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

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

51 For HIV-1, the timing of the early events during infection are well-documented and cell type-
52 dependent. Reverse transcription can take anywhere from 6 - 48 hours, and integration follows about
53 5 hours after the completion of reverse transcription.[9, 10] As these constructs do not produce the
54 reporter from full-length (unspliced) viral RNA, they require reverse transcription, integration,
55 transcription and splicing to express luciferase. Accordingly, within the first 12 hours of infection, no
56 change in signal was observed, indicating that the luciferase signal represents protein that is
57 transferred to and/or that remains associated with cells (Fig. 1E). The presence or absence of Env or
58 the RT inhibitor nevirapine (NVP) had no effect on the signal observed, although infection with the
59 destabilized reporter viruses consistently yielded less signal. Likewise, at 20 hours post-infection,
60 there was still no expression from the provirus, but expression did occur at 72 hpi, which was
61 reduced to background levels in the absence of a functional Env or RT inhibition (Fig. 1F). These data
62 show that by controlling protein stability, we can markedly reduce passive protein packaging into
63 virions and their delivery into recipient cells, minimizing the background for assessment of incoming
64 retroviral RNA translation.

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

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

80 both cases, although increased stabilization of the protein delivered passively by virions was also
81 evident (Fig. 2D; black vs gray lines). Importantly, when DD-Nluc is stabilized, there is a clear increase
82 in reporter expression over time, which is markedly reduced upon translation inhibition (Fig. 2D; red
83 vs. black lines). This difference is the result of newly synthesized reporter proteins from the incoming
84 retroviral genomic RNA in the absence of reverse transcription. Pre-treatment of virus supernatants
85 with DNase or benzonase did not alter reporter expression from the incoming retroviral RNA, even
86 though both enzymes were functional (Fig. 2E-F), ruling out nonspecific uptake of ambient DNA or
87 RNA. To rule out endogenous reverse transcripts in virions or the packaging and delivery of plasmid
88 fragments from producer cells as the source of reporter signal, we infected cells with DD-Nluc virus in
89 the presence of Shield1 and NVP, while inhibiting transcription (ActD) or translation (CHX). As
90 expected, translation inhibition markedly reduced the signal, whereas transcription inhibition did
91 not, demonstrating that the observed signal is not due to DNA transfer, which would have required
92 both of these processes (Fig.2G). Supporting these results, "Gag-less virions" produced in the
93 absence of a packaging vector or Env-less (bald) virions did not yield any signal over background (Fig.
94 2H-I). Although nucleic acid packaging into extracellular vesicles (EVs) may occur and these EVs may
95 even be adorned with Env glycoproteins (reviewed in [11]), the lack of signal in Gag-less virions also
96 argues against this possibility as the source of signal. To rule out incomplete inhibition of RT by NVP,
97 we also confirmed expression from the incoming retroviral RNA using viruses produced in the
98 presence of an RT catalytic mutant (Fig. 2J). Notably, the translation of incoming retroviral genomes
99 was observed in diverse cell lines and primary cells, indicating that the phenotype is not restricted to
100 a specific cell type (Fig. 2K).

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

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

113 immunoprecipitation (IP) with an anti-Gag antibody and western blot one day after infection to
114 maximize the accumulation of newly-synthesized viral proteins. In the absence of NVP, Pr55-Gag
115 production and proteolytic processing into p24-capsid (CA) was evident at this time point, while NVP
116 treatment decreased this signal to ~35% of WT levels (Fig. 3C). Importantly, the signal did not
117 originate from incoming CA protein, as CHX treatment completely abolished the signal (Fig. 3C, lanes
118 3 vs. 4). The NVP concentration we used (10 µM) is sufficient to prevent any signal that originates
119 from reverse-transcribed genomes (Fig. 1F). However, to rule out incomplete RT inhibition by NVP,
120 we also infected cells with an HIV-1 clone that carries an inactive RT due to a catalytic mutation (IIIB
121 ΔRT) and analyzed Gag production by western blot. The difference in band intensities of CHX-treated
122 vs. untreated cells demonstrate the amount of incoming capsid associated with viral particles
123 compared to *de novo* synthesis, respectively (Fig. 3D).

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

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

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

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

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

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

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

188 ## Methods

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

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

213 Virus stocks were produced by transfection of 293T cells with polyethylenimine (PEI),
214 followed by media change after one day and supernatant collection after two days. ΔEnv or ΔGag
215 viruses were produced by omitting the respective plasmids during the transfection step. Virus-
216 containing supernatants were filtered (0.45 µ), ultracentrifuged over a 20% sucrose cushion,
217 aliquoted and frozen at -80°C. Nuclease treatment of virus stocks were performed at room
218 temperature by DNase (Ambion) or benzonase (Millipore).
219 **Cell culture, treatments, infections.** 293T and TZM-bl cells were maintained in DMEM
220 containing 9% FBS (Gibco) and 100 µg/ml Pen/Strep (Gibco). All suspension cell lines including THP-1,
221 U937, C8166, Jurkat, PM1 and A3.01 were maintained in RPMI with 9% FBS, 100 µg/ml Pen/Strep,
222 100 µg/ml Normocin (Invivogen). PBMCs of healthy donors were isolated from buffy coats from the
223 German Red Cross using standard Ficoll separation. Monocytes were selected by adhering PBMCs in
224 RPMI with 5% pooled AB human serum (Sigma), 1 mM HEPES (Gibco) and 24 µg/ml gentamicin
225 (Sigma) for several hours, followed by extensive washing to remove unbound cells. Monocyte-
226 derived macrophages were differentiated from primary monocytes by 50 ng/ml GM-CSF (R&D
227 Systems) treatment for 6-10 days. CD4+ T-cells were isolated from PBMCs by negative selection using
228 the MACS human CD4+ T-cell isolation kit (Miltenyi). Infections were performed by spinoculation at
229 1200 x g for 60-90 mins at 25°C. Unless indicated otherwise, the following concentrations were used
230 for treatments: NVP: 10 µM (Merck), CHX: 10 µg/ml (EMD/Millipore), ActD: 2 µg/ml (Sigma), Shield1:
231 1.5 µM (Takara). With the exception of time-course experiments, Nluc activity was measured after 6
232 hours post-infection, using the Nano-Glo luciferase assay system (Promega).
233 **Polysome fractionation.** Polysome fractionation was performed as previously with some
234 modifications.[29] Cells were split the day before such that they would reach a maximum of 60-80%
235 confluency on the day of the assay. At four hours post-infection with RT-deficient viruses or with WT
236 viruses in the presence of NVP treatment, cells were treated with 50 µg/ml CHX for 10 min at 37°C,
237 washed once with ice-cold PBS + CHX (50 µg/ml), collected by scraping in PBS+CHX, pelleted, and
238 lysed in 1X polysome lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.5 % NP40, 1
239 mM DTT, 50 µg/ml cycloheximide, protease inhibitors [Roche]) on ice for 10 mins. Lysates were
240 passed through a 21-gauge needle 12 times, incubated on ice for another 5 mins and cleared by
241 spinning at 4°C, 13.000 rpm for 10 mins. Cleared lysates were then loaded on 15-45% linear sucrose
242 gradients (in 20 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 50 µg/ml CHX) prepared using a
243 BioComp gradient master in ultra-clear centrifuge tubes (14x89 mm, Beckman Coulter) and
244 centrifuged for 2:30 hours at 36.000 rpm in an SW41 rotor. Fractions (0.5 ml) were collected by a
245 piston gradient fractionator (Biocomp) with continuous UV absorbance recording at 254 nm. RNA
246 from each fraction was isolated by phenol-chloroform extraction and quantified by RT-qPCR.

247 **qPCR, RT-qPCR.** Viral RNA was isolated from virus stocks with viral RNA isolation kit (Qiagen).
248 RNA was treated with Turbo DNase and inactivation beads (Ambion), cDNA was synthesized using
249 Superscript III (Invitrogen), and qPCR was performed with the SensiFAST No-ROX Probe Master Mix
250 (Bioline) on a CFX96 qPCR machine (Bio-Rad). Primer and probe sequences used in RT-qPCR are listed
251 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

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

253 **Immunoprecipitation and western blots.** Immunoprecipitations were performed essentially
254 as described with slight modifications.[30] Protein G Dynabeads (Invitrogen) were washed and coated
255 with anti-p55+p24+p17 antibody (Abcam) by rotating for 15 mins at 4°C. After washing off unbound
256 antibody, cleared cell lysates were added to the coated beads and incubated at 4°C with rotation for
257 1 hour. Beads were then separated by a magnet, washed three times and bound proteins were
258 released from the beads by boiling in the presence of a denaturing loading buffer. Released proteins
259 were analyzed by SDS-PAGE. Western blots were performed as previously described.[31] Briefly, cells
260 were washed with PBS, scraped, transferred to a tube and washed again with PBS. The pellet was
261 lysed in NP40 lysis buffer (100 mM Tris, 30 mM NaCl, 0.5% NP-40) containing benzonase for 30 min
262 on ice. Lysates were cleared by centrifugation at 10,000 rpm for 5 min, supplemented with
263 denaturing loading buffer (Invitrogen) and ran on an SDS-PAGE. Proteins were transferred to a PVDF
264 membrane (Millipore), blocked by blocking buffer (Rockland) and incubated with primary and IRdye-
265 labeled secondary antibodies (Licor). Blots were visualized on an Odyssey scanner (Licor). Anti-p24
266 antibody used for HIV-1 capsid detection was the AG3.0 clone (NIH ARP #4121)[32] and the anti-tubulin
267 antibody was from Sigma.

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

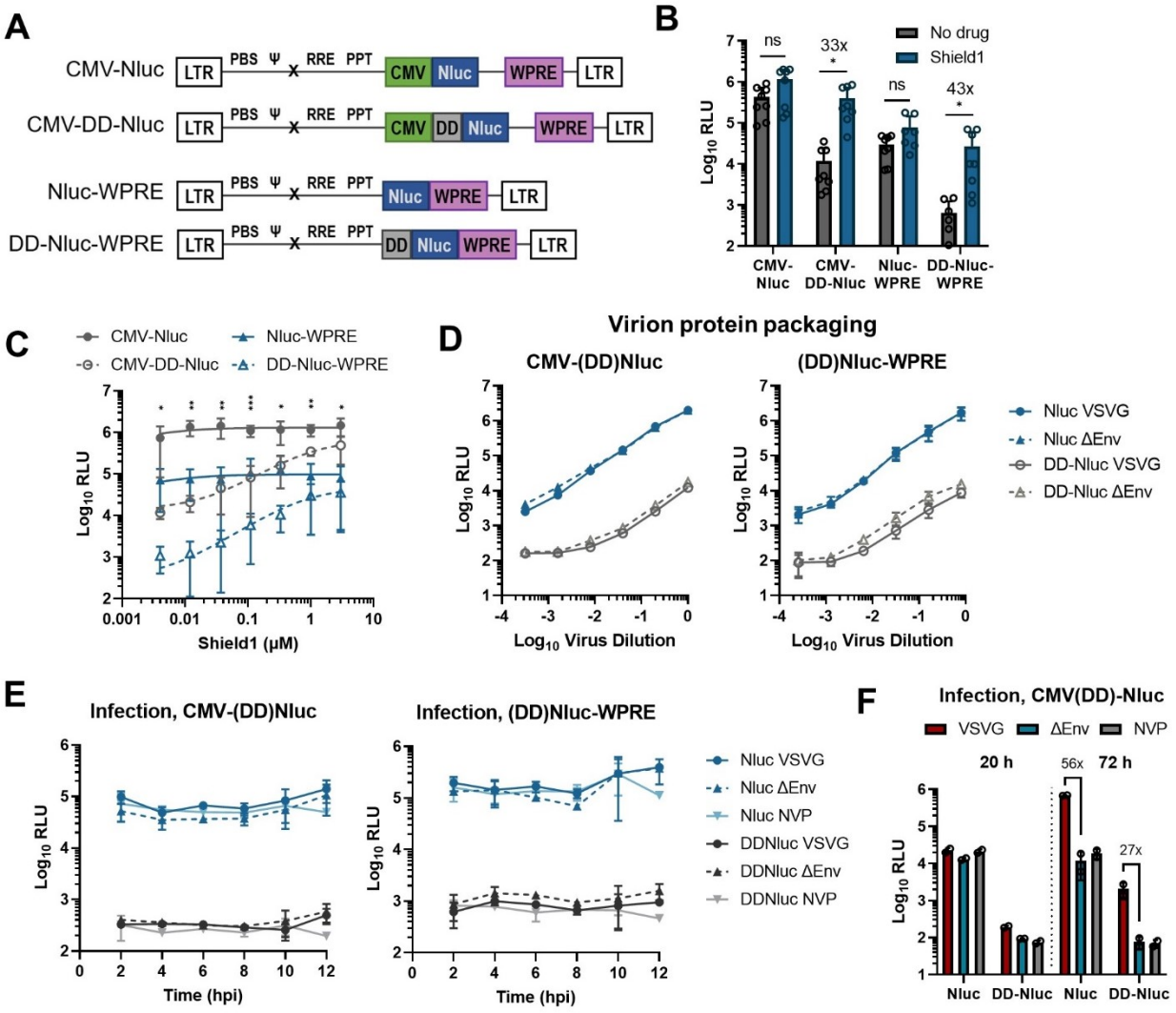
276 diluted 1:10000 was added to the wells and incubated at 37°C for 1 h. Following another three
277 washes, the secondary antibody anti-human IgG-HRP (Sigma) was added at 1:1000 dilution. After
278 three more washes, the substrate solution (12.5 ml phosphate/citrate buffer + 1 OPD tablet (5 mg;
279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

282 Acknowledgements

283 We thank all members of the Cingöz Lab and our colleagues at RKI Unit 18. We thank
284 Christine Goffinet and her lab members for valuable input, support and materials. We also thank Igor
285 Minia and Markus Landthaler for their help with polysome fractionation. This work was funded by
286 the priority program of the German Research Council (DFG) awarded to OC and NB (SPP1923; innate
287 sensing and restriction of retroviruses), intramural funds from the Robert Koch Institute, as well as
288 the KT Boost Fund awarded to OC by the Klaus Tschira Foundation (KTS) and the German Scholars
289 Organization (GSO).

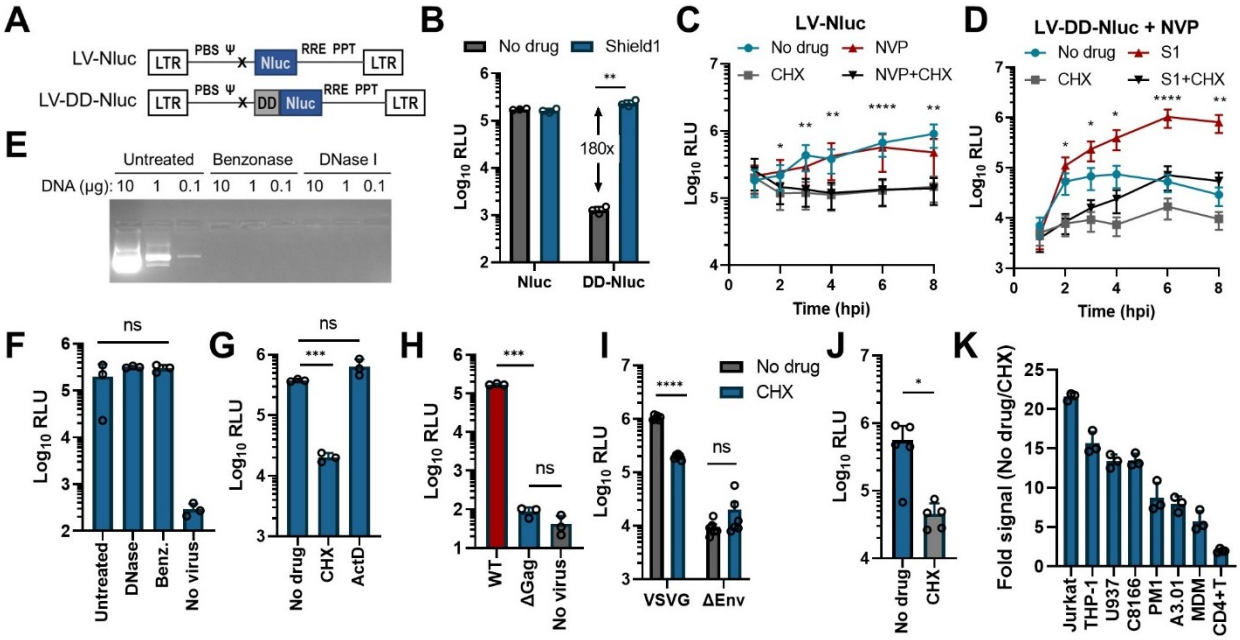
290 **Figures and figure legends**

291 **Figure 1**



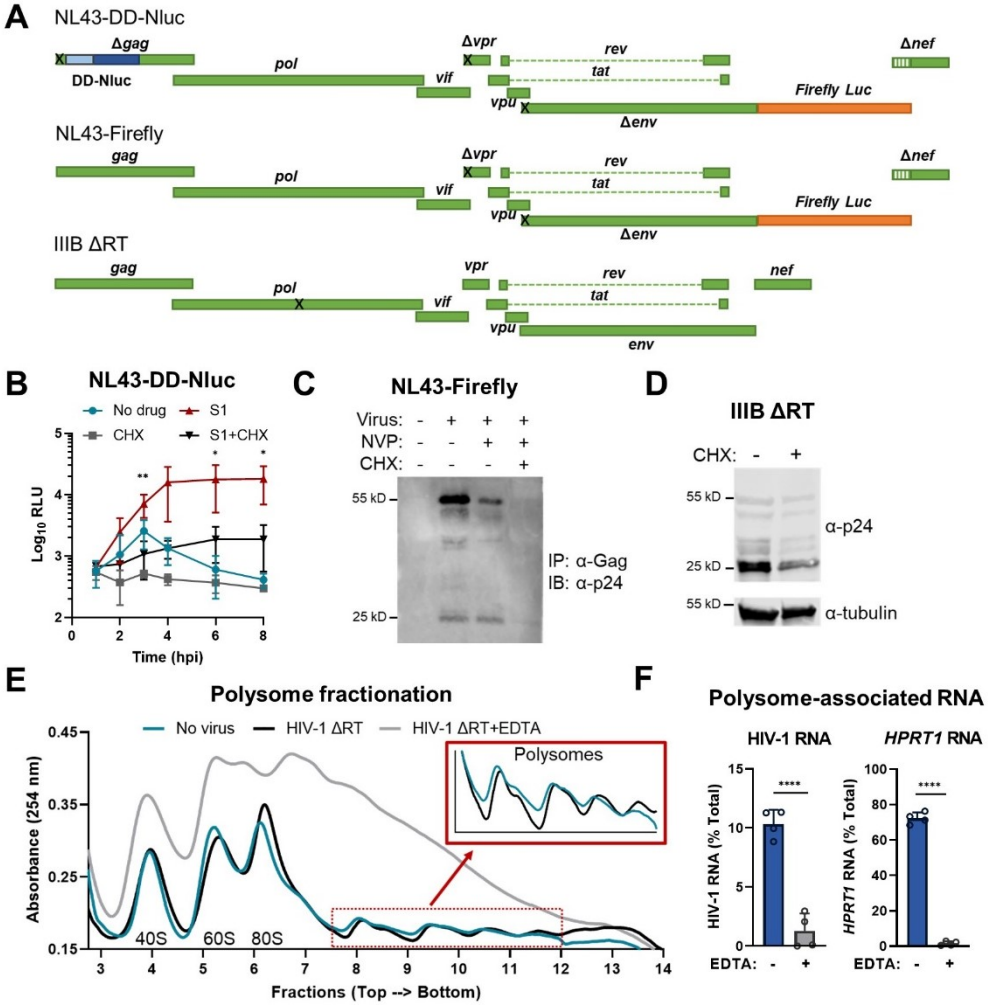
292 **Figure 1. Posttranslational regulation of protein stability reduces protein packaging into lentiviral**
293 **particles and their delivery into recipient cells.** (A) Schematic representation of the lentiviral
294 constructs used. Nano-luciferase (Nluc) was cloned under a CMV promoter to generate CMV-(DD)-
295 Nluc or without a separate promoter in a minimal lentiviral vector, with or without the destabilizing
296 domain (DD). Vector maps are not drawn to scale. 'X' denotes the mutated start codon of *gag*. (B)
297 293T cells were transfected with the constructs in A in the presence or absence of Shield1 (3 μM).
298 Nluc activity was measured after 24 hours. (C) Transfection was performed as in B with serial
299 dilutions of Shield1. Asterisks denote comparison between the solid and dashed grey lines. (D) Serial
300 dilutions of virus particles produced using the constructs in A in the presence or absence of a VSV-G
301 plasmid were assayed for Nluc activity after harvesting. (E) Infection was performed with the viruses
302 in D in the presence or absence of NVP (10 μM) and assayed for Nluc activity at the indicated time
303 points. (F) 293T cells were infected with CMV-(DD)-Nluc carrying a VSV-G Env in the presence or
304 absence of NVP (10 μM), or with bald viruses without an Env. Nluc activity was measured at 20 hpi
305 and 72 hpi. Nluc: Nano-luciferase, WPRE: woodchuck hepatitis virus posttranscriptional regulatory
306 element, PBS: primer binding site, psi (Ψ): packaging signal, RRE: rev-response element, PPT:
307 polypurine tract, NVP: nevirapine. Data represent mean +/- SD. Statistical significance was measured
308 by Student's t test, with Welch's correction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

309 **Figure 2**



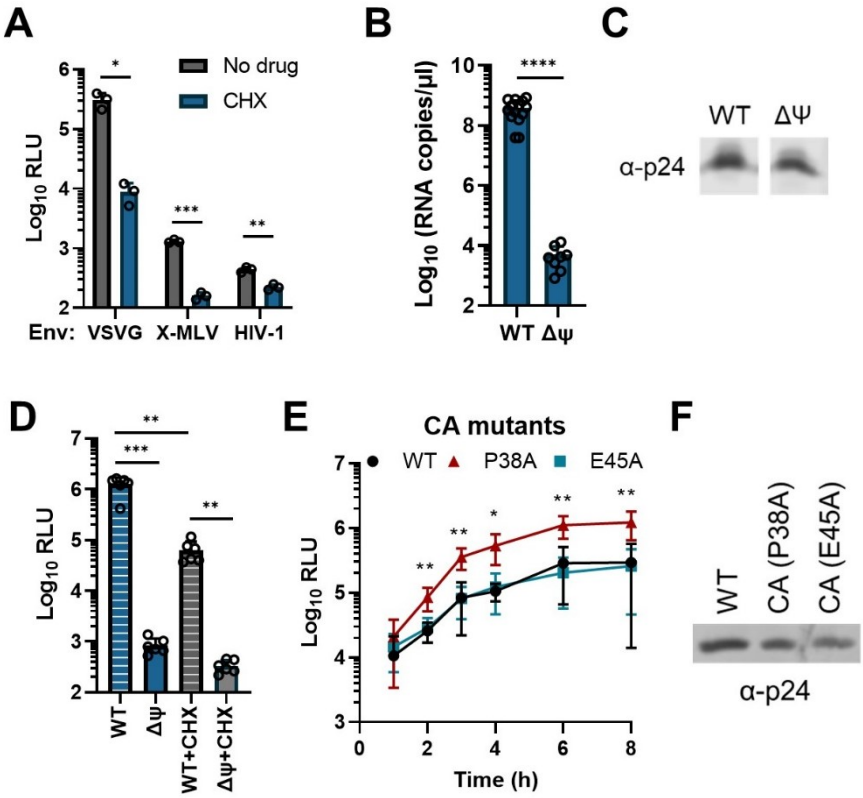
310 **Figure 2. Incoming minimal lentiviral RNA genomes are translated following entry into cells.**
311 (A) Schematic representation of the lentiviral constructs used. Nluc was cloned into a minimal
312 lentiviral vector downstream of the packaging signal (Ψ), with or without the destabilizing domain
313 (DD). Vector maps are not drawn to scale. 'X' denotes the mutated start codon of *gag*. (B) 293T cells
314 were transfected with the constructs in (A) in the presence or absence of Shield1 (S1; 1.5 µM) and
315 assayed for Nluc activity after 24 hours. (C) Cells were infected with VSV-G pseudotyped LV-Nluc virus
316 in the presence or absence of NVP and/or CHX. Luciferase activity was measured at the indicated
317 time points. (D) Infection was performed as in C, but with the DD-containing virus. (E) Different
318 amounts of plasmid DNA (0.1, 1 or 10 µg) were treated with the indicated nucleases for 30 mins or
319 left untreated and visualized on an agarose gel. (F) Equal amounts of virus stocks were treated with
320 benzonase, DNase or left untreated for 1 h prior to infection. Nluc activity was measured at 6 hpi. (G)
321 Cells were infected with VSV-G pseudotyped LV-DD-Nluc virus in the presence of both S1 and NVP,
322 with or without Actinomycin D (ActD) or CHX. Nluc activity was measured at 6 hpi. (H) Cells were
323 infected with lentiviruses produced in the presence or absence of a lentiviral packaging vector (WT
324 vs. ΔGag, respectively). Nluc activity was measured at 6 hpi. (I) 293T cells were infected with LV-Nluc
325 virus bearing VSV-G or no Env (ΔEnv) in the presence of NVP, with or without CHX treatment. Nluc
326 activity was measured at 6 hpi. (J) 293T cells were infected with LV-DD-Nluc virus that carries a
327 catalytic RT mutation (D110E) in the presence of Shield1, with or without CHX. Nluc activity was
328 measured at 6 hpi. (K) T-cell lines (Jurkat, C8166, PM1, A3.01), monocytic cell lines (THP-1, U937),
329 primary monocyte-derived macrophages (MDM) or CD4+ T-cells were infected with VSV-G
330 pseudotyped LV-DD-Nluc in the presence of NVP and Shield1, with or without CHX. Nluc activity was
331 measured at 6 hpi. Results are given as the normalized ratio of CHX-/CHX+ infections, which
332 represent de novo translated over incoming virion packaged reporter. NVP: 10 µM, Shield1 (S1): 1.5
333 µM, CHX: 10 µg/ml, ActD: 10 µg/ml. Data represent mean +/- SD. Statistical significance was
334 measured by Student's t test, with Welch's correction, and reflect the comparison of red and black
335 lines in panels C and D. hpi: hours post-infection. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns,
336 not significant.
337

338 **Figure 3**



339 **Figure 3. Incoming full-length HIV-1 genomes are translated to produce viral proteins in the**
340 **absence of productive infection.** (A) Schematic representation of the (near) full-length HIV-1
341 constructs used in the study. DD-Nluc was cloned into the NL43-Firefly construct (middle panel)
342 downstream of the packaging signal to generate NL43-DD-Nluc (top panel). A full-length IIIB clone of
343 HIV-1 carrying a catalytic mutation in RT was used for immunoprecipitation studies (bottom panel).
344 Vector maps are drawn to scale and 'X' denotes mutated codons. (B) 293T cells were infected with
345 NL43-DD-Nluc in the presence or absence of Shield1 (S1) and/or CHX. Nluc activity was measured at
346 the indicated time points. (C) Cells were infected with VSV-G-pseudotyped NL43-Firefly with or
347 without NVP or CHX treatment. Lysates were collected one day after infection, immunoprecipitated
348 with a polyclonal anti-HIV-1 Gag antibody (p17+24+55) and probed for p24. (D) Cells were infected
349 with VSV-G pseudotyped IIIB ΔRT with or without CHX. Lysates were run on an SDS-PAGE and probed
350 with anti-p24 and anti-tubulin antibodies. (E) Representative polysome fractionation profiles from
351 293T cells infected (or not) with VSV-G pseudotyped IIIB ΔRT, treated with CHX (50 µg/ml) at 4 hpi
352 and lysed in the presence or absence of EDTA (20 mM). Lysates were run on a sucrose density
353 gradient and fractions were collected from top to bottom while simultaneously measuring UV
354 absorbance. (F) RNA was isolated from each fraction by phenol extraction and the levels of HIV-1
355 genomic RNA and *HPRT1* were measured by RT-qPCR. The amount of each RNA is given as a
356 percentage of total RNA for that message. Unless indicated otherwise, the following concentrations
357 were used: NVP: 10 µM, Shield1 (S1): 1.5 µM, CHX: 10 µg/ml. Data represent mean +/- SD. Statistical
358 significance was determined by Student's t-test with Welch correction and reflect the comparison of
359 red and black lines in panel B. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

360 **Figure 4**



361 **Figure 4. Mutations in viral components can alter the translation of incoming HIV-1 genomes.**
362 (A) TZM-bl cells were infected with LV-DD-Nluc viruses pseudotyped with the indicated Env proteins
363 in the presence of Shield1 and NVP, with or without CHX treatment. Luciferase activity was measured
364 at 6 hpi. (B-C) Viral RNA content (B) and p24 content (C) of WT and ΔΨ LV-DD-Nluc virions were
365 quantified by RT-qPCR and western blot. (D) Cells were infected with of WT and ΔΨ LV-DD-Nluc
366 viruses, in the presence of NVP (10 µM) and S1 (1.5 µM), with or without CHX (10 µM). Nluc activity
367 was measured at 6 hpi. (E-F) Cells were infected with viruses carrying WT capsids or the CA stability
368 mutants P38A and E45A carrying the LV-DD-Nluc genome. Infection was performed with Shield1 and
369 NVP treatment, with or without CHX. Nluc activity was measured at the indicated time points.
370 Unless indicated otherwise, the following concentrations were used: NVP: 10 µM, Shield1 (S1): 1.5
371 µM, CHX: 10 µg/ml. Data represent mean +/- SD. Statistical significance was determined by Student's
372 t-test with Welch correction and reflect the comparison of red and black lines in panel E. $^{*}P < 0.05$,
373 $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, not significant.

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