Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

In vitro display evolution of unnatural peptides spontaneously cyclized via intramolecular nucleophilic aromatic substitutions

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Supplemental Materials and Methods

Activity analysis of 2-fluoro-5-nitrobenzoyl-tRNA^{ini} and pentafluorobenzoyl-tRNA^{ini}

Escherichia coli initiator tRNA (tRNA^{ini}) was prepared by *in vitro* transcription of appropriate DNA template using T7 RNA Polymerase (RNAP) and purified by ethanol precipitation.[1] Preparation of acyl-tRNA^{ini} with 2-fluoro-5-nitrobenzoic acid (FNO_2Ph) and pentafluorobenzoic acid (F_5Ph) cyanomethyl ester by acylation ribozyme was performed with N-cyclohexyl-2-aminoethanesulfonic acid buffer at pH 9.5 and 4°C for 24 h. FNO_2Ph-tRNA^{ini} and F_5Ph-tRNA^{ini} were purified by ethanol precipitation. Template DNA encoding model fMet-$(Tyr)_3$-Cys-Asp-Tyr-Lys-$(Asp)_4$-Lys peptide was PCR-amplified.[1] Template DNA was transcribed and translated using PURE system containing four proteinogenic amino acids (Cys, Asp, Tyr, and Lys) and FNO_2Ph-tRNA^{ini} or F_5Ph-tRNA^{ini} for 1 h at 37°C. Translation products were incubated with reverse transcription buffer at pH 8.4 for 30 min at 42°C. Expressed peptides were desalted and analyzed using Matrix Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) mass spectrometry (MS).

Template DNA encoding model fMet-$(Tyr)_3$-His-Asp-Tyr-Lys-$(Asp)_4$-Lys peptide was PCR-amplified. Template DNA was transcribed and translated using PURE system containing four proteinogenic amino acids (His, Asp, Tyr, and Lys) and FNO_2Ph-tRNA^{ini}, F_5Ph-tRNA^{ini}, m-chloromethylbenzoyl (mClPh)-tRNA^{ini}, or *N*-chloracetyl-*N*-benzylglycyl ($ClAc^{Bn}Gly$)-tRNA^{ini} for 1 h at 37°C. Translation products were incubated with reverse transcription buffer at pH 8.4 for 30 min at 42°C. Expressed peptides were desalted and analyzed using MALDI-TOF MS.

Immobilization of PCSK9

Site-specifically biotinylated human PCSK9 was immobilized on streptavidin-modified magnetic beads. Fc-fusion protein of human PCSK9 was immobilized on Protein A-modified magnetic beads. PCSK9-immobilization was confirmed with sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

SELEX of PCSK9-binding cyclic peptides by mRNA display

Eight kinds of template DNA libraries encoding 8-15 random amino acids between cyclization compounds (FNO_2Ph or F_5Ph) and downstream cysteine residue were prepared through PCR-amplification of template DNAs prepared by primer extension.[1] Prepared template DNA libraries were transcribed to mRNA libraries by run-off *in vitro* transcription using T7 RNAP. Eight kinds of prepared mRNA libraries were mixed. Libraries of mRNA-displayed FNO_2Ph-cyclized or F_5Ph-cyclized peptides were prepared by *in vitro* translation at 37°C for 25 min in release factor (RF)-free PURE system containing 0.5 mM of 19 proteinogenic amino acids (excluding Met), 3 μM mixed mRNA library, 3 μM puromycin-DNA linker, and 100 μM FNO_2Ph-tRNA^{ini} or F_5Ph-tRNA^{ini}. EDTA was added to mRNA-displayed FNO_2Ph-cyclized and F_5Ph-cyclized peptide libraries. The mRNA-displayed peptide libraries were reverse-transcribed to form double-stranded mRNA/cDNA at 42°C for 30 min using RNase H-inactivated reverse transcriptase. Reverse transcription was quenched with EDTA and neutralized with HEPES. The mRNA-displayed FNO_2Ph-cyclized and F_5Ph-cyclized peptide libraries were incubated with PCSK9-immobilized streptavidin-modified beads as positive selection. After supernatant was removed, beads were washed with HBS-T. cDNAs encoding peptides binding to PCSK9-immobilized beads were PCR-amplified using Taq DNA polymerase and used for next round of

SELEX. From second round, mRNA-displayed peptide libraries were prepared by *in vitro* transcription and translation in RF-free PURE system containing 19 proteinogenic amino acids (excluding Met), puromycin-DNA linker, FNO_2Ph-$tRNA^{ini}$ or F_5Ph-$tRNA^{ini}$, and DNA libraries obtained from prior round. mRNA-displayed peptide libraries were reverse-transcribed and mixed with streptavidin-modified beads, and supernatant was removed. This process, called negative selection, was conducted five times to remove streptavidin-modified beads-binding peptides. After positive selection, cDNAs encoding peptides binding to PCSK9-immobilized beads were PCR-amplified. cDNAs were quantified using SYBR green-based qPCR. After final round of SELEX, libraries were sequenced by Sanger method or next-generation sequencing. Template focused DNA libraries for *in vitro* affinity maturation were prepared by PCR-amplification of template DNAs prepared by primer extension. The prepared template focused DNA libraries were transcribed to focused mRNA libraries by *in vitro* transcription using T7 RNAP.

PCSK9-binding analysis of cloned mRNA-displayed cyclic peptides

Template clone DNAs encoding peptide were PCR-amplified using Taq DNA polymerase. Cloned mRNA-displayed FNO_2Ph-cyclized and F_5Ph-cyclized peptides were prepared by transcription and translation at 37°C for 25 minutes using PURE system containing template clone DNA, puromycin-DNA linker, and FNO_2Ph-$tRNA^{ini}$ or F_5Ph-$tRNA^{ini}$. Cloned mRNA-displayed FNO_2Ph-cyclized and F_5Ph-cyclized peptides were reverse-transcribed, pulled down with PCSK9-immobilized beads and analyzed by quantification of cDNA using SYBR green-based qPCR.

Chemical peptide synthesis

Peptides with C-terminal biocytin were synthesized using standard solid-phase peptide synthesis with 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids on Rink amide resin. Amino acid coupling, FNO_2Ph coupling and F_5Ph coupling were performed using HBTU, HOBt, and DIEA. Peptides were deprotected and cleaved from resin using a mixture of H_2O, triisopropylsilane, 3,6-dioxa-1,8-octanedithiol, and trifluoroacetic acid. Cleaved peptides were filtered from resin, precipitated by centrifugation in diethyl ether and dried *in vacuo*. Linear peptides were cyclized by triethylamine in DMF, analyzed by MALDI-TOF MS and quantified by measuring absorbance at 280 nm using microspectrometer.

Chemical dimers of FNO_2Ph-cyclized peptides were synthesized using N-α,ε-di-Fmoc-L-lysine. Amino acid coupling, FNO_2Ph coupling and F_5Ph coupling were performed using HATU, HOAt and DIEA. Peptides were deprotected, cleaved, cyclized, analyzed by MALDI-TOF MS and quantified according to the above-mentioned method.

PCSK9-binding analysis of chemically synthesized cyclic peptides

Chemically synthesized biotinylated cyclic peptides were mixed with streptavidin-horseradish peroxidase (HRP) conjugate. Peptide-HRP complex was pulled down with PCSK9-immobilized beads. HRP substrate was added to PCSK9-pull-downed beads, and chemiluminescence was detected using LuminoGraph III (ATTO).

Chemically synthesized biotinylated cyclic peptides were immobilized on streptavidin-modified magnetic beads. His-tagged PCSK9 was mixed with anti-His-tag antibody-HRP conjugate. PCSK9-HRP complex was pulled down with peptide-immobilized beads. HRP substrate was added to

peptide-pull-downed beads, and chemiluminescence was detected using LuminoGraph III (ATTO).

PCSK9-detection with chemically synthesized PCSK9-binding cyclic peptides on PVDF membrane

Chemically synthesized biotinylated peptides were mixed with streptavidin-HRP conjugate. PCSK9 was spotted on PVDF membrane. Blocking of the PVDF membrane was performed using 5% BSA in TBS-T. The PVDF membrane was incubated with peptide-HRP complexes and washed three times using TBS-T. HRP substrate was added to the PVDF membrane, and chemiluminescence was detected using LuminoGraph III (ATTO).

Fluorescent imaging of PCSK9 on beads with chemically synthesized PCSK9-binding cyclic peptides

Chemically synthesized biotinylated cyclic peptides were mixed with streptavidin-Qdot 605 conjugate. Peptide-Qdot complexes were pulled down with PCSK9-immobilized beads. Qdot 605 fluorescence was imaged using fluorescence microscope (Leica).

Sandwich ELISA of PCSK9 with chemically synthesized PCSK9-binding cyclic peptides and anti-PCSK9 antibody

Anti-PCSK9 antibody was immobilized on Protein G-modified magnetic beads. The anti-PCSK9 antibody-immobilized beads were incubated with PCSK9 on ice for 10 min. Chemically synthesized biotinylated cyclic peptides were mixed with streptavidin-HRP conjugate. Peptide-HRP complexes were pulled down with the PCSK9-immobilized anti-PCSK9-beads. HRP substrate was added to pull-downed beads, and chemiluminescence was detected using LuminoGraph III (ATTO).

Determination of PCSK9-binding dissociation constant (K_D) by the Bio-layer Interferometry assay

PCSK9 binding kinetics was assayed by Bio-layer interferometry (BLI) using BLItz (ForteBio, Fremont, CA, USA) and performed according to the ForteBio's protocol. Chemically synthesized biotinylated PCSK9-binding peptides were immobilized on a streptavidin (SA) biosensor that had been hydrated for 10 minutes. His-tagged PCSK9 was diluted in HBS-T to a range of concentration (2000 nM–125 nM) for analytes. Each step in the binding kinetics assay was as follows: immobilization of peptides to SA biosensor for 150 s, association (binding of peptides to PCSK9) for 150 s, and dissociation for 150 s.

Supplemental Figures

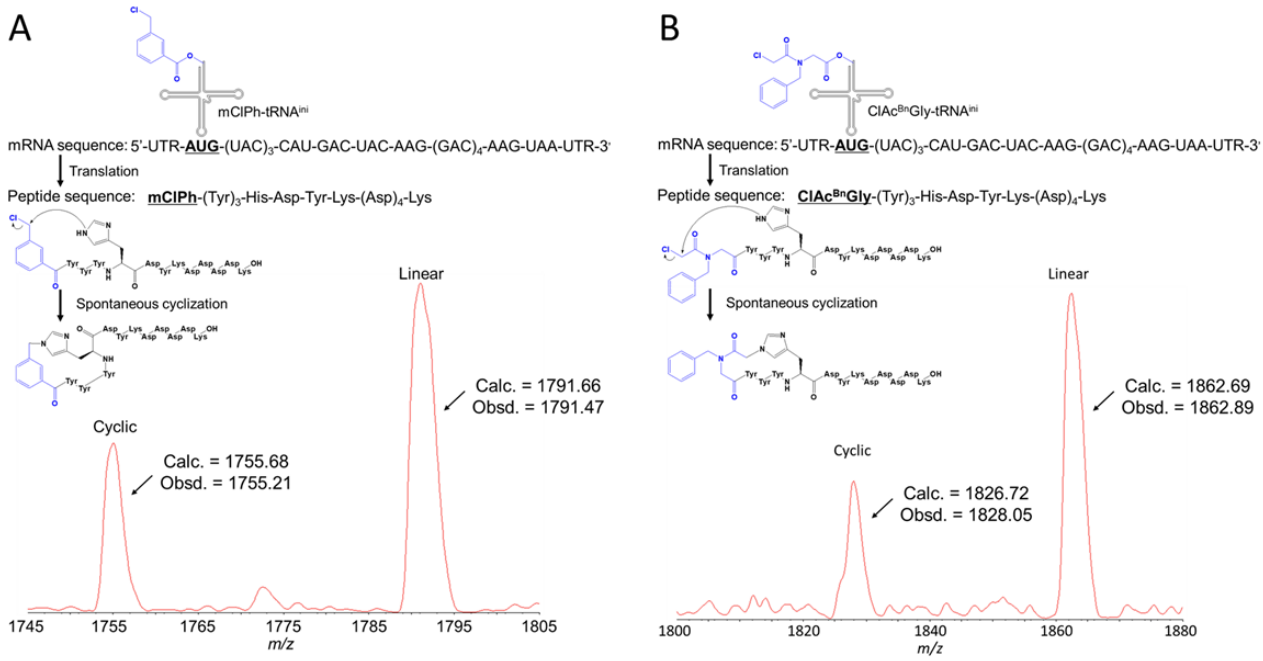


Fig. S1

Translation initiation with *m*-chloromethylbenzoic acid (mClPh) (A) and *N*-chloroacetyl-*N*-benzylglycine (ClAc^{Bn}Gly) (B) charged onto tRNA^{ini} to express mClPh- and ClAc^{Bn}Gly-initiated histidine-containing model peptides using PURE system and subsequent peptide cyclization with downstream histidine residue; MALDI-TOF mass spectrum of mClPh- and ClAc^{Bn}Gly-initiated (linear) and cyclized (cyclic) peptides. Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M+H]^+$ of linear and cyclic peptides are shown.

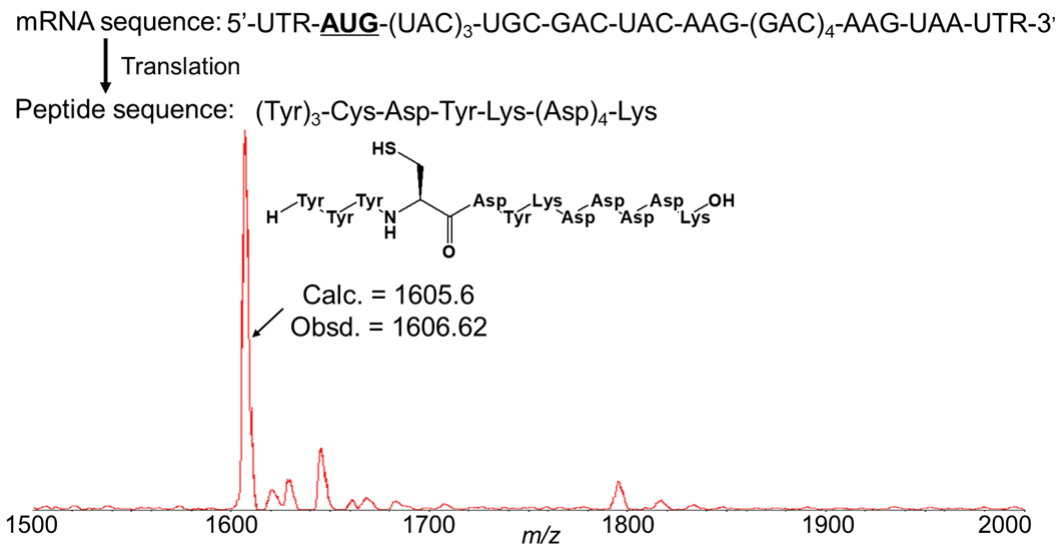


Fig. S2

Translation initiation in the absence of acyl-tRNA^{ini} to express a negative control cysteine-containing model peptide using PURE system; MALDI-TOF mass spectrum of the peptide. Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M+H]^+$ of the peptide are shown.

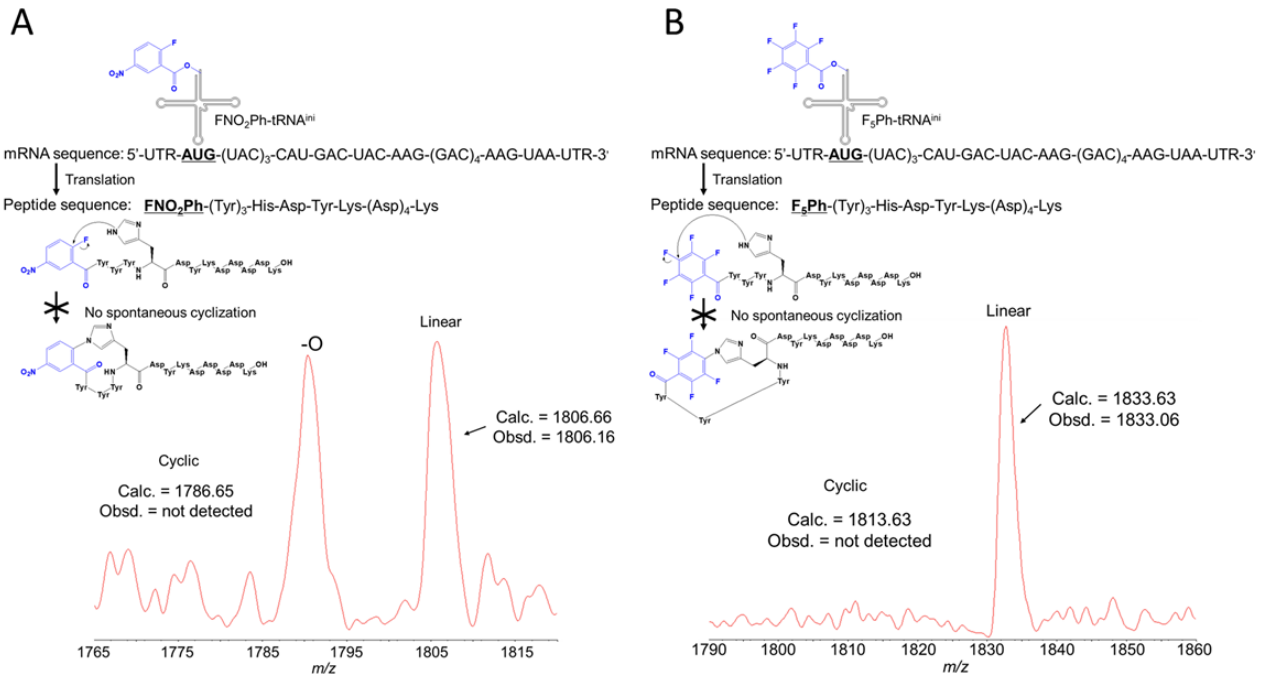


Fig. S3

Translation initiation with FNO_2Ph and F_5Ph charged onto $tRNA^{ini}$ to express FNO_2Ph- and F_5Ph-initiated histidine-containing model peptides using PURE system; MALDI-TOF mass spectrum of FNO_2Ph- and F_5Ph-initiated (linear) peptides. Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M+H]^+$ of peptides are shown. -O indicates 2-fluoro-5-nitroso-benzoic acid-initiated peptide generated by photodecomposition of NO_2 to NO during the MALDI-TOF MS analysis.

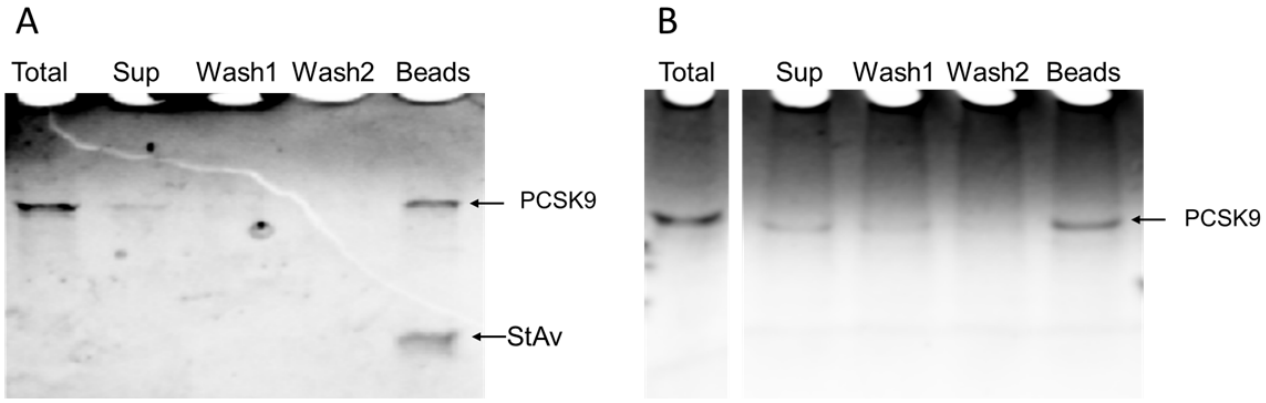
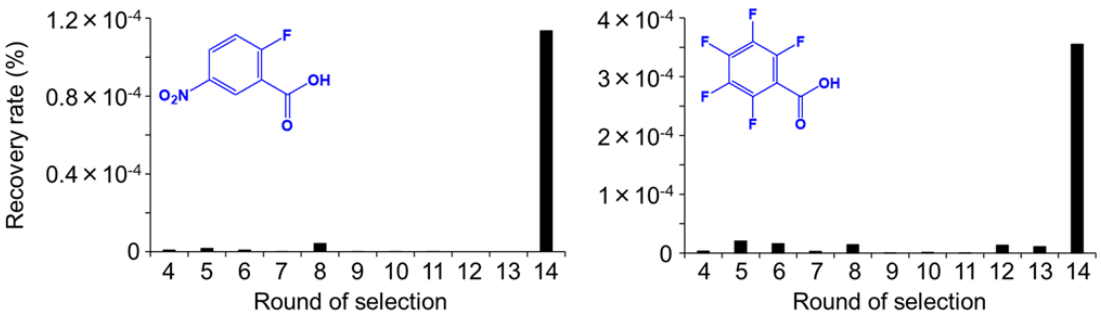


Fig. S4

Immobilization of PCSK9-Avi-His and PCSK9-Fc on beads confirmed by visualizing the protein using CBB staining after SDS-PAGE. (A) PCSK9-Avi-His, site-specifically biotinylated PCSK9 with a C-terminal Avi tag followed by polyhistidine tag was immobilized on streptavidin (StAv) beads. (B) PCSK9-Fc, PCSK9 with a C-terminal Fc region of mouse IgG1 was immobilized on Protein A beads.

A



B

Clone	Sequence	Frequency
1	FNO_2Ph-RWRFYSGPYFILAA-C	1/10
2	FNO_2Ph-RWRLYSGPYFILAA-C	1/10
3	FNO_2Ph-RWRFYSGPYFILAS-C	1/10
4	FNO_2Ph-RWRFYSGPYFILAL-C	1/10
5	FNO_2Ph-GWRFYSGPYFILAV-C	1/10
6	FNO_2Ph-VWRLYSGPYFILAV-C	1/10
7	FNO_2Ph-VWRLYSGPYFTLALS-C	1/10
8	FNO_2Ph-DWRFYSGPYFTLAWA-C	1/10
9	FNO_2Ph-GVKRIYVVYVLSV-C	1/10
10	FNO_2Ph-DGPLFLVLVVFRSSA-C	1/10

Clone	Sequence	Frequency
1	F_5Ph-RGHCWLYVYFPVRSL-C	1/13
2	F_5Ph-GGHCWLYVYFPVRSA-C	1/13
3	F_5Ph-RGHCWLYVYFPVRSV-C	1/13
4	F_5Ph-LGHCWLYVYFPVRSV-C	1/13
5	F_5Ph-VGHCWLYVYFPVRSL-C	1/13
6	F_5Ph-VGHCWLYVYFPVCSL-C	1/13
7	F_5Ph-RGHCWLYVYFPVCSL-C	1/13
8	F_5Ph-RAVIILYWRIPSL-C	1/13
9	F_5Ph-NAYSFWLYLRFPS-C	1/13
10	F_5Ph-GVIWIYFIRKRSV-C	1/13
11	F_5Ph-VGCVFVFVFYPQSS-C	1/13
12	F_5Ph-HVRIYVYFLTRTPA-C	1/13
13	F_5Ph-VGSRILCLIFLLRVV	1/13

Fig. S5

(A) Progress during SELEX of FNO_2Ph-cyclized (left) and F_5Ph-cyclized (right) peptides against PCSK9. The recovery of cDNAs in each round of SELEX was determined by RT-qPCR. (B) Peptide sequences identified by cloning and sequencing of DNA library in the final SELEX round.

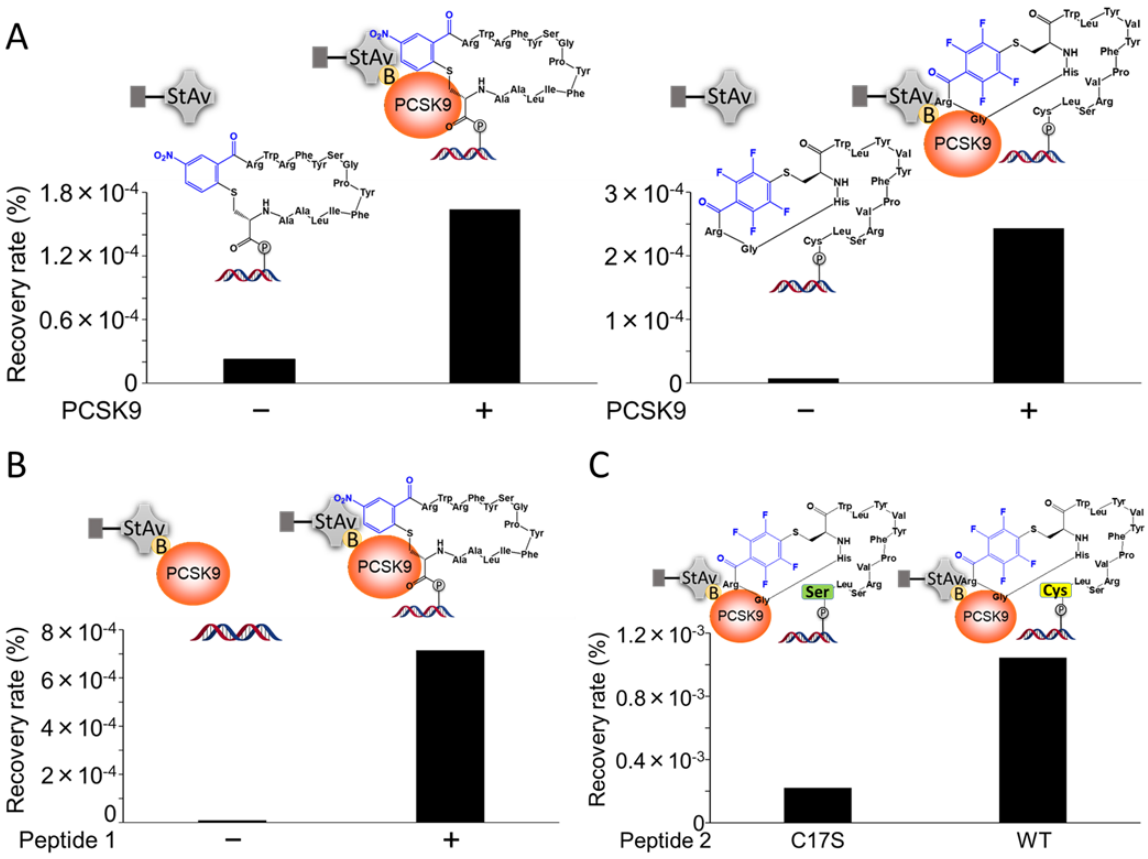


Fig. S6

PCSK9-binding analysis of mRNA-displayed FNO_2Ph-cyclized peptide 1 and F_5Ph-cyclized peptide 2 identified by SELEX. (A) PCSK9-binding analysis of mRNA-displayed FNO_2Ph-cyclized peptide 1 and F_5Ph-cyclized peptide 2 by quantification of cDNA recovered after pull-down with PCSK9-immobilized or nonimmobilized beads using qPCR. (B) PCSK9-binding analysis of mRNA-displayed FNO_2Ph-cyclized peptide 1 by quantification of cDNA recovered after PCSK9-pull-down with translation or without translation using qPCR. (C) PCSK9-binding analysis of mRNA-displayed F_5Ph-cyclized peptide 2 and C17S mutant by quantification of cDNA recovered after PCSK9-pull-down using qPCR.

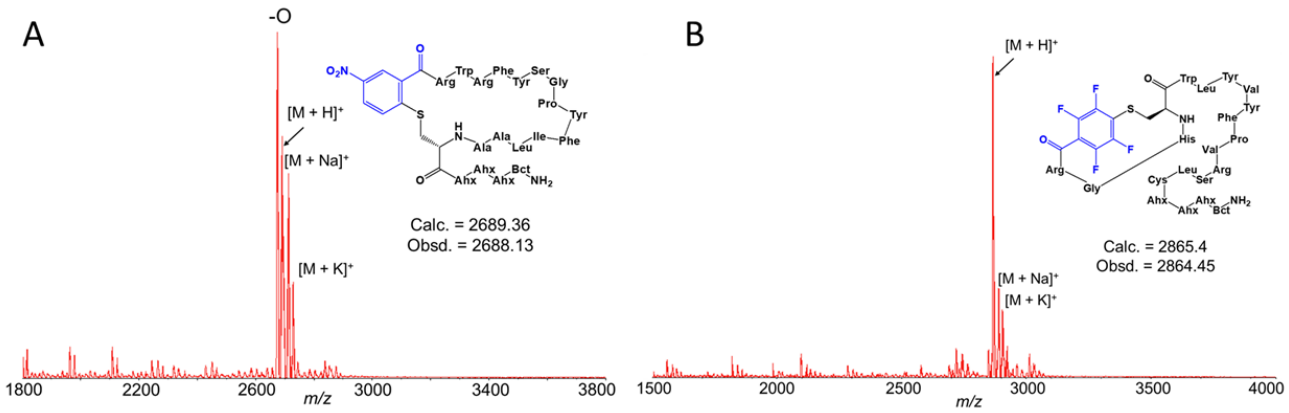


Fig. S7

MALDI-TOF MS of chemically synthesized FNO_2Ph-cyclized peptide 1 (A) and F_5Ph-cyclized peptide 2 (B) with C-terminal biocytin (Bct). Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M + H]^+$ of the desired peptides are shown. -O indicates nitroso-benzoic acid-cyclized peptide generated by photodecomposition of NO_2 to NO during the MALDI-TOF MS analysis.

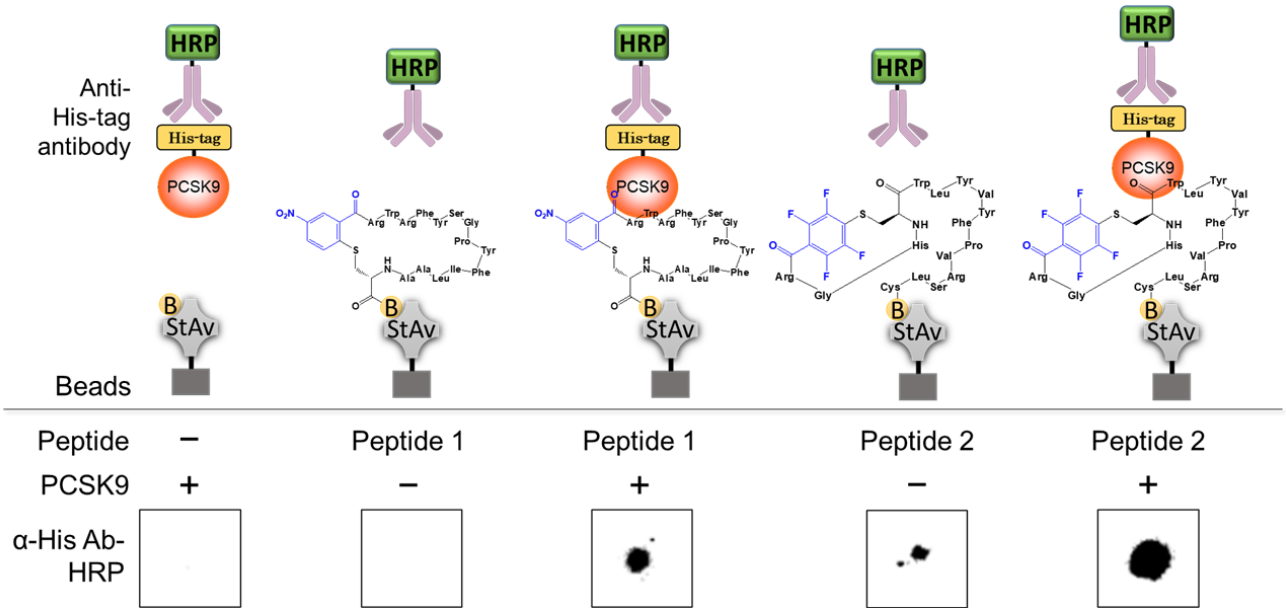


Fig. S8

PCSK9-binding analysis of FNO_2Ph-cyclized peptide 1 and F_5Ph-cyclized peptide 2 by peptide-pull-down of PCSK9 and chemiluminescent detection using HRP-labeling of PCSK9 via anti-His-tag antibody (α-His Ab).

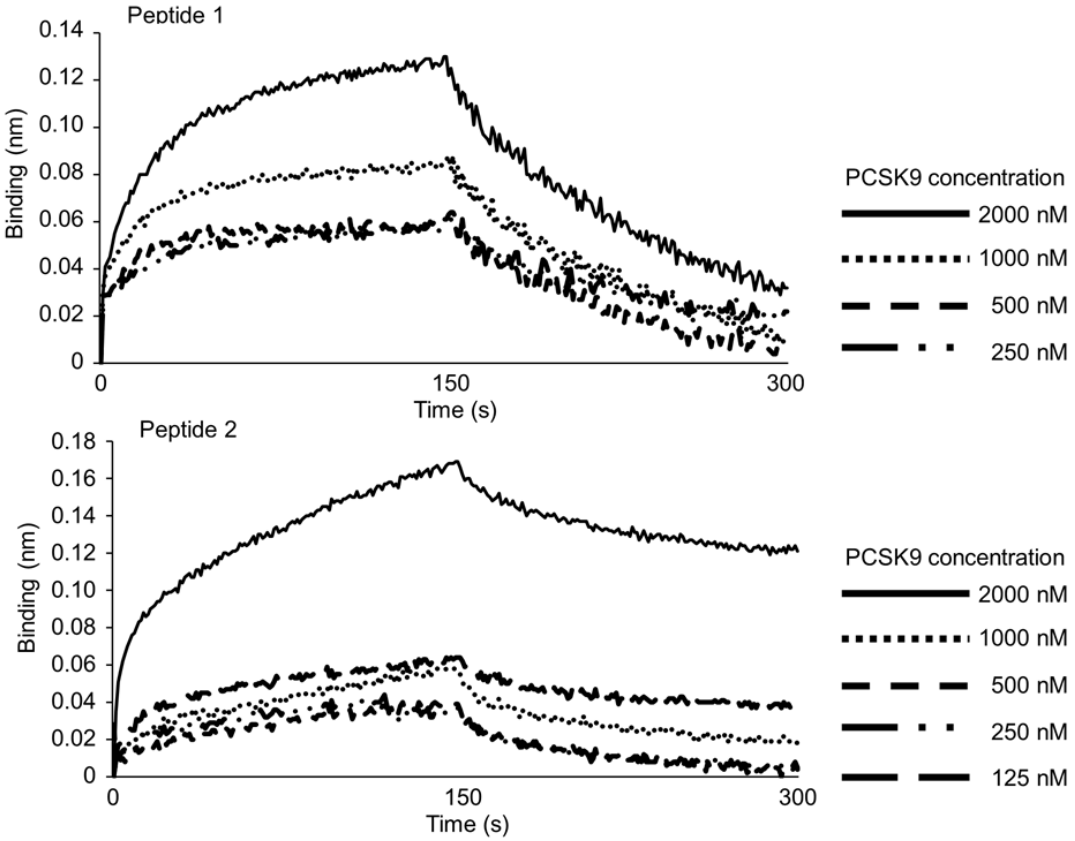


Fig. S9

PCSK9-binding kinetics of FNO_2Ph-cyclized peptide 1 and F_5Ph-cyclized peptide 2 was analyzed using the BLI assay. Representative sensorgrams obtained using BLI are shown. The dissociation constants (K_D) of the PCSK9-binding peptides were determined from the resulting BLI sensorgrams.

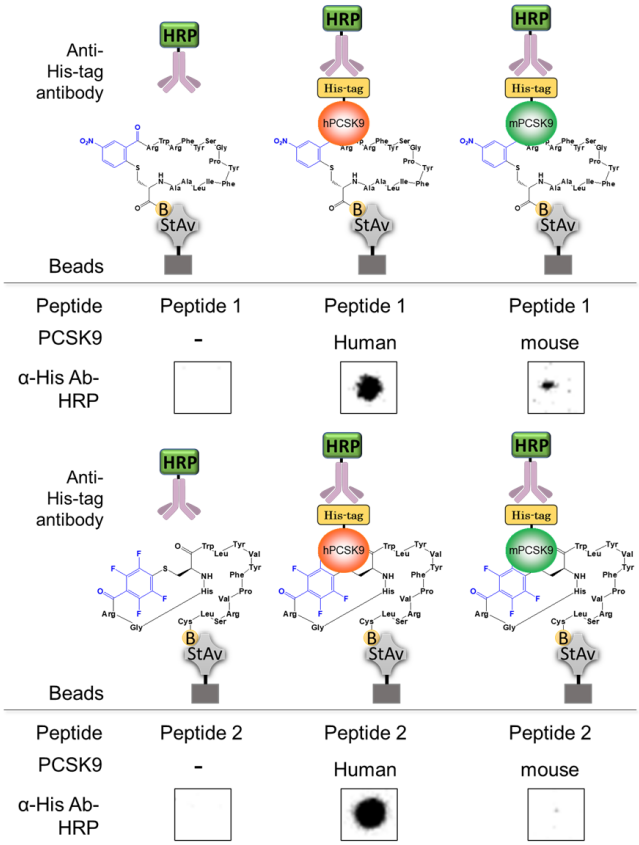


Fig. S10

PCSK9-binding analysis of FNO_2Ph-cyclized peptide 1 and F_5Ph-cyclized peptide 2 by peptide-pull-down of human PCSK9 and mouse PCSK9 and chemiluminescent detection using HRP-labeling of PCSK9 via anti-His-tag antibody (α-His Ab).

A

Original sequence	**FNO_2Ph-Arg-Trp-Arg-Phe-Tyr-Ser-Gly-Pro-Tyr-Phe-Ile-Leu-Ala-Ala-Cys**
N-terminal randomized	FNO_2Ph-X-X-X-X-X-X-X-Pro-Tyr-Phe-Ile-Leu-Ala-Ala-Cys
C-terminal randomized	FNO_2Ph-Arg-Trp-Arg-Phe-Tyr-Ser-Gly-X-X-X-X-X-X-X-Cys

B

Clone	Sequence	Frequency
1	FNO_2Ph-RWRFYSGMRNREMD-C	3513/12615
2	FNO_2Ph-RWRLYSGM	1936/12615
3	FNO_2Ph-RWRFYSGHKQEMDS-C	595/12615
4	FNO_2Ph-RWRFYSGGTKEMEY-C	438/12615
5	FNO_2Ph-RWRFYSGMRNREMA-C	276/12615
6	FNO_2Ph-RWRFYSGMRKUEMD-C	175/12615
7	FNO_2Ph-RWRFYSGUEMDSV	162/12615
8	FNO_2Ph-RWRFYSGRQKEMDV-C	153/12615
9	FNO_2Ph-RWRFYSGEPEREMA-C	150/12615

C Peptide 3 sequence: **FNO_2Ph-Arg-Trp-Arg-Phe-Tyr-Ser-Gly-Ile-Arg-Asn-Arg-Glu-Ile-Asp-Cys**

Fig. S11

In vitro affinity maturation of PCSK9-binding FNO_2Ph-cyclized peptide 1 by SELEX using mRNA-displayed focused libraries expressed by the PURE system. (A) Sequences of N and C-terminal randomized focused library. X represents random proteinogenic amino acids, excluding methionine. (B) Alignment of sequences obtained by NGS at the final SELEX round from the C-terminal randomized library. The most frequent sequence is shown above the graph. (C) Peptide 3 with the most frequent sequence determined by alignment analysis of the C-terminal randomized library at the final SELEX round.

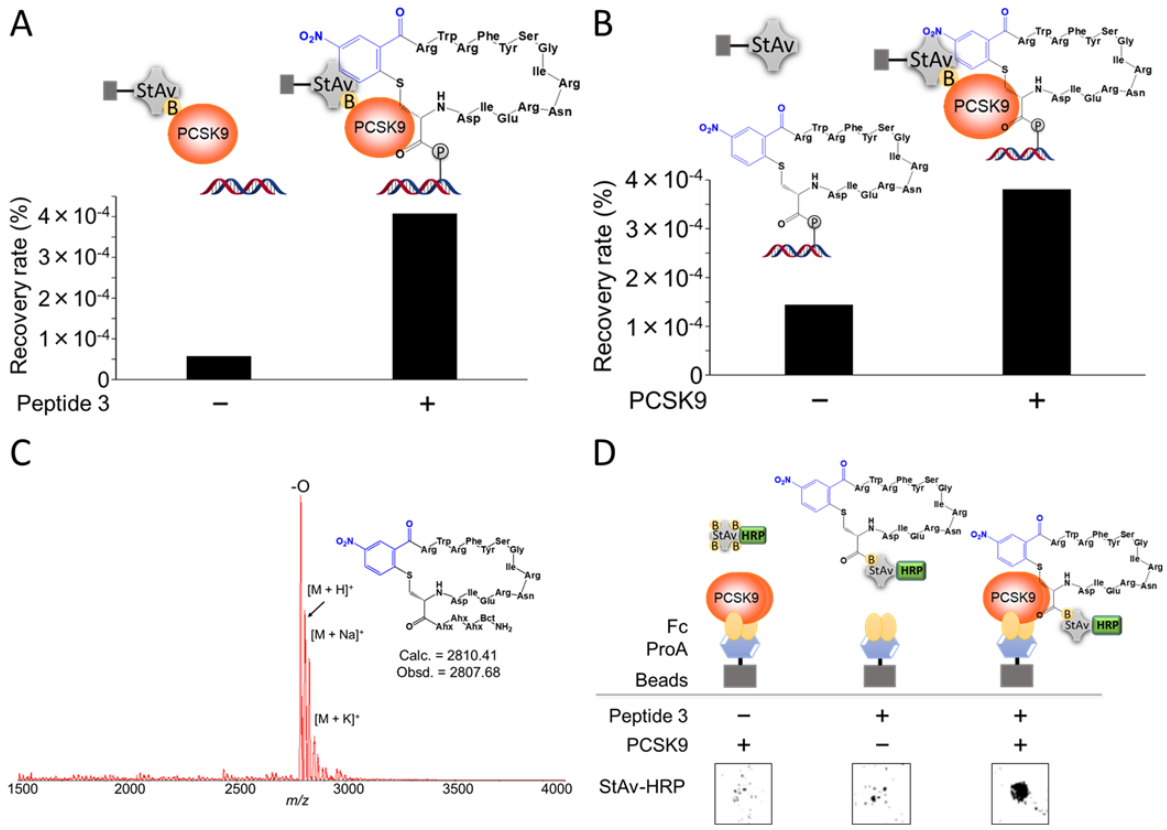


Fig. S12

PCSK9-binding analysis of unnatural FNO_2Ph peptide 3 identified by *in vitro* affinity maturation. (A) and (B) PCSK9-binding of mRNA-displayed FNO_2Ph-cyclized peptide 3 was analyzed by cDNA quantification after pull-down using qPCR. (C) MALDI-TOF MS of chemically synthesized FNO_2Ph-cyclized peptide 3 with C-terminal biocytin (Bct). Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M + H]^+$ of the desired peptide are shown. -O indicates nitroso-benzoic acid-cyclized peptide generated by photodecomposition of NO_2 to NO during the MALDI-TOF MS analysis. (D) PCSK9-binding of chemically synthesized FNO_2Ph-cyclized peptide 3 was assayed by PCSK9-pull-down and chemiluminescence detection using HRP-labeling via streptavidin (StAv).

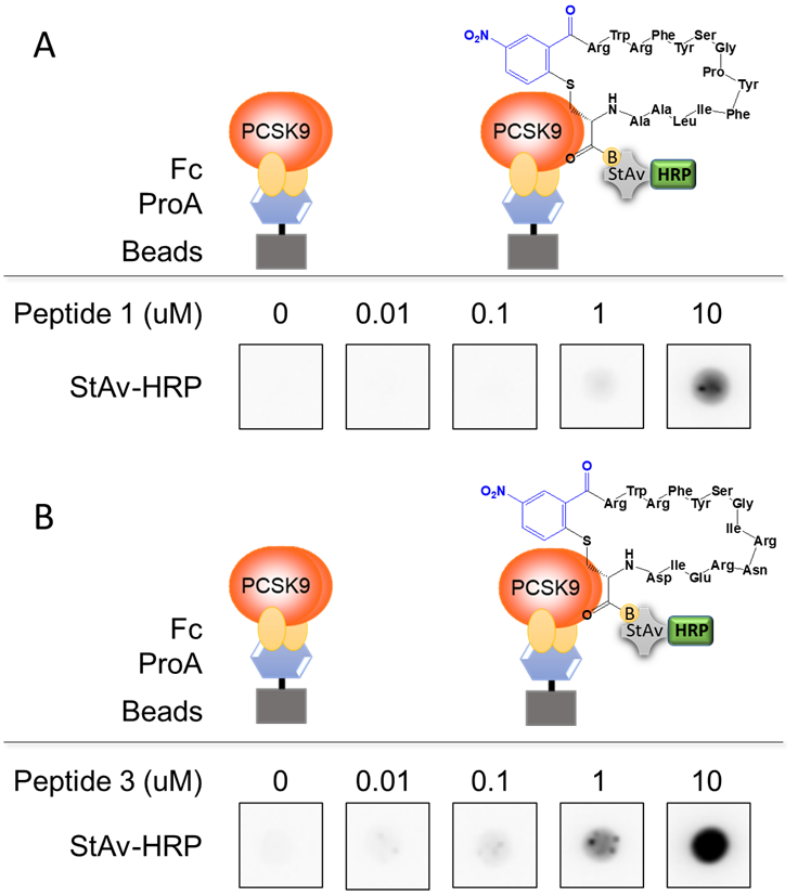


Fig. S13

Titration of PCSK9-binding FNO_2Ph-cyclized peptides 1 (A) and 3 (B) in the PCSK9-pull-down and chemiluminescence detection using HRP-labeling of the peptides.

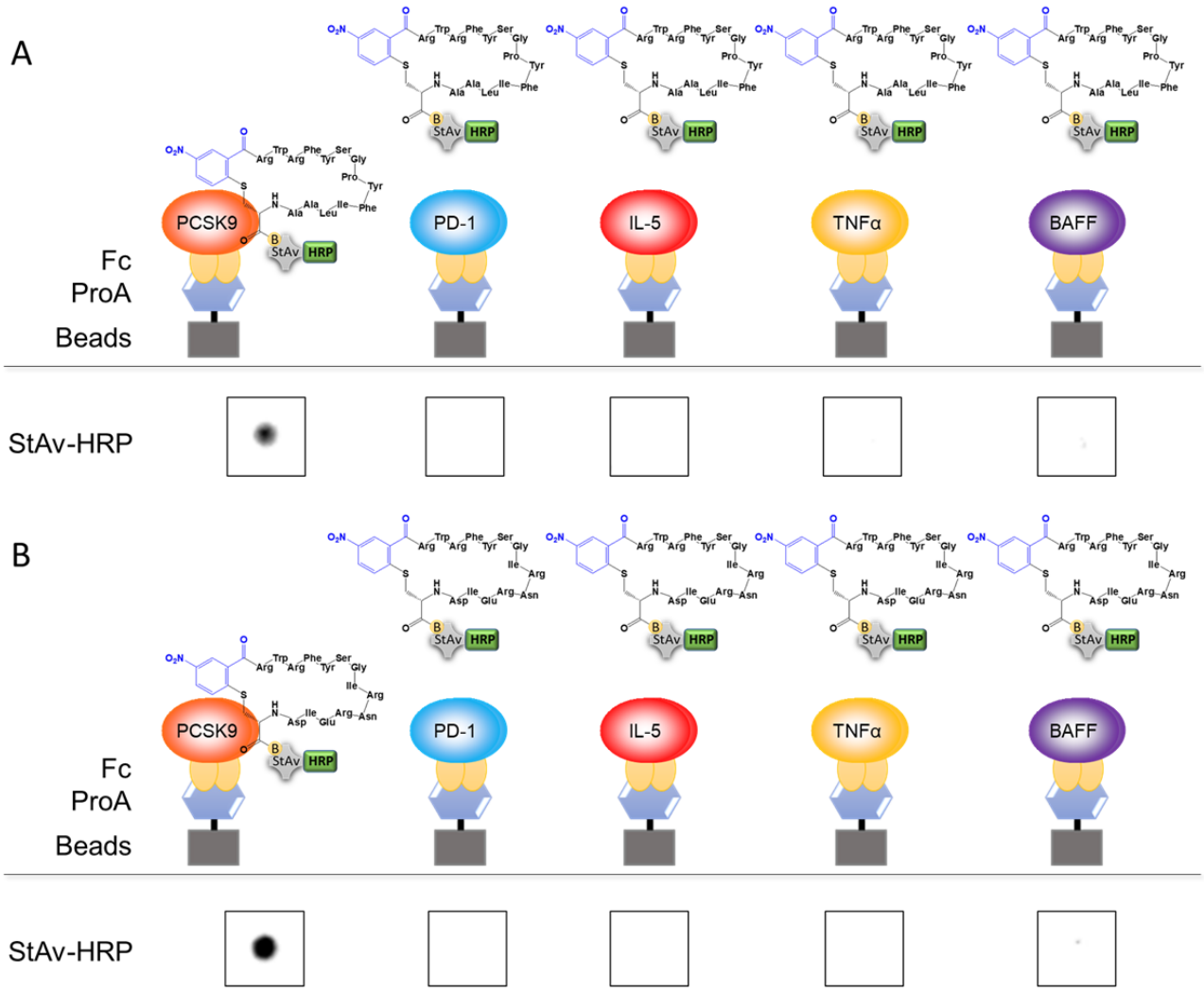


Fig. S14

Target-specificity of PCSK9-binding FNO_2Ph-cyclized peptides 1 (A) and 3 (B) assayed by the various protein-pull-down and chemiluminescence detection using HRP-labeling of the peptides.

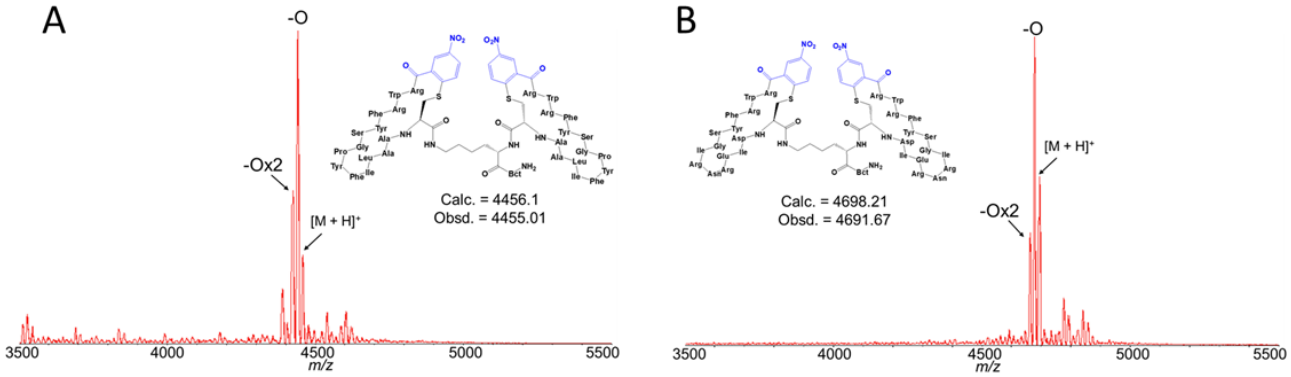


Fig. S15

MALDI-TOF MS of chemically synthesized dimers of FNO_2Ph-cyclized peptides 1 (A) and 3 (B) with C-terminal biocytin (Bct). Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M + H]^+$ of the desired peptide are shown. -O and -Ox2 indicate nitroso-benzoic acid-cyclized peptide generated by photodecomposition of one and two NO_2 to NO, respectively, during the MALDI-TOF MS analysis.

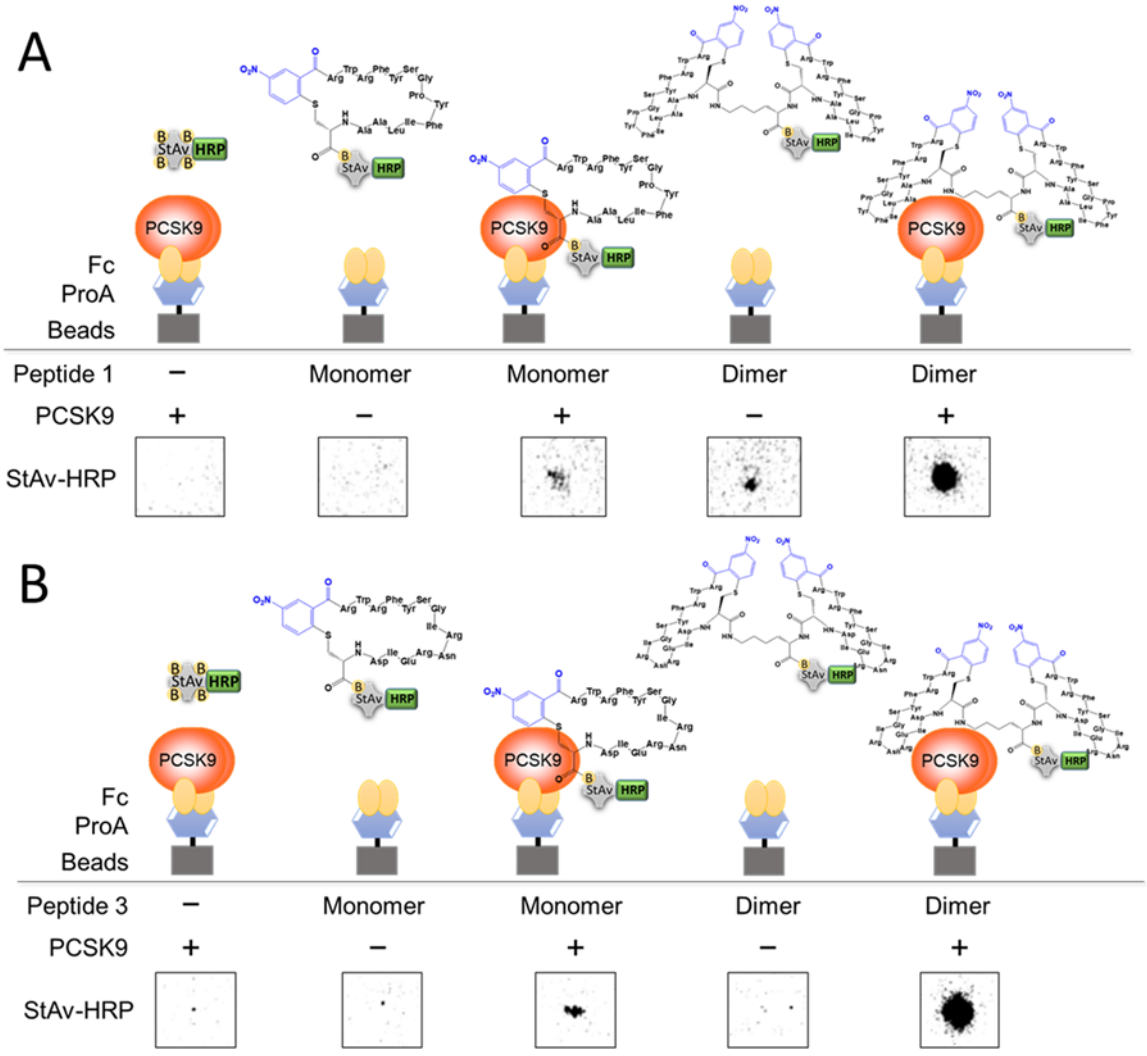


Fig. S16

(A) and (B) PCSK9-binding of chemically synthesized dimers of FNO_2Ph-cyclized peptides 1 and 3 was assayed by PCSK9-pull-down and chemiluminescence detection using HRP-labeling via streptavidin (StAv).

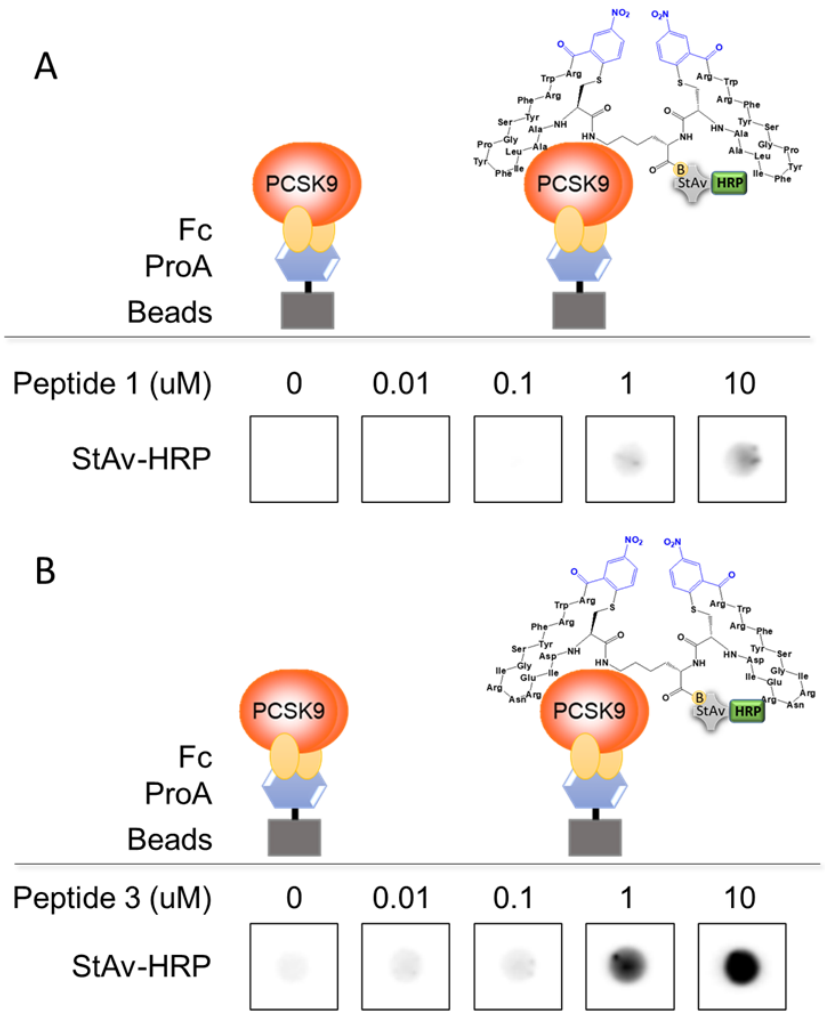


Fig. S17

Titration of dimeric PCSK9-binding FNO_2Ph-cyclized peptides 1 (A) and 3 (B) in the PCSK9-pull-down and chemiluminescence detection using HRP-labeling of the dimeric peptides.

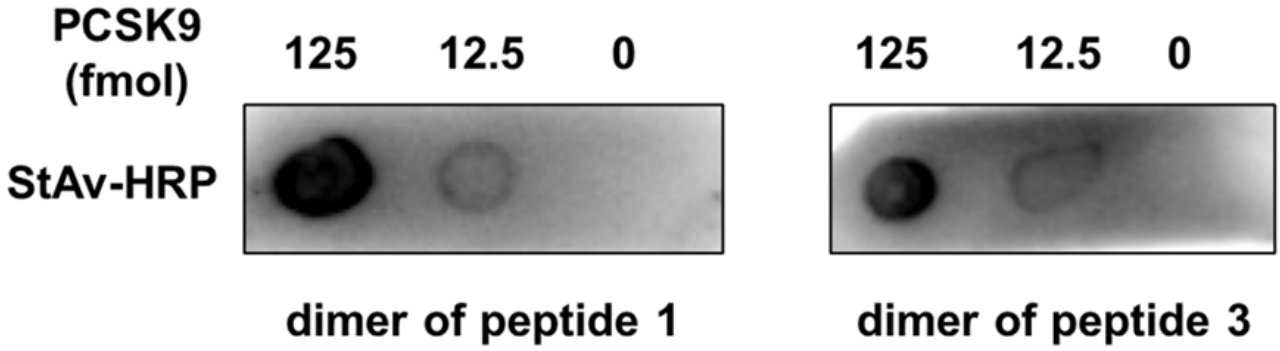


Fig. S18

Dot blotting of PCSK9 detected with chemical dimers of FNO_2Ph-cyclized peptides 1 and 3 by chemiluminescence using HRP-labeling of peptides via streptavidin (StAv).

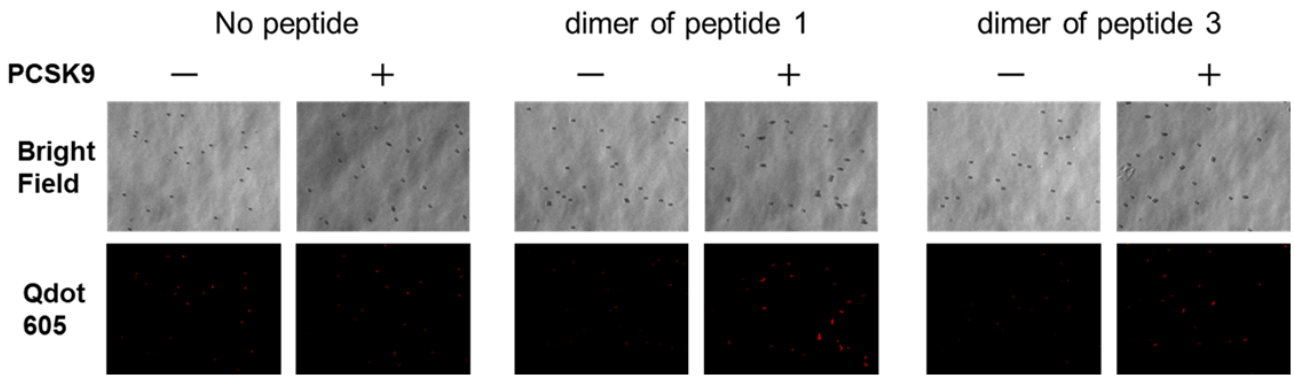


Fig. S19

Fluorescent images of PCSK9 immobilized on beads by chemical dimers of FNO_2Ph-cyclized peptides 1 and 3 labeled with Qdot 605 via streptavidin.

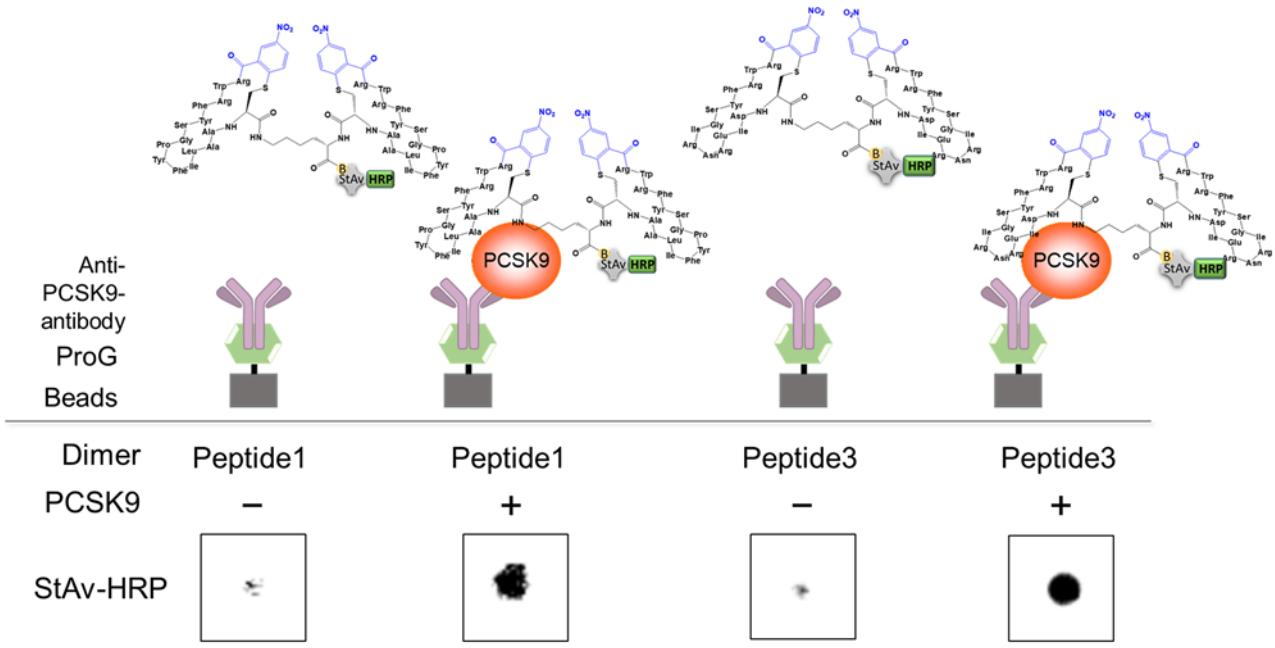


Fig. S20

Sandwich ELISA of PCSK9 with anti-PCSK9 antibody and chemical dimers of FNO_2Ph-cyclized peptides 1 and 3 detected by chemiluminescence using HRP-labeling of peptides via streptavidin (StAv).

Supplemental References

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