Supporting Information (SI)

Visualization of the Delivery and Release of Small RNAs Using Genetic Code Expansion and Unnatural RNA-binding Proteins

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Materials and Methods:

Cloning of CIRV-p19

The codon optimized CIRV p19 was designed and cloned into pTriEx-Neo-4 vector, as described before[1]. Amber codon (TAG) has been incorporated using site-directed mutagenesis resulting in the following constructs pTriEx-p19-W19AzF and pTriEX-p19-N46AzF using QuickChange Site-Directed Mutagenesis (Agilent) according to manufacturer's instructions. The obtained constructs were validated by DNA sequencing to confirm the mutated nucleotides. Sequencing to confirm mutations was performed at Génome Québec (Montreal, Canada).

Table 1

Primer	Sequence
W19TAG fwd	ctaacggtgaagctaggatggcggctcc
W19TAG rev	ggagccgccatcctagcgttcaccgttag
N46TAG fwd	ctgagtggcgcctgtattaggatgagaccaattcc
N46TAG rev	ggaattggtctcatcctaatacaggcgccactcag

Introduction of amber mutations in a triple III p19 Mutant

CIRV p19 containing three different cysteines residues (C110, C134 and C160) mutated to Isoleucine as described previously[1,2] using QuikChange Site-Directed Mutagenesis kit (Agilent). Amber codons (TAG) have been introduced resulting in the following constructs pTriEx-p19III-W19AzF and pTriEX-p19III-N46AzF using primers presented in table 1.

Bacterial overexpression of CIRV p19 and mutants

E.coli strain BL21 (DE3) (Thermofisher Scientific) transformed with the constructs were grown at 37^{o}C in the presence of 100 ug/ ml ampicillin in LB media until the optical density at 600 reached 0.5. Constructs containing the amber mutation (pTriEx-p19-W19AzF and pTriEX-p19-N46AzF, pTriEx-p19III-W19AzF and pTriEX-p19III-N46AzF, pTriEx-p19III-W19AzF and pTriEX-p19III-N46AzF) were transformed in *E.coli* Bl21 (DE3) pre-transformed with pULTRA-CNF[3], a vector bearing a polyspecific MJ tyrosyl synthetase and tRNA pair. The plasmid encoding the p-cyanophenylalanine specific aminoacyl-tRNA synthetase/suppressor tRNA pair was a kind gift from Dr. Peter Schultz (The Scripps Research Institute).

Expression of p19-TAG mutants were induced by IPTG at a final concentration of 1mM. Additionally, a final concentration of 1mM p-Azidophenylalanine (Bachem) has been added to the media upon induction. Post induction, the cultures were grown for 4 hours at 25 ^{o}C. Cells were harvested at 5000xg for 5 minutes. The pellets were suspended in lysis buffer (50 mM TRIS, 300 mM NaCl, 1mM DTT , 10 mM Imidazole, pH 8.0) and subsequently lysed by sonication for 2 minutes with one second pulse on/ off at 50% amplitude. Cells were then centrifuged at 20,000xg for 20 minutes at 4^{o}C to obtain the soluble fraction. Recombinant p19-wt and mutants were purified using Ni2+NTA column (HisTrap FF, GE Health). Proteins were washed using washing buffer (50 mM TRIS, 600 mM NaCl, 1mM DTT, 60 mM Imidazole, pH 8.0). Proteins were then eluted using elution buffer (50 mM TRIS, 300 mM NaCl, 250 mM Imidazole, 10 mM β-mercaptoethanol). The eluted fraction was then concentrated using centrifugation tubes (10 kDa MWCO, Amicon). Pure dimerized p19-wt or mutants were further fractionated using size exclusion chromatography using S200 column (FPLC ÄKTA pure). Samples were then concentrated using centrifugation tubes (10 kDa MWCO, Amicon) and the concentration was determined using Bradford assay (Bio-Rad).

Fluorescent labeling

p19-wt and p19-TAG mutants, as well p19-III and corresponding III mutants were purified as described above. Fluorescent labeling was performed for all mutants using DBCO-Cy5 (Sigma Aldrich) in 1:2 protein: dye ratio in 1x PBS buffer pH 7.4 where SPAAC reaction took place. 40 µM of p19-WT or mutants were incubated with the dye at room temperature for 3 hours. To remove excess dye, the labeled proteins were loaded on p30 gel microspin columns (Bio-Rad).

Subsequently, samples were concentrated and labeled protein was analyzed via SDS-PAGE and in-gel fluorescence to determine the labeling efficiency.

Immunoblotting

p19 expression and UAA incorporation was analyzed using SDS-PAGE and Western blotting. 20 µl of soluble fraction per sample were analyzed using 12% stain-free polyacrylamide gel electrophoresis (TGX Stain-Free Fastcast Acrylamide kit, Bio-Rad) and western blotting. A control culture, without *p*-AzF addition, was grown under the same conditions. Proteins were transferred to a PVDF membrane using the Trans-blot Turbo RTA Transfer Kit (Bio-Rad). Blots were blocked in tris-buffered saline with 0.05% Tween-20 (TBS-T) containing 5% W/V milk and washed then in TBS-T. Proteins were probed by α-histag HRP antibody (1:1000, life technologies). The antibody was detected via clarity western ECL substrate (Bio-Rad) and visualized on a ChemiDoc MP imaging system (Bio-Rad).

CD Analysis

CD measurements were performed for p19III, p19III-W19AzF and p19III-N46AzF using JASCO J-815 (JASCO) with quartz cell path length 0.01 cm at 25^{o}C. Proteins were prepared in phosphate buffered saline (PBS buffer pH 7.5) at 10 µM protein concentration. Spectra reflect an average of 8 scans recorded from 185-250 nm with a 0.2 nm step resolution, speed of 20 nm/ minute and a band width of 1 nm.

Substrates

GL2 siRNA cyanine 3 labeled guide strand (5'-Cy3-CGU ACG CGG AAU ACU UCG AUU-3') and passenger strand (5'-UCG AAG UAU UCC GCG UAC GUU-3'; Sigma-Aldrich) and GL2 siRNA FAM 3 labeled guide strand (5'-FAM-CGU ACG CGG AAU ACU UCG AUU-3') and

passenger strand (5'-UCG AAG UAU UCC GCG UAC GUU-3'; Sigma-Aldrich) were annealed at a 1:1.2 molar ratio of unlabeled/labeled RNA in a buffer containing 100 mM potassium acetate, 30 mM HEPES pH 7.5, and 2 mM magnesium acetate by heating to 95°C for 2 min and cooling to 25°C at a rate of 1°C/min.

Electrophoretic Mobility Shift Assay

Samples were prepared by incubating 1 nmol/l FAM-labelled siRNA with 0–500 μmol/l of purified p19 WT or mutants in 20 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.02% v/v TritonX-100, 2 mmol/l dithiothreitol, pH 7 for 1 hour at room temperature. The samples were analyzed by electrophoresis, where 5× Tris/Borate/EDTA (TBE) sample buffer (90 mmol/l Tris, 90 mmol/l boric acid, 2 mmol/l EDTA, 15% Ficoll type 400, 0.02% xylene cyanol) was diluted to 1× in the binding reaction and then 10 μl applied to a 6% TBE gel (Thermofisher Scientific). The gel was ran at 100 V for 45 minutes in 0.5x TBE. The gels were imaged using ChemiDoc MP System (Bio-Rad) and the densitometry was performed with ImageJ software (NIH). The fraction of RNA bound by p19 was determined by dividing the band intensity of p19-bound RNA by the sum of the band intensities from the bound and unbound RNA. The data were analyzed by plotting the fraction bound values against p19 concentration and fitted using GraphPad Prism 7 according to the following equation[4]:

$$\Delta P = \Delta P_{max}\left(\frac{K_d + np + x}{2np} - \sqrt{\left(\frac{K_d + np + x}{2np}\right)^2 - \frac{x}{np}}\right)$$

where $\triangle P$ denotes the change in fluorescence intensity, $\triangle P_{max}$ is the maximal change in fluorescence intensity, *Kd* is the dissociation constant, n is the number of equivalent sites on the

p19 dimer, p is the concentration of labelled small RNA, and x is the concentration of the p19 dimer.

Fluorescence Resonance Energy Transfer Measurements:

Measurements were carried out using SpectraMax i3 (Molecular devices). All measurements were carried out in a 96 well plate. Sample solution containing variable concentrations of p19-WT and mutants (p19-W19AzF) or p19III and corresponding mutants (p19 III-W19AzF and p19III-N46AzF) ranging from (0-250 nM) diluted in phosphate buffered saline (50 mM potassium phosphate, pH 7.5; 150 mM NaCl). Either Cy3-siRNA or unlabeled siRNA at 10 nM were added to the proteins (both Cy5 labeled proteins or unlabeled controls). After 1-hour equilibration, the fluorescence emission spectra were recorded. Samples were excited at 520 with 5 nM excitation band width and fluorescence spectra were recorded from 555 to 700 nm.

Cell culture

Huh7 cells were cultured at 37^{o}C with 5% CO_2 in DMEM (Gibco-invitrogen) with 10% fetal bovine serum and 100 nmol/l nonessential amino acids.

Imaging delivery of RNA using p19-W19AzF-III-TAT

Purified 2x-p19-W19AzF-III-TAT or 2xp19-III-TAT was concentrated using Amicon Ultra 10-kDa MWCO centrifugal filter device to 100 umol/l as determined by DC assay (Bio-Rad). DBCO-Cy5 in 1:2 protein: dye ratio in 1x PBS buffer pH 7.4. Proteins were then incubated at room temperature, protected from light for three hours to allow the labeling to procced. To remove excess dye-DBCO-Cy5, the labeled proteins were loaded on p30 gel microspin columns (Bio-Rad). Proteins were then complexed with BHQ-2 GL2 RNA (Dharmacon) in a 10:1 molar ratio

for 45 minutes at room temperature, protected from light, and applied to cells in optimem media (Gibco) at a final siRNA concentration of 500 nmol/L. Huh7 cells were seeded at 60% confluency in 8-well chamber slides (Thermofisher). After 24 hours, protein treatments were applied as described above at 3 different time points 2 hours, 5 hours or 24 hours. The cells were then washed with phosphate buffered saline and fixed using 4% formaldehyde and 4% sucrose solution in PBS. The cells were then mounted using ProLong™ Gold Antifade Mountant with DAPI (Thermofisher). The fluorescence imaging was performed using 40X objective on a Zeiss Axiophot Fluorescence Microscope (Zeiss). The images were captured at (Blue: Ex 365, Em 420) and Cy5 fluorescence (Red: 546, LP 590). The images were false coloured with imageJ and the same brightness and contrast was applied to images within the same time point.

Gene knockdown in human cell culture

Purified 2x-p19-W19AzF-III-TAT was concentrated using Amicon Ultra 10-kDa MWCO centrifugal filter device (Millipore) to 100 μmol/l and then complexed with luc2 siRNA (Thermo Scientific Dharmacon) with the sequence 5′-gga cga gga cga gca cuu cuu-3′,in a 10:1 molar ratio for 45 minutes at room temperature and applied to cells in reduced-serum transfection medium (Optimem, Invitrogen) at a final siRNA concentration of 500 nmol/l. After 4 hours, the cells were recovered by adding DMEM with 20% fetal bovine serum.

Luciferase Assay

Huh7 cells were seeded into 24-well plate at 40,000 cells/well, after 24 hours (at 70% confluency), cells were transfected with a dual luciferase reporter vector, psiCHECK-2 (Promega) using Lipofectamine2000 (Invitrogen) as per manufacturer's instructions. After 24 hours, the psiCHECK-2 expressing Huh7 cells were treated with 2x-p19-W19AzF-III-TAT: Luc2 complexes

as discussed above. Cells were lysed after 48 hours and analyzed using a Lmax luminometer microplate reader (Molecular Devices). Percent knockdown was obtained through calculating Luciferase firefly/ Renilla signal and normalizing to untreated control.

References

1. Sagan, S. M.; Koukiekolo, R.; Rodgers, E.; Goto, N. K.; Pezacki, J. P. (2007) Inhibition of siRNA Binding to a p19 Viral Suppressor of RNA Silencing by Cysteine Alkylation. *Angew. Chemie - Int. Ed.* 46, 2005–2009.
2. Cheng, J.; Koukiekolo, R.; Kieliszkiewicz, K.; Sagan, S. M.; Pezacki, J. P. (2009) Cysteine Residues of Carnation Italian Ringspot Virus p19 Suppressor of RNA Silencing Maintain Global Structural Integrity and Stability for siRNA Binding. Biochim. Biophys. *Acta* 1794, 1197–1203.
3. Young, D. D.; Young, T. S.; Jahnz, M.; Ahmad, I.; Spraggon, G.; Schultz, P. G. (2011) An Evolved Aminoacyl-tRNA Synthetase with Atypical Polysubstrate Specificity. *Biochemistry* 50, 1894–1900.
4. Danielson, D. C.; Sachrajda, N.; Wang, W.; Filip, R.; Pezacki, J. P. (2016) A Novel p19 Fusion Protein as a Delivery Agent for Short-Interfering RNAs. *Mol. Ther. Nucleic Acids* 5, e303.

Figures:

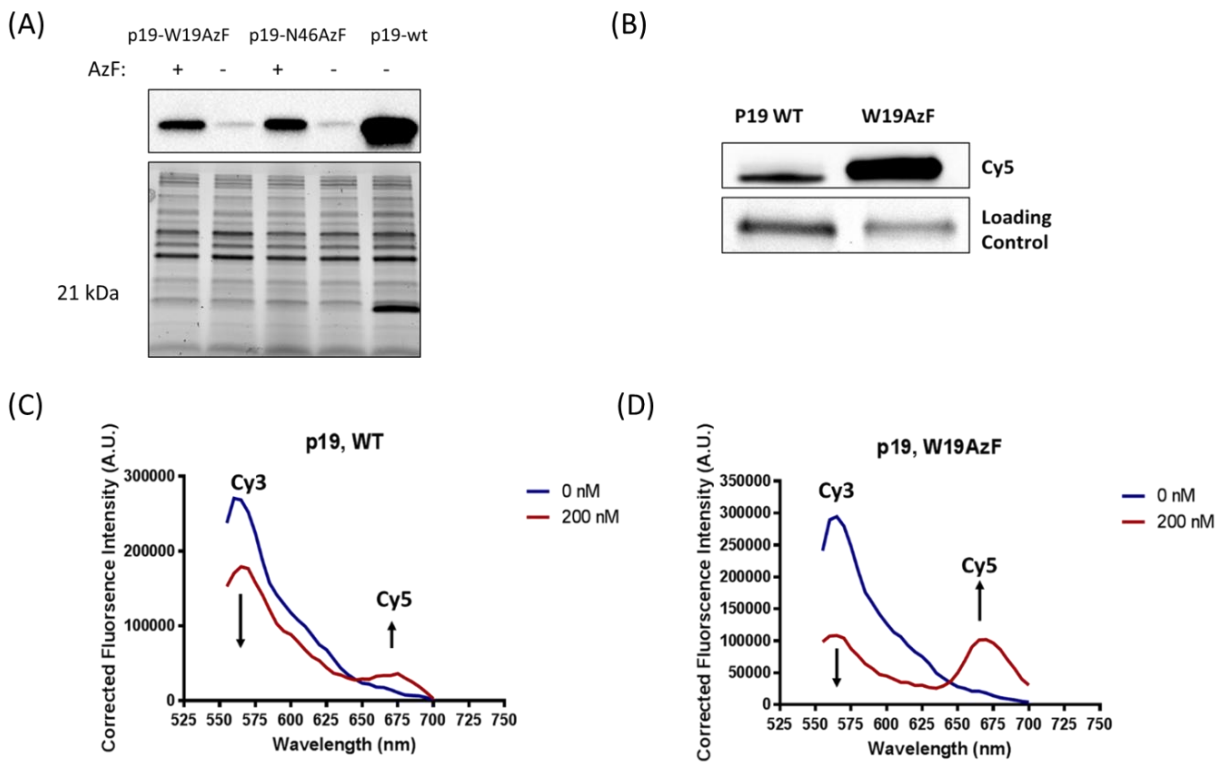

Figure S1: Expression Blot and Fluorescence spectra of Cy5 labelled mutant of p19. (A) Top panel represents a western blot depicting the expression of mutants (p19-W19AzF and p19-N46AzF) in the presence and absence of 1mM *p*-AzF from bacterial (*E.coli*) lysates. Bottom panel represents a TGX stain free SDS-PAGE gel as a loading control for bacterial cell lysate. (B) Purified p19-wt and p19-W19AzF are labeled using DBCO-Cy5 and analyzed using SDS-PAGE in-gel fluorescence (Cy5). Loading control refers to TGX stain-free gel. (C, D) 200 nM of p19-wt -Cy5 and p19-W19AzF-Cy5 are incubated with 10 nM Cy3-Gl2 siRNA duplex for 1 hour at room temperature. The spectra are recorded for p19-wt-Cy5 and p19-W19Azf-Cy5 with excitation at 520 nm with emission recorded from 555 to 700 nm.

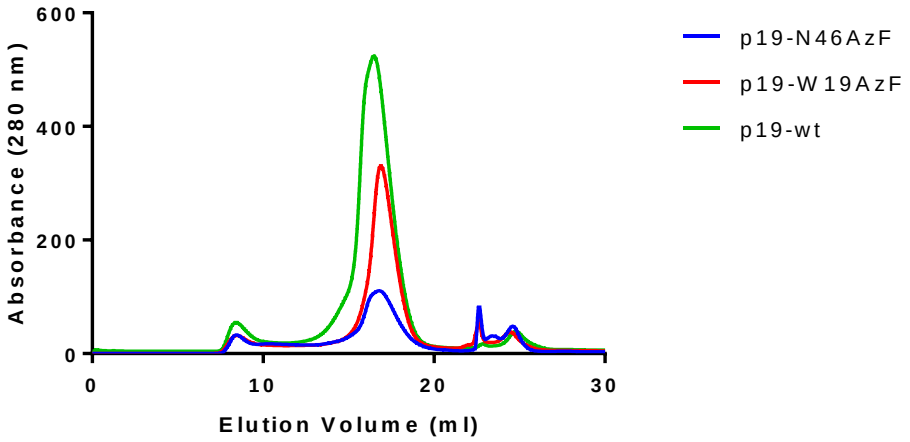

Figure S2: Size exclusion chromatography profile for p19-wt, p19-W19AzF and p19-N46AzF run in 1x PBS, pH 7.5 on a Superdex 200 column (GE).

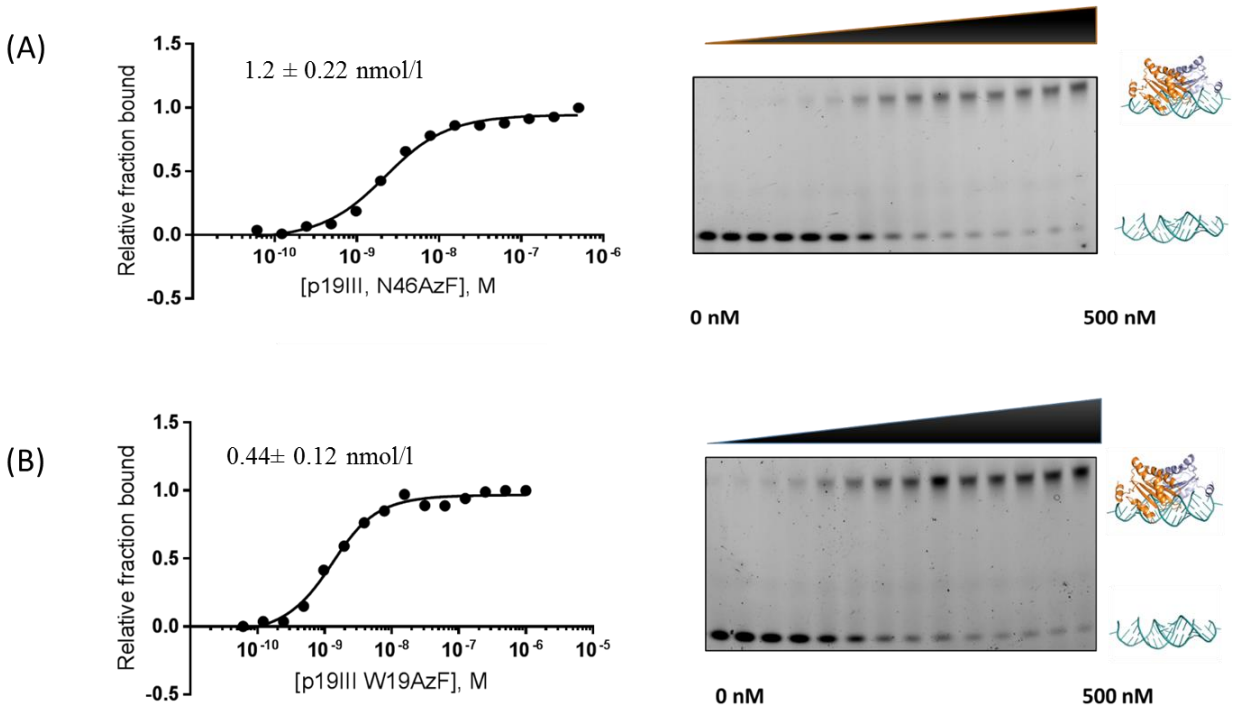

Figure S3: Binding affinity determination using electrophoretic mobility shift assay. Binding affinity of p19-III-W19AzF-Cy5 and p19-III-N46AzF-Cy5 as measured by EMSA using 1 nM 5' FAM labeled Gl2 siRNA (21 mer). Varying concentrations of protein (0 nM-500 nM) were incubated with fixed concentrations of siRNA at room temperature in EMSA buffer for 1 hour.

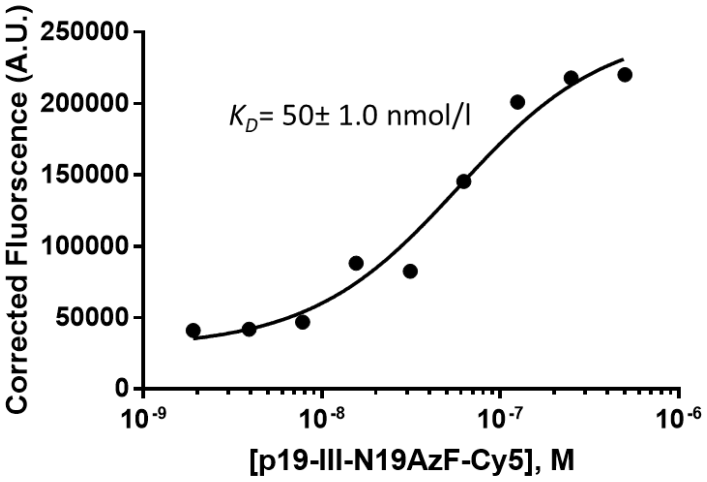

Figure S4: Binding affinity as determined by FRET. Binding affinity of p19-III-W19AzF-Cy5 as measured by FRET using 10 nM 5' Cy3-Gl2 siRNA (21 mer). Varying concentrations of protein were incubated with fixed concentrations of siRNA at room temperature for 1 hour.

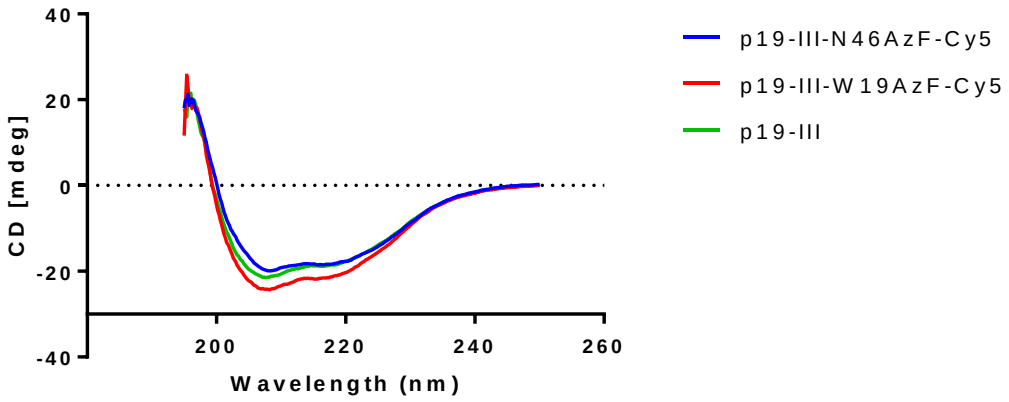

Figure S5: Circular dichroism of p19-III and labeled mutants. Far UV spectra for p19-III-N46AzF-Cy5, p19-III-W19AzF-Cy5 and p19-III is recorded from 185-250 nm.

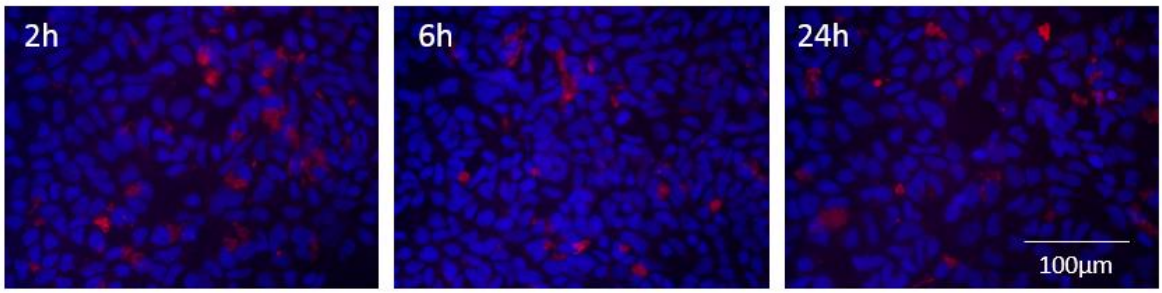

Figure S6: Fluorescence imaging of Cy5-2x-p19-III-W19AzF delivery of unlabeled siRNA in Huh7 cells. Cells were treated with 10:1 molar ratio of Cy5-2x-p19-III-W19AzF: unlabeled GL2 siRNA for 2hours, 5 hours and 24 hours. Scale bar= 100 µm. Nuclei were stained with DAPI (blue).

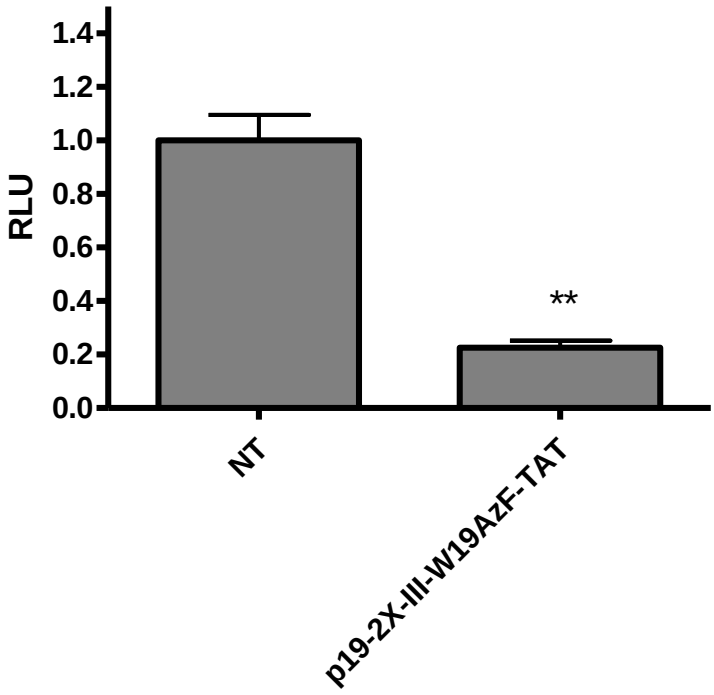

Figure S7: p19-2X-III-W19AzF-TAT delivers siRNA for gene knockdown in human cell culture. Gene knockdown reporter assay using dual luciferase reporter vector (psiCHECK-2) allow to observe luciferase gene knockdown after 48 hour treatments with p19-2X-III-W19AzF-TAT: Luc2 complex. **P<0.01, n=3.