

## Electronic Supplementary Information

### Self-assembling mini cell-penetrating peptides enter by both direct translocation and glycosaminoglycan-dependent endocytosis

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#### Abbreviations

Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; CHO cells, Chinese hamster ovary cells; CPP, cell-penetrating peptide; DAPI, 4',6-diamidino-2-phenylindole; DCC, dicyclohexylcarbodiimide; DLS, Dynamic light scattering; DMEM, Dulbecco's modified Eagle medium; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DTT, dithiotreitol; FBS, fetal bovine serum; EDTA, ethylenediamine tetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole;  $^1\text{H}$ -PKCi, non deuterated PKCi;  $^2\text{H}$ -PKCi, deuterated PKCi; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBHA-PS, 4-methylbenzhydrylamine polystyrene; Npys, 3-nitro-2-pyridinesulphenyl; PBS, phosphate buffered saline; PKCi, protein kinase C inhibitor; rt, room temperature; SEM, standard error of the mean; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

#### Reagents

Coupling reagents for peptide synthesis and protected amino acids were purchased from Novabiochem (Merck Chemicals Ltd) except for (2,2-D<sub>2</sub>, 98%)-Boc Glycine, which was obtained from Euriso-top. Solvents for peptide synthesis and TFA were obtained from SDS. Tris-HCl, Triton X-100, trypsin inhibitor and bovine serum albumin were obtained from Sigma-Aldrich. Dulbecco's modified Eagle medium, fetal bovine serum, trypsin-EDTA (0.05 % trypsin, 0.02 % EDTA) and Hank's BSS were purchased from PAA. The cell counting kit (CCK8) was from Dojindo Laboratories. Streptavidin-coated magnetic beads (Dynabeads® MyOne™ Streptavidin C1 or Dynabeads® M-280 Streptavidin) were purchased from Invitrogen. The complete mini tablets of protease inhibitors were from Roche. DAPI was from Pierce.

#### Peptide synthesis

##### *Cysteamino-Merrifield resin*

Merrifield resin (0.5 g, loading 1.09 mmol/g) was swollen in DMF. Cysteamine hydrochloride (0.186 g, 5.45 mmol) was added to an ice-cooled suspension of NaH 60 % in mineral oil (0.218 g, 5.45 mmol) in dry DMF (1.5 mL). The resulting mixture was stirred at rt until the generation of hydrogen gas stopped (about 20 min) and then added to the Merrifield resin in DMF. The mixture was shaken for 48 h at rt or heated at 50 °C under micro-waves (10 x 5 seconds, 60 W). The resin was washed with CH<sub>2</sub>Cl<sub>2</sub>, CH<sub>3</sub>OH, H<sub>2</sub>O and CH<sub>2</sub>Cl<sub>2</sub> and dried under vacuum.

##### *Synthesis of CPPs C4 to C18*

Amounts of reagent are given in equivalents (eq.) with respect to the peptidyl-resin.

CPPs were synthesised manually on the cysteamino-Merrifield resin (0.2 g, 0.218 mmol) using the Boc strategy. Boc-Arg(Tos)-OH (3 eq.) was activated by 5 min treatment with HBTU (2.9 eq.), HOBt (3 eq.) and DIEA (6 eq.) in DMF (final amino acid concentration: 0.45 M). Each coupling was performed by 5 shots of 5 s of micro-waves (50 °C, 60 W). Between each shot, the resin was cooled in liquid nitrogen. The reaction was monitored by the Kaiser test and double couplings were performed if necessary. The Boc protecting group was removed by treatment with TFA (3 x 1 min) followed by neutralisation with DIEA (20 % in DMF). Coupling of the carbon chains was performed using either the chloride derivative (3 eq.) (lauroyl chloride for C12, myristoyl chloride for C14) or the carboxylic acid derivative (3 eq.) (palmitic acid for C16, stearic acid for C18) with HBTU/DIEA activation. Reactions were performed under micro-waves (5 x 5 s, 50 °C, 60 W). Peptide cleavage from the resin was performed by treatment with HF (2 h, 0 °C) in presence of anisole (1.5 mL/g peptidyl-resin), dimethylsulfide (0.25 mL/g peptidyl-resin) and *p*-toluenethiol

(300 mg/g peptidyl-resin). After HF removal under vacuum, the peptide was precipitated in ice-cold diethyl ether. The precipitate was dissolved in degassed acetic acid (10 % in H<sub>2</sub>O), the resin eliminated by filtration and the peptide freeze-dried. Peptides were purified by reverse-phase HPLC on a semi-preparative C4 column, using a linear gradient of CH<sub>3</sub>CN in an aqueous solution containing 0.1 % (v/v) TFA. Peptides were obtained with a purity > 95 %, as assessed by analytical HPLC.

#### *Synthesis of CPP Pyr*

Amounts of reagent are given in equivalents (eq.) with respect to the peptidyl-resin.

Pyr was synthesised manually using the Fmoc strategy on cysteamino-2-chlorotrityl resin (0.3 g, 0.234 mmol). Fmoc-Arg(Pbf)-OH (3 eq.) and 1-pyrene butyric acid (3 eq.) were activated for 5 min with HCTU (2.9 eq.) and DIEA (6 eq.) in NMP (final concentration: 0.45 M). Coupling reactions were performed under micro-waves (5 x 5 s, 50 °C, 60 W). The Fmoc protecting group was removed by treatment with piperidine (20 % in NMP) for 3 x 5 min. Peptide cleavage from the resin was performed by treatment with TFA/H<sub>2</sub>O/triisopropylsilane/1,2-ethanedithiol (90:5:2.5:2.5) 1 h at 50 °C, 60 W. TFA was removed by nitrogen flushing and the peptide was precipitated in cold diethyl ether. The crude peptide was dissolved in degassed acetic acid (10 % in H<sub>2</sub>O) and freeze-dried. It was purified by reverse phase HPLC on a semi-preparative C4 column, using a linear gradient of CH<sub>3</sub>CN in 0.1 % TFA. The peptide was obtained with a purity > 95 %, as assessed by analytical HPLC.

#### *Synthesis of <sup>1</sup>H-PKCi (<sup>1</sup>H-cargo) and <sup>2</sup>H-PKCi (<sup>2</sup>H-cargo)*

Amounts of reagent are given in equivalents (eq.) with respect to the peptidyl-resin.

The peptide RFARKGALRQKNV was assembled by automated stepwise solid-phase synthesis (ABI 433A peptide synthesiser, Applied Biosystems) using the Boc strategy (0.1 mmol MBHA-PS resin with a loading of 0.51 mmol/g, amino acid activation with DCC/HOBt). The peptide-resin was then separated into two batches that were further elongated manually to give peptides <sup>1</sup>H-PKCi (Biotin(sulfone)GGGGC(Npys)RFARKGALRQKNV-NH<sub>2</sub>, elongation with non-deuterated Gly) and <sup>2</sup>H-PKCi (Biotin(sulfone)GGGGCRFARKGALRQKNV-NH<sub>2</sub>, with bi-deuterated Gly). Boc amino acids were activated with HBTU/DIEA except Boc-Cys(Npys)-OH which was activated with DCC. Peptides were cleaved from the resin by treatment with anhydrous HF (2 h, 0 °C) as described above (No *p*-toluenethiol was added during cleavage of <sup>1</sup>H-PKCi to preserve the Npys protection of Cys). Peptides were purified by reverse phase HPLC on a semi-preparative C8 column, using a linear gradient of CH<sub>3</sub>CN in 0.1 % TFA.

#### *Synthesis of the CPP-cargo conjugates via a disulfide bridge*

The CPP was dissolved in 50 mM degassed sodium acetate buffer (50 mM, pH 5.5) and mixed with 1.1 eq. of <sup>1</sup>H-PKCi (final peptide concentration: 1 to 5 mM). The reaction was monitored by HPLC. At the end of the reaction, conjugates were purified by reverse phase HPLC on a C8 column, using a linear gradient of CH<sub>3</sub>CN in 0.1 % TFA. Conjugates were obtained with a purity > 95 %, as assessed by analytical HPLC.

#### **Peptide characterisation**

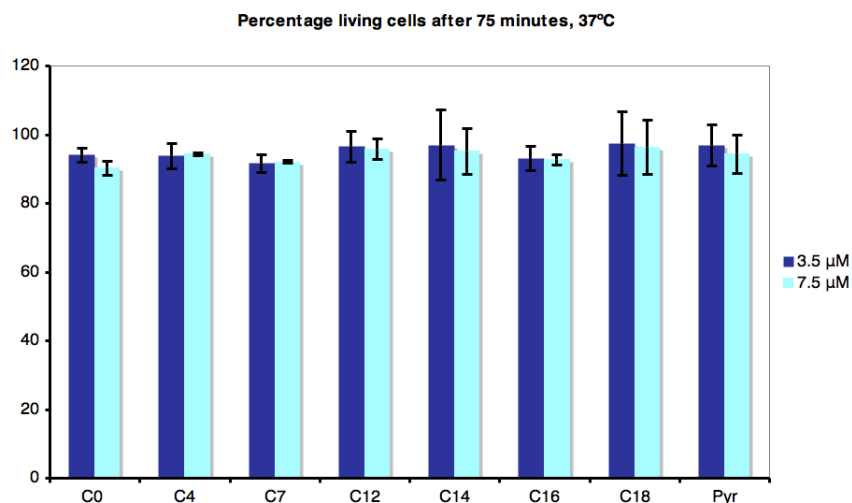
Peptides and conjugates were characterised by MALDI-TOF MS in the positive ion reflector mode on an ABI Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) using as matrix a saturated solution of CHCA in CH<sub>3</sub>CN/H<sub>2</sub>O/TFA (50:50:0.1). The *m/z* of the protonated molecules (first isotope) are given as experimental vs. (theoretical).

**Unconjugated CPPs:** C0, 702.4 (702.4); C4, 772.6 (772.5); C7, 813.5 (813.5); C12, 884.8 (884.6); C14, 912.9 (912.9); C16, 940.9 (940.7); C18, 968.9 (968.9); **Pyr**, 972.7 (972.5).

**CPP-PKCi conjugates:** C0, 2831.8 (2831.7); C4, 2901.4 (2902.6); C7, 2943.1 (2943.6), C12, 3013.9 (3014.7); C14, 3042.2 (3043.1); C16, 3070.3 (3070.8); C18, 3098.2 (3098.8), **Pyr**, 3101.5 (3101.6).

### Cell culture and cell viability assays

CHO cells were cultured in sterile conditions in DMEM supplemented with 10 % heat-inactivated FBS in a humidified atmosphere of 5 % CO<sub>2</sub>, at 37 °C. Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8). Cells were seeded in 96-well plates to obtain 50000 cells/well the day of the experiment. Cells were incubated with 100 µL of conjugate solution (3.5 or 7.5 µM in DMEM) for 75 min at 37 °C. The supernatant was removed and 100 µL of CCK8 (10 % in DMEM) was added. After 2 h incubation at 37 °C, the absorbance was measured at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH) with a reference wavelength at 620 nm. The experiments were performed in triplicates and repeated twice independently. Mean values ± SEM are given in the histogram.



**Fig S1.** Viability of cells incubated with the different CPP-cargo conjugates.

### Quantification of the internalised cargo by MALDI-TOF MS in wild type and Gag-deficient cells

Cells (CHO-K1 or CHO-pgsA745) were seeded in sterile conditions in 12-well plates 24 h before the internalisation experiment. Internalisation experiments were performed on confluent cells (10<sup>6</sup> cells/well). Cells were incubated with the free <sup>1</sup>H-PKCi peptide (no carrier) or with the <sup>1</sup>H-cargo-CPP conjugates (7.5 µM in DMEM) for 75 min at 37 °C or 4 °C. Cells were then washed 3 times with 1 mL Hank's BSS, treated for 3 min at rt with 200 µL TCEP (2 mM in 50 mM Tris-HCl buffer pH 7.5) and washed again with Hank's BSS. They were then incubated for 5 min at 37 °C with 500 µL trypsin-EDTA for internalisation experiments done at 37 °C or for 10 min at 4 °C with pronase (0.5 mg/mL in 100 mM Tris-HCl pH 7.5) for internalisation experiments at 4 °C. Trypsin inhibitor (100 µL, 5 mg/mL) or a cocktail of protease inhibitors (100 µL of a 7x solution of complete mini) and BSA (100 µL, 1 mg/mL) were added and the cell suspension was transferred to a tube. Wells were washed with 500 µL of 50 mM Tris-HCl buffer (pH 7.5). The combined suspensions were centrifuged for 2 min at 640 g. The pellet was washed with 1 mL 50 mM Tris-HCl pH 7.5, 0.1 % BSA (buffer A) and centrifuged again. The pellet was mixed with a known amount of internal

standard ( $^2\text{H}$ -PKCi) and 150  $\mu\text{L}$  of a solution containing 0.3 % Triton X-100, 1 M NaCl, 2 mM DTT. The mixture was heated for 15 min at 100  $^{\circ}\text{C}$ . The lysate was centrifuged for 5 min at 7080 g. The supernatant was mixed with 850  $\mu\text{L}$  of buffer A containing 2 mM DTT. Streptavidin-coated magnetic beads (5  $\mu\text{L}$  of Myone Streptavidin C1 or 10  $\mu\text{L}$  of M-280 Streptavidin) were added to the sample and the mixture was incubated for 1 h at rt to capture biotinylated peptides (intact PKCi cargo and cargo digests). After bead immobilisation with the magnet, the supernatant was removed and beads were washed as described below.

*Bead washing:*

Buffer A = 50 mM Tris-HCl pH 7.5, 0.1 % BSA

Buffer B = 50 mM Tris-HCl pH 7.5, 0.1 % BSA, 0.1 % SDS

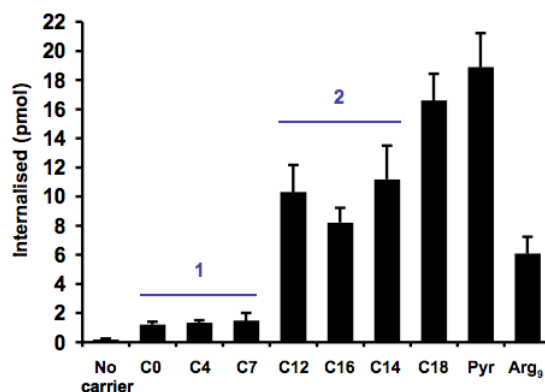
Buffer C = 50 mM Tris-HCl pH 7.5, 0.1 % BSA, 1M NaCl

Diluted (d10) Laemmli buffer: 1 % SDS, 6 % glycerol, 1 %  $\beta$ -mercaptoethanol, 0.025 % bromophenol blue, 25 mM Tris-HCl, pH 6.8.

When M-280 Streptavidin beads were used in the experiment, the following washings were performed: 2 x 200  $\mu\text{L}$  buffer A, 2 x 200  $\mu\text{L}$  buffer B, 2 x 200  $\mu\text{L}$  buffer C, 3 x 200  $\mu\text{L}$   $\text{H}_2\text{O}$ , 2 min incubation with 50  $\mu\text{L}$  biotin (10  $\mu\text{M}$ ), 1 x 50  $\mu\text{L}$   $\text{H}_2\text{O}$ , 1 x 50  $\mu\text{L}$   $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1).

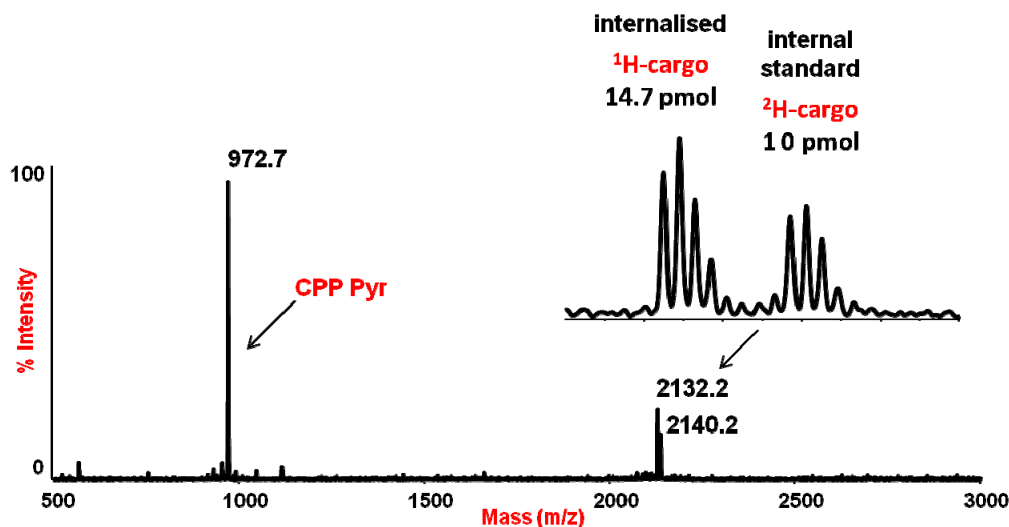
When MyOne Streptavidin C1 beads were used, the following washings were performed: 2 x 200  $\mu\text{L}$  buffer A, 10 min incubation at 50  $^{\circ}\text{C}$  with 20  $\mu\text{L}$  Laemmli buffer (d10), 2 x 200  $\mu\text{L}$  buffer C, 3 x 200  $\mu\text{L}$   $\text{H}_2\text{O}$ , 2 min incubation with 50  $\mu\text{L}$  biotin (10  $\mu\text{M}$ ), 1 x 50  $\mu\text{L}$   $\text{H}_2\text{O}$ , 1 x 50  $\mu\text{L}$   $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1).

After washing, the supernatant was removed and 3  $\mu\text{L}$  of matrix (saturated solution of CHCA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{TFA}$ , 50:50:0.1) was added. The beads were incubated 10 min at rt to elute the biotinylated peptides and 1  $\mu\text{L}$  of the supernatant was spotted on the MALDI-TOF plate. MALDI-TOF MS analyses were performed in the ion positive reflector mode on an ABI Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems). On the MALDI-TOF mass spectrum corresponding to the average of several hundreds laser shots, the area of the  $[\text{M}+\text{H}]^+$  signals (including all isotopes) of the  $^1\text{H}$ -PKCi and  $^2\text{H}$ -PKCi signals were measured and the amount of intact internalised cargo was calculated from the area ratio. All internalisation experiments were performed in triplicates and repeated at least twice independently.



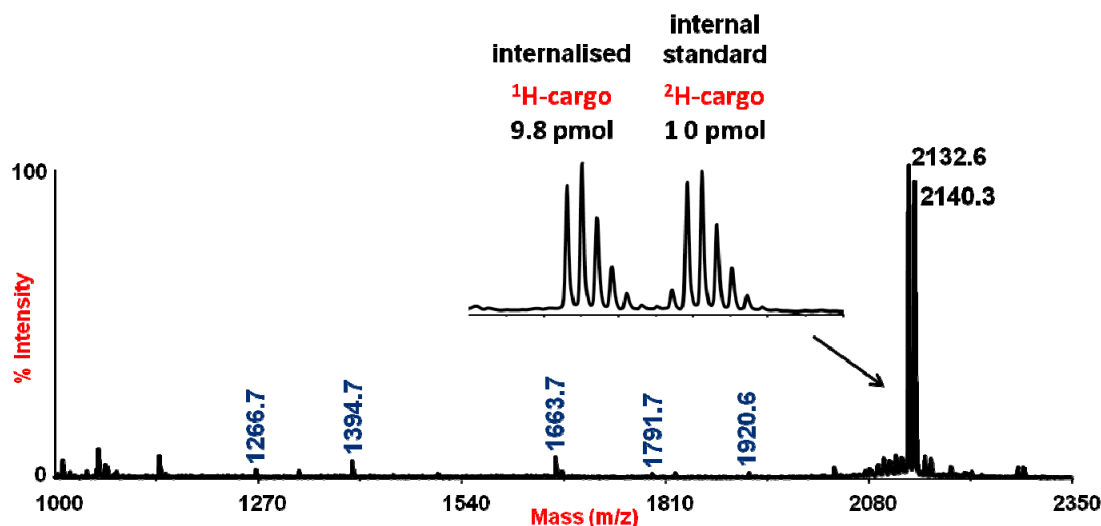
**Fig. S2** Amounts of intact PKCi cargo delivered in  $10^6$  CHO-K1 cells at 37  $^{\circ}\text{C}$  and significance. Cells were incubated for 75 min with the free  $^1\text{H}$ -cargo (No carrier) or the  $^1\text{H}$ -cargo-CPP conjugates (7.5  $\mu\text{M}$ ). The amount of intact PKCi cargo delivered in the same conditions by the widely used Arg<sub>9</sub> CPP is also shown for comparison. Unpaired two-tailed t-tests showed no significant difference between C0, C4 and C7 (population 1) ( $p_{\text{C0-C4}} = 0.35$ ;  $p_{\text{C0-C7}} = 0.34$ ). A similar result was found for C12, C14 and C16 (population 2) ( $p_{\text{C12-C14}} = 0.16$ ;  $p_{\text{C12-C16}} = 0.17$ ). The difference in uptake efficiency between the two populations is extremely significant ( $p < 0.0001$ ). Both C18 and Pyr showed to be significantly more internalised than population 2 ( $p < 0.0001$  in both cases). In addition, the p-value for C18 and Pyr shows a significant difference between these conjugates ( $p = 0.04$ ).





**Fig. S3** Mass spectrum obtained for the cellular uptake of Pyr conjugate at 37 °C in CHO-K1 cells. Insert: zoom on the intact <sup>1</sup>H-PKCi and <sup>2</sup>H-PKCi peaks, which are used for quantification. MyOne Streptavidin C1 beads were used in this experiment to extract the biotinylated peptides, beads were washed with diluted Laemmli buffer.

Non-biotinylated CPPs were found to bind non-specifically to the streptavidin-coated magnetic beads. They were detected on the mass spectra in addition to the biotinylated PKCi cargo species (Fig. S3). This can hamper the detection of the species of interest, the PKCi cargo. We have optimised the protocol of bead washing and found that incubation with diluted Laemmli buffer could remove (partially) the CPP species. In addition, we have observed less non-specific binding of the peptides on M-280 Streptavidin beads (hydrophobic bead surface) compared to MyOne Streptavidin C1 beads (hydrophilic bead surface).



**Fig. S4** Mass spectrum (zoom) obtained for the cellular uptake of C14 conjugate at 37 °C in CHO-K1 cells. Peaks that were identified as digests of <sup>1</sup>H-PKCi are labelled in blue. As expected, no digests of the internal standard (<sup>2</sup>H-PKCi) were observed.

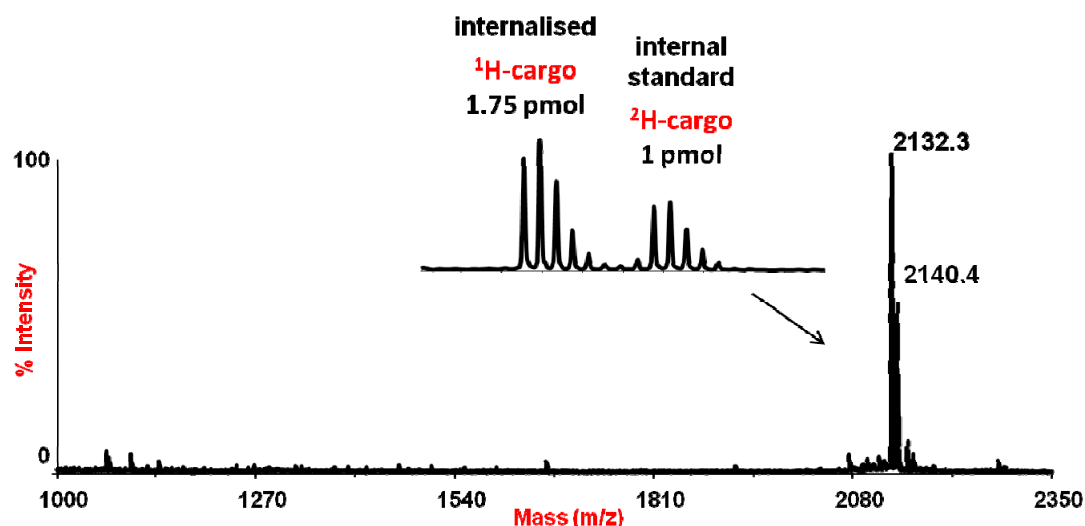


Fig. S5 Mass spectrum (zoom) obtained for the cellular uptake of C12 conjugate in CHO-K1 cells at 4 °C.

### Quantification of the internalised cargo in CHO-K1 cells in presence of heparin (conjugates C0 and C12)

The same procedure was used as above except that conjugates (7.5  $\mu$ M) were pre-incubated for 5 minutes at room temperature with heparin (25  $\mu$ g/mL) in DMEM, then the mixture was added to CHO-K1 cells and incubated for 75 min at 37 °C. Addition of heparin decreased the uptake efficiency of conjugate C12 in CHO-K1 cells by 60 % suggesting that heparin interacts with conjugate C12 and inhibits its entry. For conjugate C0, internalisation was not inhibited in presence of heparin.

### Quantification of the membrane-associated cargo

The following modifications were introduced in the protocol described for the quantification of the internalised cargo. After incubation of the cells with the conjugates, cells were washed 3 times with Hank's BSS and treated directly in the plate at 100 °C for 15 min with the lysis solution in the presence of  $^2$ H-PKCi. The cell lysate was transferred into a micro-tube and the well was washed with 850  $\mu$ L buffer A. Extraction of the peptides and MALDI-TOF MS analysis were carried out as described above.

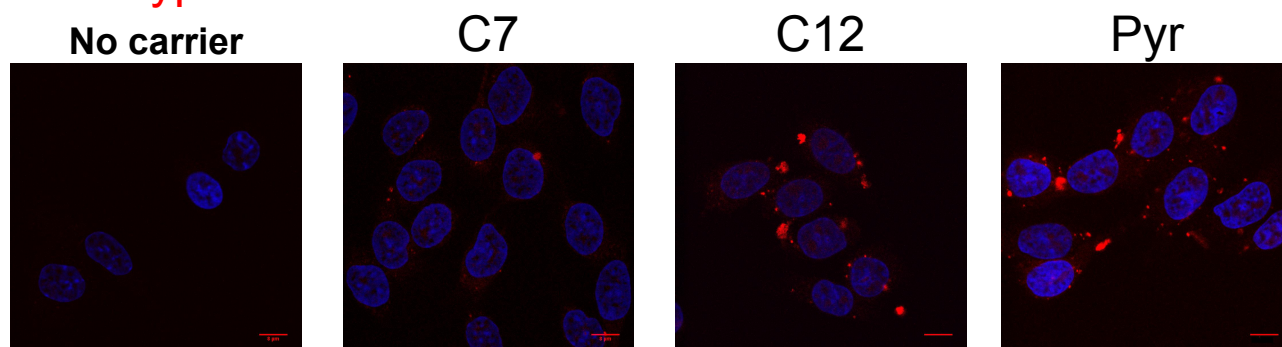
Noteworthy, this protocol omitting the TCEP/protease treatment after conjugate incubation gives access to the total amount of internalised cargo and membrane-associated cargo. Membrane-bound species potentially include  $^1$ H-cargo-CPP conjugates adsorbed on the cell membrane and  $^1$ H-cargo covalently linked *via* a disulfide bridge to cell-surface thiols. Amounts given in Fig 3A (of the main manuscript) correspond to the membrane-associated conjugate (amounts of internalised peptide have been subtracted).

### Confocal microscopy experiments

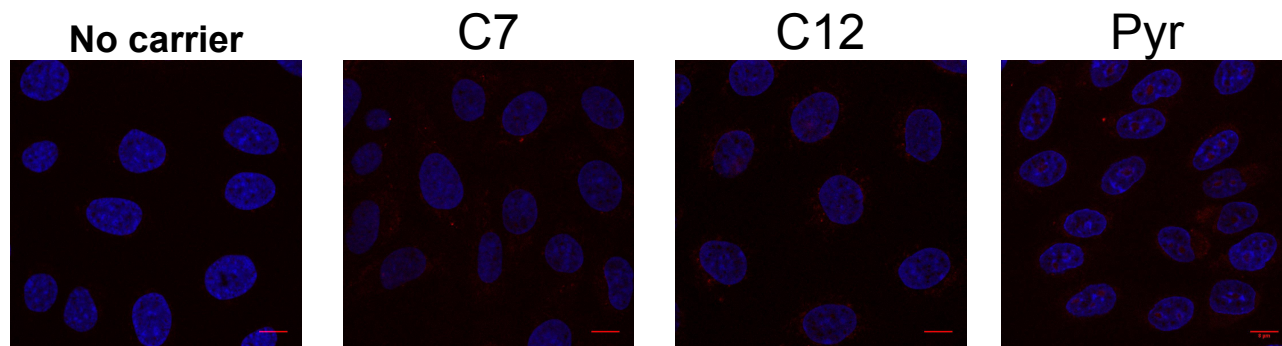
$10^5$  CHO cells (wild type or GAG-deficient) were cultured on coverglass in 24-well plates in DMEM with 10 % FCS (fetal calf serum). After washing with DMEM, cells were incubated with the conjugates (7.5  $\mu$ M in DMEM) for 75 min at 37 °C. Cells were washed 3 times with DMEM, incubated for 10 min at rt with 200  $\mu$ L unlabelled avidin (10  $\mu$ M) and washed with PBS. Cells were then incubated with 3 % paraformaldehyde (4 °C, 10 min), permeabilised with 0.1 % Triton X-100 in PBS (rt, 5 min) and incubated with 10 % FCS in PBS (rt, 30 min). Cells were incubated with streptavidin-TRITC (2  $\mu$ g/mL) for 1 h at rt, washed again with PBS and treated with DAPI (1.5

$\mu\text{g/mL}$ ) for 15 min at rt. Coverslips were mounted in Fluoromount mounting medium for observation on an inverted Leica DMI 6000.

### Wild-type cells :



### GAG-deficient cells :



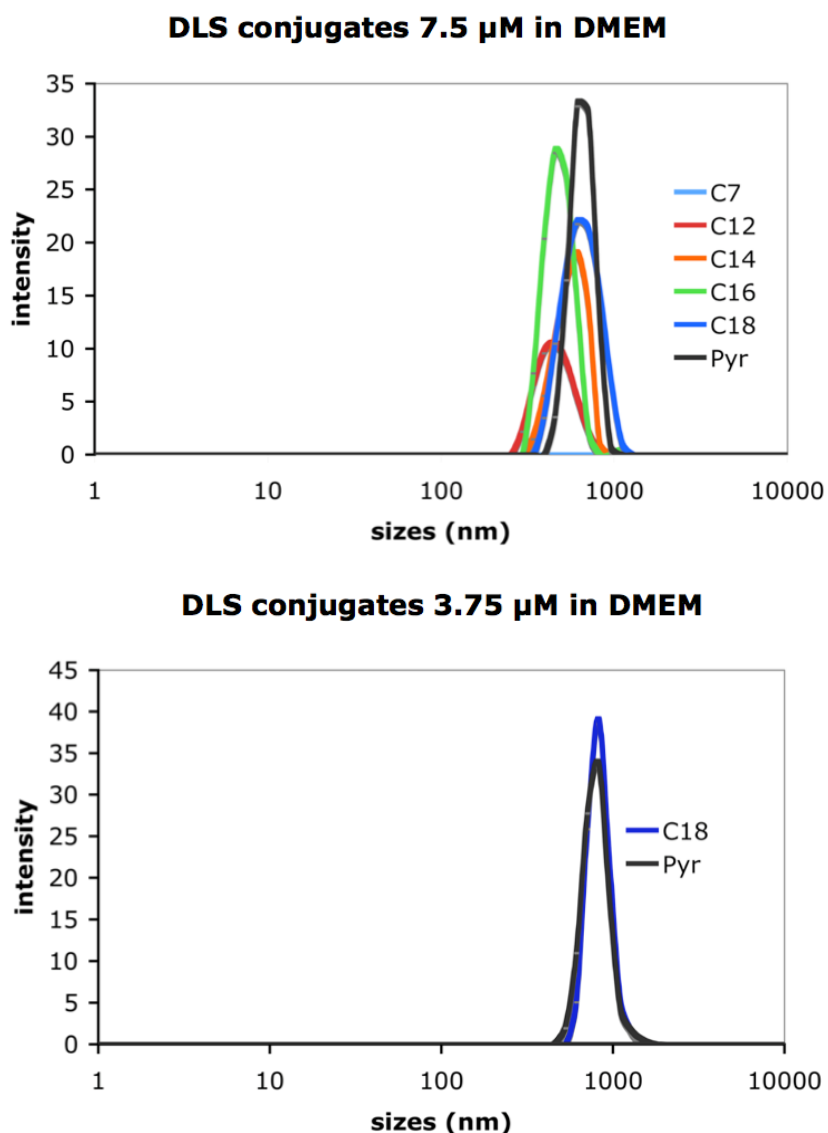
**Fig S6.** Confocal microscopy images. The internalised biotinylated cargo was revealed with streptavidin-TRITC (red). Nuclei were stained with DAPI (blue). The scale bar represents 8  $\mu\text{m}$ .

### Analysis of self-association by DLS

Conjugate solutions of 30, 7.5 and 3.25  $\mu\text{M}$  were prepared in  $\text{H}_2\text{O}$  or DMEM from 1 mM stock solutions. The solutions were filtered prior to mixing (PES, 0.22  $\mu\text{m}$ ; Millipore; Billerica, MA). DLS was performed on a Zetasizer Nano S (Malvern Instruments Ltd, England). Measurements were performed in 1 cm rectangular cuvettes at 25  $^{\circ}\text{C}$ .

No self-association was observed when the samples were prepared in water. However, conjugates with carbon chains  $\geq \text{C12}$  showed formation of self-assembled structures when prepared in DMEM at concentrations of 7.5  $\mu\text{M}$  or higher (Fig. S7). Only C18 and Pyr showed the existence of particles at a lower concentration of 3.25  $\mu\text{M}$ . DLS measurements performed on the Pyr derivatives thus suggested self-association. However, the inherent fluorescence of the pyrene moiety might interfere with the signal measured during DLS experiments.

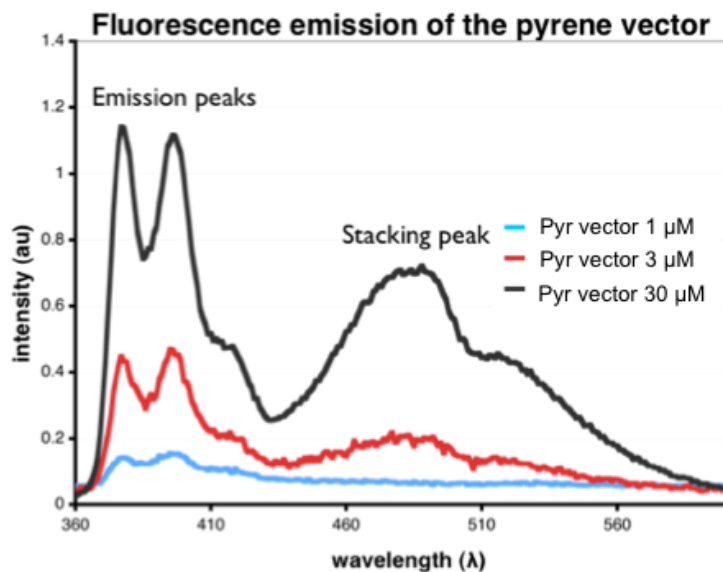
It should be noted that samples of all conjugates had a high polydispersity, which may affect the size given by the DLS. Furthermore, the diameter that is measured in DLS is a value that refers to the hydrodynamic diameter, and as a consequence, the translational diffusion coefficient of the particle is used as measure for its size. If the particle is non-spherical, the hydrodynamic diameter of the particle will be the diameter of a sphere that has the same translational diffusion speed as the particle. If the shape of a particle changes in a way that affects the diffusion speed, this could largely affect the size indicated by DLS.



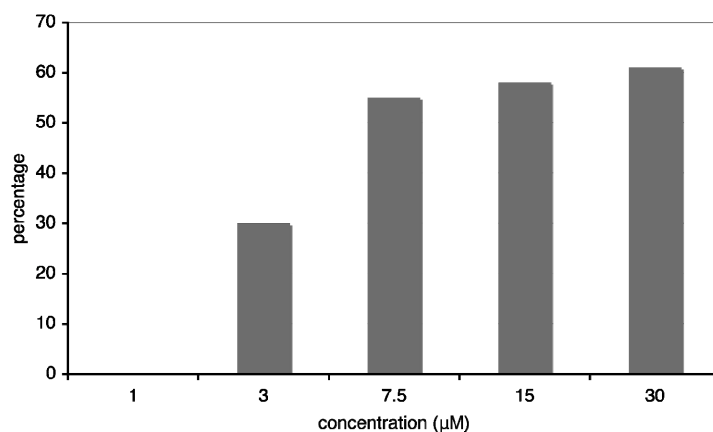
**Fig. S7** DLS analysis of the conjugates. Data is plotted as intensity versus sizes. (C7 showed no DLS signal).

### Analysis of self-association of the Pyr vector by fluorescence $\beta$

Vector solutions of 30, 15, 7.5, 3 and 1  $\mu\text{M}$  were prepared in  $\text{H}_2\text{O}$  from a 1 mM stock solution. The excitation of the Pyr vector was first analysed, then was used as excitation for the emission spectrum. Measurements were performed in microcuvettes at 25  $^\circ\text{C}$  shielded from light. For vector concentrations of 3  $\mu\text{M}$  and higher, a stacking peak ( $\lambda = 480$  nm) was observed, indicating the presence of a structure that was self-associated due to  $\pi$ - $\pi$  interactions (Fig. S8). The height of the 'stacking-signal' compared to the emission signals in percentages is given in Fig. S9 for all concentrations tested. The results suggested that at concentrations between 7.5  $\mu\text{M}$  and 30  $\mu\text{M}$  stable self-assemblies are formed, but when the concentration is lowered to 3  $\mu\text{M}$  only a fraction of the vectors are able to self-assemble. When the concentration is further decreased, no self-assemblies are observed.



**Fig. S8** Fluorescence emission spectra of the Pyr vector, excited at 343 nm, at concentrations of 1  $\mu\text{M}$  (bright blue), 3  $\mu\text{M}$  (red) and 30  $\mu\text{M}$  (dark grey).



**Fig. S9** Height percentages of the stacking peak compared to the emission peaks as shown in Fig S7. For 30, 15 and 7.5  $\mu\text{M}$  the height is around 60 %. This suggests that a self-assembled species is formed at these concentrations with similar efficiencies. When the concentration is lowered to 3  $\mu\text{M}$ , the height of the stacking peak decreases to 30 %. This means that at this concentration the self-associated structure can still be formed, but with diminished efficiency. At a concentration of 1  $\mu\text{M}$  the Pyr vector is not able to form self-assemblies.