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Supplemental Data

HIV Evades RNA Interference Directed at TAR by an Indirect Compensatory Mechanism

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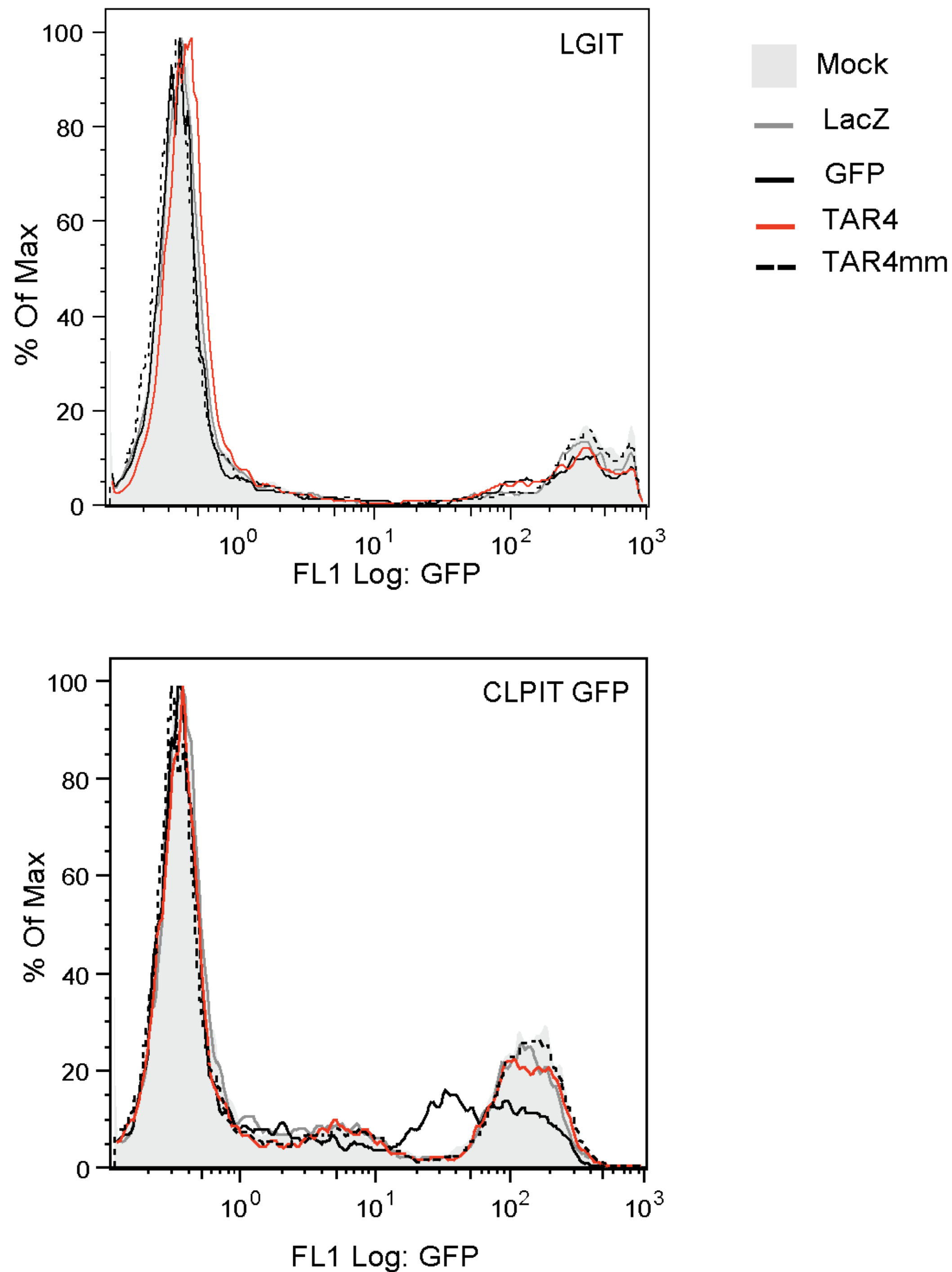

Figure S1: Transient GFP Knockdown

1×10^5 HEK 293T cells infected with LGIT or CLPIT GFP were transiently transfected with 1.5 μg of shRNA-expressing plasmid. Fluorescence was measured 3 days post-transfection. Sample flow data for each cell line is shown for mock (grey silhouette), LacZ control (solid grey line), GFP control (solid black line), TAR4 (solid red line) and the TAR4 mismatch (TAR4mm) control (dashed black line).

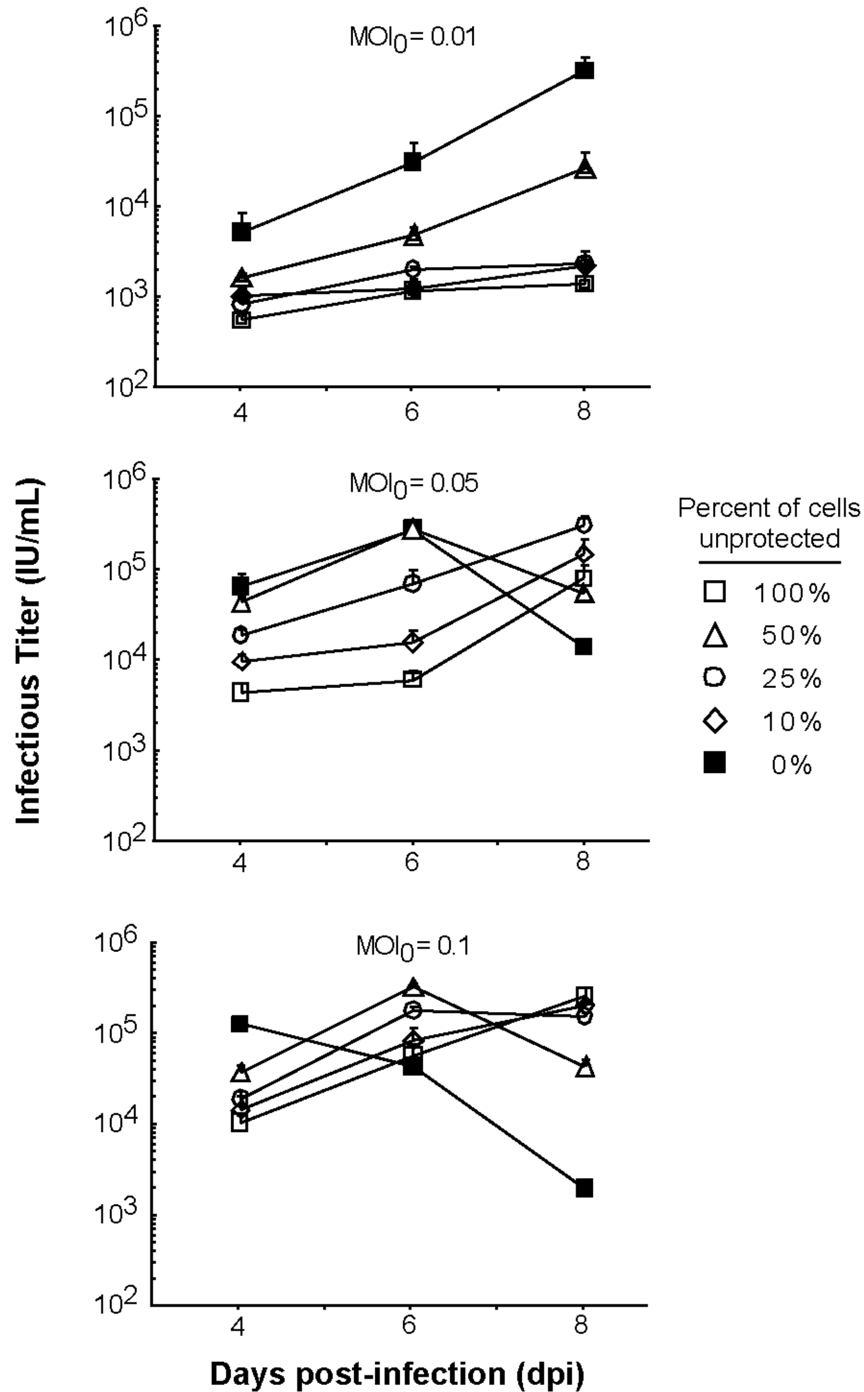

Figure S2: HIV Replication on Mixed Cell Populations

Unprotected SupT1s cells and TAR4-protected cells were mixed at the indicated ratios and challenged with WT virus at an intial MOI (MOI_0) of 0.01, 0.05 or 0.1. Most viral titers at 2 dpi were near or below the detection threshold and are not shown. Experiments were performed in biological triplicate, and error bars indicate one standard deviation.

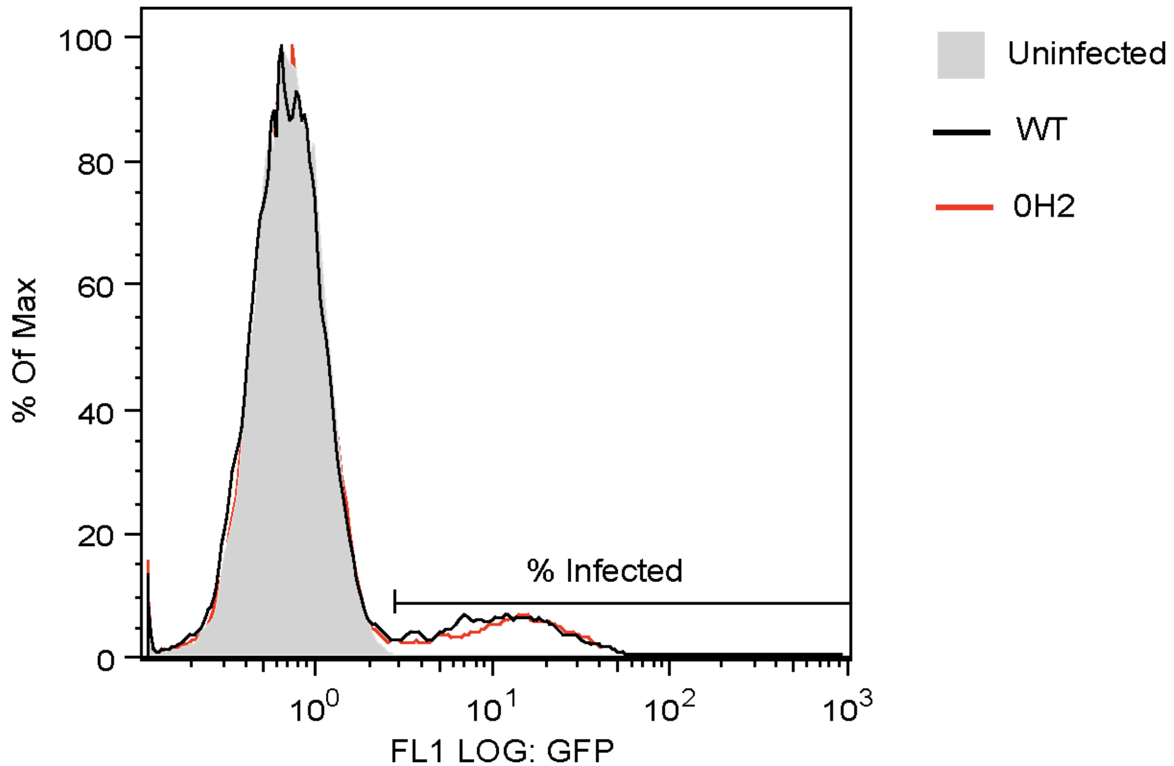

Figure S3: CEM GFP Indicator Cell Line

$1x10^5$ CEM GFP cells were infected with 100 μL of viral supernatant from the indicated HIV culture. Cells were fixed in 2% paraformaldehyde 3 dpi and analyzed using flow cytometry. Thresholds were set such that 0.05% of uninfected cells under similar culture conditions were gated in the "infected" region. This threshold was robust in response to slight differences in CEM GFP sensitivity to mutants compared to WT virus such that altering the threshold between 0.01% and 0.1% did not result in different outcomes for mutant or WT virus.

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                                                              EtsI
       -187                                              -138
        |--------- ---------- ---------- ---------- ---------|
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  0H2  TGGAAGGGCT AATTCACTCC CAAAGAAGAC AAGACATCCC ATCCGGAGTA
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 0H16  TGGAAGGGCT AATTCACTCC CAAAGAAGAC AAGATATCCC ATCCGGAGTA
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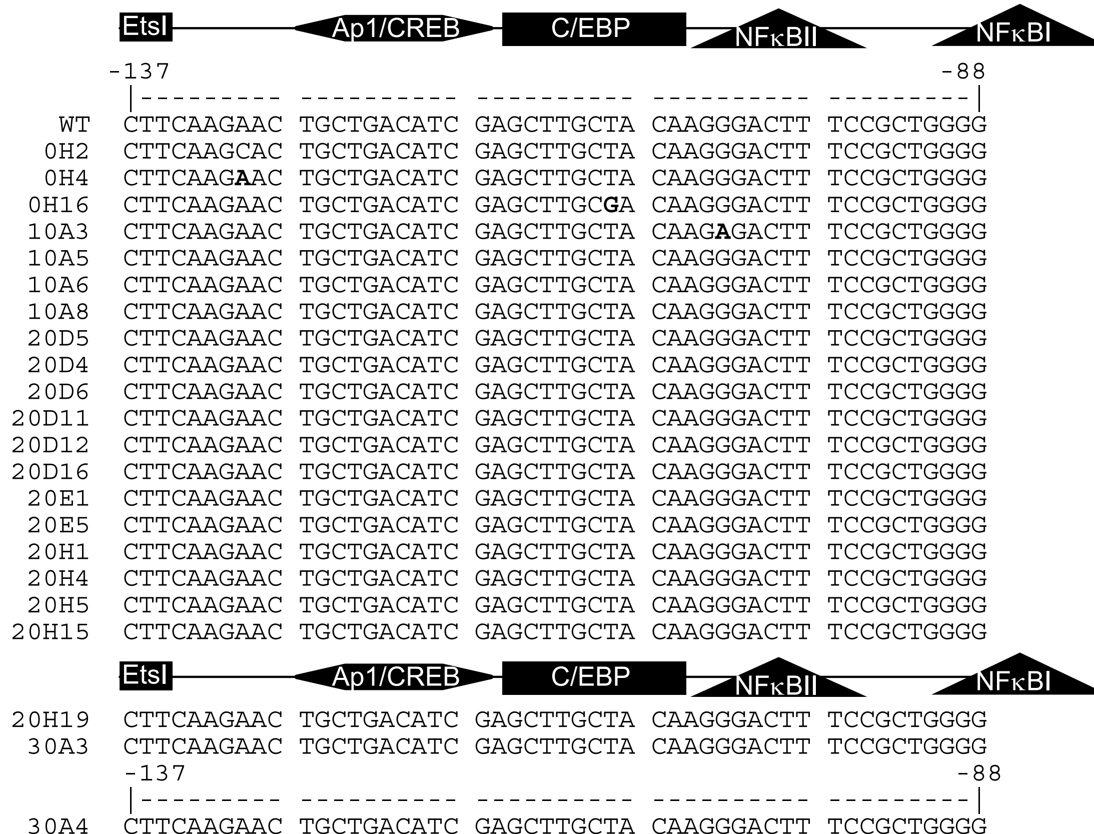


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  0H4  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
 0H16  CTTCAAGAAC TGCTGACATC GAGCTTGCGA CAAGGGACTT TCCGCTGGGG
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 10A8  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
 20D5  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
 20D4  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
 20D6  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
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20H15  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG

20H19  CTTCAAGAAC TGCTGACATC GAGCTTGCTA CAAGGGACTT TCCGCTGGGG
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        -137                                              -88
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          NFκBI         Sp1 III        Sp1 II

       -87                                                ---
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 0H16   ACTTTCCAGG GAGGCGTGGC CTGGG..... .......... ..........
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 20D6   ACTTTCCAGG GAGGCGTGGC CTGGG..... .......... ..........
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        ---                                               -50
        |--------- ---------- ---------- ---------- ---------|
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 0H16   .......... .......... .......... .......CGG GACTGGGGAG
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        ---                                               -50
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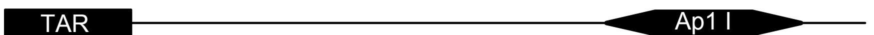

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+52 +101
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+102 +151
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0H16 AGTGTGTGCC CGTCTGTTGT GTGACTCTGG TAACTAGAGA TCCCTCAGAC
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Ap1 III NFAT/AP3

+152 +181
|--------- ---------- ---------- |
WT CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
0H2 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
0H4 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
0H16 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
10A3 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
10A5 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
10A6 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
10A8 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20D5 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20D4 CCTTTT.AGT CAGTGTGGAA AATCTCTAG**T** A
20D6 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20D11 CCTTTT.AGT CAGTGTGGAA AATCTC**C**AGC A

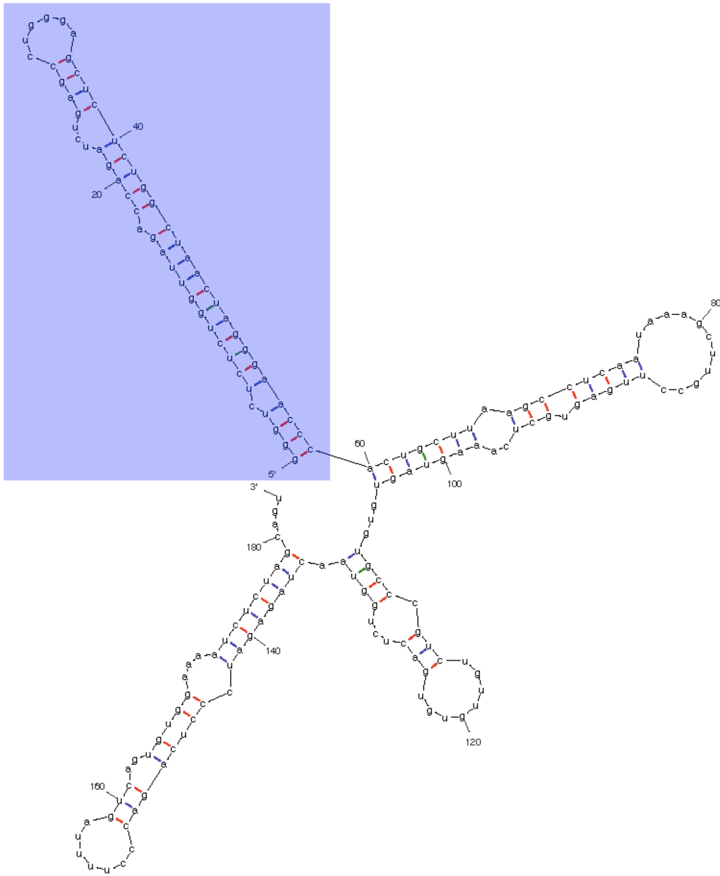
Ap1 III NFAT/AP3

+152 +181
|--------- ---------- ---------- |
20D12 CCTTTT.AGT CAGTGTGGAA AATCTCTAG**A** A
20D16 CCTTTT.AGT **A**AGTGTGGAA AATCTCTAGC A
20E1 C**T**TTTT**T**A**A**T CAGTGTGGAA AATCTCTAGC A
20E5 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20H1 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20H4 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20H5 CCTTTT.AGT CAGTGCGGAA AATCTCTAGC A
20H15 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
20H19 CCTT**G**T.AGT CAGTGTGGAA AATCTCTAGC A
30A3 CCTTTT.AGT CAGTGTGGAA AATCTCTAG**A** A
30A4 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30A5 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30A6 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30G3 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30G4 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30G5 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A
30G8 CCTTTT.AGT CAGTGTGGAA AATCTCTAGC A

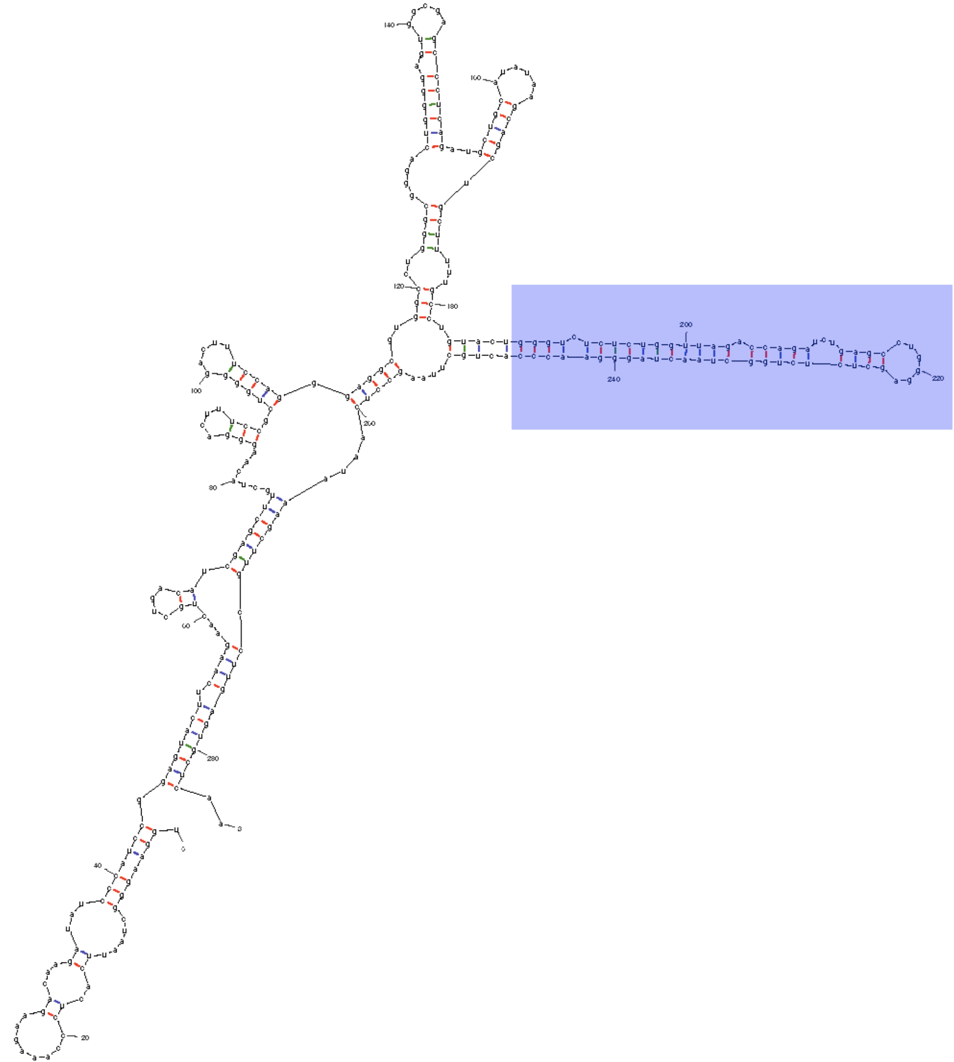
Figure S4: Complete Mutant LTR Sequences

Mutants were aligned using Vector NTI AlignX. Point mutations are indicated in bold face. Exact sequence repeats of 20H mutants, indicated in red type, suggest that Sp1 site multimers were generated through recombination. Transcription factor binding sites are indicated directly above the sequence

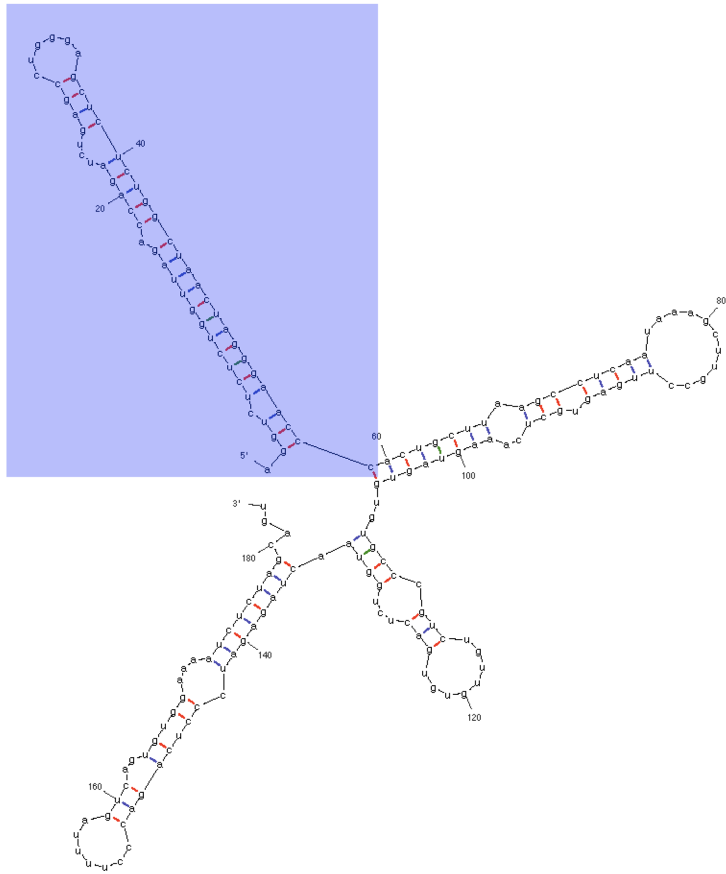
WT 5' (R-U5)



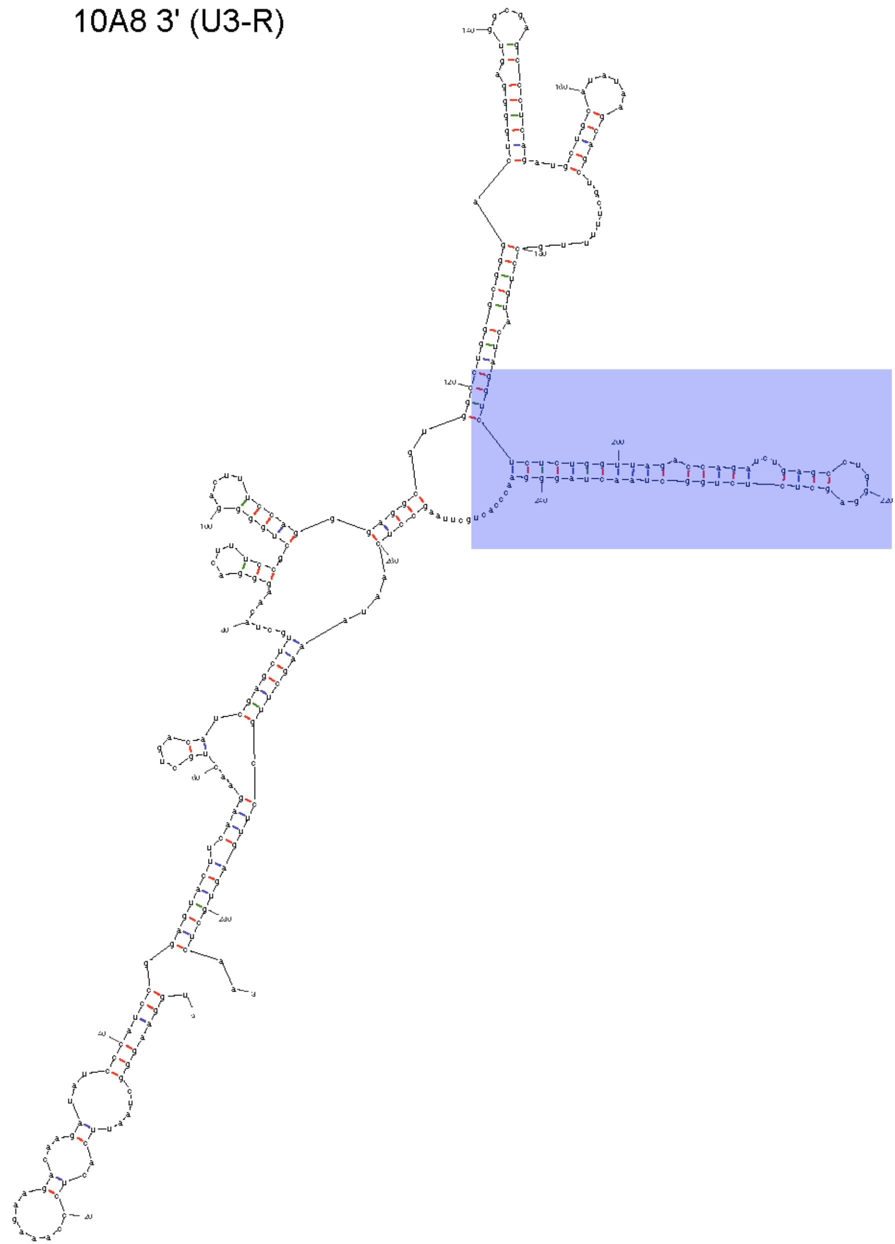
WT 3' (U3-R)



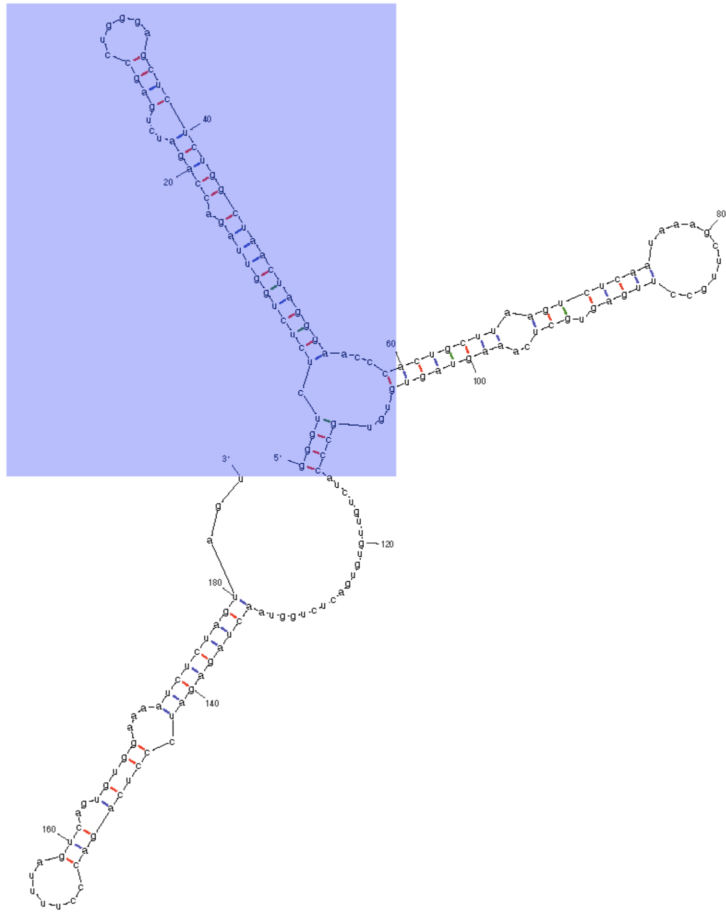
10A8 5' (R-U5)



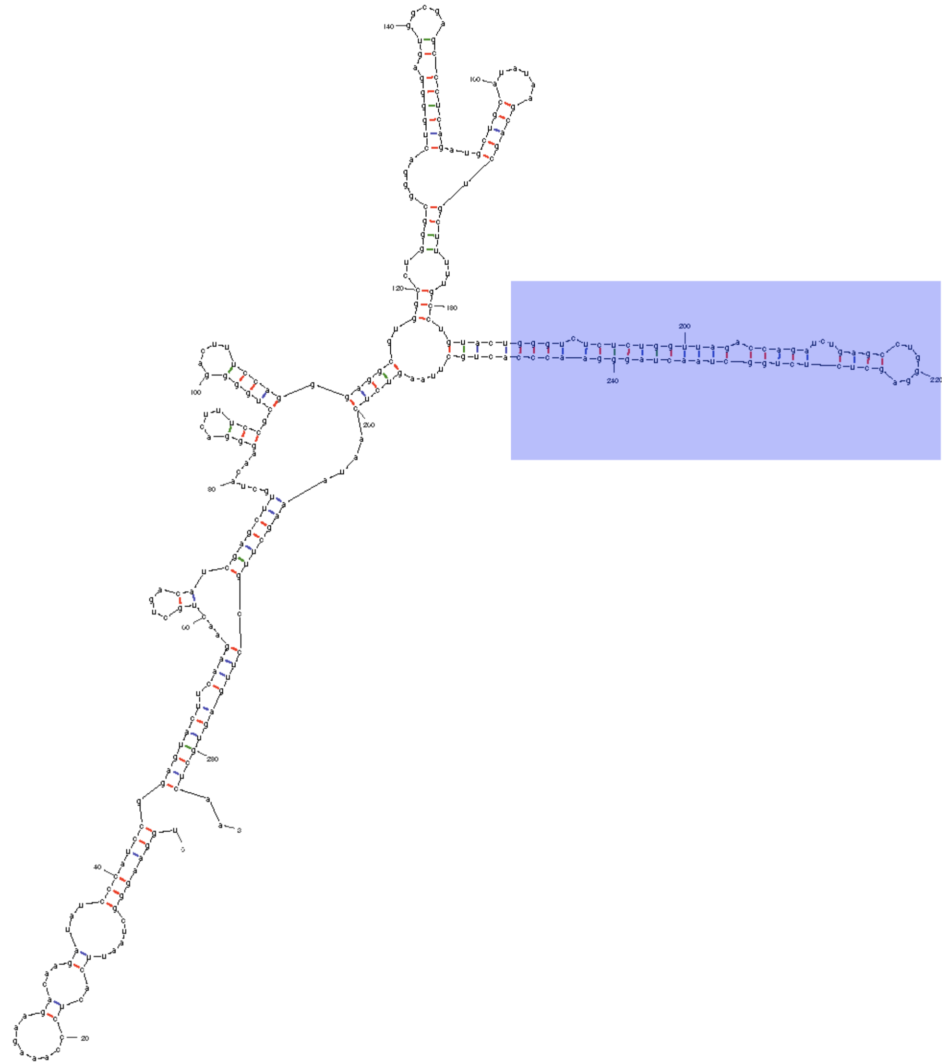
10A8 3' (U3-R)



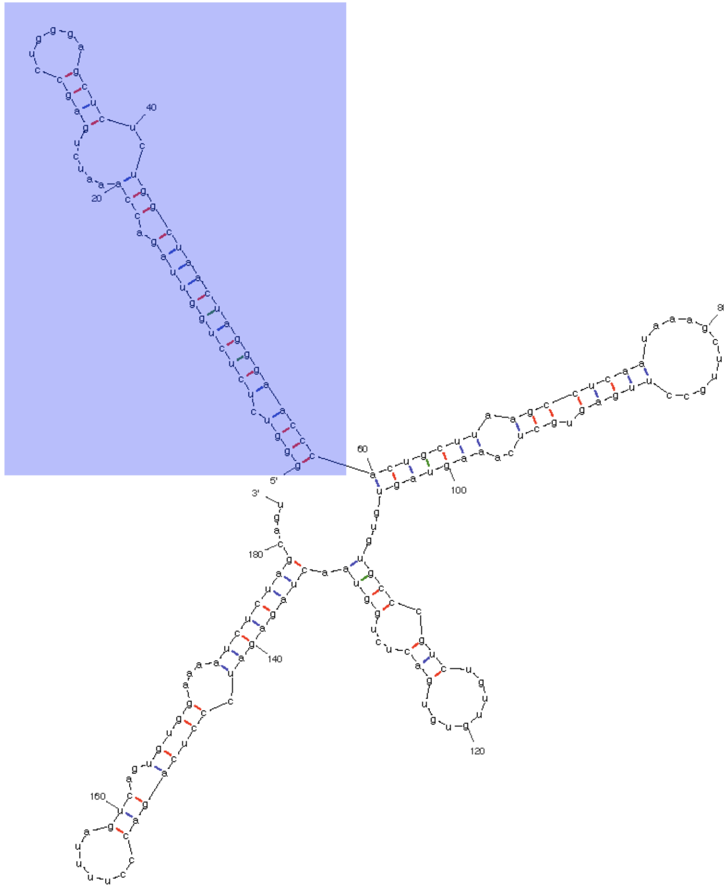
20D4 5' (R-U5)



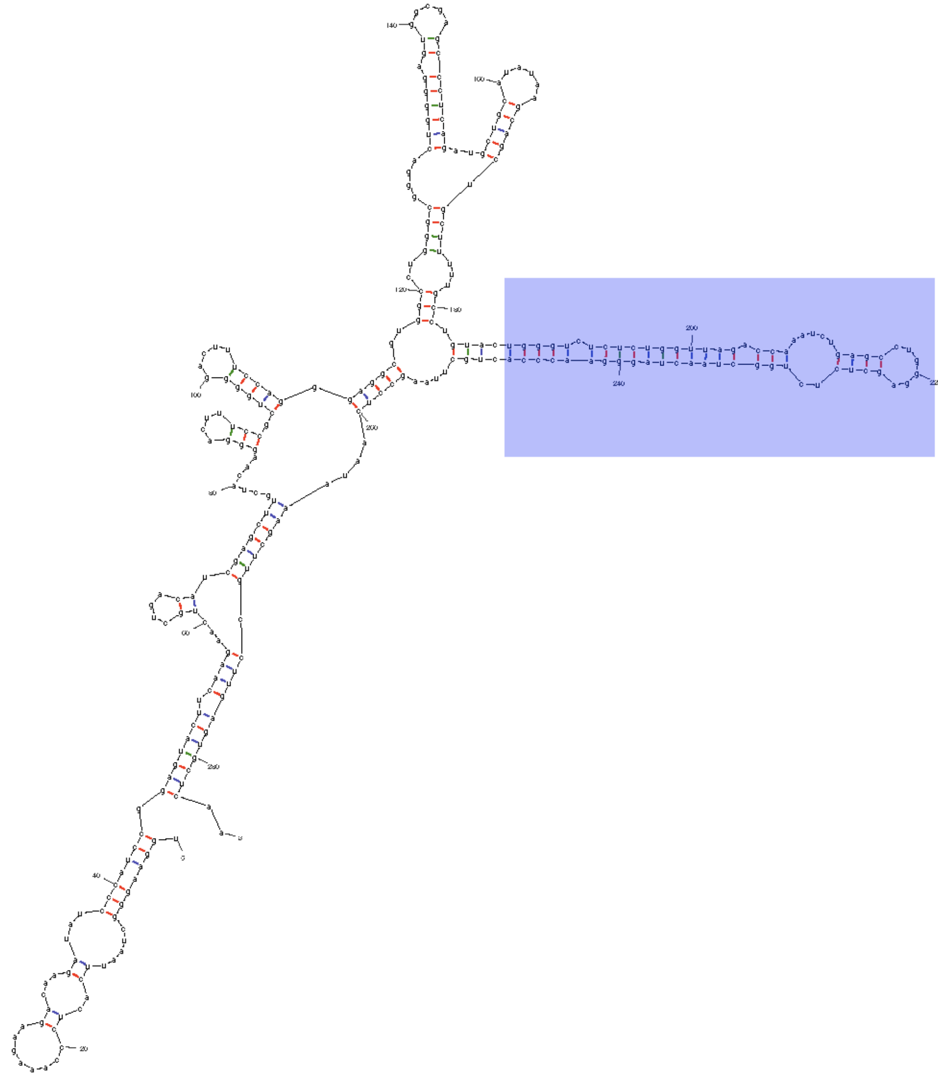
20D4 3' (U3-R)



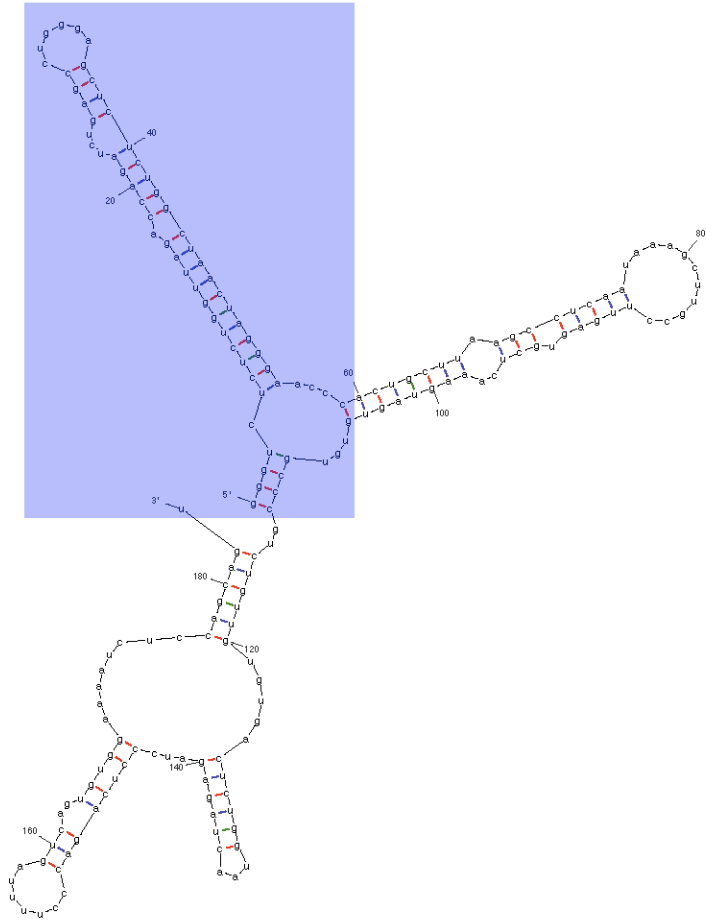
20D6 5' (R-U5)



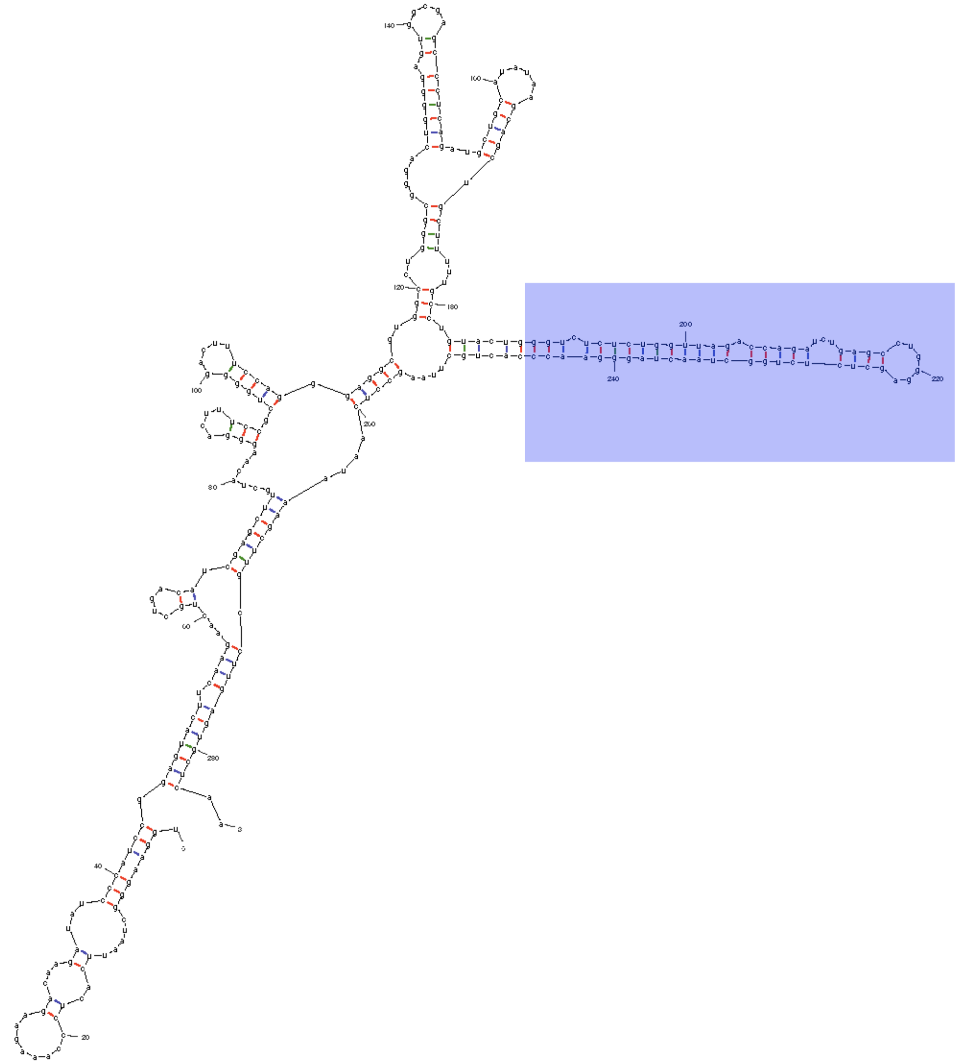
20D6 3' (U3-R)



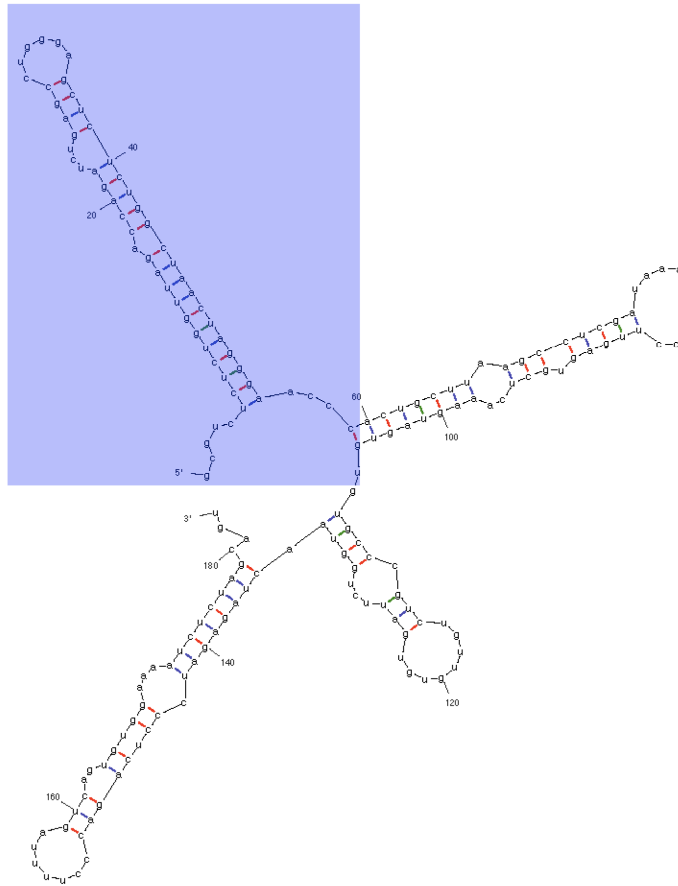
20D11 5' (R-U5)



20D11 3' (U3-R)



20H4 5' (R-U5)



20H4 3' (U3-R)

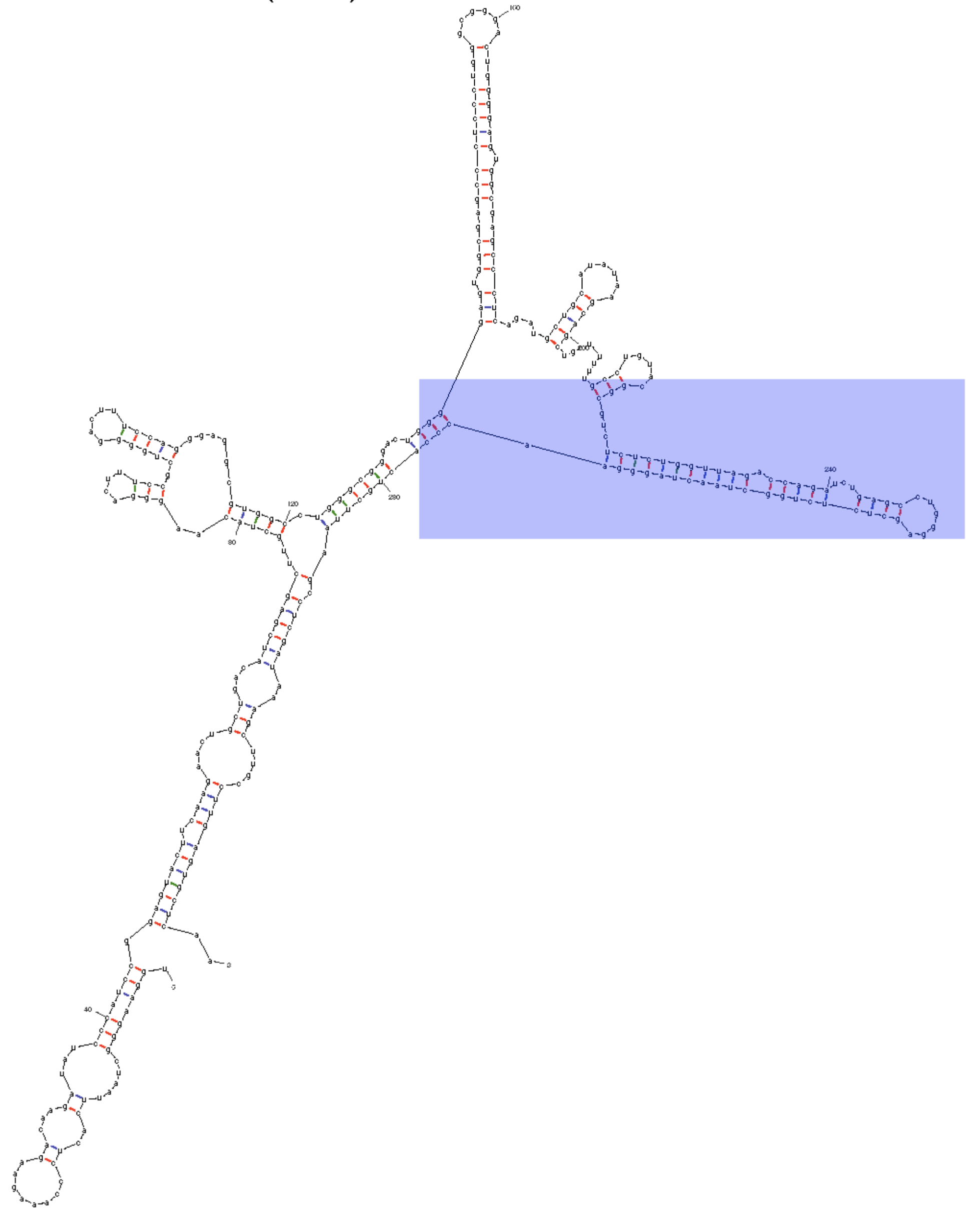


Figure S5: RNA Secondary Structure

Secondary structure of the 5' (left) and 3' (right) UTRs were predicted for WT and mutant sequences in mFold using the R and U5 regions and the U3 and R regions, respectively. The TAR hairpin is highlighted in blue. Mutants with TAR structures that vary from WT structure are shown, however both 5' and 3' structures may not vary simultaneously. Of all mutants analyzed, only mutants 10A8, 20D4, 20D11 and 20H4 had altered structures at the base of the 5' hairpin. Only mutant 10A8 had an altered structure at the base of the 3' hairpin and only 20D6 had any alterations adjacent to the RNAi-targeted region.

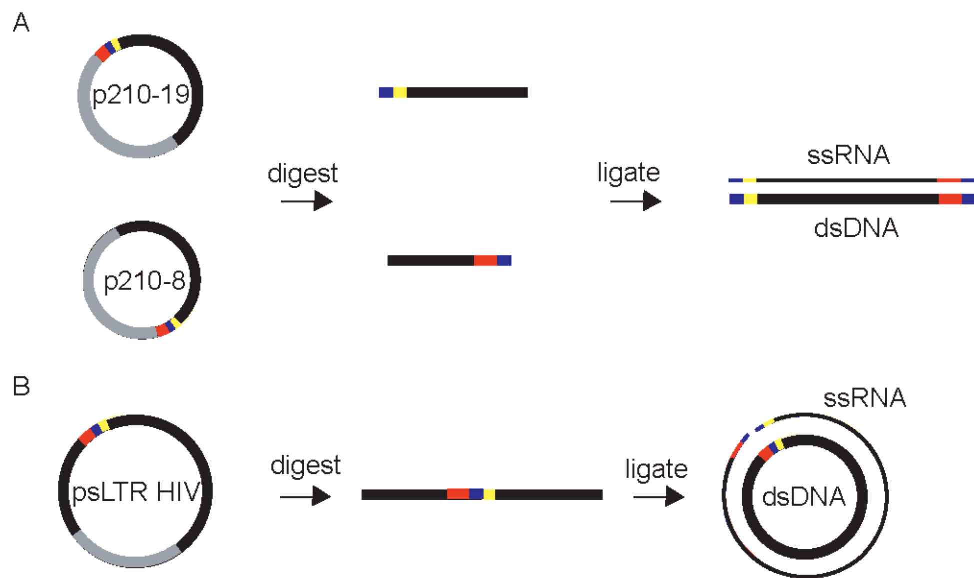


Figure S6: Generation of HIV Using a Single-LTR Platform (psLTR HIV)
(A) In the hemigenomic method, the p210-19 and p210-8 plasmids are linearized and rejoined to form linear full length HIV (black). The linear DNA is introduced into HEK 293Ts and transcribed for viral production. (B) In the psLTR HIV method the HIV genome (black) is interrupted by bacterial sequence (grey). The 3' half of the HIV genome and 3' LTR from p210-8 were fused immediately upstream of the 5' half of the genome, plasmid origin of replication and ampicillin selection gene from p210-19 (replacing the 5' LTR). Each psLTR HIV variant was digested with *Eco* RI (New England Biolabs), to excise the non-HIV sequences, and recircularized with T4 DNA ligase (New England Biolabs) to produce full-length HIV with a single LTR (U3, red; R, blue; U5, yellow). The LTR is transcribed once at the beginning and once at the end of each transcript in HEK 293Ts. GFP was also inserted into the *nef* open reading frame to track HIV replication in cell culture.

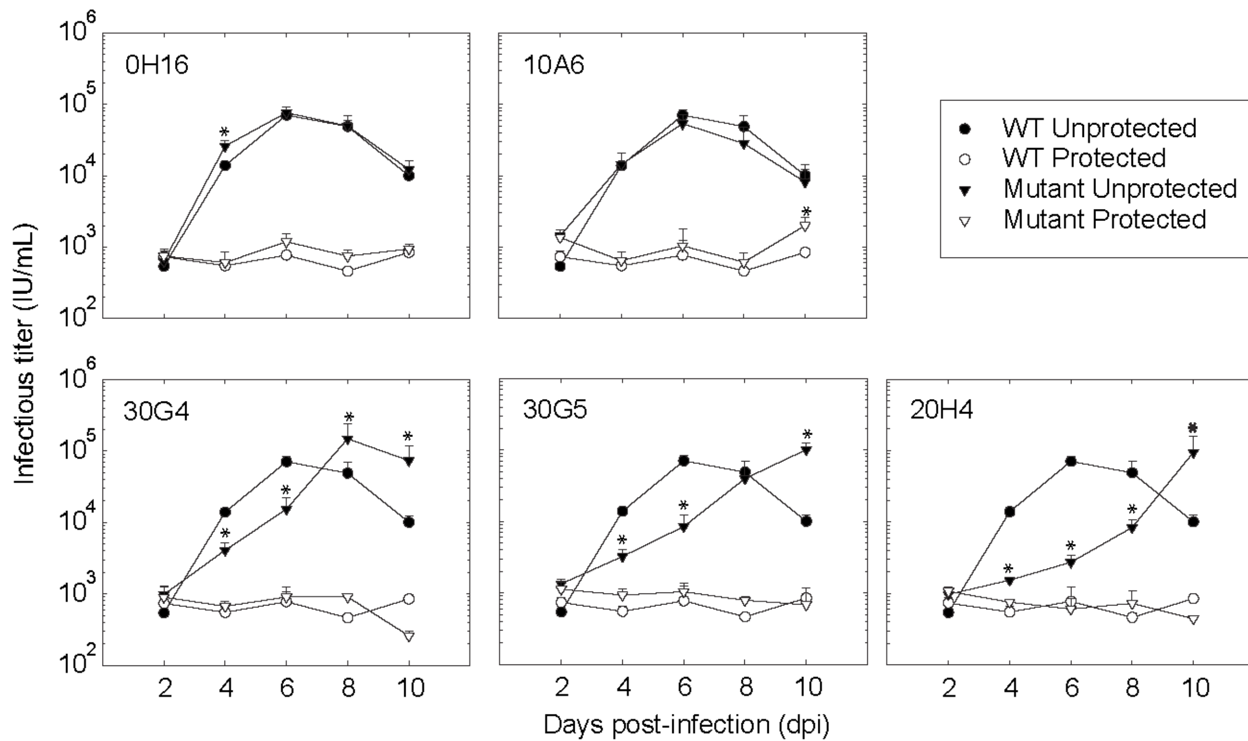


Figure S7: Replication of Nonevasion Mutants

TAR4-protected or unprotected SupT1 cells were challenged with either WT virus or a homogeneous preparation of each potential evasion mutant at a MOI of 0.015. Infections were performed in biological triplicate, error bars represent one standard deviation, and (*) indicates a significantly different titer as compared to WT virus replicating in the same cell type ($p<0.05$).

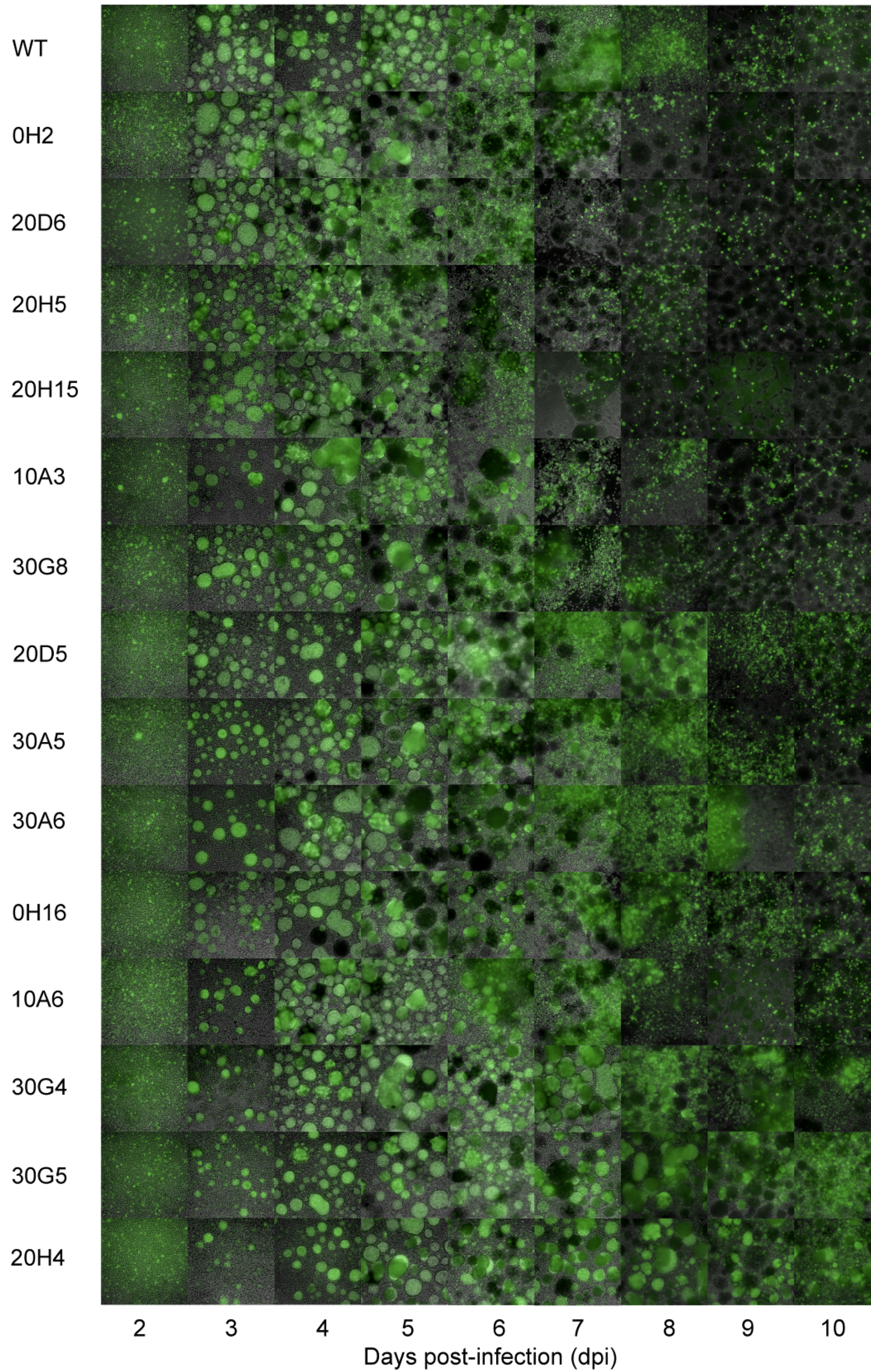


Figure S8: Viral Replication-Mediated Cell Death
Overlays of GFP and bright field images of HIV replication in unprotected cells.

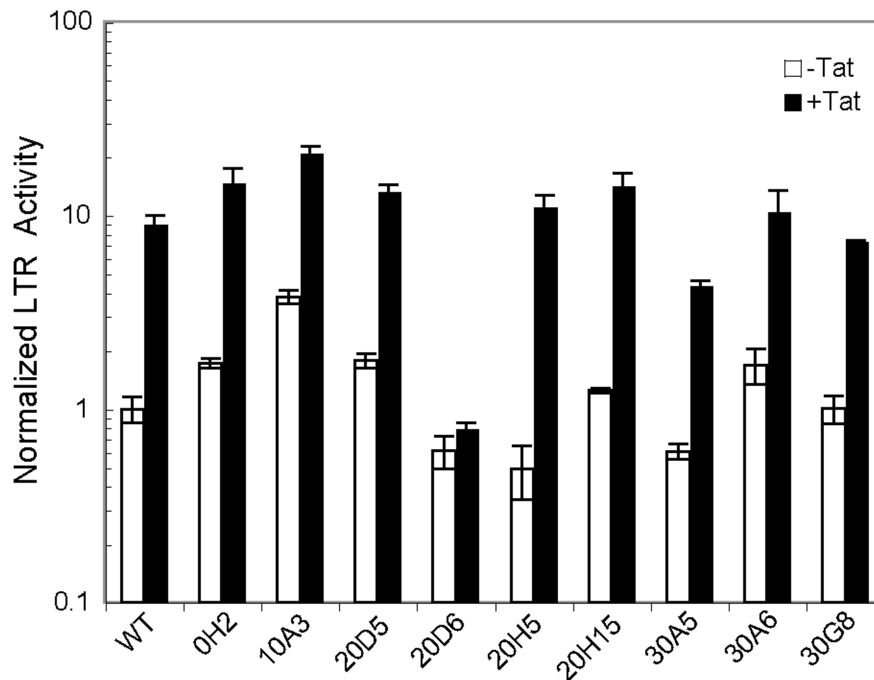


Figure S9: Transcriptional Activities Determined by Luciferase Assay
Basal and transactivated transcription was measured by luciferase assay in naive or Tat-expressing SupT1s transduced with the indicated LLIG variant. Luminescence was normalized by infection efficiency (determined by QPCR) and protein content of cell lysates. All values were then normalized to the WT luminescence in the absence of Tat (759 RLU). Experiments were performed in technical triplicate and error bars represent one standard deviation.

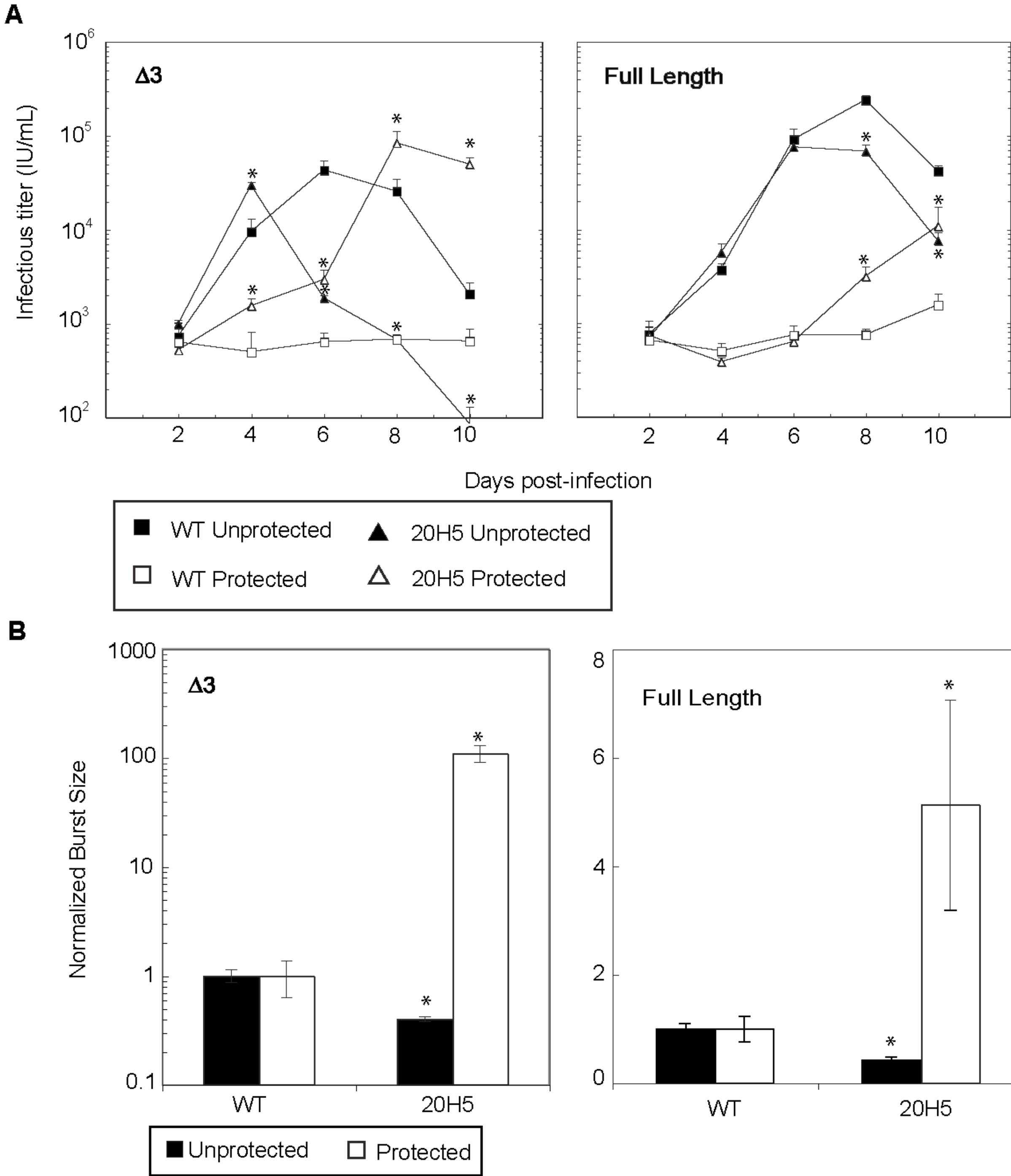


Figure S10: Enhanced Replication of Sp1 Duplication Mutants in Full-Length and Triple-Deletion (Δ3) Virus

(A) Unprotected and protected cells were infected with the indicated virus at a MOI of 0.015 and 0.002 for Δ3 and full length virus, respectively. (B) Total viral yield (burst size) was calculated by integrating the titers from (A) and normalizing to WT virus (in identical cells). Experiments were performed in biological triplicate and error bars indicate one standard deviation, and (*) indicates a significantly different titer (or burst size) as compared to WT virus replicating in the same cell type ($p<0.05$).

Primer name	Oligonucleotide sequence (5' to 3')
Universal (+)	ATAAGAATGCGGCCGCCCCGGGGATCCAAGGTCGGG
LacZ (-)	GCTCTAGAAAAAGTGACCAGCGAATACCTGTTCTCTTGAAAC AGGTATTCGCTGGTCACGGTGTTTCGTCCTTTCCAC
GFP (-)	GCTCTAGAAAAAGAAGAAGATGGTGCGCTCCTCTCTTGAAGG AGCGCACCATCTTCTTCGGTGTTTCGTCCTTTCCAC
TAR1 (-)	GCTCTAGAAAAAGACCAGATCTGAGCCTGGGTCTCTTGAACC CAGGCTCAGATCTGGTCGGTGTTTCGTCCTTTCCAC
TAR2 (-)	GCTCTAGAAAAAGATCTGAGCCTGGGAGCTCTCTCTTGAAGA GCTCCCAGGCTCAGATCGGTGTTTCGTCCTTTCCAC
TAR3 (-)	GCTCTAGAAAAAGACCAGATCTGAGCCTGGTGACAGGAAGC CAGGCTCAGATCTGGTCTCGGTGTTTCGTCCTTTCCAC
TAR4 (-)	GCTCTAGAAAAAGCCTGGGAGCTCTCTGGCTATCTCTTGAAT AGCCAGAGAGCTCCCAGGCGGTGTTTCGTCCTTTCCAC
TAR4mm (-)	GCTCTAGAAAAAGCCTGGGACGTCTCTGGCTATCTCTTGAAT AGCCAGAGACGTCCCAGGCGGTGTTTCGTCCTTTCCAC

Table S1: Primers for shRNA Construction
Each PCR reaction used the pTZU6+1 template, the Universal (+) forward primer, and a unique reverse (-) primer. Each PCR product contains the U6 promoter, the shRNA encoding sequence (sense-loop-antisense), and a TTTTT RNA Polymerase III transcription termination signal.

Mutant	Frequency	Mutant	Frequency
0H2	1/22	20E5	1/6
0H4	1/22	20H1	1/9
0H16	1/22	20H4	1/9
10A3	1/14	20H5	5/9
10A5	1/14	20H15	1/9
10A6	1/14	20H19	1/9
10A8	1/14	30A3	1/10
20D4	1/16	30A4	1/10
20D5	1/16	30A5	1/10
20D6	1/16	30A6	1/10
20D11	1/16	30G3	1/7
20D12	1/16	30G4	1/7
20D16	1/16	30G5	1/7
20E1	1/6	30G8	1/7

Table S2: Mutation Frequency per Culture

The frequency of each mutation as listed in Figure 3 is given as a fraction of the total number of samples sequenced. The remaining fraction of each culture contained no mutations within the LTR.