Supplementary Information

Translational repression using BIV Tat peptide-TAR RNA interaction in mammalian cells

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TITLE RUNNING HEAD: BIV Tat-TAR translational repression in mammalian cells

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1. Materials and Methods

Plasmids

All PCR primers (see Supplementary Table 1 for detailed sequences) and oligonucleotides (see Supplementary Table 2 for detailed sequences) used in the present study were purchased from Integrated DNA Technologies Inc. (IL, USA), unless otherwise stated. Green Fluorescent Protein (GFP) was amplified from the pRNA-T U6.1 vector (Genscript USA Inc., NJ, USA) by polymerase chain reaction (PCR). Cyan Fluorescent Protein (CFP) was PCR-amplified from pCAG-CFP, which was a gift of Connie Cepko (Addgene plasmid # 11179). A plasmid, pNo-apt, was prepared by cloning GFP in between *Bgl* II and *Xho* I sites of multiple cloning site (MCS) 2 and CFP in between *Age* I and *Mlu* I sites of MCS 1 of pVITRO2-hygro-MCS vector (InvivoGen Inc., CA, USA). Constructs pAI-13, pBI-13, pCI-13, pDI-13 and pEI-13, were prepared by inserting corresponding DNA sequences (Supplementary Table 3) AI-13, BI-13, CI-13, DI-13 and EI-13 (Genscript USA Inc., NJ, USA) in between *Bsp* EI and *Xmn* I sites of pNo-apt, such that the hairpins were at +13 position downstream of the 5'-cap.

A construct pBIV-Temp was made by inserting the DNA sequence BIV-Temp (Supplementary Table 3) into the *Bsp* EI and *Xmn* I sites of pNo-apt to introduce restriction sites *Eco* RI upstream and *Bam* HI downstream of the 5'-cap. The construct pCI-1 was made by cloning the annealed oligonucleotides CI-1 A and CI-1 B

(Supplementary Table 2) in between *Eco* RI and *Bam* HI sites of pBIV-Temp. The plasmid pCI-120 was prepared by cloning annealed oligonucleotides CI-120 A and CI-120 B (Supplementary Table 2) encoding the corresponding hairpin into the *Xmn* I site of pNo-apt. The plasmid pCII-1 was constructed by inserting annealed oligonucleotides T1 A and T1 B (Supplementary Table 2) in the *Bam* HI site of pCI-1. The plasmid pCIII-1 was constructed by inserting annealed oligonucleotides T2 A and T2 B (Supplementary Table 2) in the *Bam* HI site of pCII-1. DsRed was amplified from pSIREN-RetroQ-DsRed-Express (Clontech Laboratories Inc., CA, USA) by PCR and cloned in between sites *Age* I and *Sal* I of MCS 1 of pVITRO2-hygro-MCS vector to construct pVITRO2-DsRed. CIBN was amplified from pCIBN(deltaNLS)-pmEGFP, which was a gift of Chandra Tucker (Addgene plasmid #26867). The reverse primer used for this amplification contained sequences encoding either BIV Tat peptide or a hexa-histidine tag (Supplementary Table 1). The resulting amplification products were then inserted in between *Bgl* II and *Xho* I sites of MCS 2 of pVITRO2-DsRed to generate pCIBN-BIV Tat and pCIBN-His respectively. GFP was amplified from pRNA-T U6.1 to yield an amplification product containing an HA tag on the N-terminus of GFP. The HA-GFP was then inserted in the *Bgl* II and *Nhe* I sites of pCII-1 to construct pCII-1'

Cell culture, transfections and flow cytometry

All cell culture reagents were purchased from Life Technologies Corporation, CA, USA. 293T cells (ATCC CRL-11268) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. The cells were grown in an

incubator maintained at 37°C and 5% CO_2. Cells were trypsinized using 0.05% Trypsin-EDTA one day before transfection and plated in 96-well plates at a density of 50,000 cells/well. Transfections were carried out using Lipofectamine 2000 reagent following the manufacturer's protocol. Cells were trypsinized 48 hours after transfection and re-suspended in 200 μL of complete medium. The samples were analyzed on a BD LSR II flow cytometer (BD Biosciences, CA, USA). Cells transfected with only the reporter constructs were gated for CFP positive population. Doubly transfected cells containing both reporter as well as the peptide constructs were gated for CFP and DsRed positive population. To control for the variability in transfection efficiency, the GFP signal was normalized with the corresponding CFP signal to yield a GFP/CFP ratio. For immunoblotting, transfected cells (5×10^5 cells per lane) containing both pCII-1' and pCIBN-BIV Tat (or pCIBN-His used as control) were sorted into collection tubes using BD FACS Aria.

Immunoblotting

The 293T cells co-transfected with pCII-1' and pCIBN-BIV Tat (or pCIBN-His used as control) were lysed and resolved on a 4-12% Bis-Tris SDS-PAGE gel. The lysate was then transferred from the gel onto a PVDF membrane and probed using anti-HA (sc-7392) primary antibody from Santa Cruz Biotechnology, Inc. (TX, USA).

RNA extraction and complementary DNA synthesis

Cytoplasmic RNA was isolated using RNeasy Mini Kit (Qiagen, Inc., CA, USA), following the manufacturer's instructions. The RNA samples were treated with RNase-

free DNase I, and RNA integrity was tested on a 2% agarose gel. RNA was quantified, and 500 ng of RNA from each sample was reverse-transcribed to cDNA using the ImProm-II Reverse Transcription system (Promega Corporation, WI, USA). Final cDNA reaction product was diluted with DNase- and RNase-free water to a concentration corresponding to 10 ng/μL of starting RNA and stored at –20 °C.

Quantitative real-time polymerase chain reaction (q-RT-PCR)

GFP-specific sense and antisense primers were purchased from Integrated DNA Technologies Inc. (IA, USA). The TaqMan probes for GFP and 18S (housekeeping gene) were purchased from BioSearch Technologies Inc. (CA, USA). The nucleotide sequences of the primers and probes are shown in Supplementary Table 4. The q-RT-PCR was performed in a LightCycler 489 (Roche, Basel, Switzerland) in a 96-well plate. Brilliant q-RT-PCR Master Mix (Agilent Technologies Inc., CA, USA) was used according to the manufacturer's instructions. Briefly, 12.5 μL of master mix, 1 μL of cDNA as template, and 0.2 mM of the primers and TaqMan probe were used in a final reaction volume of 25 μL. Different samples were run in triplicate on a single plate, which included no-template controls. PCR conditions were as follows: denaturation (95 °C for 10 min) followed by 70 amplification cycles (95 °C for 15 s, 60 °C for 1 min). The q-RT-PCR analysis was repeated using RNA isolated from three independent experiments.

2. Supplementary Figures

Supplementary Figure S1

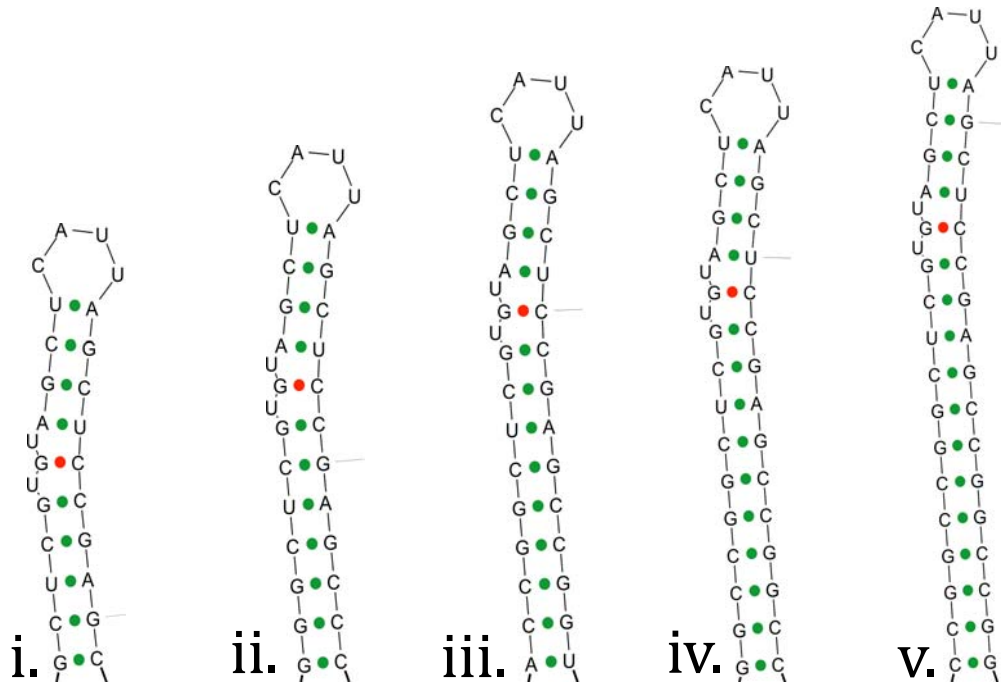

Fig. S1. Sfold predicted secondary structures of hairpins (i) A ($\Delta G_{predicted}$ = –10.4 kcal mol^{-1}), (ii) B ($\Delta G_{predicted}$ = –17.0 kcal mol^{-1}), (iii) C ($\Delta G_{predicted}$= –21. kcal mol^{-1}), (iv) D ($\Delta G_{predicted}$ = –26.1 kcal mol^{-1}) and (v) E ($\Delta G_{predicted}$ = –31.8 kcal mol^{-1}).

Supplementary Figure S2

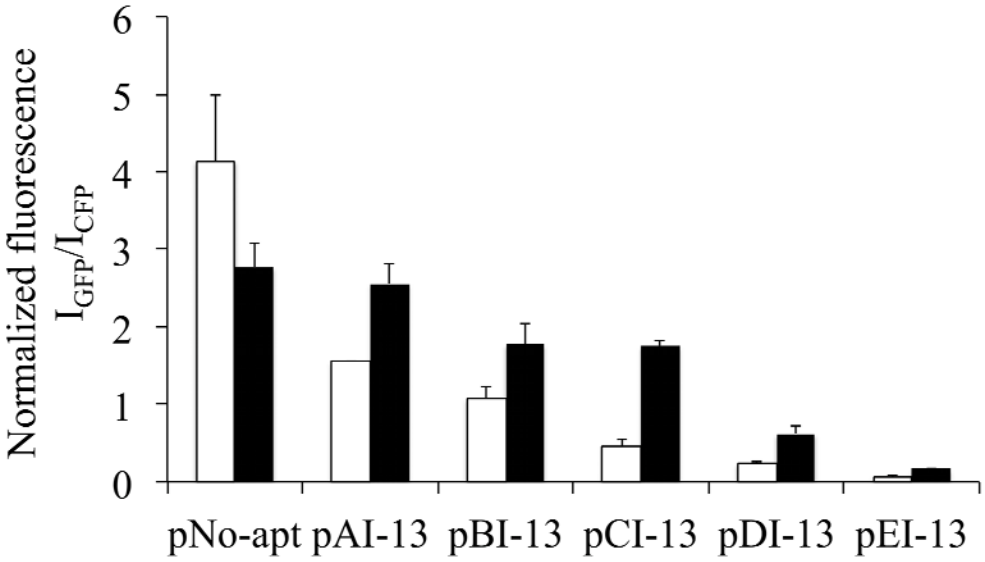

Fig. S2. Influence of hairpin thermodynamic stability on translational repression values. The normalized fluorescence intensity (I_{GFP}/I_{CFP}) for 293T cells co-transfected with either pCIBN-His (dark bars) or pCIBN-BIV Tat (white bars) and one of the reporter constructs pNo-apt, pAI-13, pBI-13, pCI-13, pDI-13, or pEI-13 (20 ng) is plotted. Error bars represent the standard deviation of three independent experiments.

Supplementary Figure S3

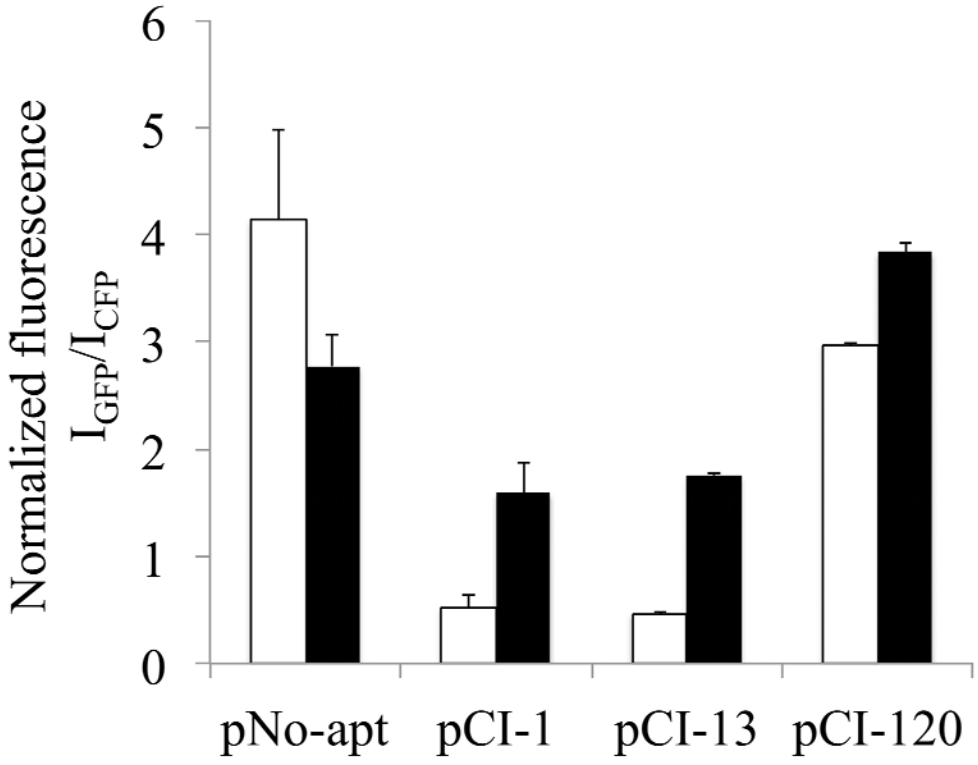

Fig. S3. Influence of hairpin location on translational repression values. The normalized fluorescence intensity (I_{GFP}/I_{CFP}) for 293T cells co-transfected with either pCIBN-His (dark bars) or pCIBN-BIV Tat (white bars) and one of the reporter constructs pNo-apt, pCI-1, pCI-13, or pCI-120 (20 ng) is plotted. Error bars represent the standard deviation of three independent experiments.

Supplementary Figure S4

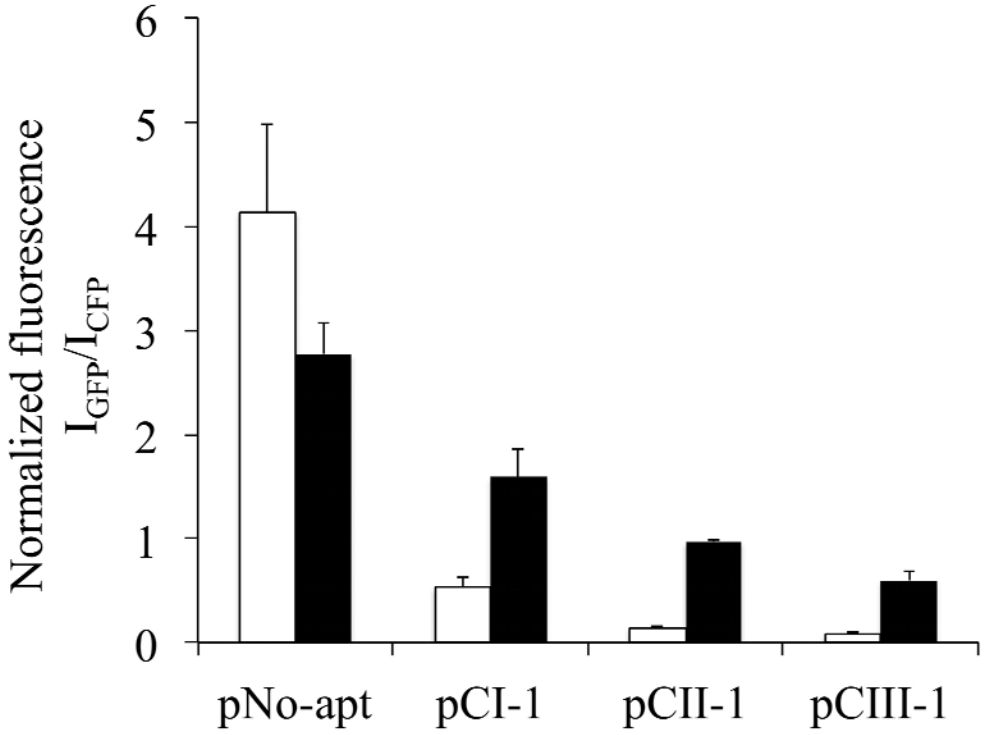

Fig. S4. Influence of the number of hairpin repeats on translational repression values. The normalized fluorescence intensity (I_{GFP}/I_{CFP}) for 293T cells co-transfected with either pCIBN-His (dark bars) or pCIBN-BIV Tat (white bars) and one of the reporter constructs pNo-apt, pCI-1, pCII-1, or pCIII-1 (20 ng) is plotted. Error bars represent the standard deviation of three independent experiments.

Supplementary Figure S5

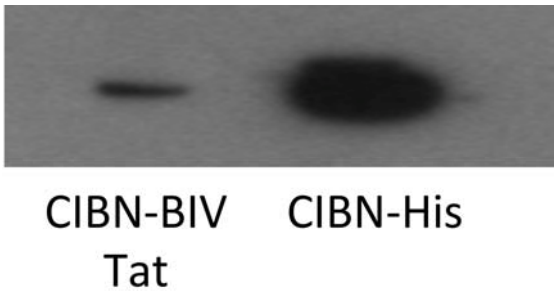

Fig. S5. Repression of translation for pCII-1' analyzed using immunoblotting. Cells co-transfected with pCII-1' and pCIBN-BIV Tat (or pCIBN-His) were probed using anti-HA antibody. Each lane contains lysate from 5 x 10^5 doubly transfected cells.

Supplementary Figure S6

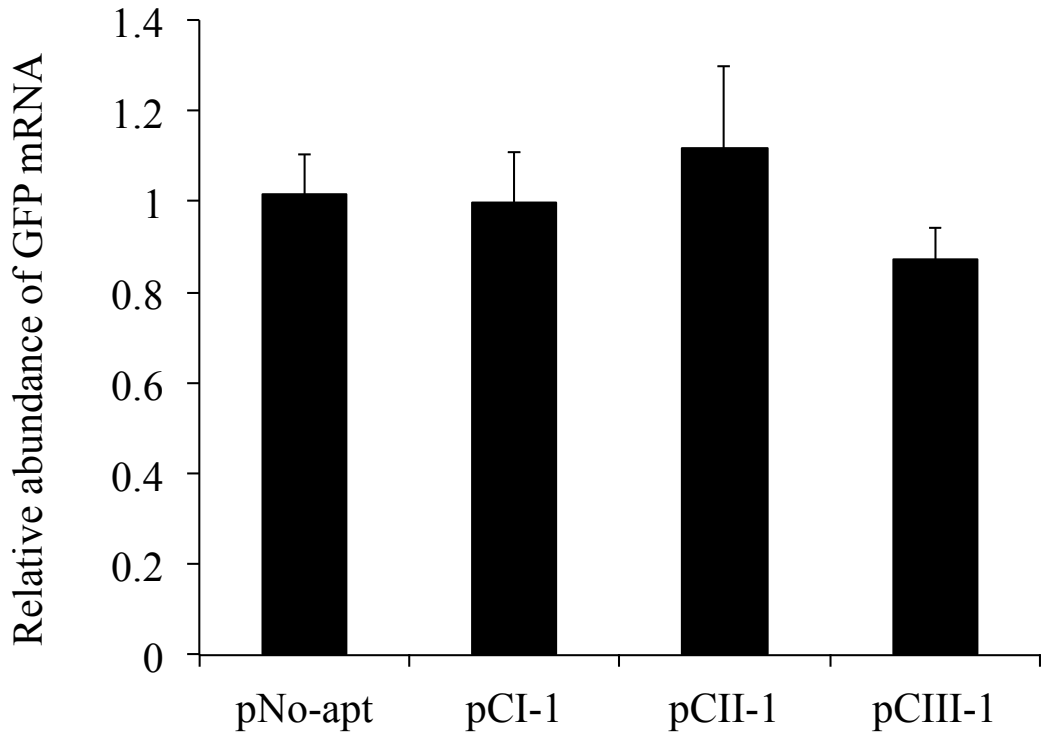

Fig. S6. Influence of the expression of ligand (pCIBN-BIV Tat) or control (pCIBN-His) on GFP mRNA transcript level. Levels of cytoplasmic mRNA for the reporter (GFP) and a housekeeping gene (18S) were quantified using q-RT-PCR. The GFP transcript level was normalized by the corresponding amount of 18S transcript to yield a normalized GFP transcript level. Relative abundance was calculated as the ratio of the normalized GFP transcript level in the presence of pCIBN-His to that in the presence of pCIBN-BIV Tat.

3. Supplementary Tables

Supplementary Table 1: Sequences of all the PCR primers used in the present study.

Primer	**Template**	**Sequence**
For-GFP	pRNA-T U6.1	5'AGATCTGGTTAATTAAATGTCTAAA CTCGAAGAGCTGTTCA 3'
Rev-GFP	pRNA-T U6.1	5'CTCGAGTCACTTGTACAGCTCGTCC ATGC 3'
For-CFP	pCAG-CFP	5'ACCGGTCGCCACCATGGTGAGCAAG GGC 3'
Rev-CFP	pCAG-CFP	5'ACGCGTTTACTTGTACAGCTCGTCC ATGCC 3'
For-DsRed	pSIREN-RetroQ-DsRed-Express	5'ACCGGTATGGCATCCTCCGAGGACG TCATCAAGGAG 3'
Rev-DsRed	pSIREN-RetroQ-DsRed-Express	5'GTCGACCTACAGGAACAGGTGGTGG CGG 3'
For CIBN-BIV Tat	pCIBN(deltaNLS)-pmEGFP	5'AGATCTATGAATGGAGCTATAGGAG GTGACCTTTTGC 3'
Rev CIBN-BIV Tat	pCIBN(deltaNLS)-pmEGFP	5'CTCGAGTTAACGACGAATACGACGG CCTTTGCCACGGGTGCCACGCGGACG ACTTCCTCCTCCACTTCCTCCTCCTAC ATGAATATAATCCGTTTTCTCCAATTC CTTC 3'
For CIBN-His	pCIBN(deltaNLS)-pmEGFP	5'AGATCTATGAATGGAGCTATAGGAG GTGACCTTTTGC 3'
Rev CIBN-His	pCIBN(deltaNLS)-pmEGFP	5'CTCGAGTTAGCCATGATGATGATGA TGATGGCTGCCACGCATACTTCCTCCT CCACTTCCTCCTCCTACATGAATATAA

		TCCGTTTTCTCCAATTCCT 3’
For HA-GFP	pRNA-T U6.1	5’AGATCTGGTTAATTAAATGTACCCA TACGATGTTCCAGATTACGCTGGATCT ATGTCTAAACTCGAAGAGCTGTTCA 3’
Rev HA-GFP	pRNA-T U6.1	5’GCTAGCTCACTTGTACAGCTCGTCC ATGC 3’

Supplementary Table 2: Sequences of all the oligonucleotides used in the present study.

Oligonucleotide	**Backbone plasmid**	**Sequence**
CI-1 A	pBIV-Temp	5'AATTCCACGTCCCCTCGACCGGC TCGTGTAGCTCATTAGCTCCGAGC CGGTAACAACAACAACCCGCGG 3'
CI-1 B	pBIV-Temp	5'GATCCCGCGGGTTGTTGTTGTTA CCGGCTCGGAGCTAATGAGCTAC ACGAGCCGGTCGAGGGGACGTGG 3'
CI-120 A	pNo-apt	5'ACTTCACCGGCTCGTGTAGCTCA TTAGCTCCGAGCCGGTGAATT 3'
CI-120 B	pNo-apt	5'AATTCACCGGCTCGGAGCTAATG AGCTACACGAGCCGGTGAAGT 3'
T1 A	pCI-1	5'GATCTCGGTGCTCGTGTAGCTCA TTAGCTCCGAGCACCGAACAACA ACAACAACAAG 3'
T1 B	pCI-1	5'GATCCTTGTTGTTGTTGTTCG GTGCTCGGAGCTAATGAGCTACAC GAGCACCGA 3'
T2 A	pCII-1	5'GATCTACGCGCTCGTGTAGCTCA TTAGCTCCGAGCGCGTAACAACA ACAACAACAAG 3'

T2 B	pCII-1	5'GATCCTTGTTGTTGTTGTTGTTAC GCGCTCGGAGCTAATGAGCTACA CGAGCGCGTA 3'

Supplementary Table 3: Sequences of all the DNA fragments used in the present study.

DNA fragment	**Backbone plasmid**	**Sequence**
AI-13	pNo-apt	5'TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCCCTAGCCACGTCCCCTCG CAACAACAACAAGCTCGTGTAG CTCATTAGCTCCGAGCCAACAA CAACAACCCGCGGGTCTGTCTC AAGCTTGCCGCCAGAACACAGG TAAGTGCCGTGTGTGGTTCCCG CGGGCCTGGCCTCTTTACGGGT TCAGGCCCTTGCGTGCCTTGAA TTACTTC 3'
BI-13	pNo-apt	5'TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCCCTAGCCACGTCCCCTCG CAACAACAACAAGGGCTCGTGT AGCTCATTAGCTCCGAGCCCCA ACAACAACAACCCGCGGGTCTG TCTCAAGCTTGCCGCCAGAACA CAGGTAAGTGCCGTGTGTGGTT CCCGCGGGCCTGGCCTCTTTAC GGGTTCAGGCCCTTGCGTGCCT TGAATTACTTC 3'
CI-13	pNo-apt	5'TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC

		CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCCCTAGCCACGTCCCCTCG CAACAACAACAAACCGGCTCGT GTAGCTCATTAGCTCCGAGCCG GTCAACAACAACAACCCGCGG GTCTGTCTCAAGCTTGCCGCCA GAACACAGGTAAGTGCCGTGTG TGGTTCCCGCGGGCCTGGCCTC TTTACGGGTTCAGGCCCTTGCG TGCCTTGAATTACTTC 3’
DI-13	pNo-apt	5’TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCCCTAGCCACGTCCCCTCG CAACAACAACAAGGCCGGCTC GTGTAGCTCATTAGCTCCGAGC CGGCCCAACAACAACAACCCGC GGGTCTGTCTCAAGCTTGCCGC CAGAACACAGGTAAGTGCCGTG TGTGGTTCCCGCGGGCCTGGCC TCTTTACGGGTTCAGGCCCTTG CGTGCCTTGAATTACTTC 3’
EI-13	pNo-apt	5’TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCCCTAGCCACGTCCCCTCG CAACAACAACAACCGGCCGGCT CGTGTAGCTCATTAGCTCCGAG CCGGCCGGCAACAACAACAAC CCGCGGGTCTGTCTCAAGCTTG CCGCCAGAACACAGGTAAGTGC CGTGTGTGGTTCCCGCGGGCCT GGCCTCTTTACGGGTTCAGGCC CTTGCGTGCCTTGAATTACTTC 3’

BIV-Temp	pNo-apt	5'TCCGGATTGGTCAGGCACGGC CTTCGGCCCCGCCTCCTGCCAC CGCAGATTGGCCGCTAGGCCTC CCCGAGCGCCCTGCCTCCGAGG GCCGGCGCACCATAAAAGAAG CCGCGAATTCCACGTCCCCTCG CAACAACAACAAGCTCGTGTAG CTCATTAGCTCCGAGCCAACAA CAACAACCCGCGGGATCCTCTC AAGCTTGCCGCCAGAACACAGG TAAGTGCCGTGTGTGGTTCCCG CGGGCCTGGCCTCTTTACGGGT TCAGGCCCTTGCGTGCCTTGAA TTACTTC 3'

Supplementary Table 4: Sequences of primers and TaqMan probes used in the present study for q-RT-PCR.

Oligonucleotide	**Gene**	**Sequence**
Sense	GFP	5'GAGGATGACGGCAACTACAA 3'
Antisense	GFP	5'CCATCTTATTGCCCAGGATGT 3'
Probe	GFP	5'-FAM490-ATACCCTGGTGAATCGCATCGAGC-BHQ-3'
Sense	18S	5'GTAACCCGTTGAACCCCATTC3'
Antisense	18S	5'CCATCCAATCGGTAGTAGCGA3'

Probe	18S	5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'