Direct translation of incoming retroviral RNA genomes

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Abstract

Viruses that carry a positive-sense, single-stranded RNA translate their genomes after entering the host cell to produce viral proteins, with the exception of retroviruses. A distinguishing feature of retroviruses is reverse transcription, where the ssRNA genome serves as a template to synthesize a double-stranded DNA copy that subsequently integrates into the host genome. As retroviral RNAs are produced by the host transcriptional machinery and are largely indistinguishable from cellular mRNAs, we investigated the potential of incoming retroviral genomes to express proteins. Here we show through various biochemical methods that HIV-1 genomes are translated after entry, in case of minimal or full-length genomes, envelopes using different cellular entry pathways and in diverse cell types. Our findings challenge the dogma that retroviruses require reverse transcription to produce viral proteins. Synthesis of retroviral proteins in the absence of productive infection has significant implications for basic retrovirology, immune responses and gene therapy applications.

Main text

All viruses, regardless of their nucleic acid type, composition or orientation, need to reach the mRNA stage for successful infection. Retroviruses carry two copies of a positive-sense, single-stranded RNA (+ssRNA) genome; however, they form a separate class from +ssRNA viruses in the Baltimore classification because their replication strategy involves reverse transcription of the ssRNA genome into a double-stranded (dsDNA) copy. Based on current knowledge most, if not all, +ssRNA viruses directly translate their RNA to synthesize viral proteins upon entry into host cells, with the exception of retroviruses, which undergo reverse transcription and degrade the original genomic RNA in the process. As retroviral genomes contain a 5' 7-methylguanosine cap and a 3' poly(A) tail and are virtually indistinguishable from cellular mRNAs, we asked: Are retroviral genomic RNAs also directly translated?

In the laboratory, retroviruses are produced by transfection of producer cells with plasmids encoding viral components. In case of viral genomes that carry a reporter gene, the reporter protein can be expressed in producer cells, packaged into virions and delivered into recipient cells, yielding false positives.[1, 2, 3, 4, 5, 6] To minimize producer cell background, which could mask the signal from *de novo* translated incoming retroviral RNAs, we employed a post-translational protein control system (ProteoTuner), where a destabilizing domain (DD) derived from a cellular gene with a very short half-life (FKBP12) is fused to the gene of interest, targeting it for rapid proteasomal degradation.[7] The unstable protein can be stabilized in a dose-dependent and reversible manner by adding a cell-permeable small molecule ligand called Shield1 that binds to the DD, allowing post-translational regulation of protein levels. We reasoned that producing viruses in the absence of the ligand would minimize reporter protein packaging into virions, whereas performing infections in the presence of the ligand would allow us to detect reporter expression from the incoming retroviral RNA genomes by stabilizing the reporter. As only two genomic RNAs per retrovirus particle are delivered into the cell upon entry, we selected the sensitive reporter nano-luciferase (Nluc) to assay incoming retroviral RNA translation, which has superior sensitivity compared to other luciferase proteins (reviewed in [8]).

Nluc was cloned with or without the DD either under a CMV promoter in a minimal lentiviral vector to produce CMV-(DD)-Nluc, or without a heterologous promoter to generate (DD)-Nluc-WPRE (Fig. 1A). Transfection of these constructs into 293T cells resulted in 30-45-fold less luciferase activity in case of DD-harboring constructs, while Shield1 treatment rescued the expression (Fig. 1B). The stabilization of DD-Nluc constructs by Shield1 was dose-dependent, whereas Nluc constructs without the responsive domain remained largely unaffected (Fig. 1C). To quantify reporter protein packaging into virions, we transfected cells with the lentiviral transfer vectors shown in Fig. 1A together with a

packaging plasmid and assayed the virus particles themselves for luciferase activity. Virions produced by DD-Nluc vectors consistently yielded 120-270-fold lower signal compared to Nluc vectors for both constructs, while the presence or absence of an envelope protein (VSV-G) did not make a difference (Fig. 1D).

For HIV-1, the timing of the early events during infection are well-documented and cell type-dependent. Reverse transcription can take anywhere from 6 - 48 hours, and integration follows about 5 hours after the completion of reverse transcription.[9, 10] As these constructs do not produce the reporter from full-length (unspliced) viral RNA, they require reverse transcription, integration, transcription and splicing to express luciferase. Accordingly, within the first 12 hours of infection, no change in signal was observed, indicating that the luciferase signal represents protein that is transferred to and/or that remains associated with cells (Fig. 1E). The presence or absence of Env or the RT inhibitor nevirapine (NVP) had no effect on the signal observed, although infection with the destabilized reporter viruses consistently yielded less signal. Likewise, at 20 hours post-infection, there was still no expression from the provirus, but expression did occur at 72 hpi, which was reduced to background levels in the absence of a functional Env or RT inhibition (Fig. 1F). These data show that by controlling protein stability, we can markedly reduce passive protein packaging into virions and their delivery into recipient cells, minimizing the background for assessment of incoming retroviral RNA translation.

To assess the translation potential of the incoming, unspliced, genomic retroviral RNA, we cloned Nluc with or without the DD into a minimal lentiviral vector, where the reporter is inserted in the location of *gag*, immediately downstream of the packaging signal (Ψ; psi) with the original gag start codon mutated (Fig. 2A). Similar to the results in Fig. 1B, transfection of DD-constructs resulted in ~180-fold decreased signal compared to Nluc, which was completely rescued by Shield1 addition (Fig. 2B). We produced virions and infected cells with LV-Nluc virus in the presence or absence of NVP or cycloheximide (CHX) to inhibit reverse transcription or translation, respectively. Even with the high background of virion-packaged protein, we did observe an increase in the luciferase signal over time, which was diminished to background levels by CHX, indicating *de novo* translation from the incoming viral genomic RNA (Fig. 2C). RT inhibition had no effect on the luciferase signal within the first 8 hours, consistent with previous results (Fig. 1E).

We then performed an infection with LV-DD-Nluc virus in the presence of NVP. The signal increased within the first 2 h, and under Shield1-stabilized conditions increased further up to 6 h, whereas in the absence of Shield1, the signal decreased after the initial hike due to the instability of the protein (Fig. 2D; red vs. blue lines). Translation inhibition by CHX drastically reduced the signal in

both cases, although increased stabilization of the protein delivered passively by virions was also evident (Fig. 2D; black vs gray lines). Importantly, when DD-Nluc is stabilized, there is a clear increase in reporter expression over time, which is markedly reduced upon translation inhibition (Fig. 2D; red vs. black lines). This difference is the result of newly synthesized reporter proteins from the incoming retroviral genomic RNA in the absence of reverse transcription. Pre-treatment of virus supernatants with DNase or benzonase did not alter reporter expression from the incoming retroviral RNA, even though both enzymes were functional (Fig. 2E-F), ruling out nonspecific uptake of ambient DNA or RNA. To rule out endogenous reverse transcripts in virions or the packaging and delivery of plasmid fragments from producer cells as the source of reporter signal, we infected cells with DD-Nluc virus in the presence of Shield1 and NVP, while inhibiting transcription (ActD) or translation (CHX). As expected, translation inhibition markedly reduced the signal, whereas transcription inhibition did not, demonstrating that the observed signal is not due to DNA transfer, which would have required both of these processes (Fig.2G). Supporting these results, "Gag-less virions" produced in the absence of a packaging vector or Env-less (bald) virions did not yield any signal over background (Fig. 2H-I). Although nucleic acid packaging into extracellular vesicles (EVs) may occur and these EVs may even be adorned with Env glycoproteins (reviewed in [11]), the lack of signal in Gag-less virions also argues against this possibility as the source of signal. To rule out incomplete inhibition of RT by NVP, we also confirmed expression from the incoming retroviral RNA using viruses produced in the presence of an RT catalytic mutant (Fig. 2J). Notably, the translation of incoming retroviral genomes was observed in diverse cell lines and primary cells, indicating that the phenotype is not restricted to a specific cell type (Fig. 2K).

The RNA genome of HIV-1 has an intricate secondary structure and several cis-acting RNA elements with diverse functions in the replication cycle, which is not present in a minimal vector.[12] To explore the contribution of such RNA elements to the process of incoming retroviral RNA translation, we cloned DD-Nluc after the Gag start codon in a near-full length HIV-1 construct based on NL4.3 encoding firefly luciferase in place of *nef* (Fig. 3A, top panel). Infection with VSV-G-pseudotyped NL43-DD-Nluc virus in the presence of NVP yielded similar results to infection with minimal lentiviral vectors (Fig. 3B vs. Fig 2D). Under stabilized conditions, the signal increased over time peaking at 4-6 hpi, whereas translation inhibition reduced the reporter activity by ~10-fold, demonstrating *de novo* protein synthesis (Fig. 3B; red vs. black lines).

As an alternative to reporter assays, we checked Gag protein production in the absence of reverse transcription. VSV-G-pseudotyped NL43-Firefly viruses were used to infect cells in the presence or absence of NVP and CHX (Fig. 3A, middle panel). Gag production was assayed by

immunoprecipitation (IP) with an anti-Gag antibody and western blot one day after infection to maximize the accumulation of newly-synthesized viral proteins. In the absence of NVP, Pr55-Gag production and proteolytic processing into p24-capsid (CA) was evident at this time point, while NVP treatment decreased this signal to ~35% of WT levels (Fig. 3C). Importantly, the signal did not originate from incoming CA protein, as CHX treatment completely abolished the signal (Fig. 3C, lanes 3 vs. 4). The NVP concentration we used (10 μM) is sufficient to prevent any signal that originates from reverse-transcribed genomes (Fig. 1F). However, to rule out incomplete RT inhibition by NVP, we also infected cells with an HIV-1 clone that carries an inactive RT due to a catalytic mutation (IIIB ΔRT) and analyzed Gag production by western blot. The difference in band intensities of CHX-treated vs. untreated cells demonstrate the amount of incoming capsid associated with viral particles compared to *de novo* synthesis, respectively (Fig. 3D).

To demonstrate the association of incoming HIV-1 RNA with polysomes - an indicator of active translation - we performed polysome fractionation on lysates from 293T cells after four hours of infection with VSV-G-pseudotyped IIIB ΔRT. Infected and uninfected cells yielded comparable polysome profiles with clearly separated peaks for ribosomal subunits, whereas EDTA treatment completely disrupted polysomes (Fig. 3E). RT-qPCR on fractions demonstrated the association of 10% and 72% of all HIV-1 RNA and the housekeeping gene hypoxanthine phosphoribosyltransferase 1 RNA (*HPRT1*) with polysomes, which was reduced to 1 and 2% in EDTA-treated controls, respectively, indicating specific association of these RNAs with polysomes (Fig. 3F).

Different retroviruses may fuse at the cell membrane (pH-independent) or in an endosomal compartment (pH-dependent). To assess whether the cellular entry pathway affects the translation of incoming retroviral RNA genomes, we used a Xenotropic Murine Leukemia Virus (X-MLV) envelope that can infect human cells and fuses in the endosomes, an HIV-1 envelope which fuses at the cell membrane, or VSV-G as a control. Infection of TZM-bl cells, which express both HIV-1 and X-MLV receptors, with reporter viruses in the presence of NVP and S1 revealed much reduced levels of infection in X-MLV and HIV-1 pseudotypes compared to VSV-G, as anticipated. Regardless of the infection level, however, the signal from the translation of incoming viral RNA was reduced to baseline levels in CHX-treated samples, indicating that genomic viral RNA can be translated regardless of the viral entry pathway (Fig. 4A).

The packaging signal (Ψ; psi) found in retroviral genomes is critical for the selective packaging of the full-length viral RNA into budding virions. The LV-DD-Nluc virus (Fig. 2A) with a deletion in psi (ΔΨ) showed a drastic reduction in the amount of packaged HIV-1 RNA compared to WT, even though the amount of the produced viral particles were comparable (Fig. 4B-C). Infection of cells with

the two viruses showed significantly reduced reporter signal in ΔΨ compared to WT, indicating that the presence of the genome is necessary for incoming viral RNA translation. Notably, CHX treatment reduced the signal during WT infection, but did not have any effect on the ΔΨ infection, which was relatively low from the beginning (Fig. 4D). It should be noted that we consistently observed markedly less reporter protein production and packaging in ΔΨ compared to WT, as evident in the difference between CHX-treated samples. This result is consistent with previous studies showing that Ψ deletions interfere with not only genome packaging, but also expression from the viral genome.[13]

As the retroviral RNA genome is found inside of the capsid, we reasoned that mutations affecting capsid stability may alter the translation kinetics. Infection of cells with reporter viruses containing hypostable (P38A) capsids indeed revealed increased incoming genome translation compared to WT, likely due to early uncoating of the capsid and increased availability of gRNA to the translation machinery (Fig. 4E-F). In contrast, incoming RNA translation was indistinguishable between the WT and hyperstable (E45A) capsids. The translation of the incoming viral genomes is likely mediated by particles containing capsids that start uncoating before reaching the nucleus, either due to a slight inherent defect or stochastic processes. Thus, making intact capsids less stable would increase translation, but rendering already intact capsids even more stable would not alter the translation kinetics, as they are not the particles to yield the signal in the first place. This scenario is also consistent with the finding that (near) intact HIV-1 capsids can be transported into the nucleus, where reverse transcription is completed.[14, 15, 16, 17] As direct translation of the genome is likely a dead-end for viral replication, it is reasonable to assume that virions that produce infectious progeny are the very few ones that successfully make it to the nucleus intact.

The production of viral proteins during HIV-1 infection in the absence of reverse transcription has major implications for host immunity and infection kinetics. Individuals who encounter HIV-1 but who do not get productively-infected, for instance due to pre- or post-exposure prophylaxis (PrEP) usage, may still be able to produce and present viral peptides to generate cell-mediated and/or humoral immune responses. In addition, as particle-to-PFU (or rather, particle-to-infectious unit) ratio of HIV-1 can vary between 10^2-10^7 according to some estimates (reviewed in [18]), an initial abortive infection may result in the recruitment and activation of T-cells, increasing the eligible target cell population locally for productive infection. During SIV infection of rhesus macaques, Gag- and Pol-specific CTL responses were identified very early (within 2h) after infection, whereas Env- or Nef-specific responses were not found until later, which was attributed to the ability of incoming viral proteins to be processed and presented.[19, 20] Our results indicate that such responses may also occur due to *de novo* translation from the viral genome. Early studies of avian and murine retroviruses

reported the detection of full-length viral genomes in polysome-containing pellets.[21, 22, 23] In support of our findings, a recent ribosome profiling study of HIV-1 infected cells revealed ribosome-protected RNA fragments in the *gag* coding region already at one hour after infection.[24] In other studies, protein expression could be detected in case of MLV-based vectors with primer binding site (PBS) mutations[25] and modified lentiviral vectors with structural rearrangements of the genome to enable direct translation, such as 5' IRES insertion,[26] although passive protein transfer cannot be ruled out in these cases. Our results challenge the textbook dogma that retroviruses require reverse transcription to produce viral proteins, warrant in vivo studies of immune responses during abortive infection and open up novel paths for safer gene therapy applications.

Methods

Cloning, constructs, virus production. As a basis for minimal lentiviral vectors the pLVX-IRES-Neo vector (Clontech) was used, which contains identical full-length LTRs. DD-Nluc was synthesized as a gBlock (IDT) and cloned between XhoI and NotI sites of pLVX-IRES-Neo, either with or without the destabilizing domain to create CMV-(DD)Nluc. Similarly, (DD)Nluc was cloned between ClaI and MluI sites of pLVX-IRES-Neo to eliminate the CMV promoter, multiple cloning site, IRES element and Neo resistance gene to create (DD)Nluc-WPRE. LV-(DD)Nluc was created first by deleting the sequences between ClaI and KpnI sites in pLVX-IRES-Neo and religating the vector onto itself, then mutating the start codon of Gag, inserting the restriction sites BstBI and PacI after the packaging signal (Ψ) and finally cloning (DD)Nluc between these restriction sites. Packaging signal deletion (ΔΨ) was introduced into LV-DD-Nluc by overlap PCR resulting in a deletion between nucleotides 741-809 based on the pLVX-IRES-Neo vector sequence. NL43-Firefly mentioned in Fig. 3 is pNL4-3 e- r- FLuc (kindly provided by Ned Landau; NIH ARP-3418). NL43-DD-Nluc was created by mutating the Gag start codon of NL43-Firefly and inserting the DD-Nluc sequence between PteI and SpeI sites. The identity of all constructs was confirmed by restriction digest and sequencing.

Reporter viruses were produced by transfecting viral plasmids together with a plasmid encoding VSV-G env (pCMV-VSV-G, kindly provided by Bob Weinberg; Addgene #8454), and in case of the minimal lentiviral vectors, also with a plasmid encoding HIV-1 gag, pol, tat and rev (psPAX2, kindly provided by Didier Trono; Addgene #12260) or with the packaging plasmid pCD/NL-BH*deltavpu/RT- that lacks RT activity due to the D110E mutation in the catalytic site (kindly provided by Jakob Reiser; Addgene: #136985). CA stability mutants P38A and E45A were kindly provided by Stephen Goff. The full-length IIIB ΔRT construct carrying mutations in the catalytic domain of RT was kindly provided by Michael Malim.[27] The HIV-1 Env plasmid was kindly provided by Yiping Zhu (University of Rochester). X-MLV Env was a codon-optimized synthetic Xenotropic MLV-Related Virus Env in a pTH plasmid, kindly provided by Alaa Ramadan.[28]

Virus stocks were produced by transfection of 293T cells with polyethylenimine (PEI), followed by media change after one day and supernatant collection after two days. ΔEnv or ΔGag viruses were produced by omitting the respective plasmids during the transfection step. Virus-containing supernatants were filtered (0.45 µ), ultracentrifuged over a 20% sucrose cushion, aliquoted and frozen at -80°C. Nuclease treatment of virus stocks were performed at room temperature by DNase (Ambion) or benzonase (Millipore).

Cell culture, treatments, infections. 293T and TZM-bl cells were maintained in DMEM containing 9% FBS (Gibco) and 100 µg/ml Pen/Strep (Gibco). All suspension cell lines including THP-1, U937, C8166, Jurkat, PM1 and A3.01 were maintained in RPMI with 9% FBS, 100 µg/ml Pen/Strep, 100 µg/ml Normocin (Invivogen). PBMCs of healthy donors were isolated from buffy coats from the German Red Cross using standard Ficoll separation. Monocytes were selected by adhering PBMCs in RPMI with 5% pooled AB human serum (Sigma), 1 mM HEPES (Gibco) and 24 µg/ml gentamicin (Sigma) for several hours, followed by extensive washing to remove unbound cells. Monocyte-derived macrophages were differentiated from primary monocytes by 50 ng/ml GM-CSF (R&D Systems) treatment for 6-10 days. CD4+ T-cells were isolated from PBMCs by negative selection using the MACS human CD4+ T-cell isolation kit (Miltenyi). Infections were performed by spinoculation at 1200 x g for 60-90 mins at 25°C. Unless indicated otherwise, the following concentrations were used for treatments: NVP: 10 µM (Merck), CHX: 10 µg/ml (EMD/Millipore), ActD: 2 µg/ml (Sigma), Shield1: 1.5 µM (Takara). With the exception of time-course experiments, Nluc activity was measured after 6 hours post-infection, using the Nano-Glo luciferase assay system (Promega).

Polysome fractionation. Polysome fractionation was performed as previously with some modifications.[29] Cells were split the day before such that they would reach a maximum of 60-80% confluency on the day of the assay. At four hours post-infection with RT-deficient viruses or with WT viruses in the presence of NVP treatment, cells were treated with 50 µg/ml CHX for 10 min at 37°C, washed once with ice-cold PBS + CHX (50 µg/ml), collected by scraping in PBS+CHX, pelleted, and lysed in 1X polysome lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.5 % NP40, 1 mM DTT, 50 µg/ml cycloheximide, protease inhibitors [Roche]) on ice for 10 mins. Lysates were passed through a 21-gauge needle 12 times, incubated on ice for another 5 mins and cleared by spinning at 4°C, 13.000 rpm for 10 mins. Cleared lysates were then loaded on 15-45% linear sucrose gradients (in 20 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 50 µg/ml CHX) prepared using a BioComp gradient master in ultra-clear centrifuge tubes (14x89 mm, Beckman Coulter) and centrifuged for 2:30 hours at 36.000 rpm in an SW41 rotor. Fractions (0.5 ml) were collected by a piston gradient fractionator (Biocomp) with continuous UV absorbance recording at 254 nm. RNA from each fraction was isolated by phenol-chloroform extraction and quantified by RT-qPCR.

qPCR, RT-qPCR. Viral RNA was isolated from virus stocks with viral RNA isolation kit (Qiagen). RNA was treated with Turbo DNase and inactivation beads (Ambion), cDNA was synthesized using Superscript III (Invitrogen), and qPCR was performed with the SensiFAST No-ROX Probe Master Mix (Bioline) on a CFX96 qPCR machine (Bio-Rad). Primer and probe sequences used in RT-qPCR are listed in Table 1.

Target	F/R/P	Sequence
HIV-1 genome	F	TCTCGACGCAGGACTCG
	R	TACTGACGCTCTCGCACC
	P	CTCTCTCCTTCTAGCCTC
HPRT1 mRNA	F	TCTTTGCTGACCTGCTGGATT
	R	TTATGTCCCCTGTTGACTGGT
	P	AGTGATAGATCCATTCCTATGACTGT

Table 1. List of primers and probes used in the study. F: forward, R: reverse, P: probe.

Immunoprecipitation and western blots. Immunoprecipitations were performed essentially as described with slight modifications.[30] Protein G Dynabeads (Invitrogen) were washed and coated with anti-p55+p24+p17 antibody (Abcam) by rotating for 15 mins at 4°C. After washing off unbound antibody, cleared cell lysates were added to the coated beads and incubated at 4°C with rotation for 1 hour. Beads were then separated by a magnet, washed three times and bound proteins were released from the beads by boiling in the presence of a denaturing loading buffer. Released proteins were analyzed by SDS-PAGE. Western blots were performed as previously described.[31] Briefly, cells were washed with PBS, scraped, transferred to a tube and washed again with PBS. The pellet was lysed in NP40 lysis buffer (100 mM Tris, 30 mM NaCl, 0.5% NP-40) containing benzonase for 30 min on ice. Lysates were cleared by centrifugation at 10,000 rpm for 5 min, supplemented with denaturing loading buffer (Invitrogen) and ran on an SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore), blocked by blocking buffer (Rockland) and incubated with primary and IRdye-labeled secondary antibodies (Licor). Blots were visualized on an Odyssey scanner (Licor). Anti-p24 antibody used for HIV-1 capsid detection was the AG3.0 clone (NIH ARP #4121)[32] and the anti-tubulin antibody was from Sigma.

p24 ELISA. p24-CA concentrations of viral stocks were determined by a homemade ELISA, as previously described.[32] Briefly, 96-well plates were coated overnight at 4°C with the AG3.0 anti-p24 antibody diluted 1:100 in carbonate/bicarbonate buffer (Sigma). The next day, plates were washed three times with wash buffer (PBS+0.05% Tween), blocked with PBS+2% milk powder at 37°C for 1 h and washed again three times. Meanwhile, the viral supernatants were inactivated by incubating with a final concentration of 0.2% Tween for 10 min at room temperature. Serial dilutions of viral supernatants in dilution buffer (PBS+2% milk powder+0.05% Tween) were pipetted into the wells and incubated at 37°C for 1 h. After three washes, the primary antibody in the form of pooled HIV+ serum

diluted 1:10000 was added to the wells and incubated at 37°C for 1 h. Following another three washes, the secondary antibody anti-human IgG-HRP (Sigma) was added at 1:1000 dilution. After three more washes, the substrate solution (12.5 ml phosphate/citrate buffer + 1 OPD tablet (5 mg; Sigma) + 12 µl 30% H_2O_2 solution) was added and incubated at room temperature for 10 mins. Reactions were stopped by adding 5% sulphuric acid (H_2SO_4). Absorbance was measured at 492 nm and 620 nm.

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Figures and figure legends

Figure 1

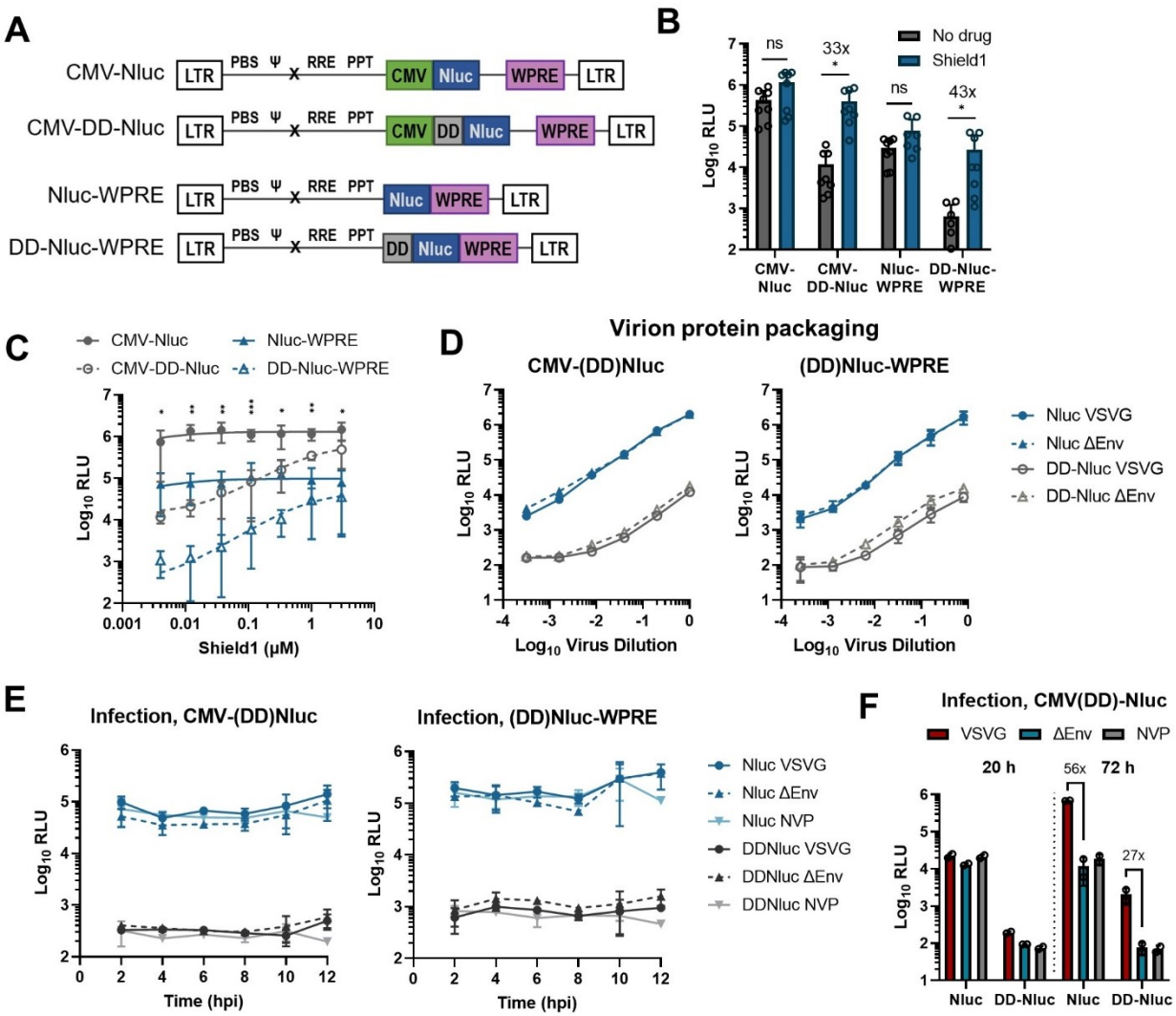

Figure 1. Posttranslational regulation of protein stability reduces protein packaging into lentiviral particles and their delivery into recipient cells. (A) Schematic representation of the lentiviral constructs used. Nano-luciferase (Nluc) was cloned under a CMV promoter to generate CMV-(DD)-Nluc or without a separate promoter in a minimal lentiviral vector, with or without the destabilizing domain (DD). Vector maps are not drawn to scale. 'X' denotes the mutated start codon of *gag*. (B) 293T cells were transfected with the constructs in A in the presence or absence of Shield1 (3 μM). Nluc activity was measured after 24 hours. (C) Transfection was performed as in B with serial dilutions of Shield1. Asterisks denote comparison between the solid and dashed grey lines. (D) Serial dilutions of virus particles produced using the constructs in A in the presence or absence of a VSV-G plasmid were assayed for Nluc activity after harvesting. (E) Infection was performed with the viruses in D in the presence or absence of NVP (10 μM) and assayed for Nluc activity at the indicated time points. (F) 293T cells were infected with CMV-(DD)-Nluc carrying a VSV-G Env in the presence or absence of NVP (10 μM), or with bald viruses without an Env. Nluc activity was measured at 20 hpi and 72 hpi. Nluc: Nano-luciferase, WPRE: woodchuck hepatitis virus posttranscriptional regulatory element, PBS: primer binding site, psi (Ψ): packaging signal, RRE: rev-response element, PPT: polypurine tract, NVP: nevirapine. Data represent mean +/- SD. Statistical significance was measured by Student's t test, with Welch's correction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

Figure 2

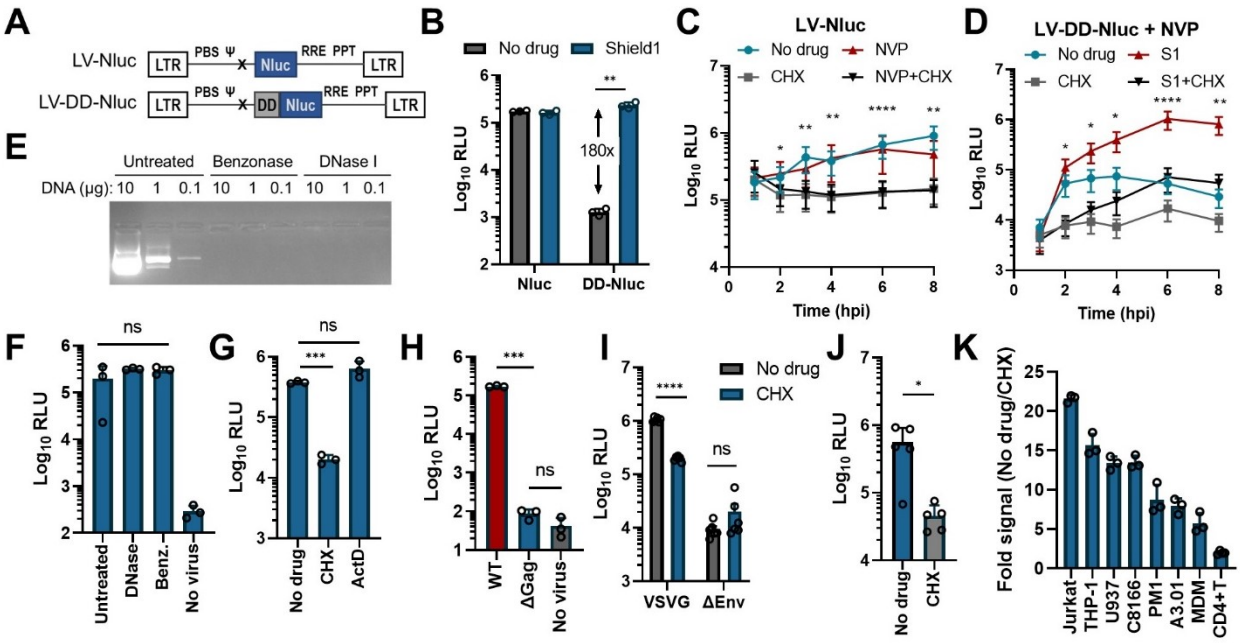

Figure 2. Incoming minimal lentiviral RNA genomes are translated following entry into cells. (A) Schematic representation of the lentiviral constructs used. Nluc was cloned into a minimal lentiviral vector downstream of the packaging signal (Ψ), with or without the destabilizing domain (DD). Vector maps are not drawn to scale. 'X' denotes the mutated start codon of *gag*. (B) 293T cells were transfected with the constructs in (A) in the presence or absence of Shield1 (S1; 1.5 µM) and assayed for Nluc activity after 24 hours. (C) Cells were infected with VSV-G pseudotyped LV-Nluc virus in the presence or absence of NVP and/or CHX. Luciferase activity was measured at the indicated time points. (D) Infection was performed as in C, but with the DD-containing virus. (E) Different amounts of plasmid DNA (0.1, 1 or 10 µg) were treated with the indicated nucleases for 30 mins or left untreated and visualized on an agarose gel. (F) Equal amounts of virus stocks were treated with benzonase, DNase or left untreated for 1 h prior to infection. Nluc activity was measured at 6 hpi. (G) Cells were infected with VSV-G pseudotyped LV-DD-Nluc virus in the presence of both S1 and NVP, with or without Actinomycin D (ActD) or CHX. Nluc activity was measured at 6 hpi. (H) Cells were infected with lentiviruses produced in the presence or absence of a lentiviral packaging vector (WT vs. ΔGag, respectively). Nluc activity was measured at 6 hpi. (I) 293T cells were infected with LV-Nluc virus bearing VSV-G or no Env (ΔEnv) in the presence of NVP, with or without CHX treatment. Nluc activity was measured at 6 hpi. (J) 293T cells were infected with LV-DD-Nluc virus that carries a catalytic RT mutation (D110E) in the presence of Shield1, with or without CHX. Nluc activity was measured at 6 hpi. (K) T-cell lines (Jurkat, C8166, PM1, A3.01), monocytic cell lines (THP-1, U937), primary monocyte-derived macrophages (MDM) or CD4+ T-cells were infected with VSV-G pseudotyped LV-DD-Nluc in the presence of NVP and Shield1, with or without CHX. Nluc activity was measured at 6 hpi. Results are given as the normalized ratio of CHX-/CHX+ infections, which represent de novo translated over incoming virion packaged reporter. NVP: 10 µM, Shield1 (S1): 1.5 µM, CHX: 10 µg/ml, ActD: 10 µg/ml. Data represent mean +/- SD. Statistical significance was measured by Student's t test, with Welch's correction, and reflect the comparison of red and black lines in panels C and D. hpi: hours post-infection. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant.

Figure 3

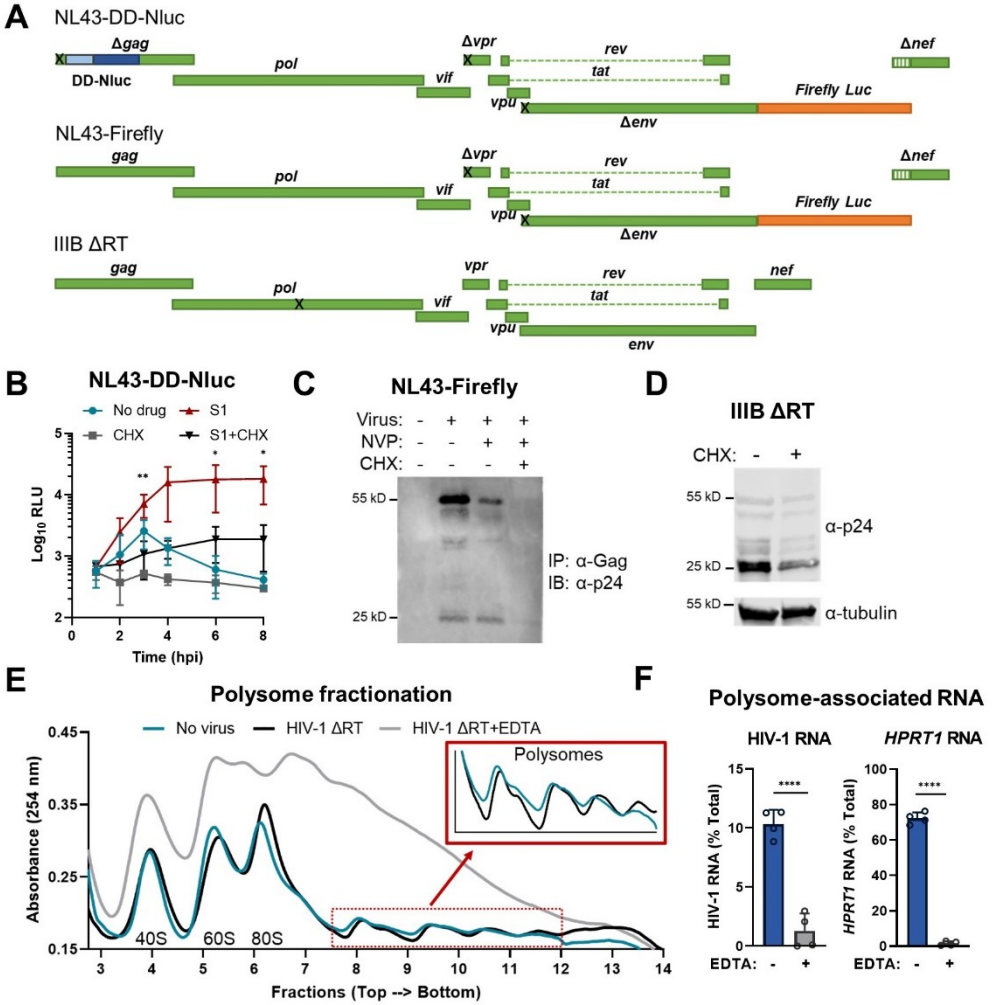

Figure 3. Incoming full-length HIV-1 genomes are translated to produce viral proteins in the absence of productive infection. (A) Schematic representation of the (near) full-length HIV-1 constructs used in the study. DD-Nluc was cloned into the NL43-Firefly construct (middle panel) downstream of the packaging signal to generate NL43-DD-Nluc (top panel). A full-length IIIB clone of HIV-1 carrying a catalytic mutation in RT was used for immunoprecipitation studies (bottom panel). Vector maps are drawn to scale and 'X' denotes mutated codons. (B) 293T cells were infected with NL43-DD-Nluc in the presence or absence of Shield1 (S1) and/or CHX. Nluc activity was measured at the indicated time points. (C) Cells were infected with VSV-G-pseudotyped NL43-Firefly with or without NVP or CHX treatment. Lysates were collected one day after infection, immunoprecipitated with a polyclonal anti-HIV-1 Gag antibody (p17+24+55) and probed for p24. (D) Cells were infected with VSV-G pseudotyped IIIB ΔRT with or without CHX. Lysates were run on an SDS-PAGE and probed with anti-p24 and anti-tubulin antibodies. (E) Representative polysome fractionation profiles from 293T cells infected (or not) with VSV-G pseudotyped IIIB ΔRT, treated with CHX (50 µg/ml) at 4 hpi and lysed in the presence or absence of EDTA (20 mM). Lysates were run on a sucrose density gradient and fractions were collected from top to bottom while simultaneously measuring UV absorbance. (F) RNA was isolated from each fraction by phenol extraction and the levels of HIV-1 genomic RNA and *HPRT1* were measured by RT-qPCR. The amount of each RNA is given as a percentage of total RNA for that message. Unless indicated otherwise, the following concentrations were used: NVP: 10 µM, Shield1 (S1): 1.5 µM, CHX: 10 µg/ml. Data represent mean +/- SD. Statistical significance was determined by Student's t-test with Welch correction and reflect the comparison of red and black lines in panel B. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

Figure 4

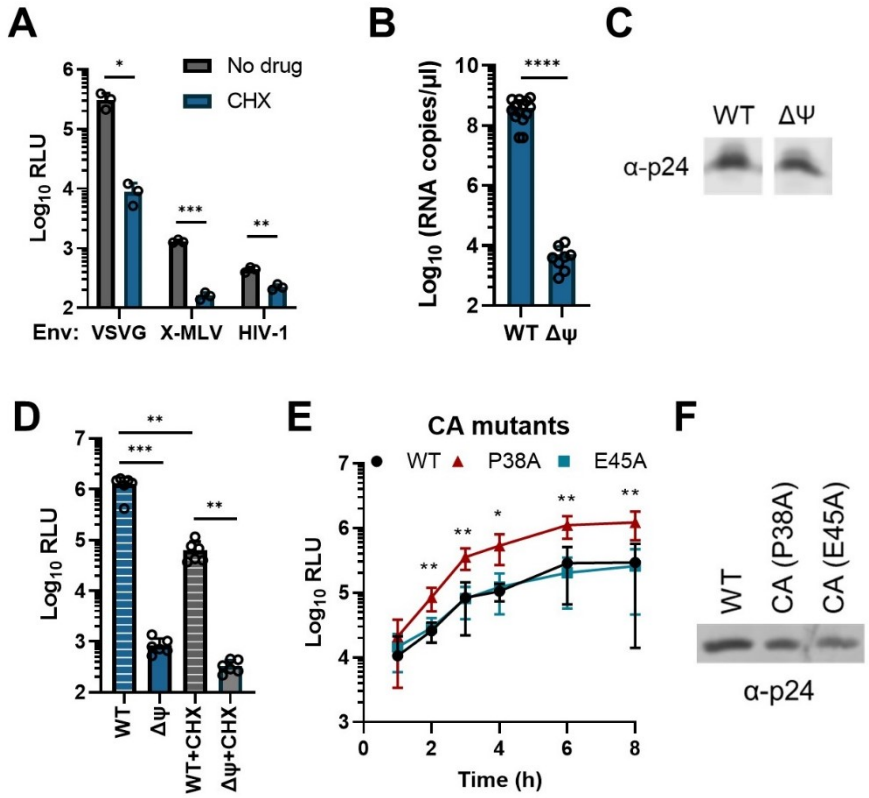

Figure 4. Mutations in viral components can alter the translation of incoming HIV-1 genomes. (A) TZM-bl cells were infected with LV-DD-Nluc viruses pseudotyped with the indicated Env proteins in the presence of Shield1 and NVP, with or without CHX treatment. Luciferase activity was measured at 6 hpi. (B-C) Viral RNA content (B) and p24 content (C) of WT and ΔΨ LV-DD-Nluc virions were quantified by RT-qPCR and western blot. (D) Cells were infected with of WT and ΔΨ LV-DD-Nluc viruses, in the presence of NVP (10 µM) and S1 (1.5 µM), with or without CHX (10 µM). Nluc activity was measured at 6 hpi. (E-F) Cells were infected with viruses carrying WT capsids or the CA stability mutants P38A and E45A carrying the LV-DD-Nluc genome. Infection was performed with Shield1 and NVP treatment, with or without CHX. Nluc activity was measured at the indicated time points. Unless indicated otherwise, the following concentrations were used: NVP: 10 µM, Shield1 (S1): 1.5 µM, CHX: 10 µg/ml. Data represent mean +/- SD. Statistical significance was determined by Student's t-test with Welch correction and reflect the comparison of red and black lines in panel E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, not significant.

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