Efficient Delivery of Cyclic Peptides into Mammalian Cells with Short Sequence Motifs

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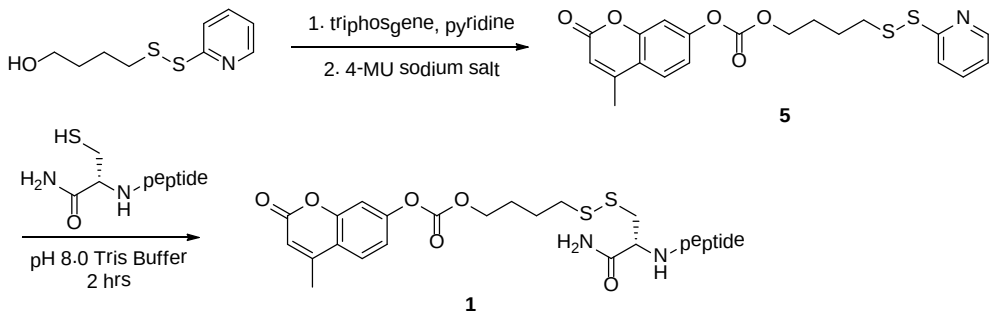
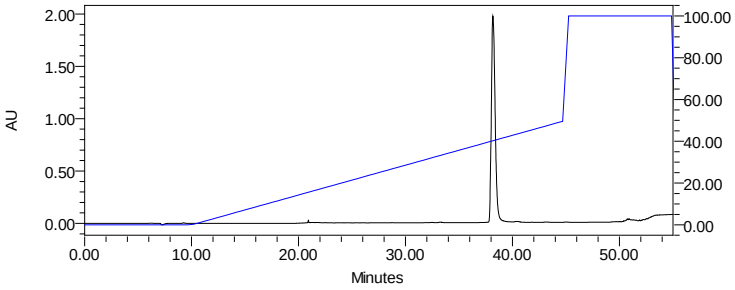
Supplementary Information

Synthesis of Labeling Reagent 5 (Caged 4-MU). To a solution of 4-S-(2'-thiopyridyl)mercaptobutanol (1) (129 mg, 0.6 mmol) in 2 mL of DCM was added dropwise to a 2 mL of cold DCM containing pyridine (48.3 µL, 0.6 mmol). The resulting solution was added dropwise over 30 min to a solution of triphosgene (0.2 mmol, 59.4 mg) dissolved in 5 mL of cold DCM, which had been chilled on an ice/brine bath. The mixture was stirred for 1 h at -10 ^{o}C. The DCM was evaporated *in vacuo* to obtain the crude chloroformate product. A solution of pyridine (96.6 µL, 1.2 mmol) in 2 mL of cold THF was added dropwise to an ice-cold solution of 4-methylumbelliferone sodium salt (119 mg, 0.6 mmol) in 5 mL of THF. The cocktail was then added dropwise into the flask containing the crude chloroformate product at -10 ^{o}C. After 1 h, the reaction mixture was washed with 30 mL of 1% TFA solution. The aqueous layer was back extracted with 30 mL of DCM and the combined organic phases were evaporated *in vacuo*. The crude product was purified by flash chromatography on a silica gel column eluted with 10 % ethyl acetate in hexane to produce a slightly yellow solid (82 mg, 33% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.46-8.43 (m, 1H), 7.74-7.57 (m, 3H), 7.22-7.05 (m, 3H), 6.27-6.25 (s, 1H), 4.26 (t, 2H, *J* = 6.08 Hz), 2.84 (t, 2H, *J* = 6.72 Hz), 2.42 (s, 3H), 1.85-1.82 (m, 4H) HRMS: calculated for $C_{20}H_{20}NO_5S_2$ ($M+H^+$) 418.0783; found 418.0784.

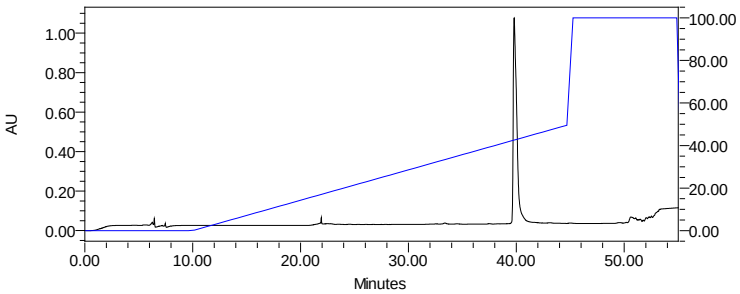


Synthesis of 4-MU-Labeled CPPs (Compound 1). Peptides R_9, $F\Phi R_4$, and $cF\Phi R_4$ were synthesized by standard Fmoc chemistry to contain a cysteine added to either the C-terminus or the side chain of invariant Gln residue. After deprotection, the peptides were purified by HPLC; the fraction containing the desired peptide was neutralized with pH 8.0 250 mM Tris buffer and directly added to a solution of 2 equiv of reagent **5** in a minimal volume of THF. The resulting mixture was shaken under argon protection for 2 h. Then the reaction mixture was concentrated *in vacuo* and fractionated by HPLC on a C18 column (eluted with a linear gradient of acetonitrile in water containing 0.05% TFA) to isolate the labeled peptides. The authenticity of each conjugated peptide was confirmed by MALDI-TOF mass spectrometry.

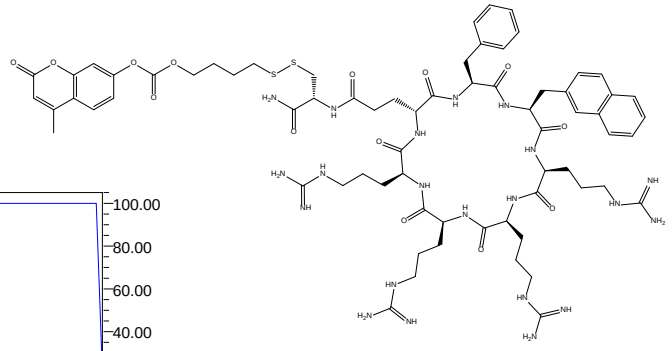
Ac-R_9-Cys(4-MU)-$CONH_2$
Purity: 99% (by analytical HPLC)
MALDI-MS: Calculated for $C_{74}H_{133}N_{38}O_{16}S_2$
$[M+H^+]$ 1874.02; found 1874.45

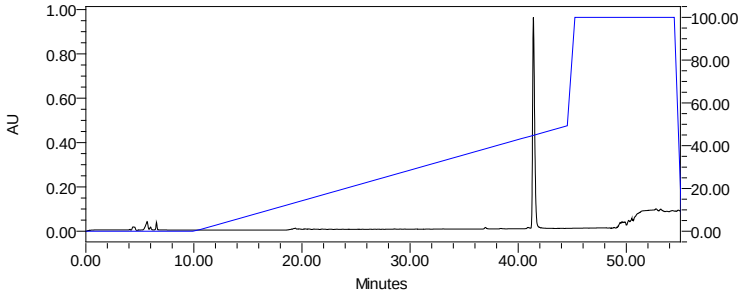


Ac-FΦR_4-Cys(4-MU)-$CONH_2$
Purity: >99% (by analytical HPLC)
MALDI-MS: Calculated for $C_{74}H_{104}N_{21}O_{16}S_2$
$[M+H^+]$ 1606.74; found 1607.08

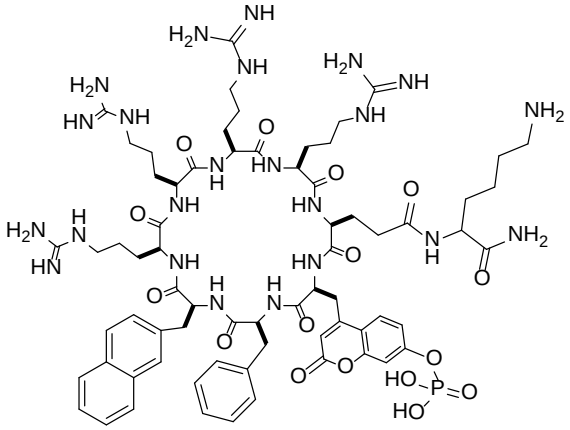


cFΦR_4-Cys(4-MU)-$CONH_2$
Purity: >99% (by analytical HPLC)
MALDI-MS: Calculated for $C_{69}H_{96}N_{21}O_{14}S_2$
$[M+H^+]$ 1506.69; found 1506.82





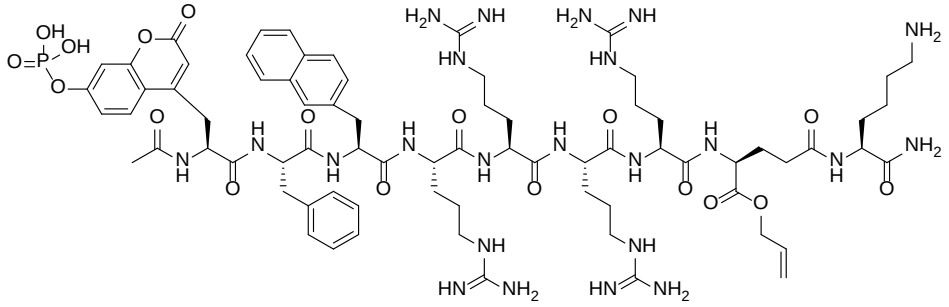
Cyclo[(pCAP)FΦRRRRQ] (Cyclic pCAP)
Purity: >99% (by analytical HPLC)
MALDI-MS: Calculated for $C_{69}H_{98}N_{23}O_{16}P$
$[M+H^+]$ 1537.63; found 1537.50



Ac-(pCAP)FΦRRRRQ (Linear pCAP)

Purity: >99% (by analytical HPLC)

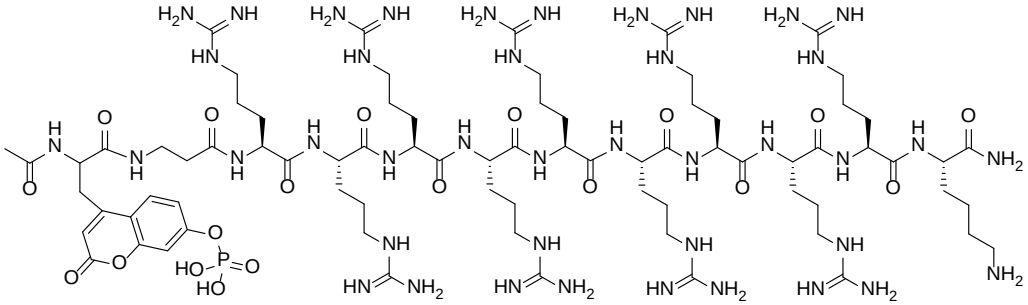
MALDI-MS: Calculated for $C_{74}H_{106}N_{23}O_{18}P$ [M+H$^+$] 1637.75; found 1637.30



Ac-(pCAP)-βAla-RRRRRRRRRK (R_9-pCAP)

Purity: >99% (by analytical HPLC)

MALDI-MS: Calculated for $C_{77}H_{140}N_{41}O_{19}P$ [M+H$^+$] 1975.17; found 1975.75



Chemical Formula: $C_{77}H_{140}N_{41}O_{19}P$

Molecular Weight: 1975.1726

Figure S1. Comparison of the cellular association efficiencies of FITC-labeled linear (No. 1, 3, 5, 7, and 9) and cyclic peptides (No. 2, 4, 6, 8, and 10) by HEK293 cells (as measured by the conventional assay). The data reported are relative to that of R_9 (peptide No. 1) and represent the mean ± SD for three parallel experiments.

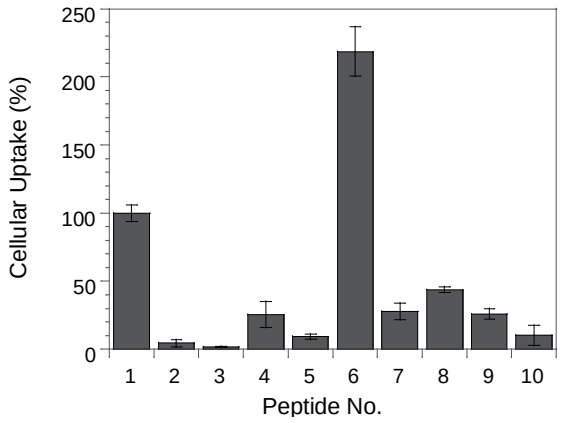


Figure S2. Internalization kinetics of peptides cFΦR$_4$, FΦR$_4$, and R$_9$ (5 μM) into MCF-7 cells at 37 ºC (by the 4-MU assay). Data are presented to show the kinetics after 150 min.

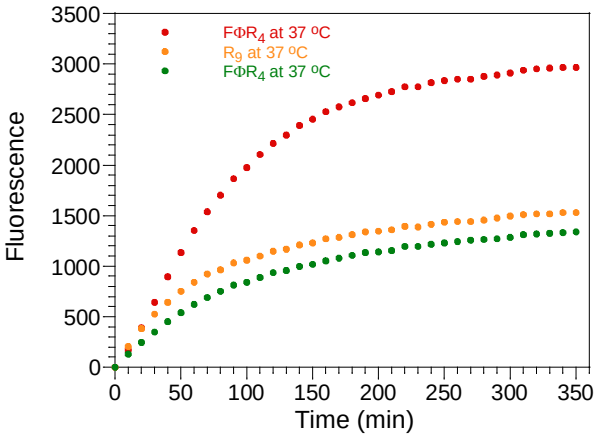


Figure S3. Release of 4-MU from compound **1** triggered by DTT (cell free). Peptide Ac-R$_9$-Cys(4-MU)-CONH$_2$ in PBS was rapidly mixed with DTT stock solution to give final solution of 1 mM DTT, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2.0 mM KH$_2$PO$_4$ (pH 10) and the increase in absorbance at 360 nm was monitored on a Perkin Elmer Lambda 20 UV/VIS spectrometer. The absorbance reached plateau value within 5-10 min, indicating complete decomposition.

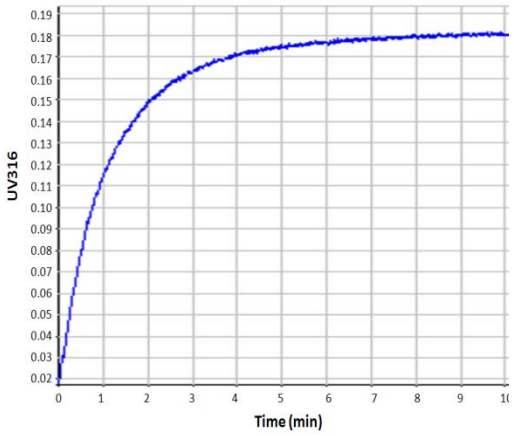
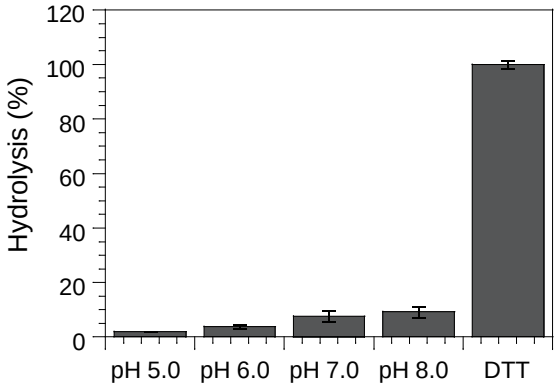


Figure S4. Background hydrolysis of the carbonate ester of compound **1** at different pH. Ac-R$_9$-Cys(4-MU)-CONH$_2$ (1 μM) was incubated for 2 h at 37 °C in 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2.0 mM KH$_2$PO$_4$ of different pH. After the incubation, the pH was adjusted to 8.0 and the amount of released 4-MU was measured on a Molecular Devices Spectramax M5 plate reader (with excitation and emission wavelengths at 360 and 450 nm, respectively). Experiments are performed in triplicates and a positive control experiment was performed in the presence of 1 mM DTT. The reported amounts of hydrolysis were relative to that of DTT treatment (100%).



Procedure for Figures S5 and S6: Approximately 5 x 10^4 A549 cells were seeded in 35-mm glass-bottomed microwell dish (MatTek) containing 1 mL of phenol-red free media and cultured overnight. On the day of experiment, growth media was removed and the cells were gently washed with PBS twice. The cells were preincubated with 75 mM sodium azide in serum-free media for one hour to deplete ATP synthesis. In the case of low temperature study, the cells were preincubated at 4 ^{o}C for 30 min. Subsequent live-cell confocal microscopy was performed as described in the main text.

Figure S5. Live-cell confocal microscopic images of A549 cells treated with cFΦR$_4$-FITC (5 μM) and endocytosis marker rhodamine B-dextran (1 mg/mL) at 4 ºC, followed by the nuclear stain DRAQ5. A, nuclear stain with DRAQ5; B, red fluorescence of rhodamine B-dextran; C, green fluorescence of cFΦR$_4$-FITC; and D, a merge of images A-C. The experiment showed that cells did not have detectable uptake of cyclic peptide or endocytosis marker at 4 ºC.

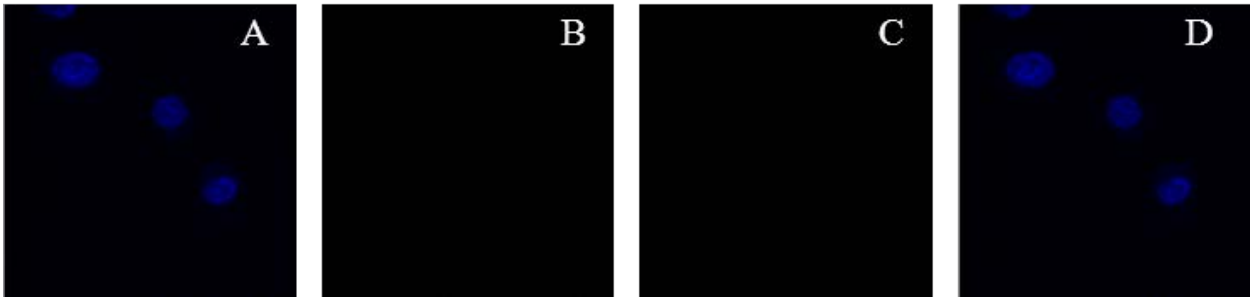
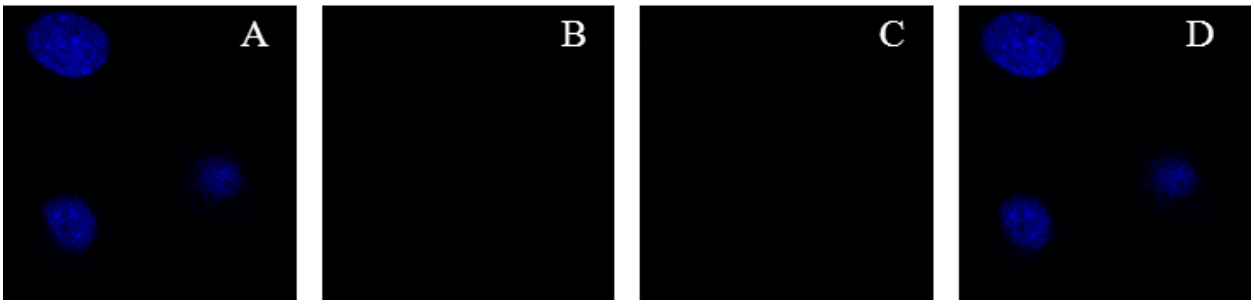


Figure S6. Live-cell confocal microscopic images of A549 cells which had been pretreated with 75 mM NaN_3 for 1 h and then with cFΦR$_4$-FITC (5 μM) and endocytosis marker rhodamine B-dextran (1 mg/mL) at 37 ^{o}C, followed by the nuclear stain DRAQ5. A, nuclear stain with DRAQ5; B, red fluorescence of rhodamine B-dextran; C, green fluorescence of cFΦR$_4$-FITC; and D, a merge of images A-C. The experiment demonstrated that pretreatment with NaN_3 blocked the uptake of cyclic peptide or endocytosis marker.



Procedure for Figures S7 and S8: Approximately 8 x 10^3 MCF-7 cells (or 3.5 x 10^3 HEK293 cells) were seeded in a 4-well chamber slide (Lab-Tek) containing 1 ml of media and cultured for two days. On the day of experiment, 500 μL of an FITC-labeled peptide solution (2 μM) in HKR buffer was added to the cells and incubated at 37 °C for 30 min in the presence of 5% CO_2. In a parallel experiment, rhodamine B-labeled dextran (1 mg/ml) was added along with the peptide. The peptide solution was removed by aspiration and the cells were gently washed twice with 1 mL of HKR buffer (three times for the rhodamine B-dextran sample) and twice with 1 mL of PBS. The resulting cells were fixed by treatment with 500 μL of 4% paraformaldehyde for 20 min and washed with PBS (4 times). In a parallel experiment, Hoechst dye 33342 was added to a final concentration of 3 μg/ml in PBS. After incubation for 10 min at room temperature, the cells were washed twice with PBS and twice with doubly distilled water. The chamber slide was sealed with a coverslip and subjected to confocal imaging on an Olympus FV1000-Filter Confocal Microscope.

Figure S7. Fixed-cell confocal microscopic images of MCF-7 cells treated with different FITC-labeled CPPs and markers. (A) Cell treated with cF_2R_4-FITC, Hoescht dye 33342, and endocytosis marker rhodamine B-dextran in the same Z-section. I, DIC image; II, nuclear stain with Hoescht 33342; III, red fluorescence of rhodamine B-dextran; IV, green fluorescence of internalized cF_2R_4-FITC; and V, merge of images I-IV. (B) Cells treated with F_2R_4-FITC. I, DIC; II, green fluorescence of F_2R_4-FITC; and III, merge of I and II. (C) Cells treated with R_9-FITC. I, DIC; and II, fluorescence of R_9-FITC.

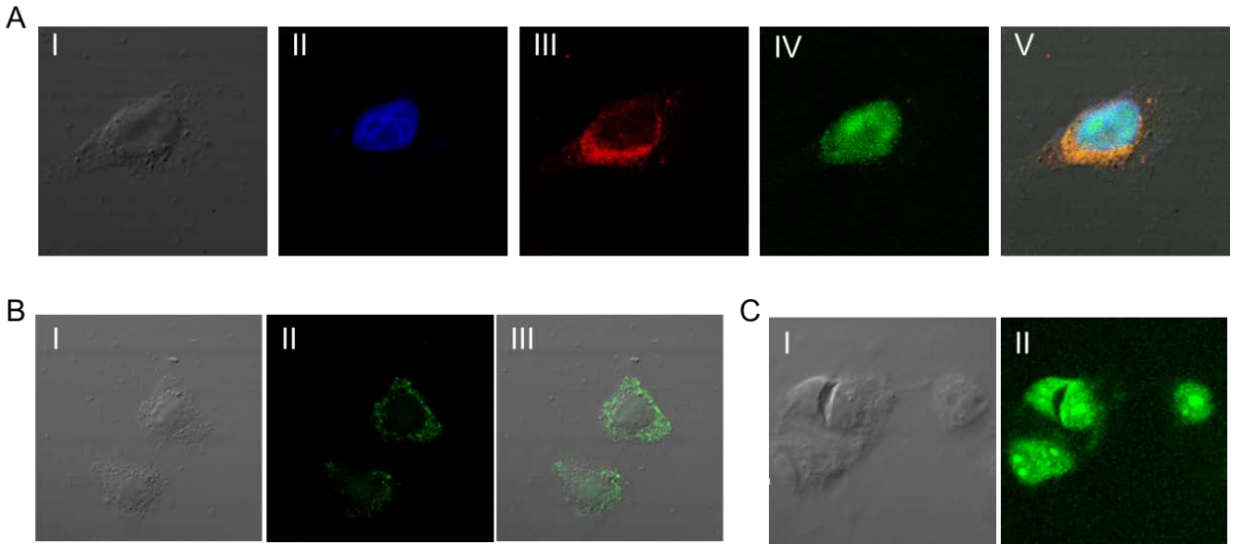


Figure S8. Fixed-cell confocal microscopy of HEK293 cells treated with cF_2R_4-FITC, Hoescht dye 33342, and rhodamine B-dextran. A, DIC; B, nuclear stain by Hoescht 33342; C, rhodamine B-dextran distribution in cells; D, cF_2R_4 distribution in cells; and E, a merge of D1-D4.

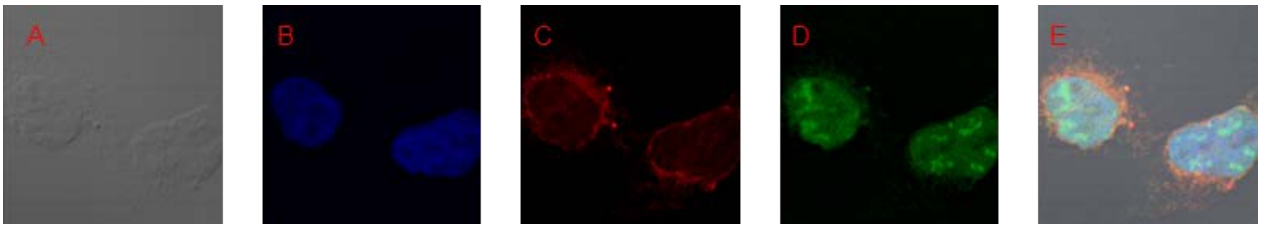


Figure S9. Fixed-cell confocal microscopic images of HEK293 cells treated with pCAP-containing peptides (5 μM cyclic or linear pCAP). (A) Cells treated with cyclic pCAP and DRAQ5 in the same Z-section. I, DIC image; II, nuclear stain with DRAQ5; III, blue fluorescence of CAP (dephosphorylation product); IV, merge of images II and III. (B) Cells treated with linear pCAP. I, DIC image; II, nuclear stain with DRAQ5; III, blue fluorescence of CAP; IV, merge of images II and III. (C) Cells treated with 1 mM sodium pervanadate and then cyclic pCAP. I, DIC image; II, nuclear stain with DRAQ5; III, blue fluorescence of CAP; IV, merge of images II and III.

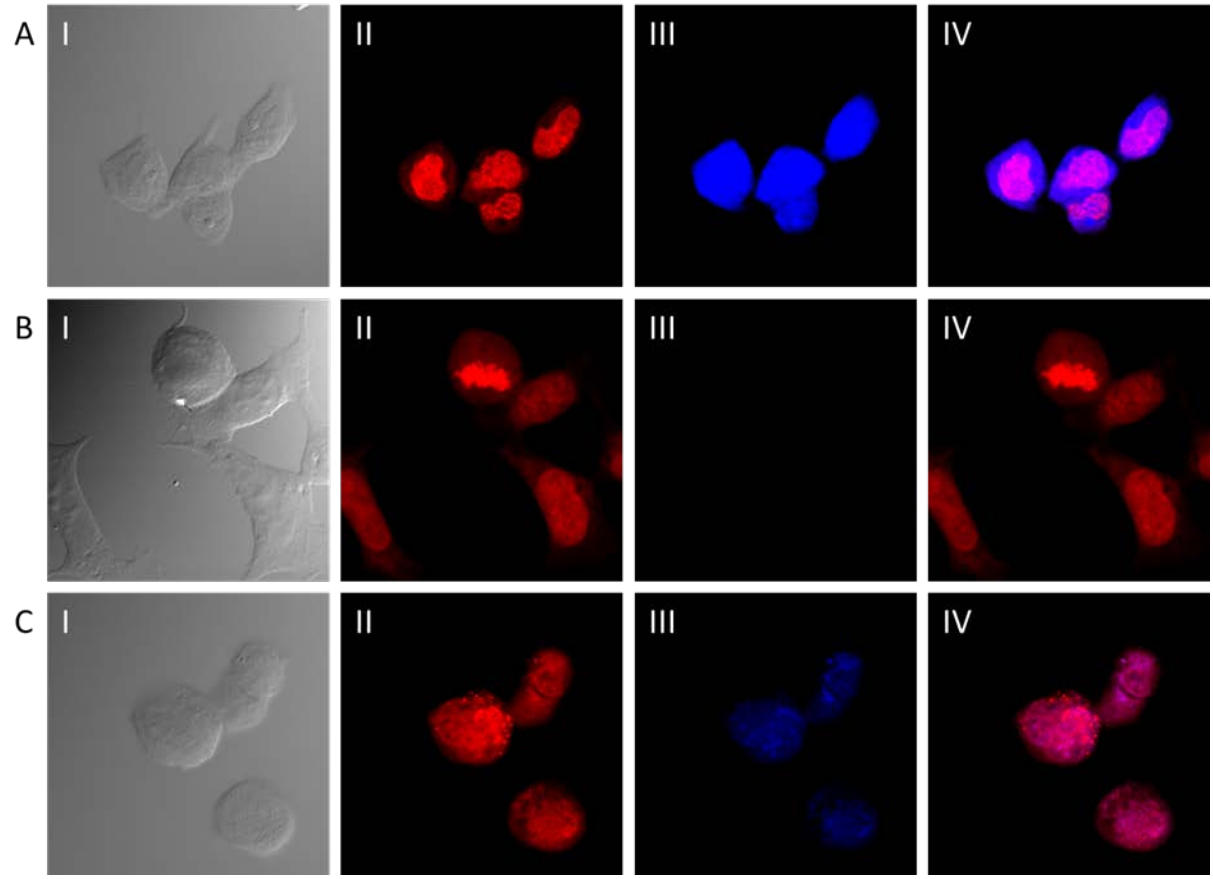


Table S1. Catalytic Activities (k_{cat}/K_M , M^{-1} s^{-1}) of Recombinant PTPs toward pCAP-Containing Peptides[a]

PTP	Cyclic pCAP	Linear pCAP	R_9-pCAP
PTP1B	4,300	540	499
TCPTP	1,240	670	147
SHP2	381	214	50
RPTPα	322	822	236
VHR	10,400	26,000	5,900

[a]Recombinant PTPs were expressed in *Escherichia coli*, purified, and assayed against peptide substrates as described previously[1].

1. Ren, L., Chen, X., Luechapanichkul, R., Selner, N. G., Meyer, T. M., Wavreille, A., Chan, R., Iorio, C., Zhou, X., Neel, B. G., Pei, D. (2011) Substrate specificity of protein tyrosine phosphatases 1B, RPTPα, SHP-1, and SHP-2. *Biochemistry 50*, 2339-2356.