Supplemental Information

An L- to D-amino acid conversion in the cell penetrating peptide dfTAT influences proteolytic stability, endocytic uptake, and endosomal escape

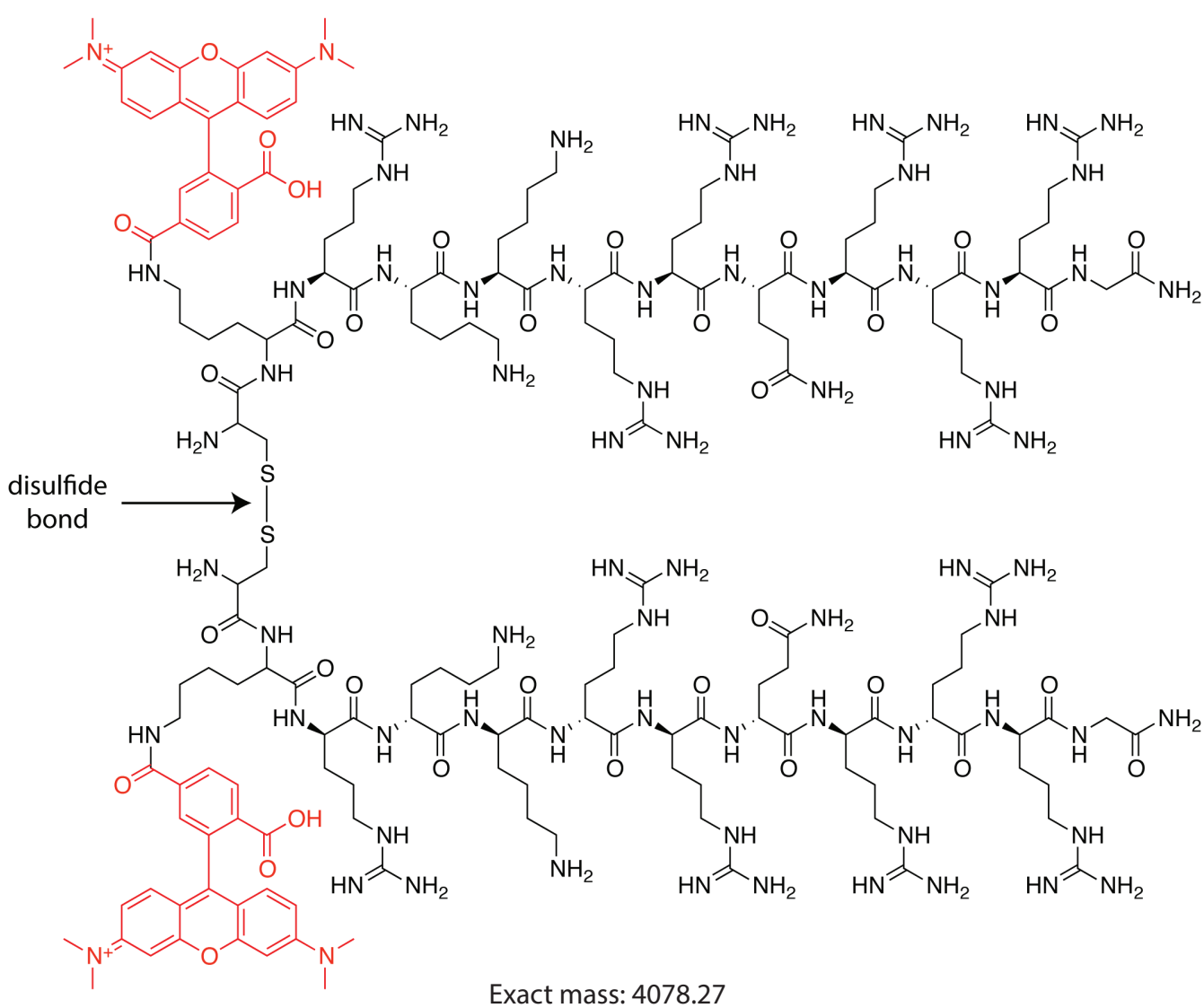
Kristina Najjar, Alfredo Erazo-Oliveras, Dakota J. Brock, Ting-Yi Wang & Jean-Philippe Pellois*

Running Title: *Interplay between CPP activity and proteolysis*
From Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843

*To whom correspondence should be addressed: Prof. Jean-Philippe Pellois, Department of Biochemistry and Biophysics, Texas A&M University, Biochemistry and Biophysics Building, Room 430, 300 Olsen Boulevard, College Station, Texas 77843-2128, Telephone: (979) 845- 0101; FAX: (979) 862-4718; E-mail: pellois@tamu.edu

Keywords: Protease-resistant cell-penetrating peptide, endocytosis, endosomal escape, cytosolic delivery

A



B

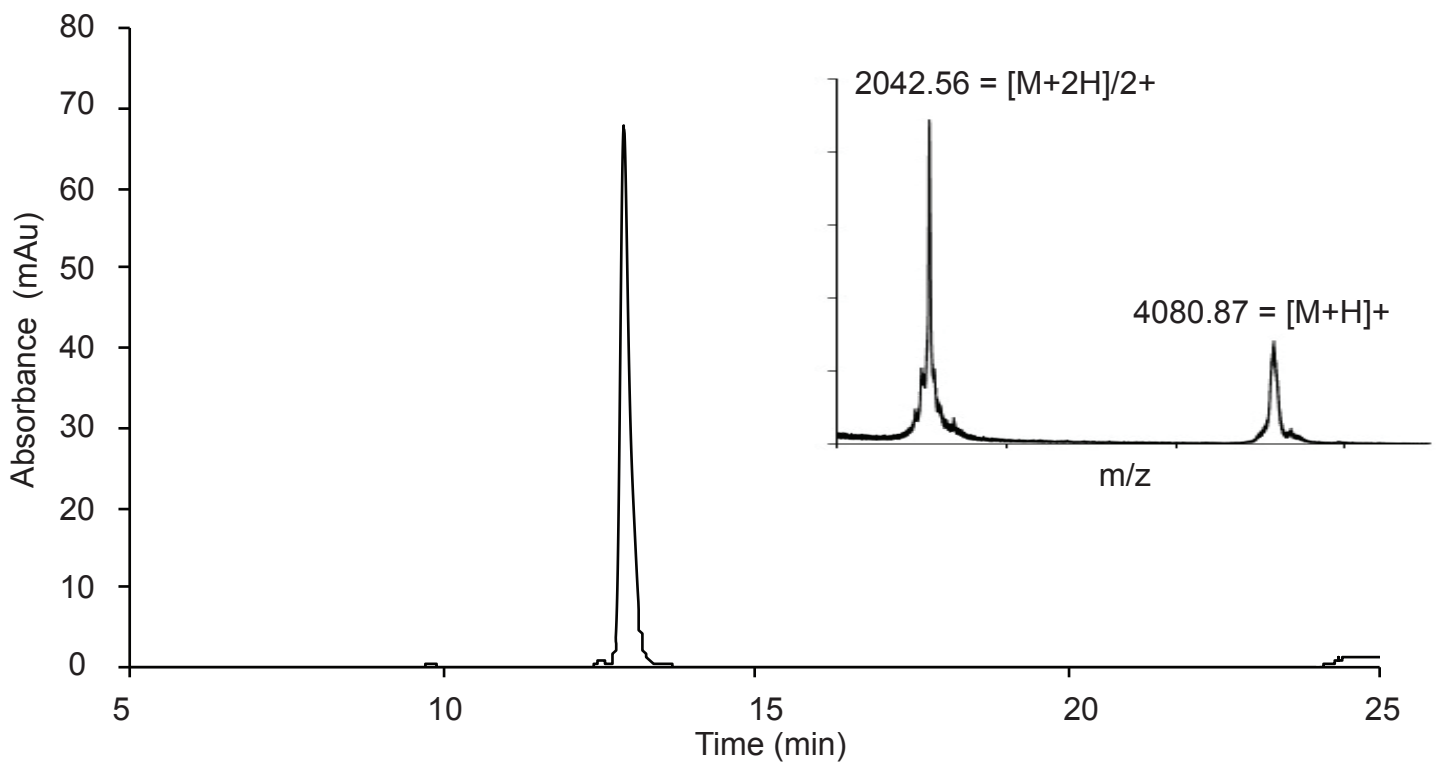
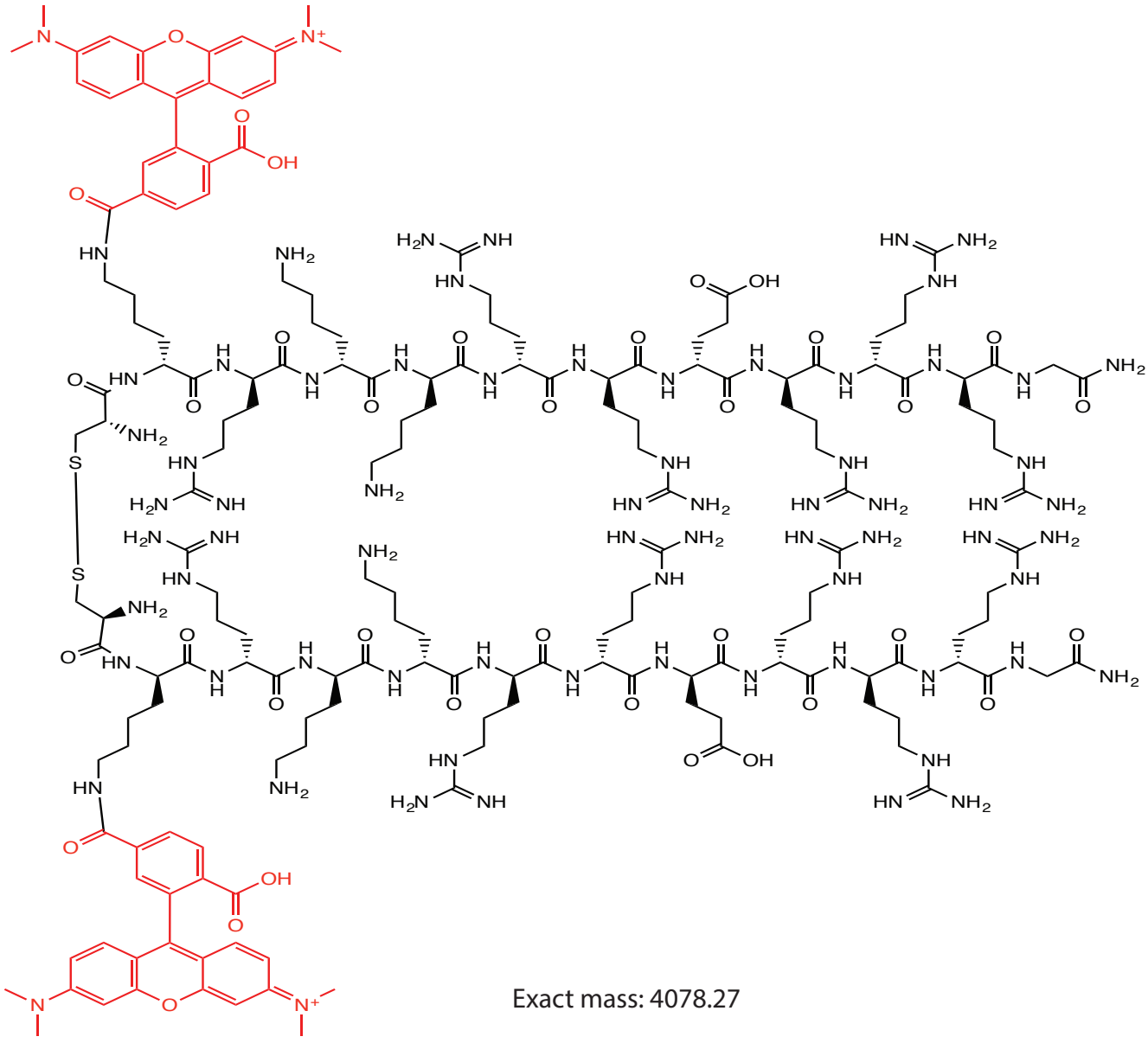


Figure S1
Characterization of dfTAT. A)HPLC and B)MALDI-TOF MS spectrum of dfTAT (retention time, rt=12.94 min). Mass Spectrum of dfTAT: observed [M+H]+ = 4080.87, [M+2H]/2+ = 2042.56
predicted Exact Mass (M) = 4078.27 Da.

A



Exact mass: 4078.27

B

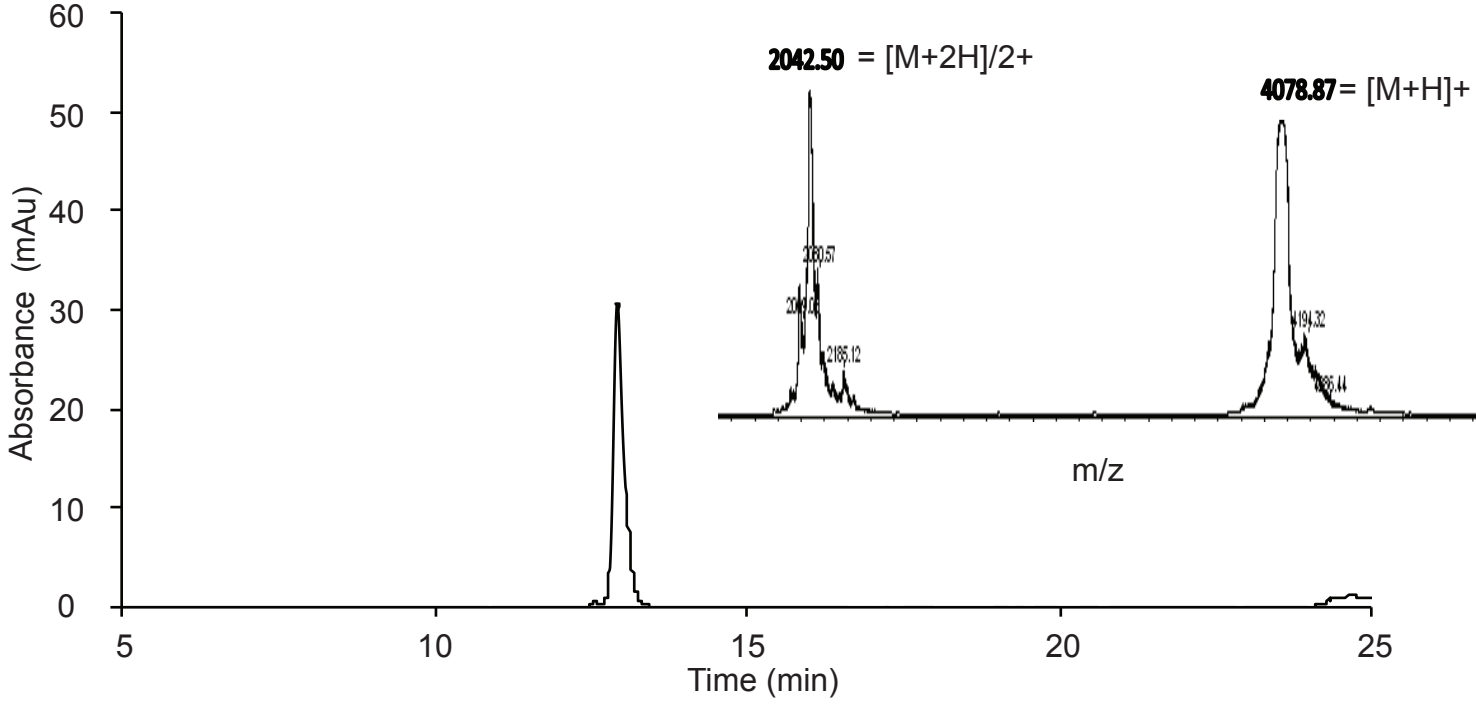


Figure S2
Characterization of D-dfTAT. HPLC and MALDI-TOF MS spectrum of D-dfTAT (rt=12.91 min). Mass Spectrum of D-dfTAT: observed [M+H]+= 4078.87, [M+2H]/2+= 2042.50 predicted M = 4078.27 Da.

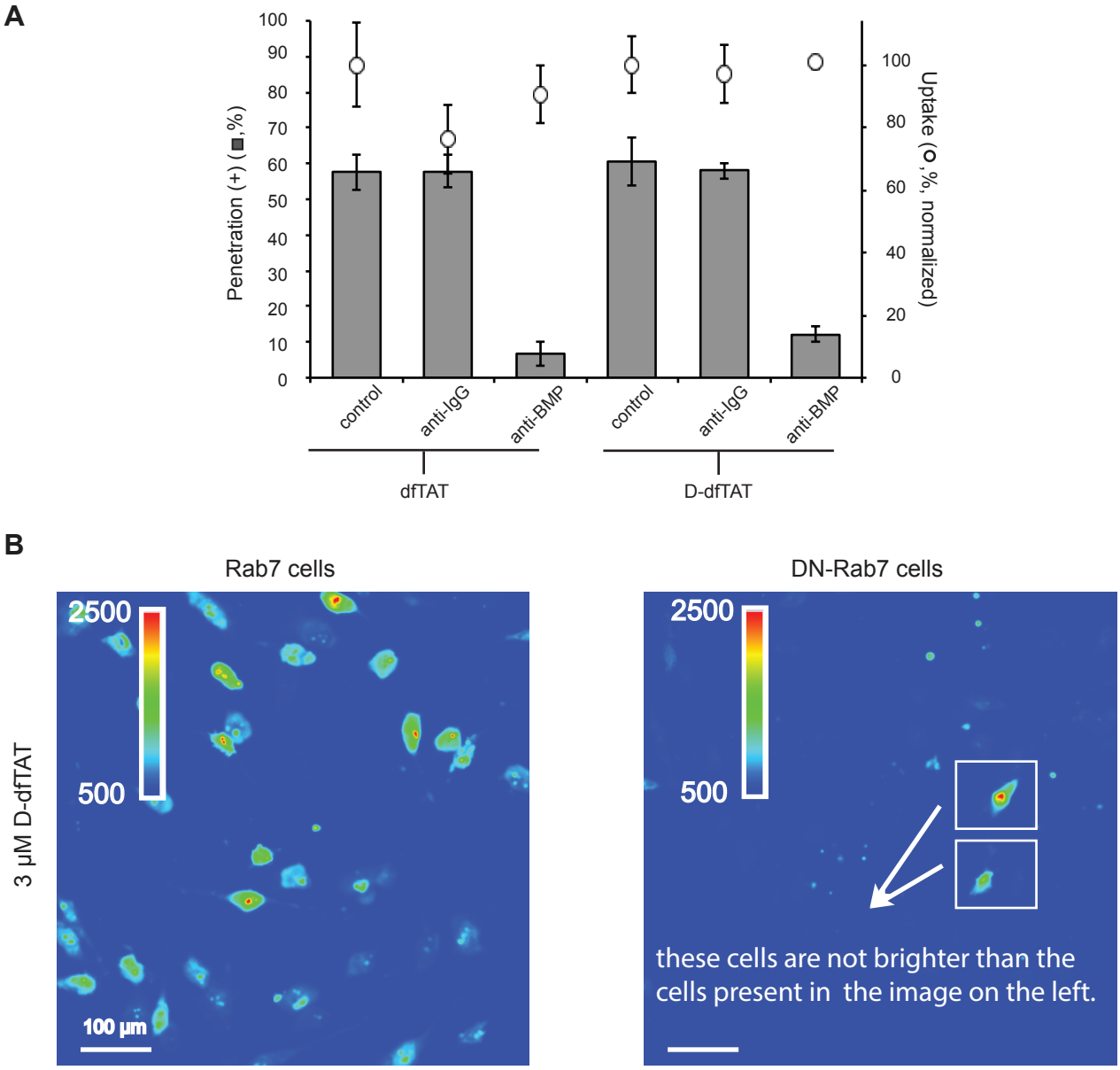
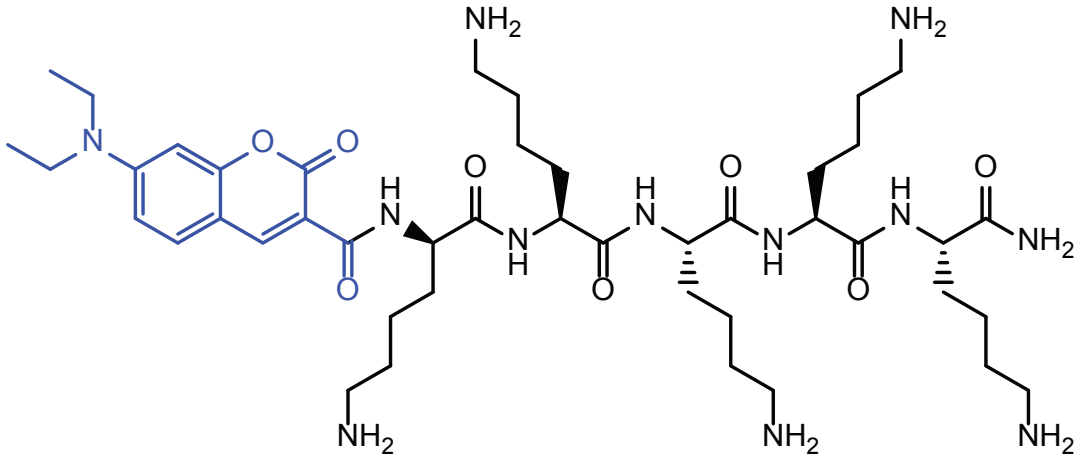


Figure S3
D-dfTAT major route of cellular entry is via endocytosis followed by endosomal escape in a manner similar to dfTAT. A) Cytosolic penetration of D-dfTAT is blocked by the anti-BMP antibody. HeLa cells were pre-incubated with anti-BMP mAb or the control anti-IgG mAb for 30 min. dfTAT or D-dfTAT (3 µM) was then incubated with cells for 1 h and cell penetration was quantified. The total fluorescence of cell lysates was also measured to assess the impact of each treatment on peptide endocytic uptake. B) Given the possibility of a secondary mode of penetration in a minority of cells (<5%, as described in Figure 2), the uptake data presented in Figure 4 may include endocytosis-dependent and endocytosis-independent uptake. In order to address how much signal may be contributed by endocytosis-independent cytosolic entry, the fluorescence intensity of cells positive for peptide penetration in the presence of DN-Rab7 was estimated (these are the cells in which uptake cannot be fully accounted for by endocytosis). HeLa cells were transfected with Rab7 or dominant negative Rab7 (DN-Rab7). Cells were then incubated with D-dfTAT at 3 µM. Images are pseudocolored based on the fluorescence intensity of D-dfTAT in the cells (Scale bars, 100 µm). These representative data confirm that the number of cells positive for peptide penetration decreases when DN-Rab7 is expressed (as shown in Figure 2b). However, the overall fluorescence intensity of the few cells positive for peptide penetration in the presence of DN-Rab7 is equivalent (as shown by the pseudocolored scale) to the fluorescence intensity of cells positive for peptide penetration in the presence of wild type Rab7 (as exemplified with cells highlighted with a white box; this observation was made in 10 images taken of the DN-Rab7 condition, the fluorescence intensity of 30 cells surveyed). Therefore, we estimate that endocytosis-independent uptake, if present in 5% of cells, may contribute 5% or less of the total signal reported.

A



Exact mass: 900.59

B

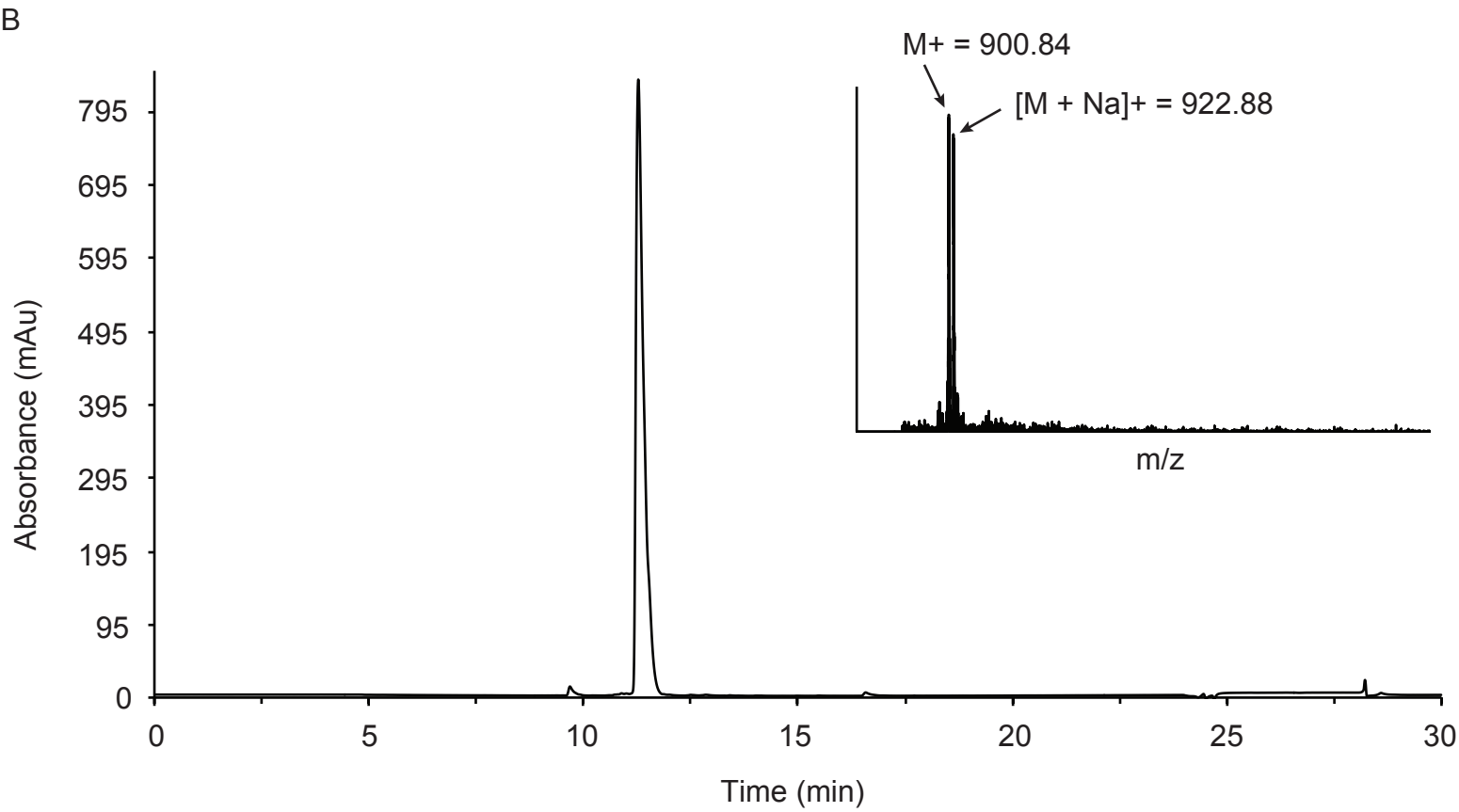


Figure S4
Characterization of DEAC-k5. A) Structure and expected mass of DEAC-k5. B) The HPLC spectrum of DEAC-k5 (rt= 11.8 min) and the MALDI-TOF mass spectrum are shown. Observed mass are [M]+= 900.84 and [M+Na]+ = 922.88, predicted mass of 900.59 Da.

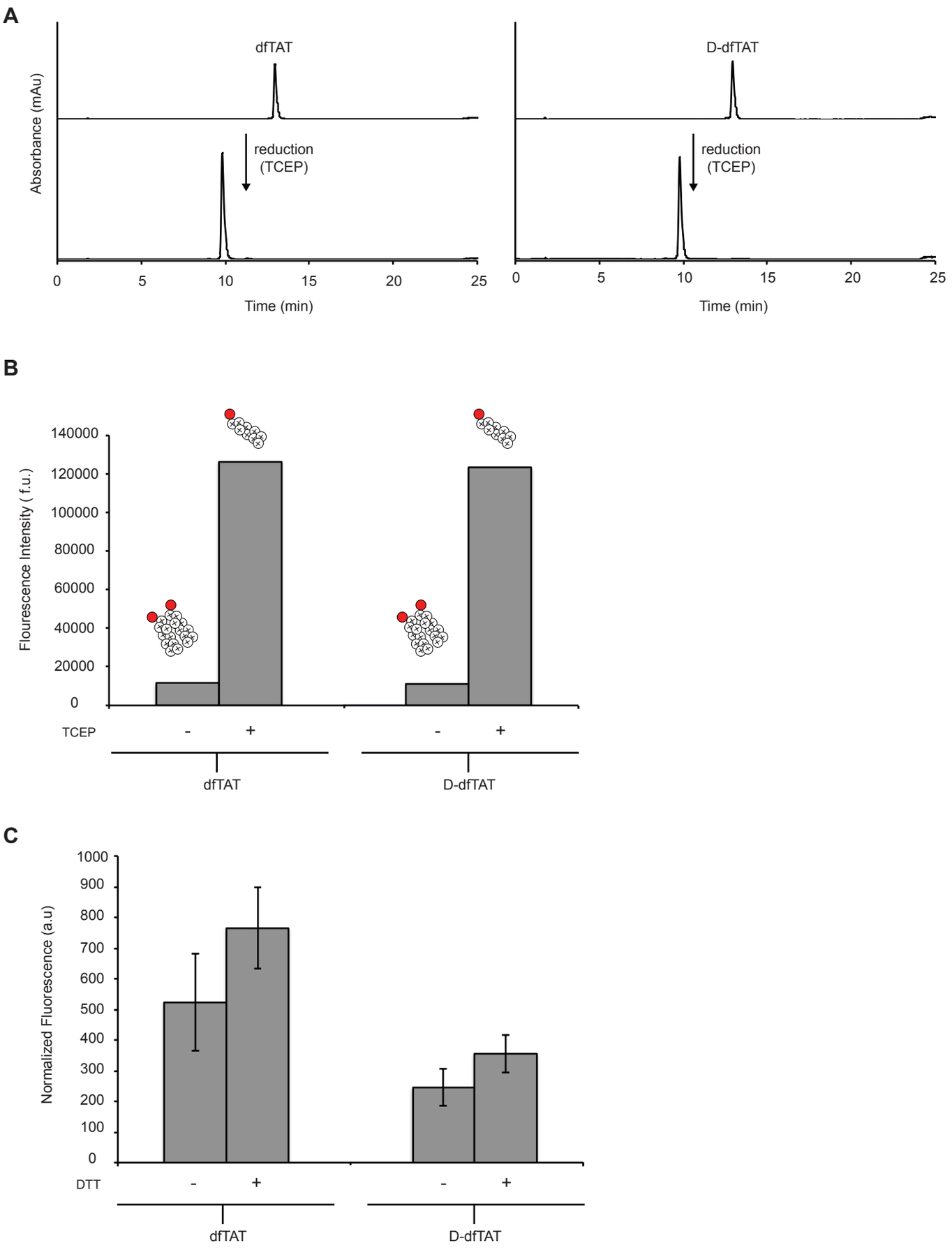


Figure S5

The fluorescence emission of dfTAT and D-dfTAT increases upon reduction of the disulfide bond. A) HPLC analysis of purified dfTAT and D-dfTAT in absence and presence of the reducing agent tris(2-carboxyethyl)phosphine (TCEP). Pure dfTAT and D-dfTAT was mixed with a solution of TCEP (50 mM) in water and allowed to react for 15 min. The HPLC chromatogram show a peak with rt = 12.9 min for both dfTAT and D-dfTAT and rt= 9.7 min for the reduced peptides. B) Bar graph of the fluorescence emission of dfTAT and D-dfTAT (1.25 µM) in the absence and presence of the reducing agent TCEP (50 mM). The samples were excited at 556 nm and the emission was scanned between 560-760nm. C) Quantification of the uptake fluorescence intensity, with or without DTT, in cells treated with dfTAT and D-dfTAT. HeLa cells were incubated with 5 µM dfTAT or D-dfTAT. Cells were then trypsinized and lysed in presence or absences of DTT (2mM). The overall amount of peptide internalized by cells (endosomal + cytosolic) was assessed by measuring the bulk fluorescence of cell lysates.

Together, these results indicate that the fluorescence of dfTAT and D-dfTAT is partially quenched when the peptides are in their dimeric form. As indicated in the main text, we therefore concluded that the reduction of cell lysates was necessary to obtain quantitative data of peptide uptake into cells. However, the contribution of the dimer fluorescence is relatively small (see -/+ DTT comparison), indicating that the peptides are presumably already reduced after entry into the cytosolic space (this is consistent with the observation that disulfide bonds are reduced in a matter of second by once inside cell) [1].

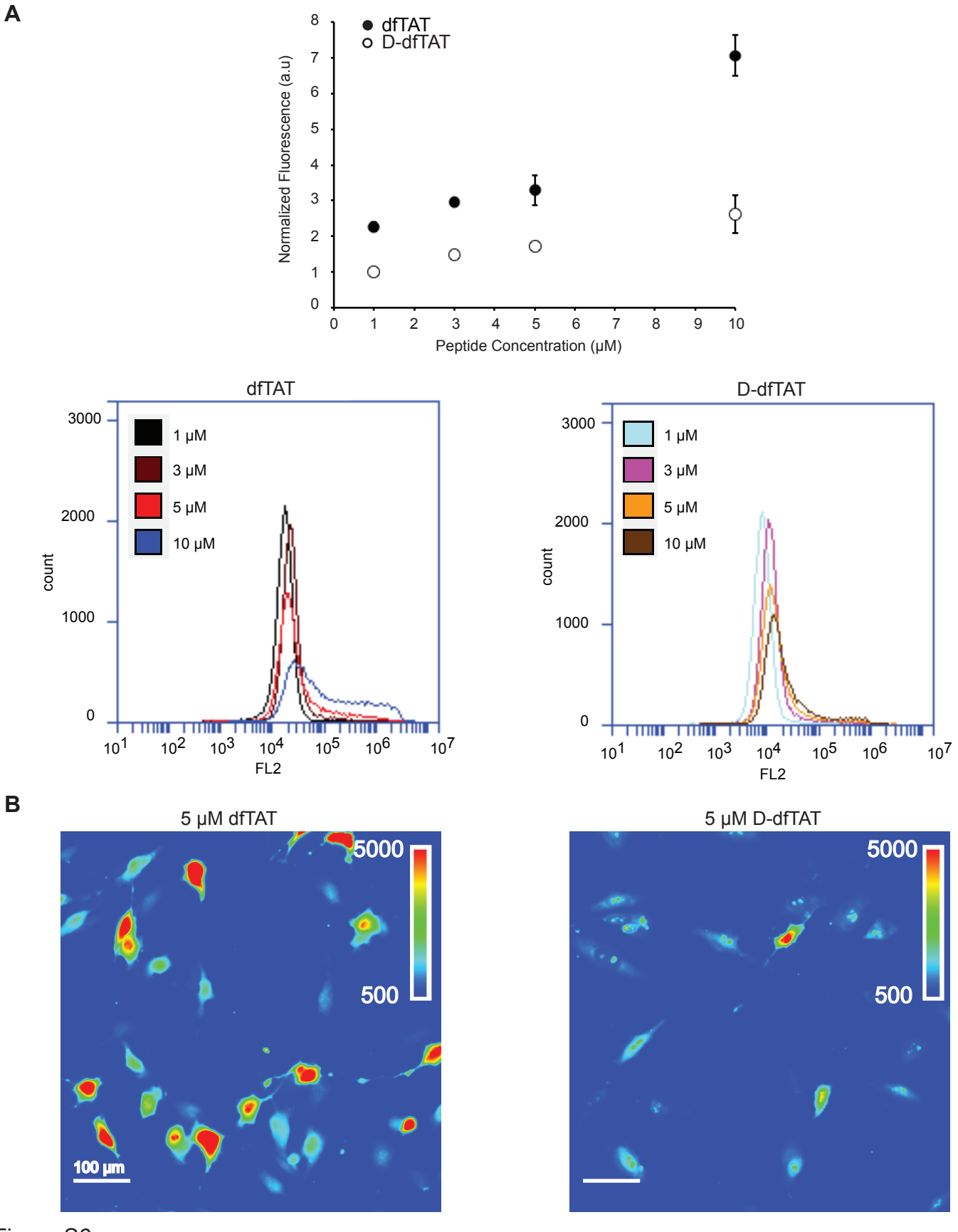


Figure S6
Comparison of the cellular uptake of dfTAT and D-dfTAT. A) The overall amount of peptide internalized by cells (endosomal + cytosolic) was measured using flow cytometry. HeLa cells were incubated with dfTAT or D-dfTAT at different concentrations (1-10 μM). Cells were then analyzed and fluorescence intensity was quantified as a function of concentration. b) The overall amount of peptide internalized by cells was quantified using fluorescence microscopy. HeLa cells were incubated with dfTAT or D-dfTAT at 5 μM. Images are pseudocolored based on the fluorescence intensity of dfTAT and D-dfTAT in the cells. Scale bars, 100 μm.

We anticipate that the data obtained by flow cytometry and fluorescence microscopy are not as quantitative as the cell lysate assays described in Figure 3 and Figure S5 because of the partial quenching of dimeric peptides present inside cells. Yet, these data are consistent with the cell lysate results presumably because the quenched peptide population is small (see Figure S5).

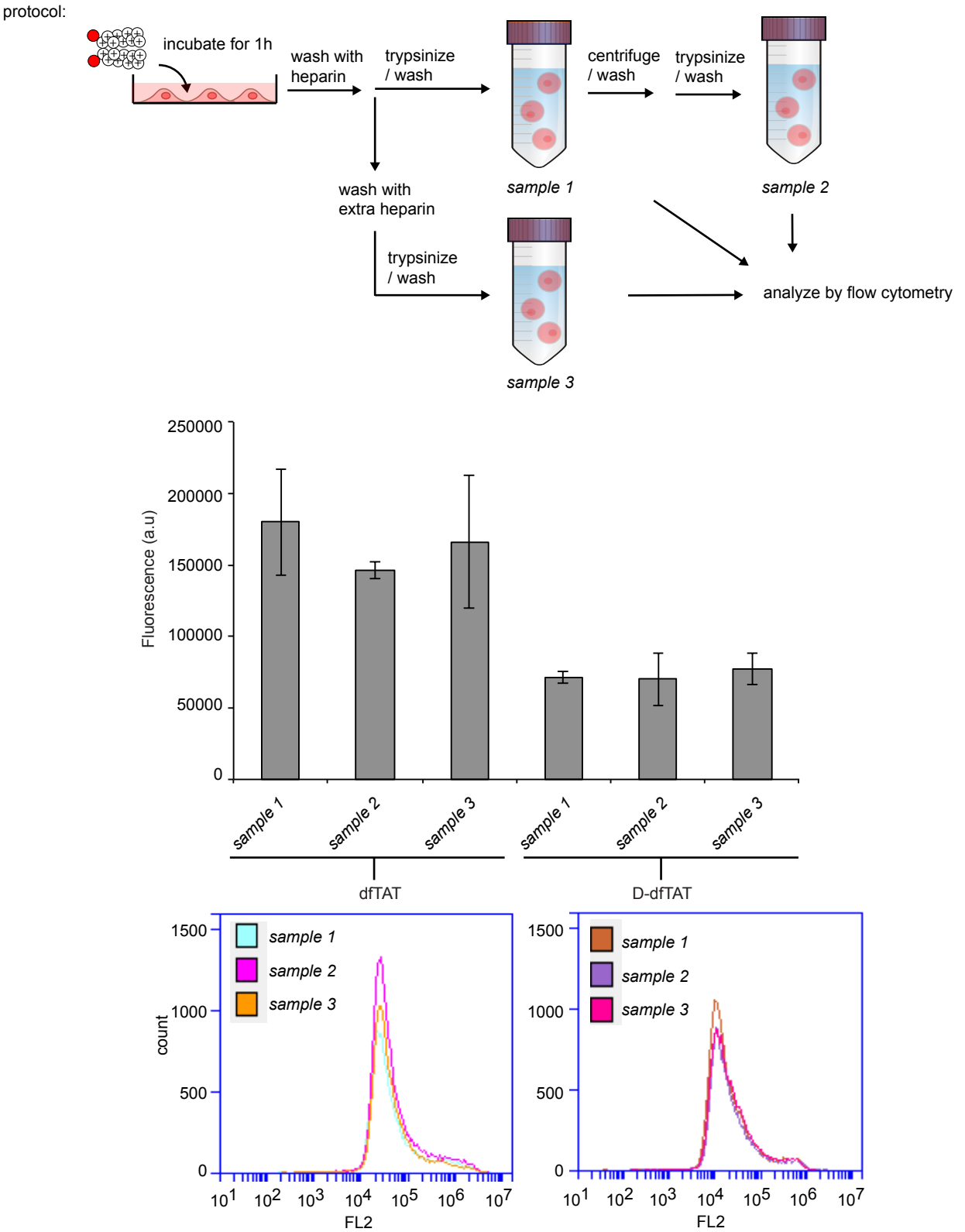


Figure S7
The uptake fluorescence intensity of dfTAT and D-dfTAT is not a consequence of extracellular membrane binding. Cells were treated with dfTAT and D-dfTAT at 5 μM for 1 h. The cells were then washed using three separate protocols that insure removal of peptide bound extracellularly. For all protocols, cells were first washed three times with L-15 supplemented with heparan (1 mg/mL) and once with nrL-15. Sample 1: control cells were then trypsinized and resuspended in nrL-15 for flow cytometry. For sample 2: The cells were then washed again three times with L-15 supplemented with heparan (1 mg/mL) and once with nrL-15. For sample 3: The cells were then trypsinized and resuspended in nrL-15 and centrifuged. The supernatant was removed and the cell pellet was trypsinized (50 μL) and resuspended once again in nrL-15 for flow cytometry analysis. The bar graph shows the average mean fluorescence intensity values from the flow cytometry (each condition was performed in triplicate). An example of the flow cytometry histogram for each condition is shown below the bar graph.

Because the cell fluorescence is independent of the various treatments used, we conclude that portion of extracellular peptide that could potentially contribute to signal is negligible.

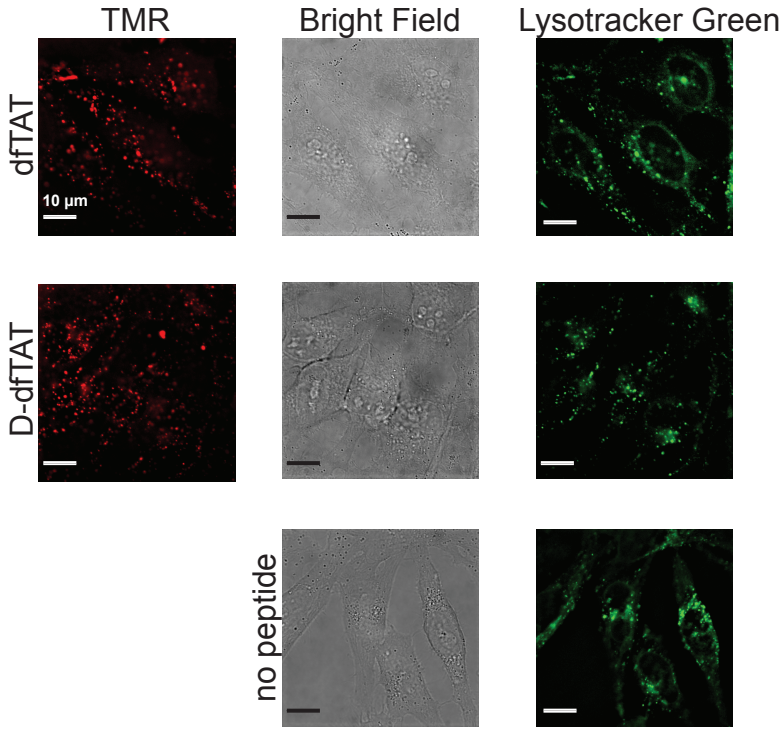


Figure S8
The number of endosomes present in cells treated with dfTAT is similar to that present in cells treated with D-dfTAT. HeLa cells were treated with 2 µM dfTAT or D-dfTAT respectively for 1h. Cells were then washed and incubated with 500 nM lysotracker green for 10 min. The fluorescent images show a punctate distribution for both the peptide and lysotracker green. Scale bar, 10 µm.

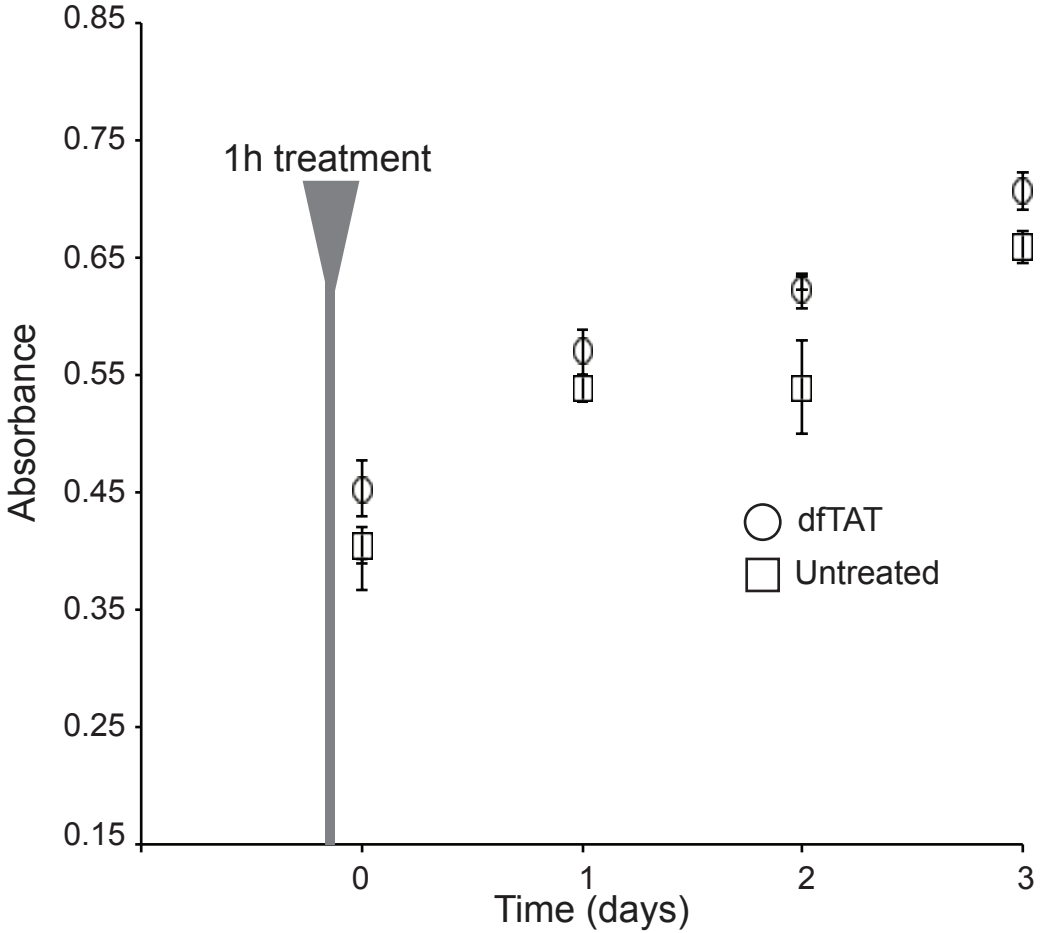


Figure S9
Proliferation assay. dfTAT treated HDF cells proliferate at a similar rate to untreated cells. HDF cells were incubated with 5 μM dfTAT or D-dfTAT in nrL-15 for 1h. Cells were then incubated in growth media (DMEM/10%FBS) and cellular proliferation was monitored over a period of 72h using a MTT assay. Experiments were done in triplicates with average and standard deviation indicated.

MCH58 Cells : D-dfTAT treated* in comparision to untreated cells

List of transcripts associated with cell death:

ABCC9*↑ ABL2*↑ ACTC1*↓ ADD3*↓
AFP↑ AGO2↑ AKR1C3↓ ALDOC↓
ANGPTL4*↑ ANKRD1↑ ARHGEF2↑ ARL6IP5↓
ATF3*↑ ATF4*↑ BCL6*↑ BDKRB1↑
BEX2↑ BIRC3*↑ BNIP3↓ CAT*↓
CCDC86↑ CCL2↑ CD55↑ CDH6*↓
CDK6↑ CEBPB↑ CEBPG↑ COL1A1↓
CRABP2↓ CRIP1↓ CRYAB↓ CSRNP1↑
CXCL1↑ CXCL12*↓ CXCL2↑ CXCL8*↑
DCN*↓ DDIT3↑ DEFB103A/DEFB103B*↑ DHRS2*↑
DLC1*↑ DNASE1L1*↓ DUSP1↑ EDN1↑
EGR1↑ EGR2↑ EPAS1↑ EPHA2↑
ERN1*↑ ETS1*↑ ETS2↑ F2RL1*↓
F3*↑ FBN2↓ FBXO32*↑ FLNA*↑
FOSB↑ FOXO3*↑ GADD45A*↑ GADD45B↑
OSR1↓ PAWR↑ PDE5A*↓ PDGFRA*↓
PDGFRB↓ PHLDA1*↑ PIM3*↑ PITX2*↓
PMAIP1*↑ PPP1R15A↑ PPP2R2A↑ PPP2R2B*↓
PPP3CC↑ PRKCDBP↓ PRKCH↑ PTGES↓
PTGIS↓ PTGS2*↑ PTPN13*↓ PTPRR*↑
RAB32↑ RABGGTB*↑ RCAN1*↑ RCAN2*↓
RECK*↓ RELB↑ RGMB*↑ RND3↑
S1PR1*↑ SAT1↑ SCD↓ SERPINB2*↑
SERPINH1↓ SESN2↑ SLC25A24*↑ SLC2A3↑
SLC3A2*↑ SMAD6↓ SOCS2*↑ SOD2*↑
SPARC↓ SPHK1*↑ SQSTM1↑ SRPK2*↑
STK17B*↑ SULF1↓ TEX10↑ TFPI2↑
THRA*↓ THUMPD2↑ THY1↓ TIAF1*↓
TMEM109↓ TMEM14A↓ TMEM158↑ TNFAIP3↑
TNFRSF10B*↑ TNFRSF12A↑ TNFRSF19*↓ TRAF1*↑
GALNT5↓ GAS1*↓ GDF15↑ GEM*↑
GLIPR1↑ GNG2↓ HBEGF↑ HDAC9*↑
HIST1H1C↑ HRASLS↑ HSPA2*↓ HSPA9↑
HSPB8↑ HSPBAP1↑ ID2*↓ IDH2↓
IER3↑ IFRD1*↑ IGFBP3*↓ IL11↑
IL12A↑ IL1A↑ IL1B↑ IL1RL1*↑
IL32*↑ IL6↑ ING3*↑ IRAK2↑
IRF1↑ IRF7*↑ IRS2↑ JUN↑
JUNB↑ KLF10*↑ KLF6*↑ LDLR↓
LIF↑ LIPA↓ LUM*↓ MAFG*↑
MAGED1*↓ MAGEH1*↓ MAP4K2↓ mir-302*↑
MXD3↓ MYC*↑ NFKB1↑ NFKB2*↑
NFKBIA↑ NFKBIB*↑ NFKBIZ*↑ NGF↑
NKX3-1↑ NOV↓ NPAS2↑ NPC1↑
NPTX1*↑ NR1H4↑ NUPR1*↑ ODC1↑
TRIB3↑ TSC22D1*↑ TXNIP↑ VGF↑
WDR4*↑ WIPF1*↓ WNT2↓ YARS↑
ZC3H12A↑

List of transcripts associated with proliferation:

ABCC9*↑ ABL2*↑ AFP↑ AGO2↑
AKR1C1/AKR1C2*↓ AKR1C3↓ ANGPTL4*↑ ARHGEF2↑
ARL6IP5↓ ATF3*↑ ATF4*↑ BCL6*↑
BDKRB1↑ BEX2↑ BIRC3*↑ BNIP3↓
CA12*↓ CAT*↓ CCL2↑ CCL20↑
CCNE2*↓ CD248↓ CD55↑ CD83*↑
CDK6↑ CEBPB↑ CHN1*↓ CLDN1↑
CLK1*↑ COL1A1↓ COL1A2*↓ CRABP2↓
CRIP1↓ CRY1*↑ CRYAB↓ CTHRC1*↓
CXCL1↑ CXCL12*↓ CXCL2↑ CXCL5*↑
CXCL8*↑ DCN*↓ DDIT3↑ DLC1*↑
DOK5↑ DUSP1↑ DUSP5↑ E2F5↑
EDN1↑ EGR1↑ EGR2↑ EPAS1↑
EPHA2↑ ERN1*↑ ERRFI1↑ ESM1*↑
ETS1*↑ ETS2↑ F2RL1*↓ F3*↑
FBN2↓ FLNA*↑ FOSB↑ FOXO3*↑
FZD7*↓ GABARAPL1↑ GADD45A*↑ GADD45B↑
GAS1*↓ GDF15↑ GEM*↑ GNG2↓
HBEGF↑ HLX*↑ HNRNPA2B1*↑ HRASLS↑
HSPB8↑ ID2*↓ IDH2↓ IER3↑
IGFBP3*↓ IL11↑ IL12A↑ IL1A↑
IL1B↑ IL1RL1*↑ IL32*↑ IL6↑
IRF1↑ IRS2↑ JUN↑ JUNB↑
KIF20A↓ KLF10*↑ KLF6*↑ LIF↑
LIPA↓ LUM*↓ MAFF*↑ MAFG*↑
MAGED1*↓ MCM3*↓ MN1*↓ MXD3↓
MXD4↓ MYC*↑ NFKB1↑ NFKB2*↑
NFKBIA↑ NFKBIB*↑ NGF↑ NKX3-1↑
NOV↓ NPC1↑ NPTX1*↑ NR1H4↑
NREP↓ NUPR1*↑ ODC1↑ OSR1↓
P4HA1*↓ P4HA2*↓ PAPPA↑ PAPSS2↓
PAWR↑ PCYOX1*↓ PDE5A*↓ PDGFRA*↓
PDGFRB↓ PDIA5↓ PHLDA1*↑ PIM3*↑
PITX2*↓ PMAIP1*↑ PPP1R15A↑ PPP2R2B*↓
PRKCDBP↓ PRKCH↑ PRRX1*↓ PTGES↓
PTGS2*↑ PTPN13*↓ PTPRR*↑ PTX3↑
RCAN1*↑ RCAN2*↓ RECK*↓ RELB↑
RND3↑ S1PR1*↑ SAT1↑ SCD↓
SCG2↑ SERPINB2*↑ SERPINH1↓ SLC22A1*↑
SLC3A2*↑ SLC7A5↑ SMAD6↓ SOCS2*↑
SOD2*↑ SPARC↓ SPHK1*↑ SQSTM1↑
SRPK2*↑ SSR1↓ STK17B*↑ STX3↑
SULF1↓ TAF1D*↑ TFPI2↑ THRA*↓
THY1↓ TNFAIP3↑ TNFRSF10B*↑ TNFRSF12A↑
TNFRSF19*↓ TRAF1*↑ TRIB3↑ TSC22D1*↑
TXNIP↑ UPP1*↑ VGF↑ WARS*↑
WIPF1*↓ WNT2↓ YARS↑ ZP3*↑

* Treated: (5 μM, 1 h) + 24 h

Table S1

Table showing the list of names of gene transcripts related to cell death and proliferation that are affected after treatment with D-dfTAT. The upward red arrow indicates an up regulation compared to control while the downward green arrow indicates a down regulation using a two fold threshold. Data were analyzed through the use of QIAGEN's Ingenuity ® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiage.com/ingenuity).

REFRENCES

1. Lee, Y. J., Datta, S., and Pellois, J. P. (2008) Real-time fluorescence detection of protein transduction into live cells, *Journal of the American Chemical Society 130*, 2398-2399.