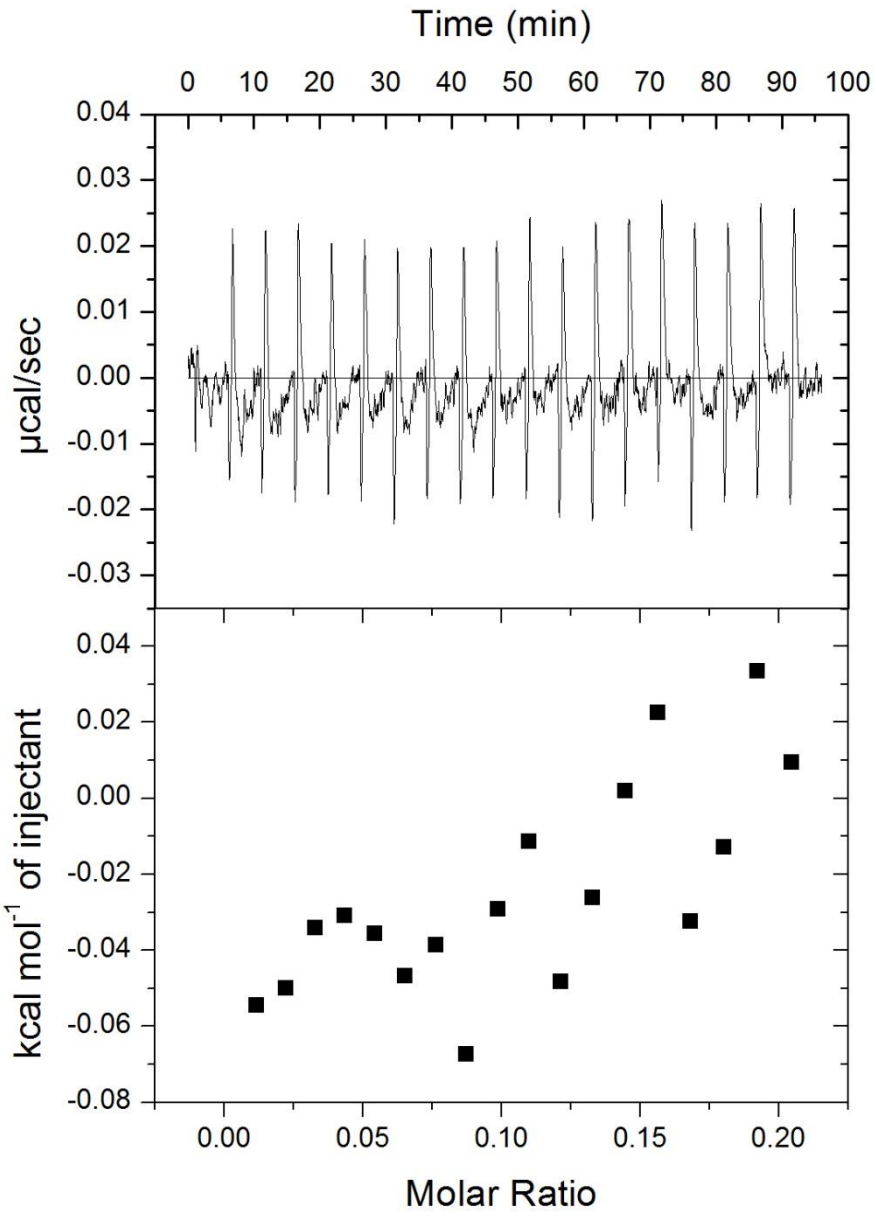

Figure S6: Titration of buffer A supplemented with 500 mM imidazole (15 µL per injection) to a sample cell containing buffer A supplemented with 500 mM imidazole (1.5 mL sample cell volume).

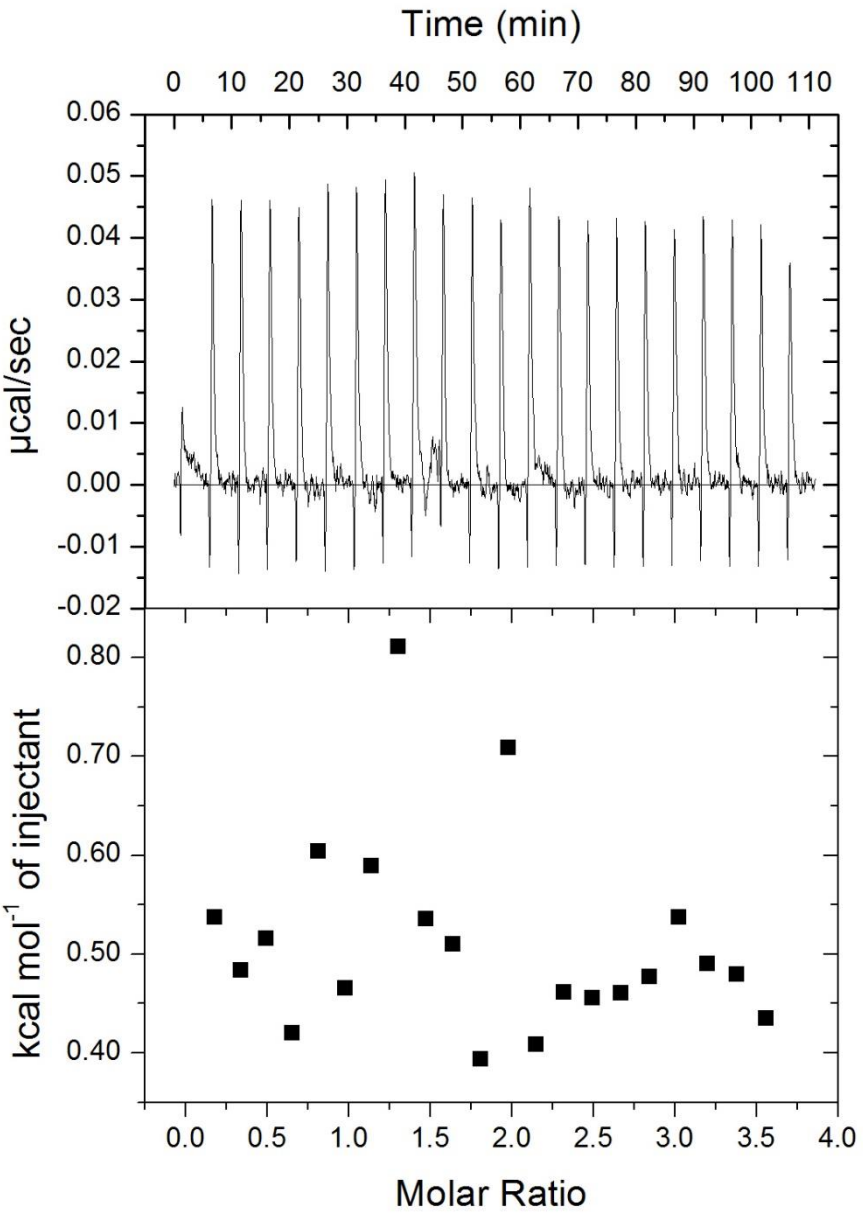

Figure S7: Titration of buffer A supplemented with 500 mM imidazole (15 µL per injection) to a sample cell containing 15 µM purified polyhistidine-thioredoxin-tagged WT S15 (1.5 mL sample cell volume).

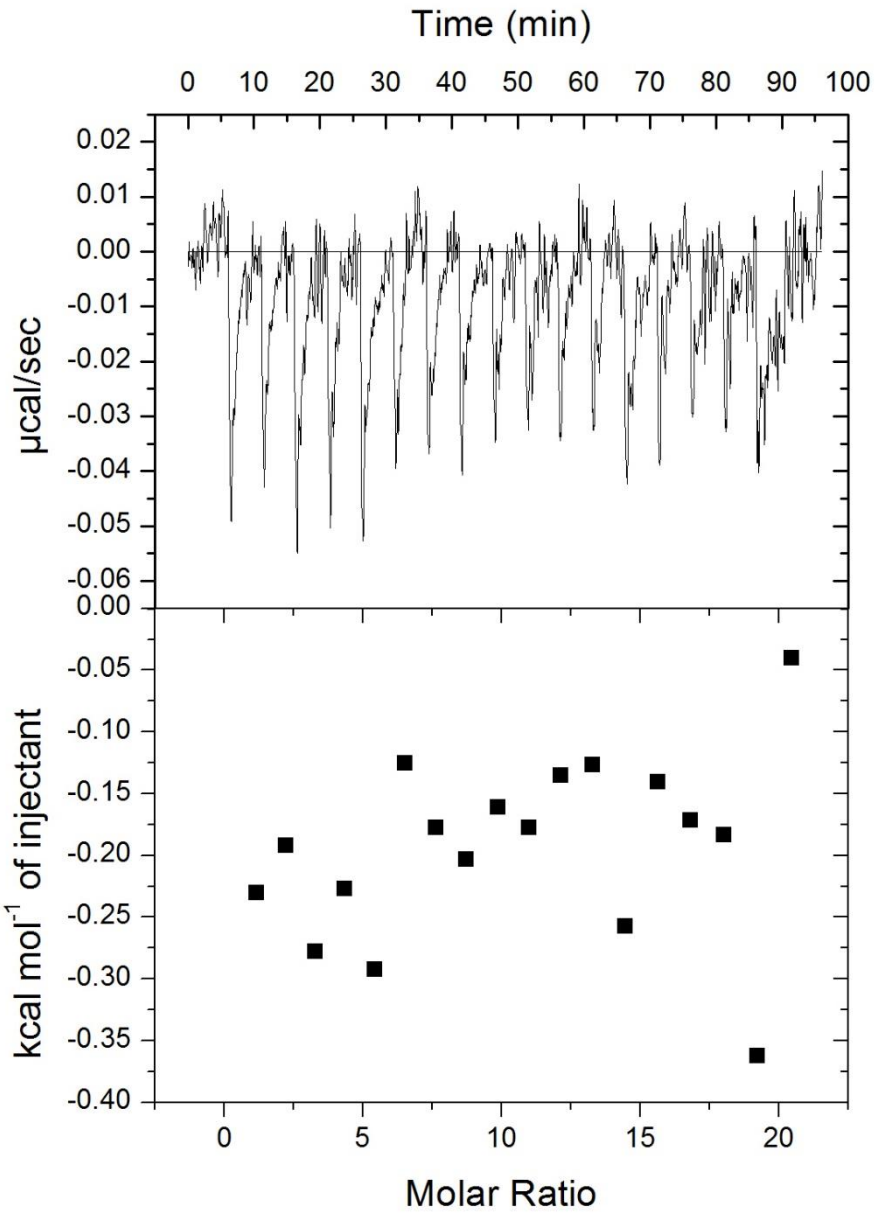

Figure S8: Titration of buffer A supplemented with 500 mM imidazole (15 µL per injection) to a sample cell containing 15 µM purified polyhistidine-thioredoxin-tagged WT S18 (1.5 mL sample cell volume).

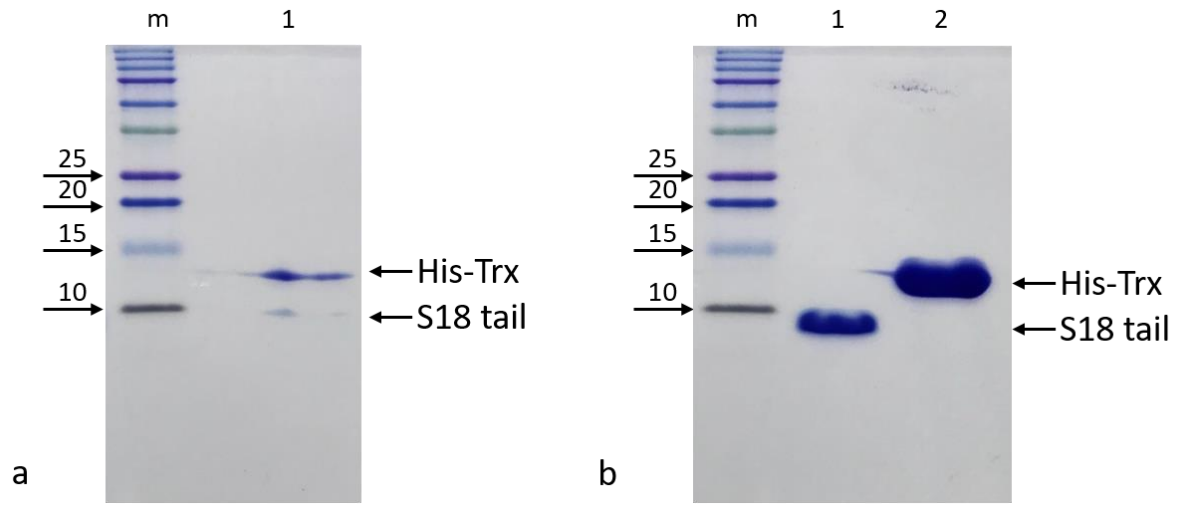

Figure S9: SDS-PAGE analysis of S18 tail RP-HPLC purification. The sequence of the S18 tail is SGLRHFWGLRVRGGQHTKTTGRRGRTVGVSKKK (the S in the N-terminal end is encoded by the plasmid), which was expressed in the pNH-TrxT vector that encodes a polyhistidine-thioredoxin tag and a TEV cleavage site to the N-terminal of the recombinant protein. (a) Gel showing the sample that was being injected into the HPLC for separation, including the polyhistidine-thioredoxin tag (13.9 kDa) and the cleaved S18 tail (3.7 kDa) (Lane 1); (b) Gel showing samples of the purified cleaved S18 tail (Lane 1) and polyhistidine-thioredoxin tag (Lane 2). Molecular weight protein marker (m) is indicated by arrows on the left side of the gels. Arrows on the right side of the gels indicate protein bands.

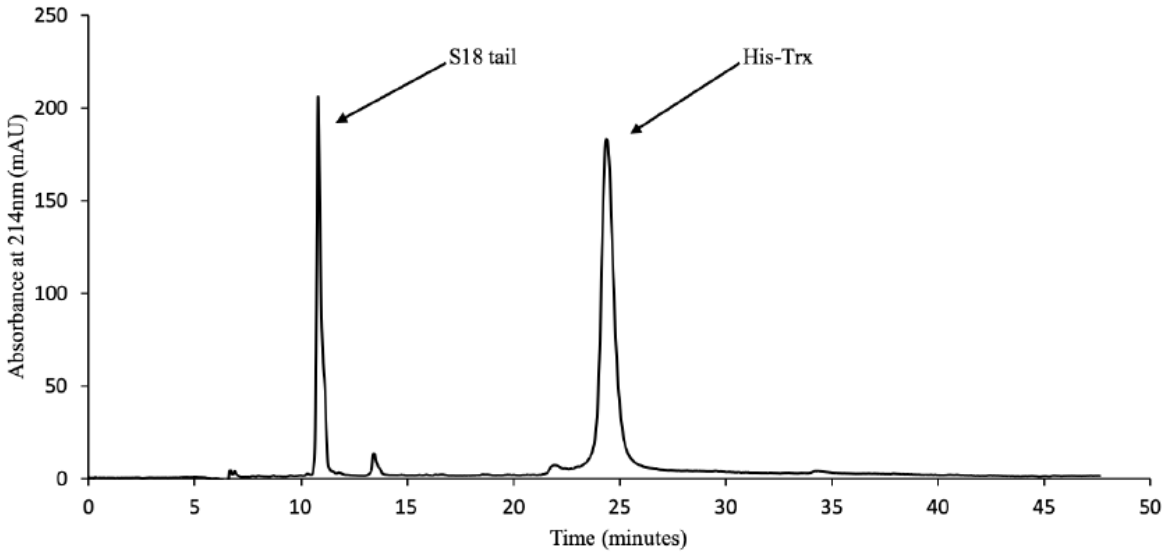

Figure S10: Reversed phase-high pressure liquid chromatography chromatogram (absorbance at 214 nm) of S18 tail peptide and the cleaved polyhistidine-thioredoxin tag (His-Trx).

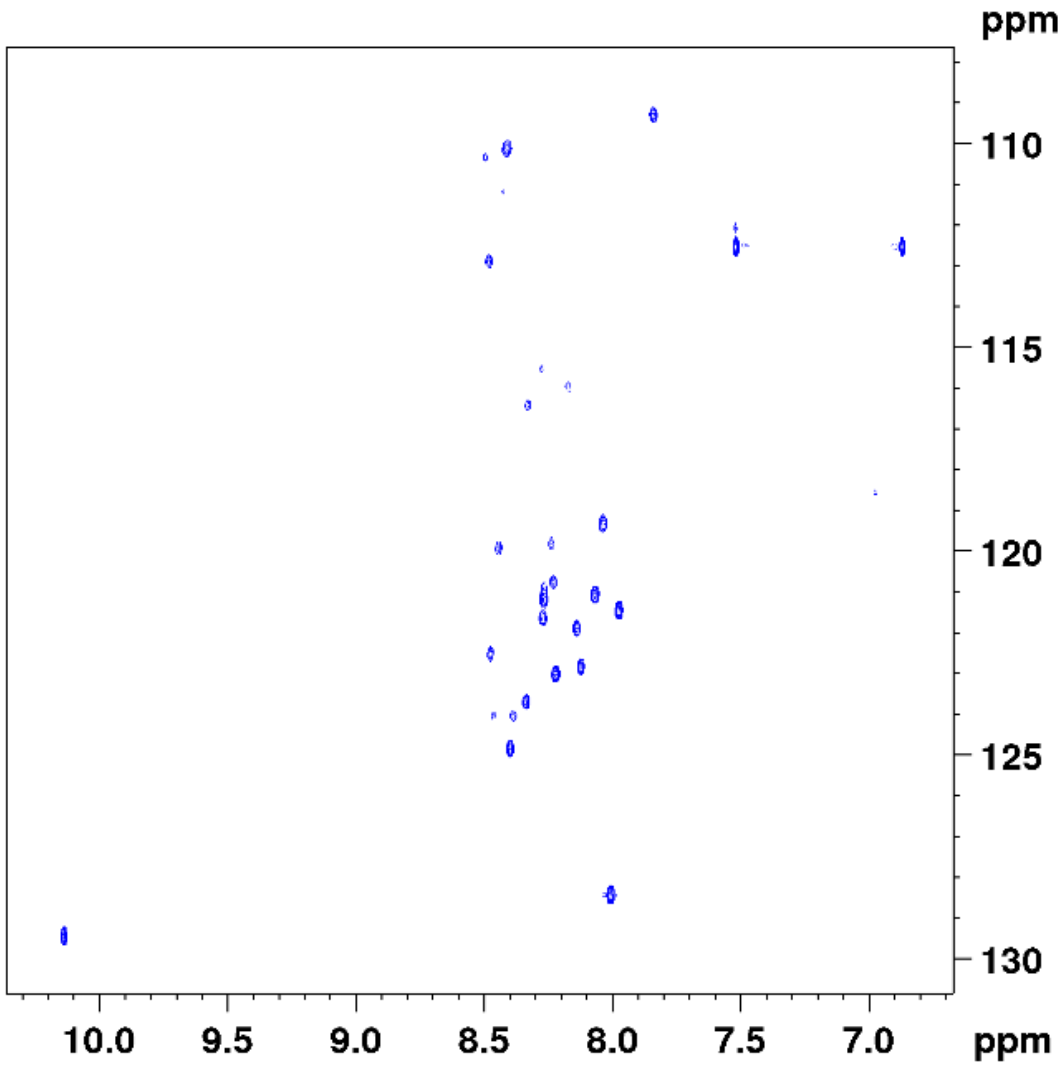

Figure S11: ^{1}H-^{15}N HSQC spectrum of the S18 tail peptide (sequence: SMGLRHFWGLRVRGGQHTKTTGRRGRTVGVSKKK, in which SM were encoded by the expression vector). Sample contained ~100 µM ^{15}N-labelled peptide, 50 mM Tris/Tris-D11 pH 6.6, 500 mM NaCl, 2 mM 2-mercaptoethanol, 5% glycerol in 10% D_2O and 90% H_2O. Sample volume was 160 µL and temperature was 298 K.

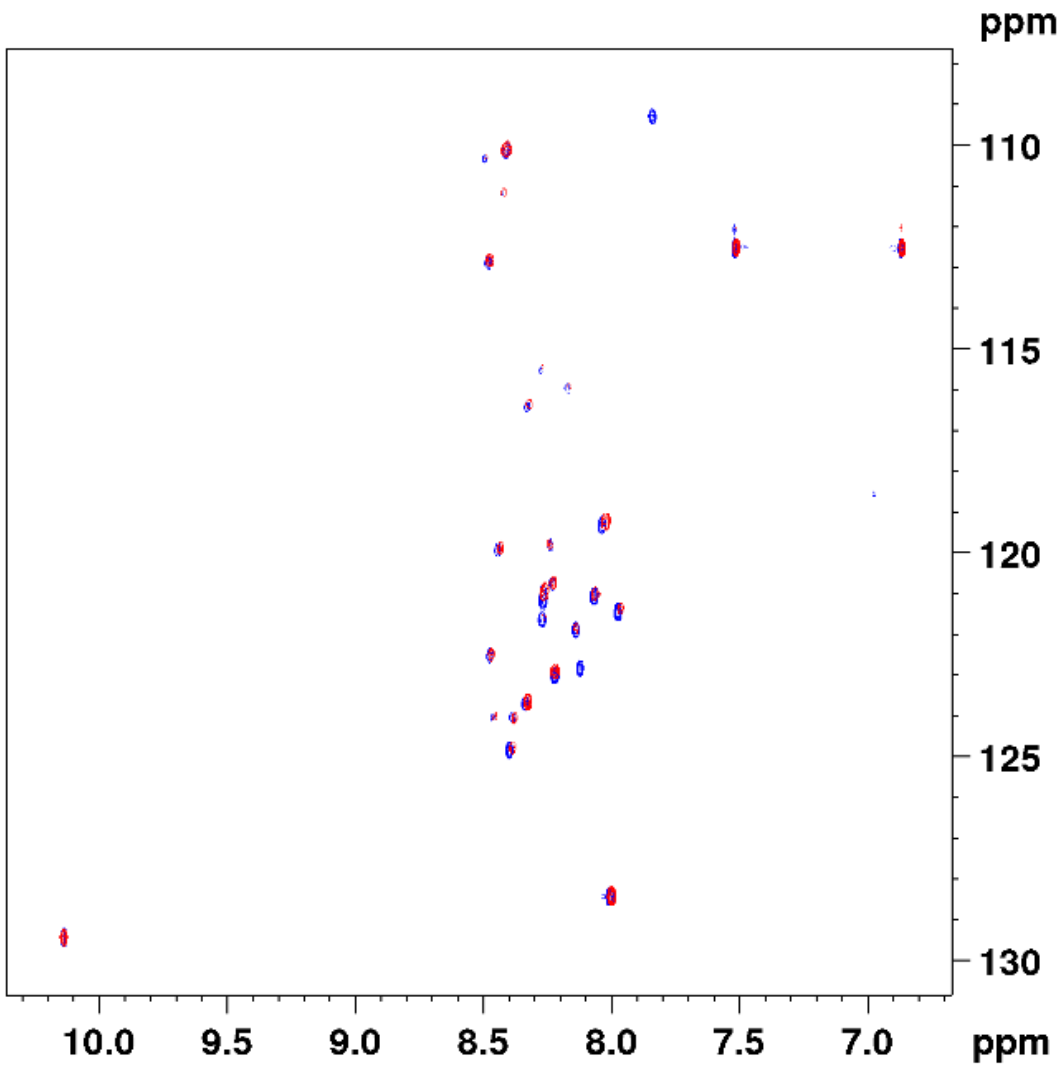

Figure S12: ^{1}H-^{15}N HSQC spectrum of the S18 tail peptide (sequence: SMGLRHFWGLRVRGGQHTKTTGRRGRTVGVSKKK, in which SM were encoded by the expression vector) in the absence (blue) and presence (red) of unlabelled N-terminal polyhistidine-thioredoxin-tagged WT S15. Sample contained ~100 μM ^{15}N-labelled peptide, ~200 μM unlabelled S15, 50 mM Tris/Tris-D11 pH 6.6, 500 mM NaCl, 2 mM 2-mercaptoethanol, 5% glycerol in 10% D_2O and 90% H_2O. Sample volume was 160 μL and temperature was 298 K.

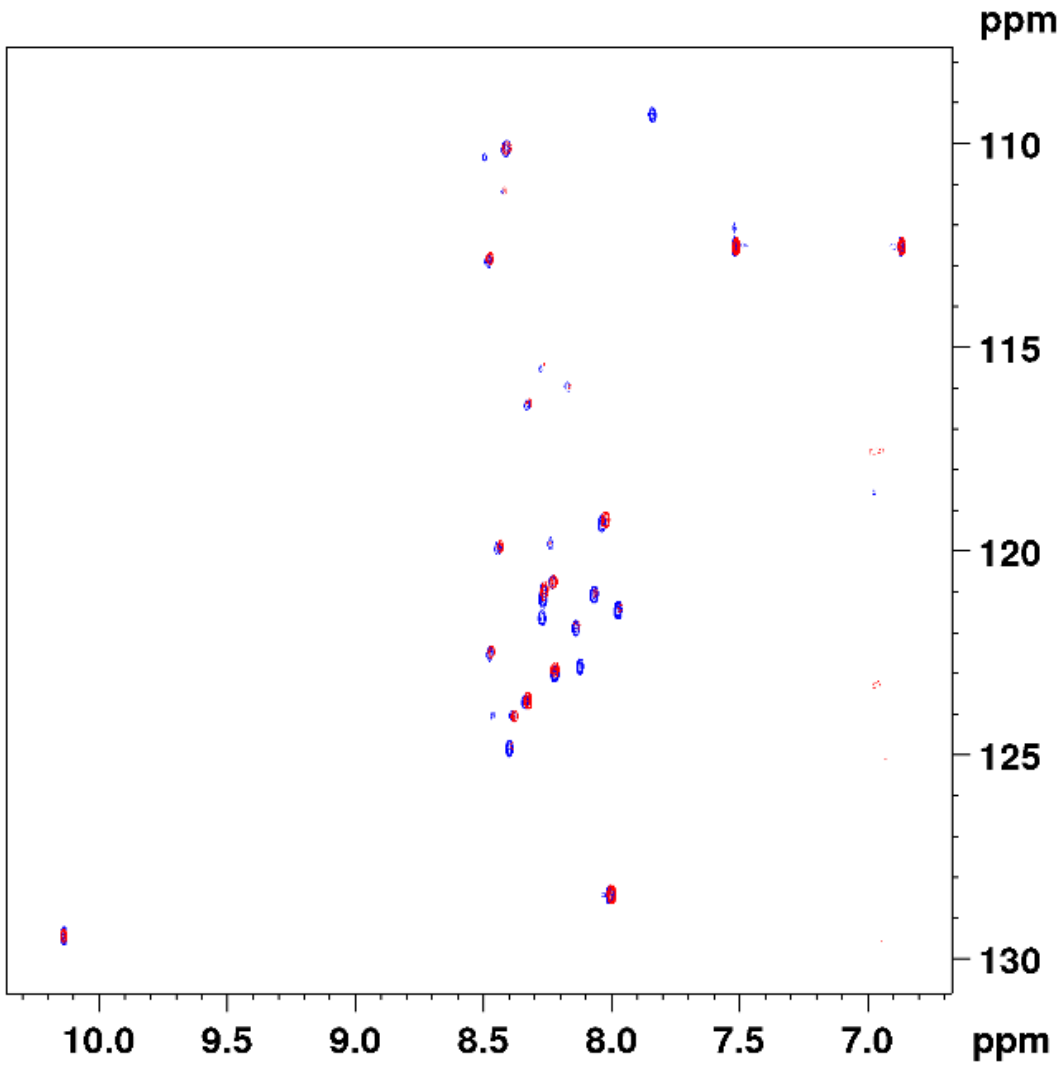

Figure S13: ^{1}H-^{15}N HSQC spectrum of the S18 tail peptide (sequence: SMGLRHFWGLRVRGGQHTKTTGRRGRTVGVSKKK, in which SM were encoded by the expression vector) in the absence (blue) and presence (red) of unlabelled N-terminal polyhistidine-thioredoxin-tagged T136D S15. Sample contained ~100 μM ^{15}N-labelled peptide, ~200 μM unlabelled S15, 50 mM Tris/Tris-D11 pH 6.6, 500 mM NaCl, 2 mM 2-mercaptoethanol, 5% glycerol in 10% D_2O and 90% H_2O. Sample volume was 160 μL and temperature was 298 K.

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1     M  A  E  V  E  Q  K  K  K  R  T  F  R  K  F  T  Y  R  G  V
1     ATGGCAGAAGTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTACCGCGGCGTG
21    D  L  D  Q  L  L  D  M  S  Y  E  Q  L  M  Q  L  Y  S  A  R
61    GACCTCGACCAGCTGCTGGACATGTCCTACGAGCAGCTGATGCAGCTGTACAGTGCGCGC
41    Q  R  R  R  L  N  R  G  L  R  R  K  Q  H  S  L  L  K  R  L
121   CAGCGGCGGCGGCTGAACCGGGGCCTGCGGCGGAAGCAGCACTCCCTGCTGAAGCGCCTG
61    R  K  A  K  K  E  A  P  P  M  E  K  P  E  V  V  K  T  H  L
181   CGCAAGGCCAAGAAGGAGGCGCCGCCCATGGAGAAGCCGGAAGTGGTGAAGACGCACCTG
81    R  D  M  I  I  L  P  E  M  V  G  S  M  V  G  V  Y  N  G  K
241   CGGGACATGATCATCCTACCCGAGATGGTGGGCAGCATGGTGGGCGTCTACAACGGCAAG
101   T  F  N  Q  V  E  I  K  P  E  M  I  G  H  Y  L  G  E  F  S
301   ACCTTCAACCAGGTGGAGATCAAGCCCGAGATGATCGGCCACTACCTGGGCGAGTTCTCC
121   I  T  Y  K  P  V  K  H  G  R  P  G  I  G  A  T  H  S  S  R
361   ATCACCTACAAGCCCGTAAAGCATGGCCGGCCCGGCATCGGGGCCACCCACTCCTCCCGC
141   F  I  P  L  K  *
421   TTCATCCCTCTCAAGTAA
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Figure S14: Amino acid and nucleotide sequences of ribosomal protein S15. In order to facilitate cloning, the sequence tacttccaatcc was added to the 5’ end and taacagtaaaggtggata was added to the 3’ end in the synthetic gene fragment.

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1     M  A  E  V  E  Q  K  K  K  R  T  F  R  K  F  T  Y  R  G  V
1     ATGGCAGAAGTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTACCGCGGCGTG
21    D  L  D  Q  L  L  D  M  S  Y  E  Q  L  M  Q  L  Y  S  A  R
61    GACCTCGACCAGCTGCTGGACATGTCCTACGAGCAGCTGATGCAGCTGTACAGTGCGCGC
41    Q  R  R  R  L  N  R  G  L  R  R  K  Q  H  S  L  L  K  R  L
121   CAGCGGCGGCGGCTGAACCGGGGCCTGCGGCGGAAGCAGCACTCCCTGCTGAAGCGCCTG
61    R  K  A  K  K  E  A  P  P  M  E  K  P  E  V  V  K  T  H  L
181   CGCAAGGCCAAGAAGGAGGCGCCGCCCATGGAGAAGCCGGAAGTGGTGAAGACGCACCTG
81    R  D  M  I  I  L  P  E  M  V  G  S  M  V  G  V  Y  N  G  K
241   CGGGACATGATCATCCTACCCGAGATGGTGGGCAGCATGGTGGGCGTCTACAACGGCAAG
101   T  F  N  Q  V  E  I  K  P  E  M  I  G  H  Y  L  G  E  F  S
301   ACCTTCAACCAGGTGGAGATCAAGCCCGAGATGATCGGCCACTACCTGGGCGAGTTCTCC
121   I  T  Y  K  P  V  K  H  G  R  P  G  I  G  A  D  H  S  S  R
361   ATCACCTACAAGCCCGTAAAGCATGGCCGGCCCGGCATCGGGGCCGACCACTCCTCCCGC
141   F  I  P  L  K  *
421   TTCATCCCTCTCAAGTAA
```

Figure S15: Amino acid and nucleotide sequences of ribosomal protein T136D S15. In order to facilitate cloning, the sequence tacttccaatcc was added to the 5’ end and taacagtaaaggtggata was added to the 3’ end in the synthetic gene fragment.

```
1     M  S  L  V  I  P  E  K  F  Q  H  I  L  R  V  L  N  T  N  I
1     ATGTCTCTAGTGATCCCTGAAAAGTTCCAGCATATTTTGCGAGTACTCAACACCAACATC
21    D  G  R  R  K  I  A  F  A  I  T  A  I  K  G  V  G  R  R  Y
61    GATGGGCGGCGGAAAATAGCCTTTGCCATCACTGCCATTAAGGGTGTGGGCCGAAGATAT
41    A  H  V  V  L  R  K  A  D  I  D  L  T  K  R  A  G  E  L  T
121   GCTCATGTGGTGTTGAGGAAAGCAGACATTGACCTCACCAAGAGGGCGGGAGAACTCACT
61    E  D  E  V  E  R  V  I  T  I  M  Q  N  P  R  Q  Y  K  I  P
181   GAGGATGAGGTGGAACGTGTGATCACCATTATGCAGAATCCACGCCAGTACAAGATCCCA
81    D  W  F  L  N  R  Q  K  D  V  K  D  G  K  Y  S  Q  V  L  A
241   GACTGGTTCTTGAACAGACAGAAGGATGTAAAGGATGGAAAATACAGCCAGGTCCTAGCC
101   N  G  L  D  N  K  L  R  E  D  L  E  R  L  K  K  I  R  A  H
301   AATGGTCTGGACAACAAGCTCCGTGAAGACCTGGAGCGACTGAAGAAGATTCGGGCCCAT
121   R  G  L  R  H  F  W  G  L  R  V  R  G  Q  H  T  K  T  T  G
361   AGAGGGCTGCGTCACTTCTGGGGCCTTCGTGTCCGAGGCCAGCACACCAAGACCACTGGC
141   R  R  G  R  T  V  G  V  S  K  K  K  *
421   CGCCGTGGCCGCACCGTGGGTGTGTCCAAGAAGAAATAA
```

Figure S16: Amino acid and nucleotide sequences of ribosomal protein S18.

```
1     M  S  L  V  I  P  E  K  F  Q  H  I  L  R  V  L  N  T  N  I
1     ATGTCTCTAGTGATCCCTGAAAAGTTCCAGCATATTTTGCGAGTACTCAACACCAACATC
21    D  G  R  R  K  I  A  F  A  I  T  A  I  K  G  V  G  R  R  Y
61    GATGGGCGGCGGAAAATAGCCTTTGCCATCACTGCCATTAAGGGTGTGGGCCGAAGATAT
41    A  H  V  V  L  R  K  A  D  I  D  L  T  K  R  A  G  E  L  T
121   GCTCATGTGGTGTTGAGGAAAGCAGACATTGACCTCACCAAGAGGGCGGGAGAACTCACT
61    E  D  E  V  E  R  V  I  T  I  M  Q  N  P  R  Q  Y  K  I  P
181   GAGGATGAGGTGGAACGTGTGATCACCATTATGCAGAATCCACGCCAGTACAAGATCCCA
81    D  W  F  L  N  R  Q  K  D  V  K  D  G  K  Y  S  Q  V  L  A
241   GACTGGTTCTTGAACAGACAGAAGGATGTAAAGGATGGAAAATACAGCCAGGTCCTAGCC
101   N  G  L  D  N  K  L  R  E  D  L  E  R  L  K  K  I  R  A  H
301   AATGGTCTGGACAACAAGCTCCGTGAAGACCTGGAGCGACTGAAGAAGATTCGGGCCCAT
121   R  G  L  R  H  F  W  G  L  R  V  R  G  Q  H  T  K  T  T  G
361   AGAGGGCTGCGTCACTTCTGGGGCCTTCGTGTCCGAGGCCAGCACACCAAGACCACTGGC
141   R  R  G  R  T  V  G  V  S  *
421   CGCCGTGGCCGCACCGTGGGTGTGTCCTAA
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Figure S17: Amino acid and nucleotide sequences of ribosomal protein S18 mutant.

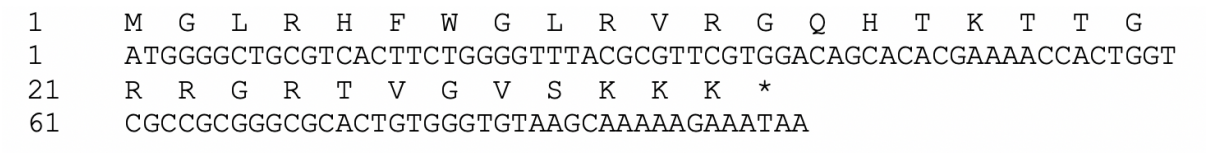

Figure S18: Amino acid and nucleotide sequences of S18 tail. In order to facilitate cloning, the sequence tacttccaatcc was added to the 5’ end and taacagtaaaggtggata was added to the 3’ end in the synthetic gene fragment.

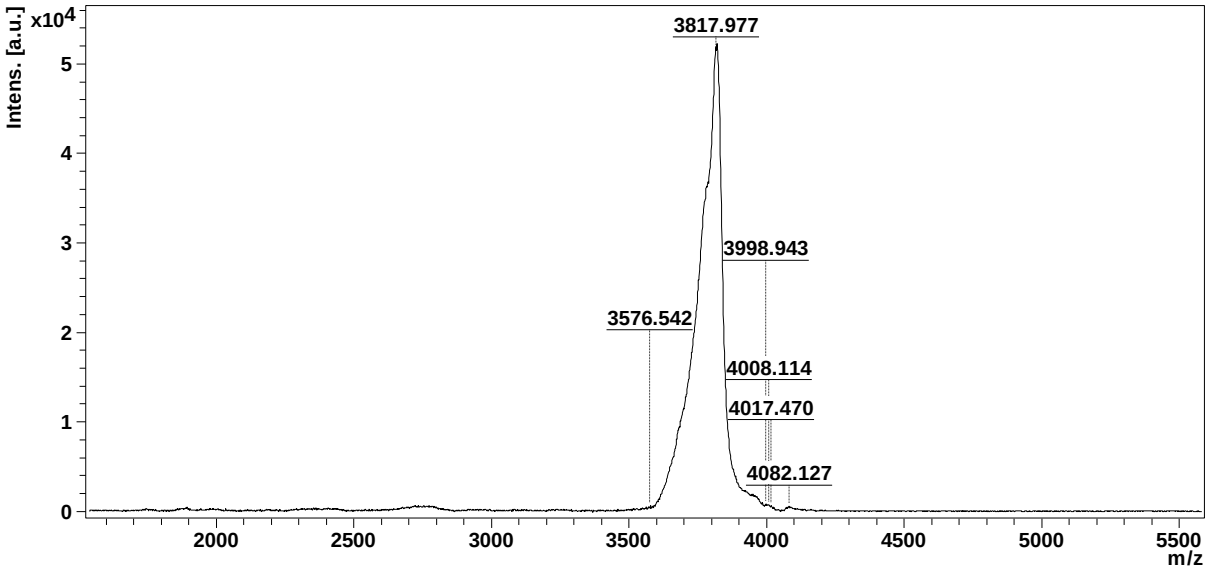

Figure S19: MALDI mass spectrum of the ^{15}N-labelled S18 tail (SGLRHFWGLRVRGGQHTKTTGRRGRTVGVSKKK, in which the S in the N-terminal end is encoded by the plasmid). MALDI-TOF-MS: m/z calculated for ^{15}N-labelled S18 tail $[M+K]^+$ 3818.09, found 3817.97.

Supporting references

1. Savitsky, P.; Bray, J.; Cooper, C. D.; Marsden, B. D.; Mahajan, P.; Burgess-Brown, N. A.; Gileadi, O. J. Struct. Biol. 2010, 172, 3.
2. Gileadi, O.; Burgess-Brown, N. A.; Colebrook, S. M.; Berridge, G.; Savitsky, P.; Smee, C. E.; Loppnau, P.; Johansson, C.; Salah, E.; Pantic, N. H. Methods Mol. Biol. 2008, 426, 221.
3. Correddu, D.; Montaño López, J. d. J.; Vadakkedath, P. G.; Lai, A.; Pernes, J. I.; Watson, P. R.; Leung, I. K. H. Sci. Rep. 2019, 9, 8884.
4. Wu, P. S. C.; Otting, G. J. Magn. Reson. 2005, 176, 115–119.
5. Li, Y.; McLarin, M.-A.; Middleditch, M. J.; Morrow, S. J.; Kilmartin, P. A.; Leung, I. K. H. Biochimie 2019, 165, 40–47.
6. Correddu, D.; Montaño López, J. d. J.; Angermayr, S. A.; Middleditch, M. J.; Payne, L. S.; Leung, I. K. H. IUBMB Life 2020, 72, 266–274.