Electronic Supplementary Material (ESI) for Molecular BioSystems

Mutagenesis Modulates the Uptake Efficiency, Cell-Selectivity, and Functional Enzyme Delivery of a Protein Transduction Domain

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Materials and Instrumentation

Materials

Phosphate buffered saline (PBS) - Hyclone/Thermo Scientific
0.25% Trypsin - Hyclone/Thermo Scientific
Brilliant Blue R-250 - J.T.Baker
Bovine serum albumin - Sigma Aldrich
Fetal bovine serum (FBS) - PAA Laboratories
Triton X-100 - Fisher Scientific
Dulbecco's modified Eagle medium (DMEM) - Hyclone/Thermo Scientific
F-12K Nutrient Mixture (Kaighn's Mod.) - Cellgro/Corning
RPMI-1640 media - Hyclone/Thermo Scientific
Mammalian cell culture dishes - Fisher Scientific
B-PER Bacterial Protein Extraction Reagent - Thermo Scientific
Imidazole - Sigma Aldrich
Modified Lowry Protein Assay Kit - Pierce/Thermo Scientific
Nano-Glo® Luciferase Assay - Promega
TACS MTT reagent- Trevigen
PageRuler Prestained Protein Ladder - Thermo Scientific

All water was obtained from a Milli-Q water purification system.

Instrumentation

All flow cytometry data was carried out on a MoFlo Flow Cytometer and High Speed Cell Sorter with a solid-state iCyt 488nm laser.
Relative luciferase units were measured on a Synergy Mx Microplate Reader from BioTek.
MTT assay absorbance was measured on Synergy Mx Microplate Reader from BioTek.
Fluorescence microscopy images were taken with EVOS FL from Advanced Microscopy Group.

Experimental Data

Experimental Procedures

Mammalian cell culture. Human prostate adenocarcinoma cells (PC-3) cells were cultured in F12K with 10% Fetal Bovine Serum (FBS) and HEK293T cells cultured in high glucose Dulbecco's modified Eagle medium (DMEM) with 10% Fetal Bovine Serum (FBS). All cells were incubated at 37 °C with 5% CO_2 environment. All cells were obtained from ATCC.

Cloning. All plasmids were constructed on a pETDuet-1 backbone. All peptides and GGS linkers on the N-terminus and C-terminus of sfGFP were assembled from a set of overlapping oligonucleotides. The peptides were then amplified with the sfGFP or nLuc proteins and the plasmids were ligated into *Nco*I and *Kpn*I restriction enzyme cleavage sites in the pETDuet-1 plasmid.

Protein Purification. Cells were grown in 500 mL LB cultures at 37 °C to OD_{600}= ~0.6 and induced with 1 mM IPTG at 30 °C overnight. Cells were then collected by centrifugation and stored at -20 °C. Frozen pellets were thawed and 20 mL B-PER was added to lyse cells. The lysate was cleared by centrifugation (17000 rpm, 30 min.) and the supernatant was mixed with 1 mL of Ni-NTA agarose resin for 1 hour. The resin was collected by centrifugation (4950 rpm, 10 min.). The resin was washed with 50 mL of PBS with 300 mM NaCl and 20 mM imidazole. The protein was then eluted with 5 mL PBS containing 300 mM NaCl and 500 mM imidazole. The proteins were dialyzed against PBS and analyzed for purity by SDS-PAGE staining with Coomassie Blue. The proteins were then quantified using a modified Lowry protein assay kit. nLuc proteins were purified in the sample way, except washed with Tris buffers (25 mM Tris-HCl, 100 mM NaCl, pH=8.0) instead of phosphate buffers.

Flow Cytometry Analysis. Mammalian cells were grown to 90% confluency in a 12-well plate. Cells were then washed once with PBS and 500 µL of diluted protein in PBS was added. The cells were incubated with the protein solution for 3 hours at 37 °C, 5 % CO_2 environment. After the incubation period, cells were then washed once with PBS and two times with PBS-HS (heparin sulfate 20 U/mL) for 10 minutes at 37 °C/ 5% CO_2. The cells were then removed from dish with 0.5 mL of 0.25%Trypsin and collected by centrifugation. The cells were then resuspended in PBS-HS and taken for flow cytometry analysis.

Live Cell Fluorescence Microscopy. Mammalian cells were grown to 90% confluency in a 12-well plate. Cells were then washed once with PBS and 500 µL of 5 µM protein in PBS was added. The cells were incubated with the protein solution for 3 hours at 37 °C, 5 % CO_2 environment. After the incubation period, cells were then washed once with PBS and three times with PBS-HS (heparin sulfate 20 U/mL) for 10 minutes at 37 °C/ 5% CO_2. The cells were then imaged on the EVOS FL fluorescence microscope.
For 4 ˚C experiments, the PC-3 cells were incubated at 4 ˚C for 30 minutes prior to the addition of the diluted protein. The incubation period was carried out at 4 ˚C and washed as described above.

MTT Assay. PC-3 cells were grown to 90% confluency in a 12-well plate. Cells were then washed once with PBS and incubated with the protein in PBS for 3 hours at 37 °C/5% CO_2. The solution was removed and the cells were washed twice with PBS-HS (heparin sulfate 20 U/mL). The cells were then incubated with 0.5 mL medium with 25 μL of MTT reagent for 4.5 hours. After the incubation, 250 μL detergent was added to the cells and they were incubated for an addition 30 minutes. MTT assay readings were taken with a Synergy Mx microplate reader at 570 nm.

NanoGlo Luciferase Assay. PC-3 cells were grown to ~80% confluency in a 24-well plate (clear bottom, black well). The nLuc proteins were diluted in TBS (25 mM Tris-HCl, 150 mM NaCl, pH=7.0) and added to the PC-3 cells. Cells were incubated with each solution for 3 hr at 37 ˚C under 5% CO_2 environment. The cells were then washed with TBS, TBS-0.1% tween-20, and TBS-HS (heparin sulfate 20 U/mL). This washing procedure was repeated a total of two times. Then, the cells were incubated with 200 μL TBS and 200 μL Nano-Glo Luciferase Assay Reagent for 10 minutes. Luminescence was measured on a Synergy Mx microplate reader.

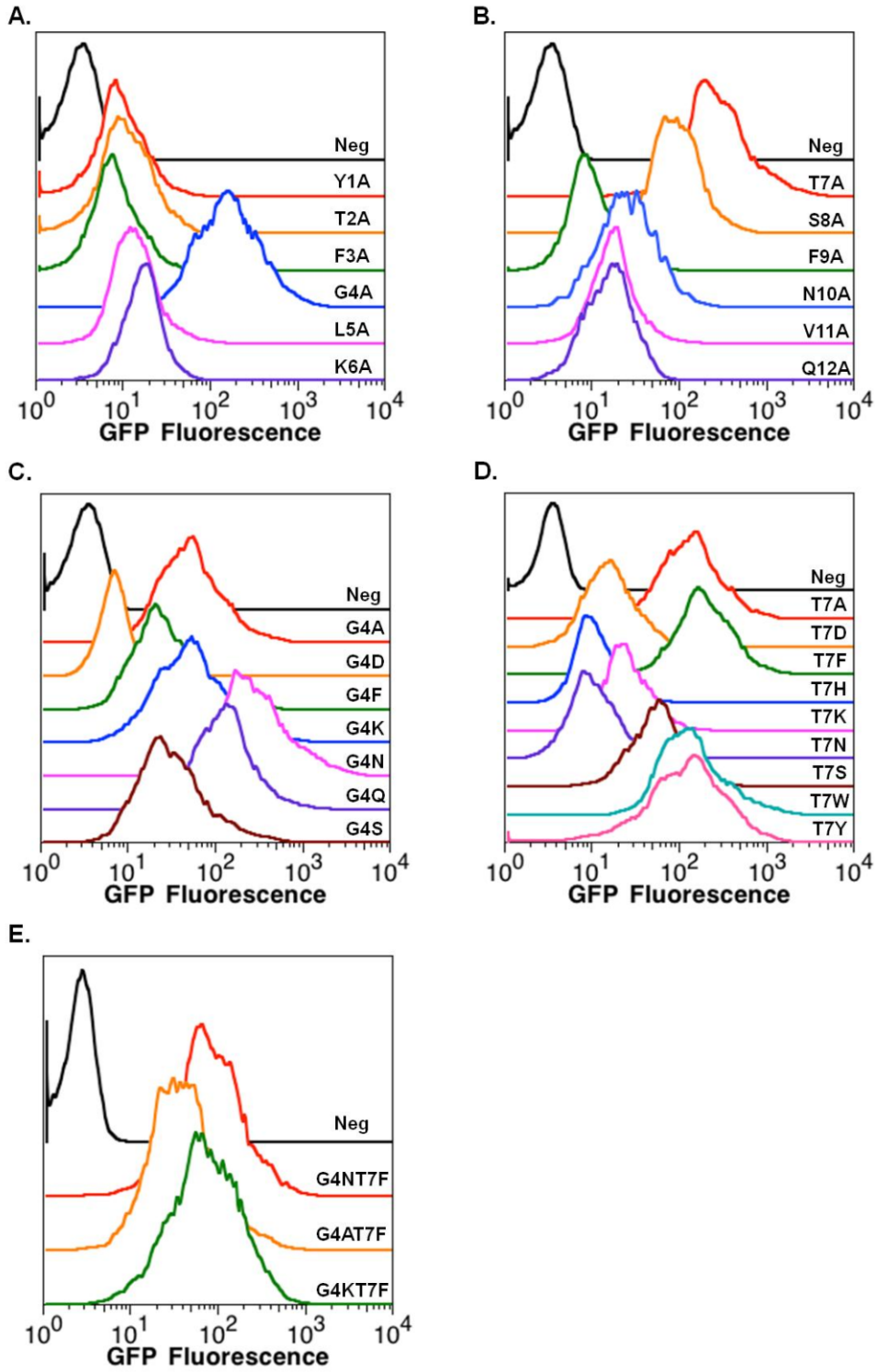

Figure S1. Representative flow cytometry data from Figure 1. Flow cytometry data showing (A-B) GFP uptake for alanine mutants of Ypep-GFP. (C) GFP uptake for Ypep-GFP mutants at residue 4. (D) GFP uptake of Ypep-GFP mutants at residue 7. (E) GFP uptake of Ypep-GFP double mutants at residue 4 and 7. (A-E) PC-3 cells treated with 5 μM mutant Ypep-GFP for 3 hours at 37 ˚C, then washed as described in the Experimental Procedures.

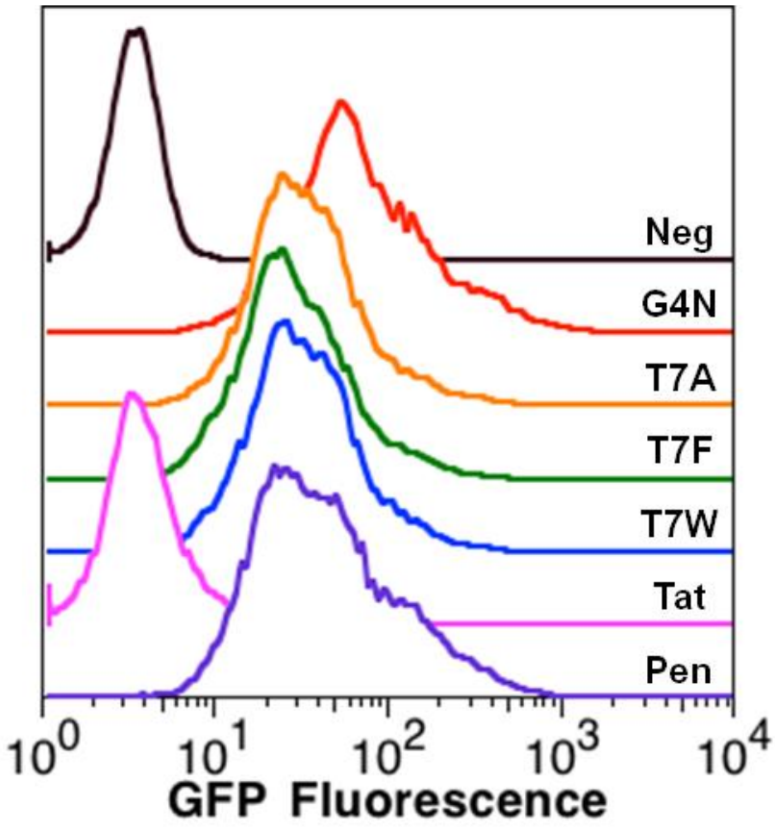

Figure S2. Representative flow cytometry data from Figure 3. Flow cytometry data showing GFP internalization of Ypep(mutant)-GFP fusions, Tat-GFP, and penetratin-GFP. PC-3 cells were treated with 1 μM of each of the proteins for 3 hours at 37 ˚C, then washed as described in the Experimental Procedures.

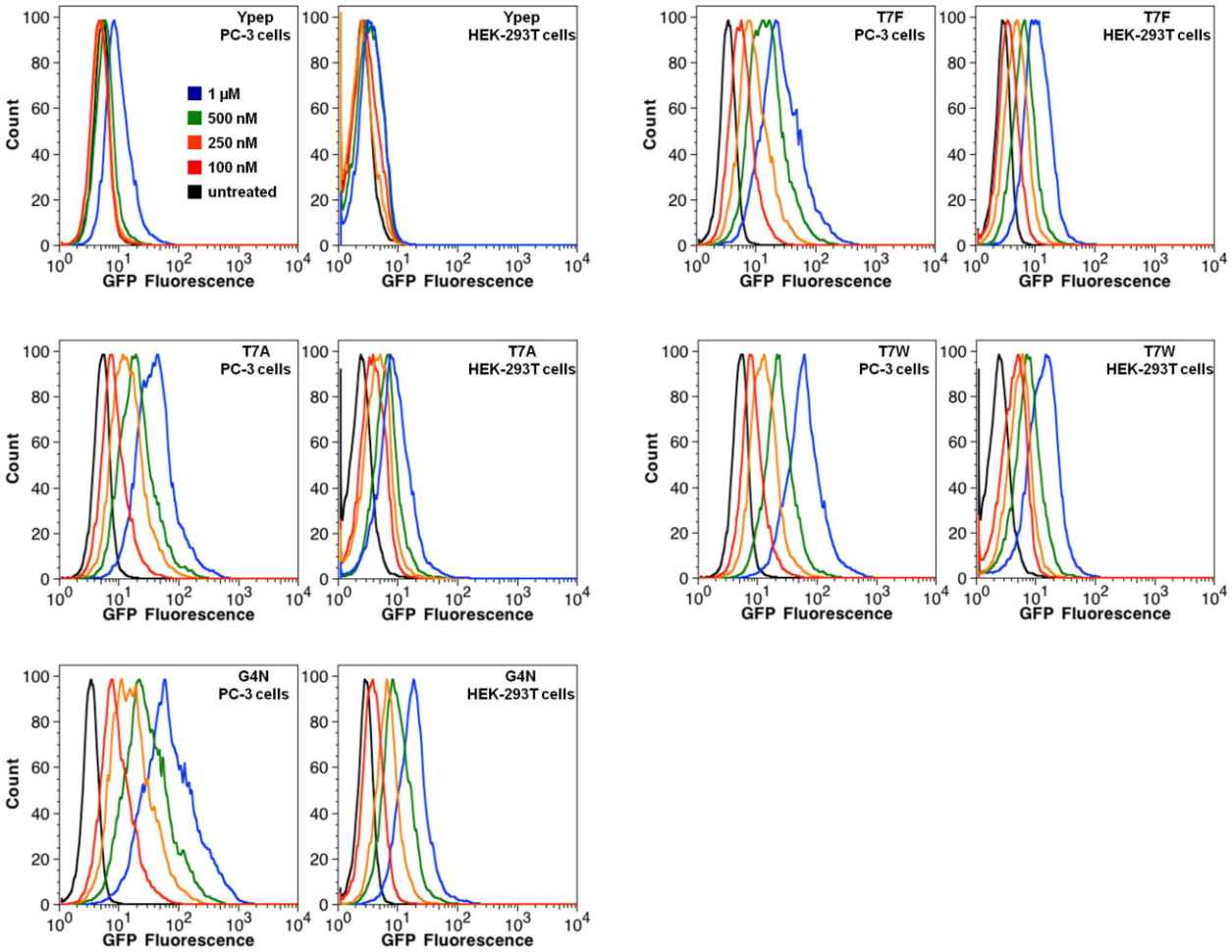

Figure S3. Representative flow cytometry data from Figure 4. Flow cytometry data showing the amount of internalized GFP in PC-3 cells or HEK-293T cells following treatment with 100 nM, 250 nM, 500 nM, or 1 μM Ypep-GFP, Ypep(T7F)-GFP, Ypep(T7A)-GFP, Ypep(T7W)-GFP, or Ypep(G4N)-GFP for 3 hours at 37 ˚C in PBS.

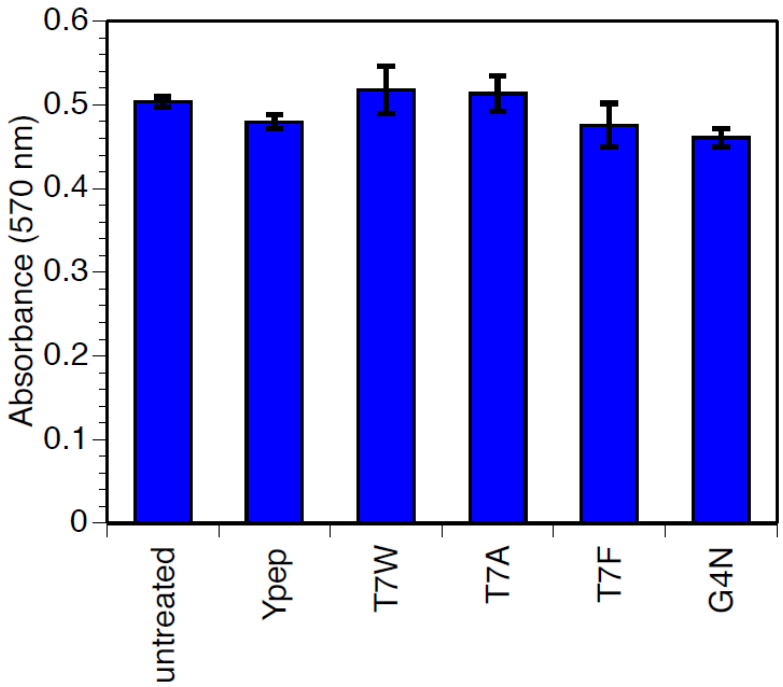

Figure S4. MTT cell viability assay data. PC-3 cells in 12-well plates were treated with treated with 5 μM protein in PBS or in PBS alone (untreated sample) for 3 hours at 37 ˚C with 5 % CO_2. The cells were then washed with PBS-heparin sulfate twice, followed by one wash with PBS. The MTT assay was then performed on the cells and the absorbance was read on Synergy Mx Microplate Reader from BioTek. Error bars represent the standard deviation from three independent experiments.

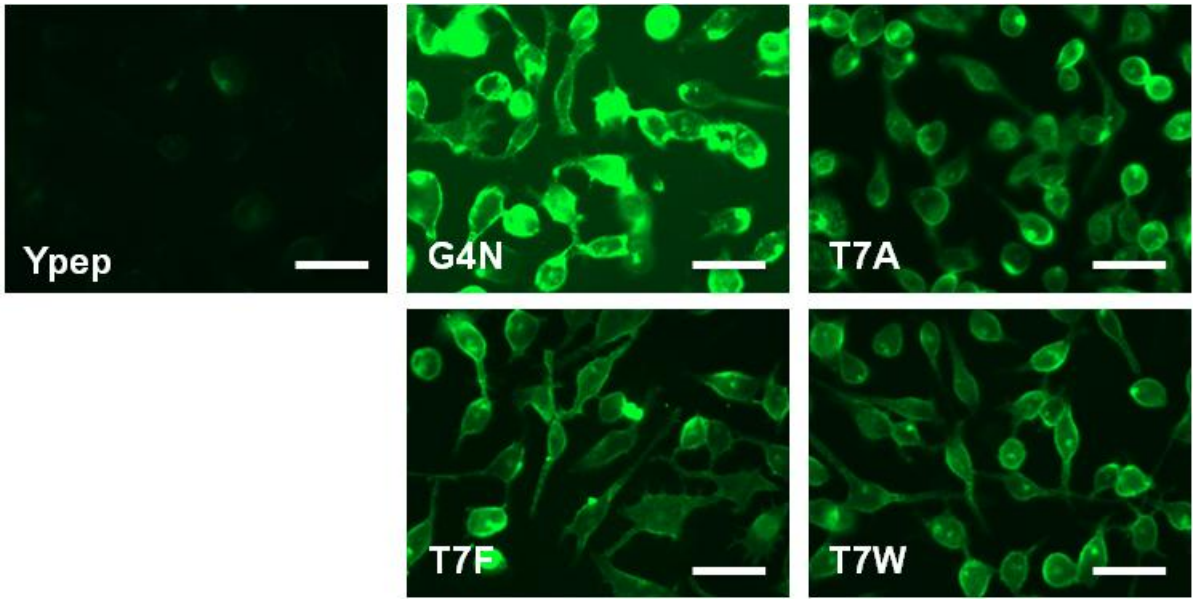

Figure S5. Fluorescence microscopy comparison of Ypep-GFP to mutant Ypep-GFP fusions. Live cell fluorescence microscopy images of PC-3 cells following treatment with 5 μM of the most efficient mutant Ypep-GFP fusions, then washed to remove cell surface-bound protein. Green color represents internalized GFP. The scale bar is 50 μm. Lamp intensity was set at 50%, with a 250 msec exposure for all images.

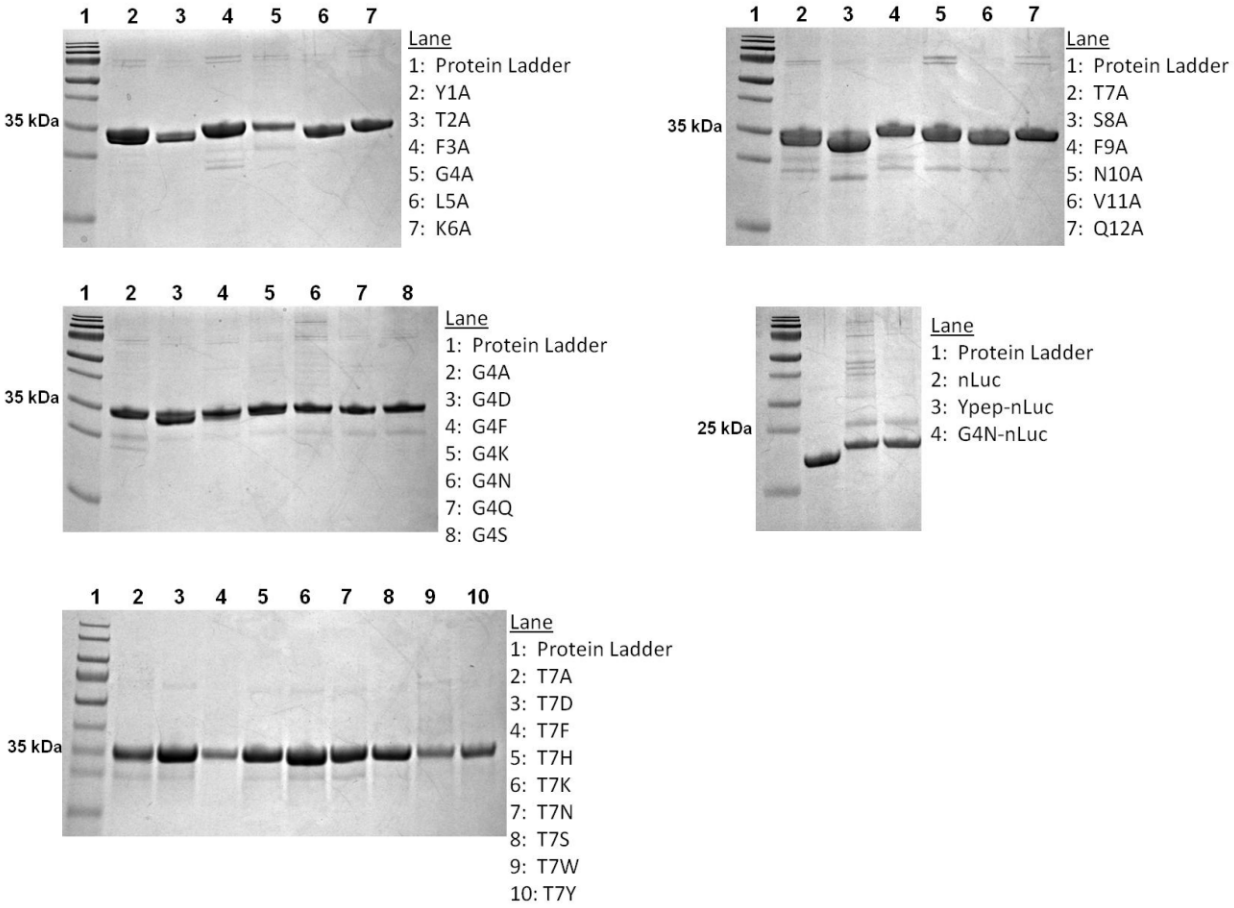

Figure S6. PAGE analysis of all proteins used in this work. Proteins were run on 15% Tris-HCl gels (BioRad), and stained with Coomassie Blue.

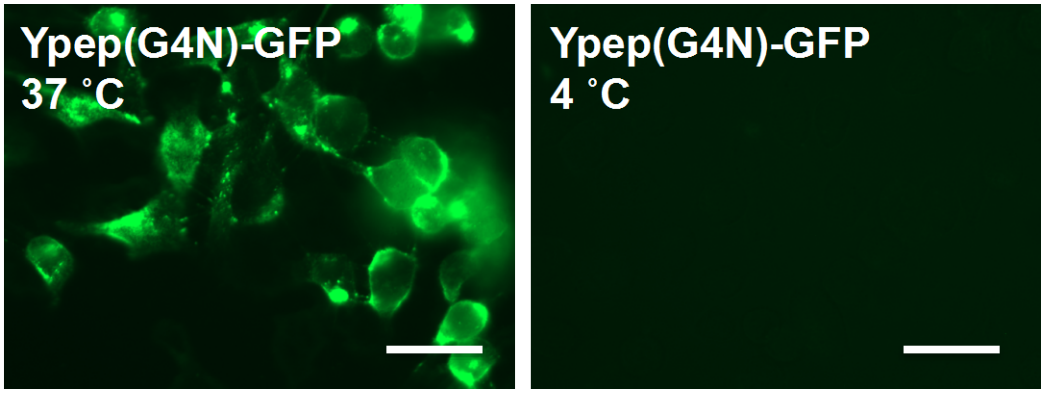

Figure S7. Cell penetration of G4N-GFP at 37 °C or 4 ˚C. Live cell fluorescence microscopy images of PC-3 cells following treatment with 5 μM of the (G4N) Ypep-GFP fusions, at either 37 °C or 4 °C for 30 minutes. Cells were then washed to remove cell surface-bound protein. Green color represents internalized GFP. The scale bar is 50 μm. Lamp intensity was set at 50%, with a 250 msec exposure for all images.

Figure S8. Sequence information for all proteins used in this work.

Ypep-GFP
MGYTFGLKTSFNVQGGSGGSGGSGGSMGGASKGEELFTGVVPILVELDGDVNGHKFSVRGE
GEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQE
RTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGI
KANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFV
TAARITHGMDELYKHHHHHH

Tat-GFP
MGYGRKKRRQRRRGGSGGSGGSGGSMGGASKGEELFTGVVPILVELDGDVNGHKFSVRGE
GEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQE
RTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGI
KANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTPSALSKDPNEKRDHMVLLEFV
TAAGITHGMDELYKHHHHHH

Pen-GFP
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GYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITAD
KQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHM
VLLEFVTAAGITHGMDELYKHHHHHH

nLuc
MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPYE
GLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVT
GTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILAHHHHHH

Ypep-nLuc
YTFGLKTSFNVQGGSALALGMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTP
IQRIVLSGENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMID
YFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILAHHHH
HH