Supporting information for

A new strategy for the in vitro selection of stapled peptide inhibitors by mRNA display

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Materials and Methods.

General. (*R*)-NH-Fmoc-S-Trityl-C$^{\alpha}$-Methyl Cysteine-OH was synthesized as described.[1] Unprotected α-MeCys was purchased from Nagase Chemical company. 5(6)-carboxyfluorescein-succinimidyl ester was prepared according to the literature procedure.[2] MALDI-TOF spectra were acquired on a Voyager DE-Pro instrument in reflectron positive mode with external standard calibration.

Cloning of Trx-E2 and Avi-Tag E2. The construction of the pGEX-4T-2 GST-E2 plasmid has previously been described.[3] In order to construct the Trx-E2 plasmid, the GST-E2 plasmid was digested with BamHI and XhoI, and the E2 insert was purified via electrophoresis. The insert was ligated into a pET-32a vector containing thioredoxin and a C-terminal hexa-His purification tag. Plasmids were transformed into E coli BL21(DE3) cells and stored as 50% glycerol stocks at -80°C. The Avi-Tagged version of E2 was prepared by BamHI and XhoI digestion of this plasmid and ligation into a modified pET32 vector where a gene encoding the Avi-tagged sequence (LNDIFEAQKIEWHE) was inserted immediately upstream of the Trx.

Expression and purification of Trx-E2. Transformed cells were grown in LB media containing ampicillin (100 µg/mL) at 37°C in a shaking incubator. Once the OD600 reached 0.6, the temperature was lowered to 15°C. Protein expression was induced when the OD600 reached 0.8-0.9 by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM final concentration) and allowed to continue overnight. Cells were harvested via centrifugation (4,000 rpm for 20 min). Lysis buffer (1X PBS pH 7.4, 5mM dithiothreitol (DTT), 1 mg/mL lysozyme and protease inhibitors) was added to the pellet and allowed to rock at 4°C for 1 hour. Lysate was maintained at 4°C for remainder of purification protocol. Lysate was sonicated (6 x 30 s), then centrifuged at 15,000 rpm for 30 min. The supernatant was incubated with Ni-NTA agarose resin for 1 hr. The resin was washed with wash buffer (50 mM HEPES pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM DTT) and the Trx-E2 protein was eluted with elution buffer (50 mM HEPES pH 8.0, 300 mM NaCl, 500 mM imidazole, 1 mM DTT). The eluted protein was dialyzed into storage buffer (1X PBS pH 7.4, 1 mM DTT, 25% glycerol) and stored at -80°C.

Expression of and Purification Avi-Tag E2 protein. The plasmid was transformed into BL21 cells already transformed with pBirAcm, and colonies were grown on LB agar containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. A single colony was picked and used to inoculate 10 mL of LB media supplemented with antibiotics as above and grown overnight at 37°C and 240 rpm. Two 500 mL cultures were each inoculated with 5 mL of the starter, and grown until the OD600 was 0.6-0.8, at which point both plasmids were induced with IPTG (0.5 mM final concentration). The culture was supplemented with 50 µM D-biotin as substrate for the BirA ligase. Induced cultures were grown for 18 hours at 18°C and pelleted by centrifugation. Cell pellets were resuspended in 25 mM HEPES-KOH (pH 8.0), 300 mM NaCl, 2 mM EDTA, 20 mM imidazole, 1 mg/mL egg white lysozyme, and 5 mM DTT with His-compatible protease inhibitor cocktail and lysed using sonication: 30 s bursts at 0.8 with 30 s rest on ice for a total of 3 min of sonication. After centrifugation at 15,000 rpm, the His-tagged protein was purified from the

cleared supernatant with Ni-NTA resin according to the manufacturer's specifications. Eluted fractions were dialyzed overnight into 50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 7 mM DTT, and 30% glycerol. Aliquots of the dialysate were flash frozen in liquid N_2 and stored at -80°C until use.

Analysis of biotinylation. To assess biotinylation, 20 μM of the Avi-Tagged Trx-E2 protein was incubated across a gradient of purified streptavidin and assessed via gel shift non-denaturing PAGE. The protein was determined to be 84% biotinylated using ImageLab 6.0 for densitometry analysis.

Peptide synthesis. Peptides were synthesized via standard Fmoc chemistry with HATU as an activator on a Liberty automated peptide synthesizer. The N-terminal Fmoc protecting group was not removed during the final step, and the resin was treated with acetic anhydride in dichloromethane (DCM) to cap any unreacted amines. For the hydrocarbon-stapled peptide, cyclization was performed in the dark under argon in a sealed, fritted polyethylene tube. A solution of Grubbs first generation catalyst in 1,2-dichloroethane (4.3 mg/mL) was added to the peptide resin in the tube and placed in a hot water bath (80°C) for 3 hours. Reaction was checked for completion via mass spectrometry, and the cyclization conditions were repeated if needed. The resin was washed with DCM/methanol (MeOH) to remove the catalyst. The Fmoc group was removed with 20% piperidine in dimethylformamide (DMF) before labeling with 5(6)-carboxyfluorescein N-hydroxysuccinimide (*vide infra*).

Peptide labeling with 5-FAM. 5(6)-carboxyfluorescein-succinimidyl ester (125 mg, 0.26 mmol, 3eq) in 8 mL N-Methyl-2-pyrrolidone (NMP) was added to peptidyl resin. After addition of N,N-diisopropylethylamine (DIPEA, 750 μL), the resin was rocked for 4 hours. After rinsing the resin with DCM/MeOH, the peptides were cleaved from resin using 92.5% trifluoroacetic acid (TFA): 2.5% water, 2.5% triisopropylsilane (TIS), 2.5% 2,2'-(ethylenedioxy)diethanethiol (DODT). Peptides were precipitated with cold diethyl ether, pelleted, resuspended in 1:1 acetonitrile (MeCN)/water and filtered, then lyophilized overnight.

Peptide cyclization by cysteine alkylation. The procedure was adapted from Diderich et al[4a]. Crude peptides (1 mM final concentration) were dissolved in 1:1 MeCN/20 mM NH_4HCO_3. Tris(2-carboxyethyl)phosphine (TCEP) was added (5 mM final concentration), then either m-dibromoxylene (DBX) or cis-1,2-dichlorobutene (CDB) was added (3 mM final concentration) and the reaction was incubated at 37°C overnight. Reaction was checked via MALDI-TOF MS for completion. The crude reaction mixture was filtered and lyophilized. Peptides **B** and **C** were instead cyclized on-resin using our standard protocol[4b].

Peptide purification. Crude peptides were resuspended in DMSO and purified via reverse-phase HPLC using an MeCN:water gradient with 0.1% TFA on a 250 mm column. As the labeled peptides were very hydrophobic, the HPLC was equilibrated to 50% MeCN:water to prevent precipitation and clogging of the instrument. The gradient was 50-100% MeCN:water over 30 min. Fractions containing peptides were frozen and lyophilized. Dried peptides were resuspended in DMSO and concentration determined via a spectrophotometer (NanoDrop) using the extinction coefficient of fluorescein as a reference. For the less hydrophobic peptides

A-F, we used a wider gradient purification strategy (10% for 0-5min, 10%-100% for 5-45min).

Fluorescence polarization assay. Trx-E2 protein was dialyzed overnight at 4°C into assay buffer (1X PBS pH 7.4, 1 mM DTT, 0.004% Triton X-100). The protein concentration was determined via Bradford assay. All preparation steps involving the FAM-labeled peptides were performed in the dark to prevent photobleaching. Stocks of the peptides were prepared in DMSO, then diluted 2-fold with assay buffer (400 nM). In 96-well plates, 5 µL of peptide (40 nM final concentration) was added to 45µL of serial dilutions of Trx-E2 protein in assay buffer and incubated at room temperature with gentle rocking. The fluorescence polarization was obtained using Flexstation 3 spectrophotometer. Controls containing protein alone or peptide alone were analyzed in parallel. Data analysis was performed with SigmaPlot software.

Circular dichroism. CD spectra were recorded on a JASCO spectrometer (Model J-1500). A total of three scans from 190 to 260 nm in 0.5 nm increments were averaged to obtain each spectrum using a 1 mm path length cell. The CD spectra was provided as wavelength vs millidegree, which was converted to mean residue ellipticity (θ, degree cm^2 $dmol^{-1}$) using the JASCO spectra analysis software. Peptide concentrations for CD were: **A** 30 µM, **B** 15 µM, **C** 30 µM, **D** 30 µM, **E** 6.3 µM, **F** 30 µM. Concentrations were determined using the Pierce Quantitative Fluorometric Peptide Assay Kit (Thermo Scientific 23290) in a 40% MeCN: 60% water solution.

AARS assay α-MeCys. The MALDI-TOF screening assay was performed as previously described.[5] Briefly, 10mM of α-MeCys (pH adjusted to 7.4 with 3 M KOH) was incubated with 0.5 µM ValRS T222P for 30 min in charging assay buffer (30 mM HEPES-KOH pH 7.4, 15 mM $MgCl_2$, 25 mM KCl, 2 mM 2-mercaptoethanol (BME), 6 mM ATP, 0.09 mg/mL bovine serum albumin (BSA, previously dialyzed into ddH_20), 0.02 units/µL inorganic pyrophosphatase (PPiase), 25 µM $tRNA^{Val}$, ddH_20 to 50 µL). Reactions were quenched by addition of 0.1 volume NaOAc (3.0 M, pH 5.2) followed by 25:24:1 PCI pH 4.3 extraction and ethanol precipitation. tRNA pellet was resuspended 200 mM NaOAc pH 5.0. Water (3.75 µL) was added to the resulting solution (6 µL) along with 30 mM (4-formylphenoxyproyl)triphenylphosphonium bromide in MeOH , 20 mM $NaCNBH_3$ in 50 mM NaOAc (pH 5.0) and tumbled at 37°C for 2 h. The reaction was quenched with 0.1 volume of 4.4 M NH_4Oac pH 5.0 and 3 volumes of ethanol. The resulting pellet was resuspended in 2.25 µL of 200 mM NH_4Oac and digested with 0.25 µL of 1 U/µL Nuclease P1 (in 200 mM NH_4Oac). Digestion was allowed to proceed for 20 min at room temperature and quenched by placing on ice. One microliter was mixed with saturated α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 1:1 MeCN:1% TFA (9 µL). One microliter of the resulting suspension was spotted on a MALDI plate and analyzed.

***In vitro* translation experiments.** *In vitro* translation experiments were performed as previously described for the addition of α–methyl amino acids into peptides.[6] Briefly, synthetic $tRNA^{Val}$ was precharged with α-MeCys via ValRS T222P. This precharged tRNA was added to a custom translation mixture lacking wild type ValRS and valine for a final concentration of 25 µM α-MeCys-$tRNA^{Val}$. Translations were initiated by addition of relevant mRNA template and incubated at 37°C for 1 h, quenched with binding buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl,

5 mM BME), bound to Ni-NTA resin for 1 h, and eluted with 1% TFA. Peptides were further concentrated via ZipTip© purification and analyzed via MALDI-TOF. For experiments involving DBX cyclization of α-MeCys, the reducing agent in the binding buffer was changed to 1 mM TCEP. For experiments being analyzed by MALDI-TOF, cyclization was performed after 1 h immobilization on Ni-NTA resin followed by addition of 500 µL cyclization buffer (20 mM Tris-HCl pH 7.8, 0.66 M NaCl, 3 mM a, α,α'-dibromo-*m*-xylene, 33% MeCN (v/v), 0.5 mM TCEP.[7]

Preparation of mRNA-peptide fusions. T7 *in vitro* transcription was performed with equimolar mixes of our *i, i + 4* libraries after preannealing the T7 Forward primer (70°C for 5 min followed by 0.1°C/s decrease to 25°C) along with an addition of 1:100 molar ratio of DNA coding for our optimized cyclized E2-binding peptide containing a ClaI restriction site (the linear selection used a 1:10 molar ratio). Both the library mRNA and E2 mRNA were psoralen photo-crosslinked to the linker containing puromycin at the 3' end as previously described.[8] Translations were performed on a 400 µL scale containing 40 mM HEPES-KOH pH 7.6, 2 mM spermidine, 10 mM KOAc, 6 mM $MgOAc_2$, 1 mM DTT, 40 ug/mL creatine kinase, 1 µM PPiase, 100 µL 6R,S)-5,10-formyl-5,6,7,8-tetrahydrofolic acid, 30 mM creatine phosphate, 1.5 mM ATP, 1.5 mM GTP, E. coli total tRNA 2.4 mg/mL, 0.52 µM EF-G, 8 µM Ef-Ts, 10 µM EF-Tu, 0.3 µM RF-1, 0.5 µM RR-F, 0.17 µM RF-3, 2.7 µM IF-1, 0.4 µM IF-2, 1.5 µM IF-3, 1.2 µM ribosomes, 0.6 µM MTF, methionine (3 µM plus additional 0.3 µM ^{35}S-methionine), 100 µM of each canonical amino acid except valine which was excluded entirely, and 0.1-0.8 µM of each aminoacyl-tRNA synthetase[9] except ValRS which was excluded entirely. Reactions were initiated by the addition of 1 µM of crosslinked mRNA that that contained a mixture of the naïve library and E2 binder. Translations were allowed to proceed at 37°C for 70 min, supplemented with 550 mM KCl and 55 mM $MgCl_2$, returned to 37°C for 1.5 h, and frozen overnight at -80°C.[10] The RNA-peptide fusions were diluted 5-fold with oligo(dT) binding buffer (20 mM Tris-HCl pH 7.8, 10 mM EDTA, 1 M NaCl, 0.2% Triton X-100, 0.5 mM TCEP) and added to 1 translation volume of pre-equilibrated oligo(dT) beads (Thermo 61002). Fusions were bound to resin for 30 min at 4°C, washed twice with wash buffer (20 mM Tris-HCl pH 7.8, 0.3 M NaCl, 0.1% Triton X-100, 0.5 mM TCEP). Cyclization was performed by the addition of 1 mL of cyclization buffer (20 mM Tris-HCl pH 7.8, 0.66 M NaCl, 3 mM a, α,α'-dibromo-*m*-xylene, 33% MeCN (v/v), 0.5 mM TCEP) and rotated at RT for 30 min. The beads were washed once with oligo(dT) wash buffer with 5 mM BME (to quench unreacted linker) and once with oligo(dT) wash buffer with 0.5 mM TCEP. Fusions were eluted with 100 µL fractions ddH_20 containing 0.5 mM TCEP. Fractions containing significant radioactivity were pooled and precipitated using 0.1 vol 3 M KOAc pH 5.2 and 0.001 vol 5 mg/mL glycogen. The pellet was resuspended into 50 µL H_20, quantified by scintillation counting, and used as a template for reverse transcription. Reverse transcription was performed using SuperScript III (Invitrogen 18080093) as per manufacturer protocol followed by 5-fold dilution with Ni-NTA denaturing binding buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 6 M guanidinium hydrochloride, 0.2% Triton X-100, 5 mM BME, pH 8.0). The quenched reverse transcription reaction was added to 100 µL of Ni-NTA agarose resin (McLab) in a 0.2 µM centrifugal filter (VWR 82031-356) and allowed to bind for 1 h at RT. The resin was washed twice with Ni-NTA wash buffer (100 mM NaH_2PO_4, 300 mM NaCl, 0.2% Triton X-100, 5 mM BME, pH 8.0) and eluted into 100 µL fractions of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 350 mM imidazole, 0.2% Triton X-100, 5 mM BME, pH 8.0). Fractions containing significant

radioactivity were pooled, ethanol precipitated, and stored at -20°C until use.

Selection against Trx-E2. Recombinant biotinylated Trx-E2 was immobilized on magnetic streptavidin beads (M280 Dynabeads) by incubating the protein with beads in binding buffer (50 mM Tris-HCl pH 7.9, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 0.1% v/v Triton X-100) for 1 h at 4°C with tumbling to yield the positive selection beads. An identical amount of beads were also equilibrated with binding buffer lacking protein to act as a negative selection. Peptide-mRNA fusions were resuspended in 50 µL of the same binding buffer and first incubated with the negative selection beads for 1 h at 4° C. The supernatant from the negative selection was then applied to the positive selection beads for 1 h at 4° C. The final concentration of immobilized biotinylated Trx-E2 was 1 µM. Beads were washed eight times with 500 µL binding buffer with five-minute incubations with tumbling during each wash. cDNA of the fusions was eluted by boiling the beads in PCR buffer with 100 µL fractions. Elutions were subsequently PCR amplified, extracted with phenol chloroform, ethanol precipitated, resuspended in water, and used for the ClaI restriction enzyme digest assay.

ClaI digestion assay. Library DNA and E2 DNA alone were both incubated with ClaI (NEB R0197S) as per manufacturer's protocol, as well as pre- and post-selection recovered DNA. The resulting digest was resolved on a 2.8% agarose gel. DNA was visualized with ethidium bromide staining.

Analysis of mRNA display experiments on gel. The agarose gel containing cut DNA was visualized on BioRad ChemiDoc gel imager and bands were quantified by intensity using ImageJ.

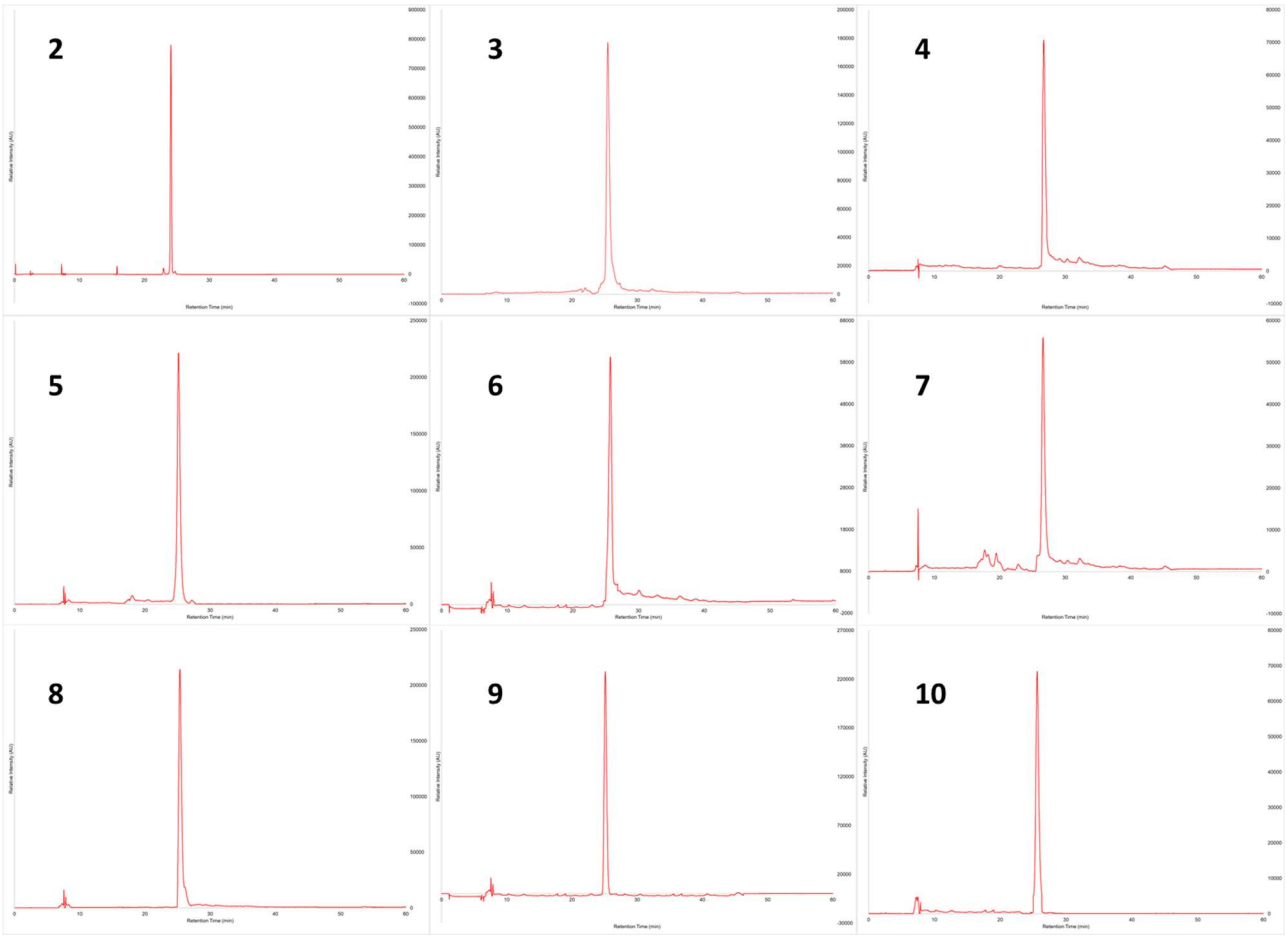

Figure S1. Reverse-Phase HPLC traces for peptides 2-10. After injection of the peptide at 5 min and at 50% MeCN/50% H_2O with 0.1% TFA, a 30 min gradient from 50-100% MeCN was initiated between 10 and 40 min, followed by 20 min at 100% MeCN with 0.1% TFA. Traces are at 443 nm.

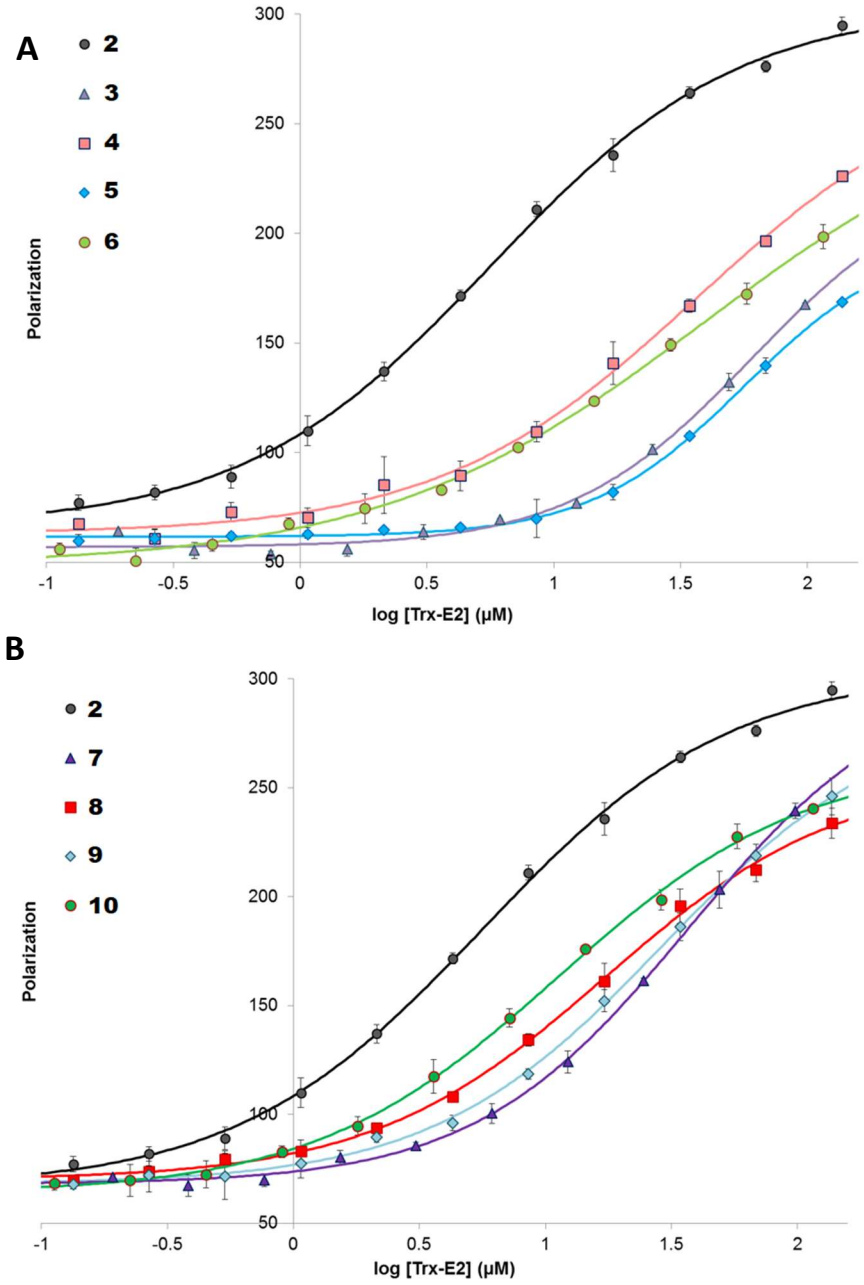

Figure S2. Fluorescence polarization binding data for peptides 2-10. Peptides were added at a concentration of 40 nM with increasing concentration of Trx-E2 protein. Peptide numbering is given in Table 1. Assays were performed in triplicate and error bars signify the standard deviation from the mean.

A

	Structure	Sequence
A	Lin-Cys	H_2N–IDZNFCSDLCSIFEEN–NH_2
B	DBX-Cys	H_2N–IDZNFCSDLCSIFEEN–NH_2
C	CDB-Cys	H_2N–IDZNFCSDLCSIFEEN–NH_2
D	Lin-Me2	H_2N–IDZNFCSDLCSIFEEN–NH_2
E	DBX-Me2	H_2N–IDZNFCSDLCSIFEEN–NH_2
F	CDB-Me2	H_2N–IDZNFCSDLCSIFEEN–NH_2

B

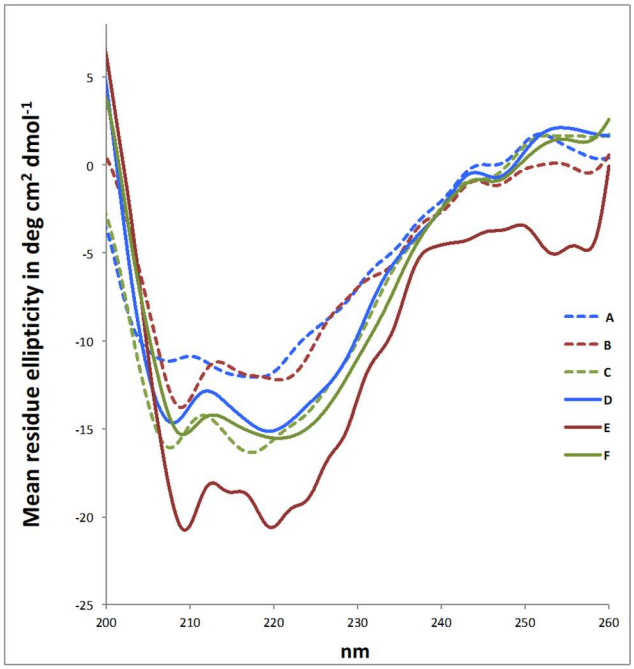

Figure S3. α-MeCys incorporation and cyclization improve helicity. (A). List of unlabeled peptides prepared for the CD experiments shown in. (B) Circular dichroism spectra of each of the peptides was taken in 50% 2,2,2-trifluoroethanol, 50% 10 mM phosphate buffer at 25°C. Peptide concentrations ranged from 6-30 μM. Peptides indicated with dashed lines contain cysteine and solid lines contain α-MeCys.

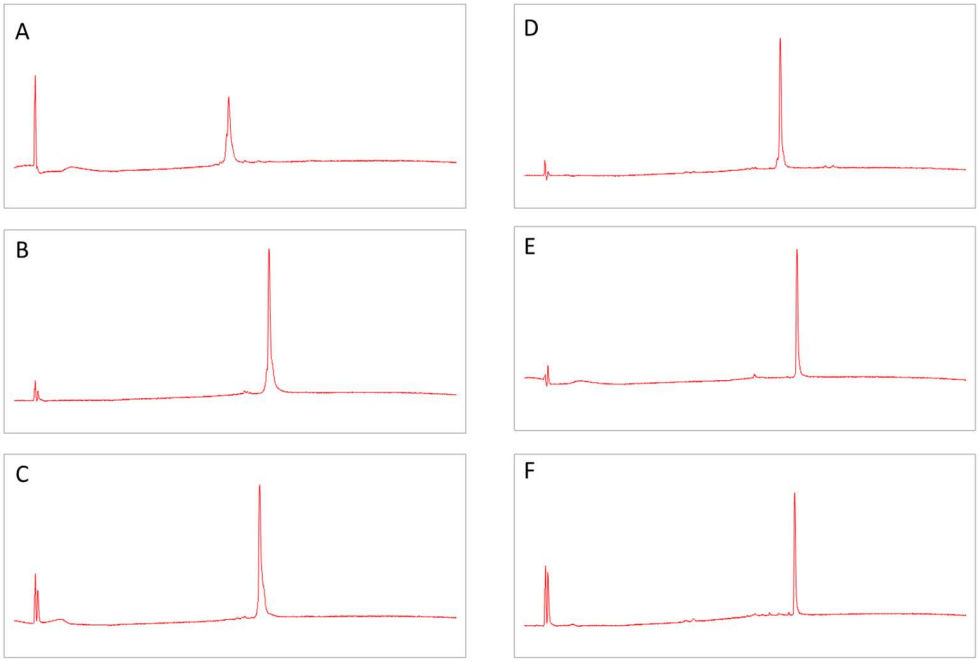

Figure S4. HPLC traces for peptides A-F prepared for the CD experiments. After injection of the peptide at 5 min at 10% MeCN/90% H_2O with 0.1% TFA, a 40 min gradient from 10-100% MeCN was created, followed by 10 min at 100% MeCN with 0.1% TFA. Traces are at 215 nm.

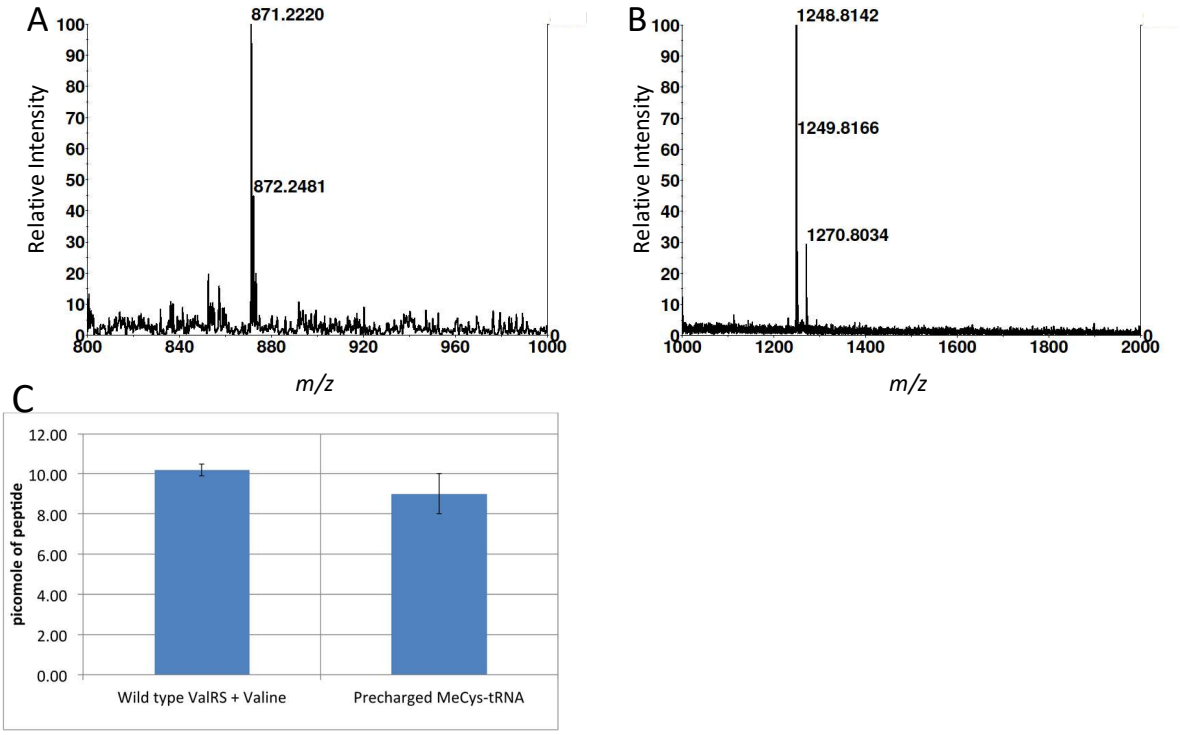

Figure S5. Evidence for incorporation of α–MeCys A) MALDI-TOF charging assay of α–MeCys with ValRS T222P. Expected $[M+H]^+$: 871.244 Observed $[M+H]^+$: 871.222. B) MALDI-TOF of an *in vitro* translated peptide containing a single α–MeCys. The mRNA template encodes M<u>V</u>MH_6. Expected $[M+H]^+$: 1248.47 Observed $[M+H]^+$: 1248.81. The second peak shown is $[M+Na]^+$. (C) Comparison of yields of 50μL *in vitro* translation reactions with added Val/ValRS and pre-charged α–MeCys-tRNA^{Val}. Error bars represent the standard deviation of three replicates.

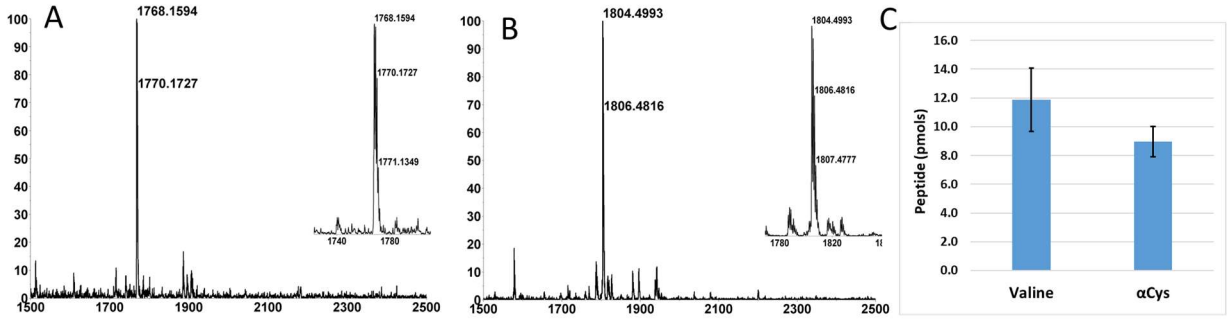

Figure S6. Further evidence for double-incorporation of α–MeCys. Each panel uses a template encoding MH_6<u>V</u>AAA<u>V</u>EP. A) MALDI-TOF of an *in vitro* translated peptide containing wild-type valine. Expected $[M+H]^+$: 1768.79 Observed $[M+H]^+$: 1768.16. B) MALDI-TOF of an *in vitro* translated peptide lacking valine and supplemented with precharged α–MeCys-tRNA^{Val} $[M+H]^+$: 1804.70. Observed $[M+H]^+$: 1804.50. (C) Comparison of yields of 50 μL *in vitro* translation

reactions with added Val/ValRS and pre-charged α–MeCys-tRNA^{Val}. Error bars represent the standard deviation of three replicates.

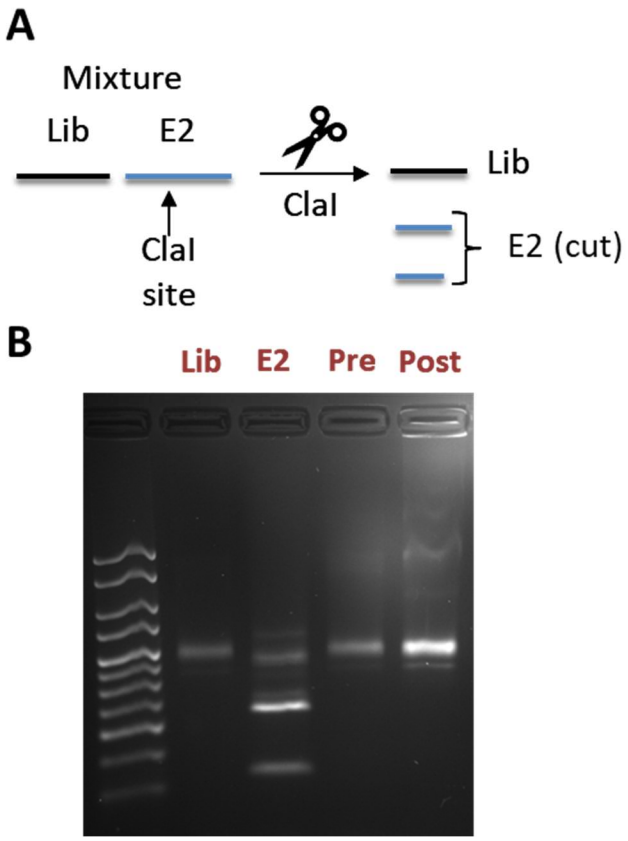

Figure S7. In vitro selection with a linear, non-cyclized E2 peptide shows no enrichment. (A) Scheme illustrating how a ClaI site can be used to distinguish between DNAs encoding the naïve library (lib) or our E2-binding peptide derived from **10** (E2) (B) Agarose EtBr stained gel showing DNAs corresponding to the library (Lib) and E2 binder (E2) alone, as well as a mixture of both before (Pre) and after (Post) one round of *in vitro* selection (right). This study differs from that in the main text, in that the peptide library was not treated with DBX for cyclization.

Table S1. MALDI-MS (m/z) spectra for peptides 1-10 and A-F. Calculated values are for $(M+H)^+$ for **1-10** and $(M-H)^-$ for **A-F**.

	Mass (Calculated)	Mass (Experimental)
1	2384.02	2382.63
2	2634.23	2633.80
3	2339.92	2338.90
4	2353.93	2352.72
5	2353.93	2352.97
6	2367.95	2365.91
7	2389.93	2389.02
8	2403.95	2402.79
9	2403.95	2402.43
10	2417.96	2416.76

	Mass (Calculated)	Mass (Experimental)
A	1858.80	1859.13
B	1960.85	1959.71
C	1910.83	1909.14
D	1886.83	1886.40
E	1988.88	1987.09
F	1938.86	1938.20

Table S2. Oligonucleotide sequences. Name and sequences of DNA libraries, E2 binding peptide oligo containing a ClaI restriction site, and primers used for RT and PCR.

Name	Sequence
MeCys4-8.n14	5’-CTAGCTACCTATAGCCGGTGGTGATGGTGATGAT GGCCACCGCCSNNSNNSNNSNNSNNSNNTACSNNSNNS NNTACSNNSNNCATATGTATATCTCCTTCTTAAAGT TAACCCTATAGTGAGTCGTATTAATTTCG-3’
MeCys6-10.n14	5’-CTAGCTACCTATAGCCGGTGGTGATGGTGATGAT GGCCACCGCCSNNSNNSNNSNNTACSNNSNNSNNTAC SNNSNNSNNSNNCATATGTATATCTCCTTCTTAAAGT TAACCCTATAGTGAGTCGTATTAATTTCG-3
MeCys8-12.n14	5’-CTAGCTACCTATAGCCGGTGGTGATGGTGATGAT GGCCACCGCCSNNSNNTACSNNSNNSNNTACSNNSNN SNNSNNSNNSNNCATATGTATATCTCCTTCTTAAAGT TAACCCTATAGTGAGTCGTATTAATTTCG-3’
MeCysRT.84bp	5’-TTTTTTTTTTTTTTTGATACGTAATTCATTACCA TTCAGCCTAGCTACCTATAGCCGGTGGTGATGGTG ATGATGGCCACCGCC-3’
UniExtT7FWD.43bp	5’-CGAAATTAATACGACTCACTATAGGGTTAACTTT AAGAAGGAG-3’
MeCysRev.25bp	5’-GATACGTAATTCATTACCATTCAGC-3’
E2.ClaI	5’-CTAGCTACCTATAGCCGGTGGTGATGGTGATGATG GCCACCGCCGTTTTCCTCGAAAATGCTTACCAGGTC

	GCTTACAAAATTCATATCGATCATATGTATATCTCC TTCTTAAAGTTAACCCTATAGTGAGTCGTATTAATT TCG-3’

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