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Short and long-range interactions in the HIV-1 5'UTR regulate genome dimerization and packaging

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12 **Abstract**

13 Genome dimerization is a conserved feature of retroviral replication and a critical step in the HIV-1
14 life cycle, but how it is regulated is incompletely understood. Here, we developed FARS-seq
15 (Functional Analysis of RNA Structure) to comprehensively identify sequences and structures
16 within the HIV-1 5'UTR influencing dimerization. We found nucleotides important for dimerization
17 throughout the HIV-1 5'UTR and identified distinct structural conformations in monomeric and
18 dimeric RNA. The dimer displayed TAR, PolyA, PBS, and SL1-SL3 as stem-loops. In the monomer,
19 SL1 was dramatically reconfigured into long- and short-range base-pairings with polyA and PBS,
20 respectively. The polyA-SL1 interaction disrupts the major packaging motifs, and the PBS-SL1
21 interaction functionally couples the primer binding site with dimerization and Pr55^{Gag} binding.
22 Altogether, our data provide insights into late stages of HIV-1 life cycle and a mechanistic
23 explanation for the link between RNA dimerization and packaging.

Introduction

HIV-1, like all retroviruses, packages two copies of its genome into viral particles. These genomes are non-covalently associated at an RNA motif called the dimerization initiation site (DIS). This association, known as dimerization, impacts multiple steps of the HIV-1 life cycle^{1,2}. Dimerization is assumed to be a prerequisite for genome packaging into virions, although the mechanistic relationship between dimerization and packaging is still under debate³⁻⁶. It also plays a role in genome integrity and evolution by bringing two genomes in close proximity for strand-switch recombination^{3,7-10}. Finally, it is linked to a structural switch that may regulate genome packaging and translation within cells^{5,11-16} (**Fig. 1a**). Despite its importance to HIV-1 biology, the RNA signals that regulate genome dimerization, and the molecular mechanism leading to the selection of the genome dimer from monomer for packaging are incompletely understood.

An extensive body of work maps the DIS to stem-loop 1 (SL1) of the HIV-1 5'UTR¹⁷⁻¹⁹ (**Fig. 1b**). SL1 contains a six-nucleotide long GC-rich palindromic sequence that initiates dimerization through an inter-molecular “kissing loop” interaction²⁰⁻²². Although SL1 is widely considered the primary dimerization motif, numerous studies indicate that genome dimerization is also modulated by sequences outside of SL1^{4,17,18,23-26}. For example, dimerization is promoted by a long-range base pairing between nucleotides overlapping the *gag* start codon (AUG) and the unique 5' element (U5)^{12,26,27} (**Fig. 1b**). Alternatively, it is inhibited when the region containing the AUG folds into a small hairpin, in turn freeing U5 to form a pseudoknot interaction with SL1^{12,28} (**Fig. 1b**). The U5-SL1 pseudoknot interaction was originally proposed as a liable interaction between the loop region of SL1 and U5, but a recent NMR study uncovered a more extensive base-pairing between U5 and SL1^{13,14,28}. Furthermore, intrinsic transcriptional start site heterogeneity, which produces transcript variants beginning with different counts of G residues (1G, 2G or 3G), has been shown to regulate dimerization by shifting the equilibrium between mutually exclusive structures containing either an U5-AUG, or a U5-SL1 interaction^{13,14,29}: 1G transcripts expose the DIS for dimerization and sequester the 5' cap, whereas 3G variants conceal the DIS whilst exposing the cap to enhance translation¹⁴. In addition to the U5-AUG and U5-SL1 conformations, over 20 structural models of

the HIV-1 genome have been proposed, suggesting that the 5'UTR may dynamically adopt multiple conformational states^{30,31}. It seems therefore likely that other structural forms of the HIV-1 genome exist to regulate genome dimerization, or other critical aspects of HIV-1 biology.

Sequences required for dimerization largely overlap with other conserved functional elements, such as those involved with genome packaging. Indeed, this genetic overlap between dimerization and packaging signals is a major reason why dimerization is considered to be a pre-requisite for packaging, even though the precise molecular mechanism underlying this phenomenon is unclear. In this study, we disentangled genome dimerization from other steps of the viral life-cycle and comprehensively mapped structure determinants of HIV-1 genome dimerization using a novel high-throughput approach that we call Functional Analysis of RNA Structure (FARS-seq). Using FARS-seq, we found nucleotides throughout the HIV-1 5'UTR influencing dimerization and identified distinct structural conformations in monomeric and dimeric RNA. The dimeric RNA folded into a 'canonical' structure of the 5'UTR that displayed TAR, PolyA, PBS, and SL1-SL3 as stem-loops, and contained a long range U5-AUG interaction. In monomeric RNA, SL1 formed interactions with polyA and PBS. The PBS-SL1 interaction functionally couples primer binding with dimerization and the polyA-SL1 long-range interaction disrupts the major packaging motifs for Pr55Gag³²⁻³⁴. All in all, our data provide a mechanistic explanation for how RNA dimerization can be a prerequisite for packaging.

Results

Functional and structural analysis of RNA dimerization. HIV-1 genome dimerization largely depends on the stem of SL1 and its GC-rich palindromic loop sequence. Nevertheless, evidence suggests that RNA sequences and structures outside of SL1 also play a role^{6,14,23,31,35-37}. We therefore devised a strategy to exhaustively survey the 5'UTR for nucleotides influencing dimerization whilst at the same time generating information about RNA structure. We call this approach the Functional Analysis of RNA Structure (FARS-seq) (**Fig. 1c**). Fundamentally, FARS-seq uses mutational interference to generate complete, unbiased, quantitative profiles of RNA function at single nucleotide resolution^{38,39} (**Fig. 1d**). These functional profiles are generated by

physical separation of mutant RNA populations according to functionality followed by next generation sequencing and the analysis of mutation frequencies in the 'functional' and 'non-functional' populations. Simultaneously, structural profiles are obtained by treating the fractions with dimethyl sulfate (DMS), which is a chemical widely used for probing RNA structure^{40,41}. DMS reacts with unpaired adenosine and cytosine bases to form adducts that can be read out as mutations on next generation sequencing machines^{42–44}. Normally, DMS only provides information on whether a nucleotide is base paired, and not the identity of the base pairing partner. However, when DMS modification is performed on mutational libraries it enables the direct detection of RNA stems (**Fig. 1f**)^{45,46}. That is, when a mutation in the library occurs within a stem, it creates an unpaired nucleotide at the position facing the mutation. This newly unpaired residue becomes more accessible for DMS modification leading to correlated mutations in the sequencing data. Thus, FARS-seq combines two different mutational read outs to experimentally couple RNA structural and functional information.

To physically separate mutants according to their effects on dimerization, we took advantage of the observation that RNA transcripts containing dimerization signals are able to spontaneously associate *in vitro*, producing a dimeric RNA species that can be physically separated from the monomeric species on native agarose gels (**Fig. 2a**). Similar gel-based assays have been instrumental in the discovery of dimerization motifs in HIV-1^{17–19} and other viruses^{47–49}. This setup also disentangles the effect of RNA structure on dimerization from other factors, such as the binding of protein or other co-factors. To assess the effect of transcription start site heterogeneity on the dimerization properties of the HIV-1 genome we tested three transcript variants beginning with 1G, 2G or 3G^{13,50} (**Fig. 2a**). For each of these transcript variants, we also tested whether capping affected dimerization and assessed their dimerization properties under low salt and high salt buffers favoring monomerization and dimerization, respectively (**Fig. 2a**). After physical separation on a native gel, bands corresponding to monomeric and dimeric RNA populations were excised and either left untreated or soaked in DMS. For the DMS sample (and its control), RNA was reverse transcribed in the presence of Mn²⁺ to allow mutagenic bypass of the modified nucleotides by the reverse transcriptase^{44,51}. In the absence of DMS, mutation frequencies in the mutated and non-

mutated control library were 5.4×10^{-3} and 3.7×10^{-4} , respectively, and the mutational interference libraries with a signal to noise $D_m(i) > 2$ (**Sup. Fig. 1**; details in **Sup. Methods**). In the DMS treated samples, we saw an additional increase in mutation frequencies at the expected A and C residues indicating a successful modification of RNA (3.4- and 7.8-fold increase at C and A, respectively, **Sup. Fig. 2**).

RNA dimerization depends not only on SL1, but also on other regions within the HIV 5'UTR. We first asked which regions of the RNA were required for dimerization using mutational interference mapping (MIME) to calculate K^{dimer} values for each nucleotide position. This metric is related to the ratio of mutation frequencies in the monomer vs dimer RNA. For computational analysis however, these ratios are corrected for errors introduced during library preparation and sequencing (mechanistic derivation in **Sup. Methods**). Thus, K^{dimer} is a quantitative measure of the relative effect of each mutation on dimerization. Across all samples and conditions, median $\log_2(K^{\text{dimer}})$ values were heavily skewed towards positive values indicating that most mutations inhibited, rather than enhanced, dimerization indicating that the HIV-1 genome is highly optimized to dimerize as a key part of its life cycle (**Fig. 2b**). By segregating K^{dimer} values by structural domain we found that **most** dimerization inhibiting mutations mapped to SL1 (**Fig. 2b**). Although less prominent than SL1, many other domains exhibited skewed distributions. Mutations to SL3, SL4, and polyA were biased towards inhibiting dimerization whereas mutations to TAR and SL2 preferentially enhanced dimerization. In contrast, mutations to the inter-domain regions were largely neutral with a narrow distribution centered around zero (**Fig. 2b**).

We next plotted median $\log_2(K^{\text{dimer}})$ values at each nucleotide position for capped and uncapped transcript variants measured under the two buffer conditions (**Fig. 2c, 2d**; **Sup. Fig. 3**). All conditions exhibited a very large peak that localized to SL1, as well as a smaller double peak mapping to SL3 (**Fig. 2c, 2d**). In high salt buffer most mutations inhibited dimerization, whereas under low salt conditions it was possible to distinguish additional dimerization enhancing or inhibiting regions (**Fig. 2c, 2d**). Notably, sequences surrounding the AUG start codon and mapping to U5 were both required for dimerization in low salt buffer, suggestive of a functionally important U5-AUG interaction (**Fig. 2d**). A double peak also emerged within the region 122-141 in low salt

buffer (**Fig. 2d**). This region contains the primer activation sequence (PAS) which hints that structural changes in the PBS domain may regulate RNA dimerization^{52,53}. Conversely, we found regions within TAR, polyA, PBS and SL2 that enhanced dimerization upon mutation (**Fig. 2d**). The strongest of these regions mapped to the 3' end of PBS and SL2. Taken together, these data reinforce the key importance of SL1 for genome dimerization, but also reveal sequences outside of SL1 participate in the dimerization process.

1G and 3G RNAs have different dimerization properties. Because the HIV-1 transcription start site has been reported to alter the structure of the HIV-1 5'UTR, we next tested which RNA sequences were important for dimerization within the 1G, 2G, and 3G uncapped variants (**Fig. 2e-f, Sup. Fig. 4**). We did this by plotting the absolute difference between the median $\log_2(K^{\text{dimer}})$ values of each variant to the mean values of the three transcripts. In high salt buffer, most positions were unchanged in the 1G, 2G and 3G variants (less than $\Delta 0.25 \log_2(K^{\text{dimer}})$ variant – mean) (**Sup. Fig. 4, Fig. 2e**). The only exception were nucleotides mapping to the SL1, which were functionally more important in the 3G variant, and less important in the 1G variant. Upon performing a similar analysis for the low salt condition, distinct functional profiles for the 1G and 3G transcript variants emerged, with divergence across regions compared to the mean of the three transcripts (**Sup. Fig 4, Fig. 2f**). The 3G variant had increased dependence on a region spanning the U5 and PAS (nts 105-117 and nts 125-131) and sequences surrounding the AUG start site (nts 335-344). Increased dependencies of smaller magnitudes were also observed in the tRNA primer binding site (nts 182-200), the anti-PAS (nts 217-223), regions flanking SL1 such as the CU rich motif (nts 228-247), a region in SL2 (nts 299-300), and a G rich region downstream of the AUG start codon (nts 360-366). We note that the regions in TAR, PBS, and SL2 that enhanced dimerization upon mutation in low salt conditions behaved identically in 1G, 2G, and 3G variants, meaning that they impact dimerization in a way that is unrelated to transcription start site selection. We also remarked that the 1G and 2G transcripts variants behaved similar in both buffer conditions (**Sup. Fig. 4**), with a reduced dependency on regions external to SL1 for dimerization. Our interpretation is that the 1G and 2G transcripts readily fold into a dimer promoting conformation, whereas the 3G variant has a reduced capacity to dimerize. Capped and uncapped transcripts had near identical functional

profiles (**Sup. Fig. 3**). The only region that differed mapped to polyA, providing indirect evidence of a functional interaction between the 5' cap structure and polyA (**Sup. Fig. 4**).

Distinct structural signals in monomeric and dimeric RNA. So far, the analysis of the functional profiles demonstrate that sequences involved in genome dimerization map to distinct regions of the HIV-1 5'UTR. These sequences may fold into RNA structures that are necessary for genome dimerization itself, or indirectly regulate genome dimerization by altering folding pathways. We therefore next determined RNA structural motifs present in monomers and dimers by analyzing the DMS reactivities of the FAR-seq data.

As before, we analyzed capped and uncapped 1G, 2G, 3G transcript variants in both monomer and dimer buffers. Correlations between DMS reactivities at each position amongst all conditions were very high (**Fig. 3a**; Kendall rank correlation coefficients, mean 0.84, min 0.70, max 1.0) suggesting that significant portion of the 5'UTR was folded into a similar conformation under all conditions. Nevertheless, hierarchical clustering of the DMS reactivities revealed a clear structural distinction between monomer and dimer, as well as between the 1G/2G and 3G transcript variants (**Fig. 3a**). In contrast to the functional profiling, where buffer conditions had a very large effect on the functional profiles, structural information obtained under both conditions were highly correlated (correlation coefficients; low salt 0.84, high salt 0.85), as were uncapped and capped RNAs (correlation coefficients; capped 0.85, uncapped 0.84). The first branchpoint separated 1G/2G dimer structures from the 1G/2G monomer and 3G structures. Subsequent branching grouped 1G/2G monomer structures away from the 3G structures. Finally, 3G structures separated into monomer and dimer subclusters. These four structural groupings were also supported by principal component analysis (PCA) of DMS reactivities, which separated monomer from dimer, and 3G variants from 1G/2G variants (**Fig. 3b**). Guided by the PCA and hierarchical clustering, we pooled DMS reactivity data into 4 structural groups: 3G dimer, 3G monomer, 1G/2G monomer, 1G/2G dimer. Interestingly, across all samples, variance in DMS reactivities localized mainly to polyA and SL1 (**Fig. 3c**). To further explore this, we used a statistical approach to compare DMS reactivities in the 1G/2G dimer cluster with the 3G monomer cluster as these were the most structurally divergent samples (correlation coefficient 0.740) (**Fig. 3d**). Between these clusters, we found

statistically significant changes in reactivity that again remained localized to polyA and SL1 (**Fig. 3d and Sup. Files**).

To obtain information on RNA secondary structure differences between these structural classes we used pooled DMS reactivities as soft constraints to guide *in silico* RNA folding^{54,55} (**Fig. 4, Sup. Fig 5 and Sup. Fig 6**). For the 1G/2G dimer class we obtained an RNA structure that closely resembled the ‘canonical’ HIV-1 5’UTR (**Fig. 4a and Sup. Fig 6**). This structure contains the TAR, PolyA, PBS, SL1 and SL3 stem loops, as well as the AUG-U5 interaction. The basal portion of SL1 folded into an extended form containing unpaired purines that are important for genome packaging^{32,56}. SL2, which can fold into alternative stem loop structures, folded as an imperfect stem-loop that exposes part of the U1snRNA binding site within the loop, and SL3 folded into its canonical short stem loop structure. We then assessed the robustness of this prediction by computing Shannon entropies of base pairing probabilities at each position in the 5’UTR (**Fig. 4b, Sup. Fig. 5**). Low entropy values throughout the 5’UTR indicated high confidence in the prediction and a well-ordered structure with only some ambiguity in the base pairing at the basal portion of SL1. This was confirmed by dot plots of base pairing probabilities **and a bootstrapping analysis** showing high confidence stem loop structures for the TAR, PolyA, PBS, SL1 and SL3 stem loops, as well as the AUG/U5 interaction (**Fig. 4c and Sup. Fig. 5**).

We next analyzed the structure of the 3G monomer sample, finding that it was dramatically reorganized (**Fig. 4d**). The most striking changes were seen in the polyA, AUG-U5, and SL1. PolyA and SL1 no longer folded into their canonical stem-loops. Instead, these stem loops were reorganized into a long-distance interaction, with the GCGCGC palindromic loop of SL1 base pairing with the apical portion of the polyA stem. The AUG-U5 interaction was also no longer present; U5 now base paired with the 5’ stem of SL1, and the AUG containing region fold into a stem loop structure also referred to as SL4. Finally, we observed a new long-distance interaction between polyA and a region within the Gag coding sequence (nts 358-367). The SL1-PolyA reorganization was well supported by the DMS reactivity changes (**Fig. 4 and Sup. Fig. 6**). In particular, the unpaired adenosine 263A in the SL1 loop, which was highly reactive in the dimer structure, became unreactive in the monomer due to base pairing with U87. Similarly, nucleotides

C84 and C85 in polyA, which were reactive in the dimer structure, became unreactive in the monomer due to base pairing with 265G and 266G in the SL1 stem. Finally, A89 in the stem of polyA, which was unreactive in the dimer structure, became unpaired in monomer structure and reactive to DMS. Shannon entropies, base pairing and bootstrapping probabilities at the predicted PolyA-SL1 interaction indicated some uncertainty in the prediction, especially within U5 and the 5' portion of SL1 (Fig. 4f and Sup. Fig 5). Remarkably, despite the dramatic reorganization of PolyA and SL1, a large proportion of the 5'UTR folded identically in 1G/2G dimer and 3G monomeric populations, with PBS, SL2 and SL3 unchanged. Interestingly, TAR was present in all predictions, but in the 3G monomer the first nucleotides in the base of TAR became single stranded, and potentially more available for the translation machinery.

As expected, the 3G dimer population folded into the canonical 5'UTR structure containing SL1 as a stem-loop and the 1G/2G monomer population folded into the alternative polyA-SL1 containing structure (Sup. Fig. 5). However, these two structural classes showed increased Shannon entropies in polyA, U5, SL1 and the Gag coding sequence when compared to the 1/2G dimer and 3G monomer structures. Thus, 3G dimer and 1/2G monomer populations are structurally less uniform, even though we selected for pure dimer and monomer structures in the native gels. The most likely explanation is that these structures partially return to equilibrium after isolation, probably during the probing reaction at 37°C.

Altogether, these data support a novel structural rearrangement of the HIV 5'UTR leading to extensive base pairing between SL1 and the polyA-U5 region. This monomeric rearrangement appears to be favored in the 3G populations, whereas the 1G/2G population tend towards the dimer structure.

Two-dimensional RNA structural probing detects structural differences in monomer and dimer populations. The incorporation of information from RNA structural probing experiments improves the accuracy of RNA structure predictions, but structural elements can still be incorrectly predicted because data from chemical probing experiments typically provide information on whether a nucleotide is base-pair or not, but not its base pairing partner^{57,58}. FARS-seq enables a

more powerful model-free approach to RNA structure determination by exploiting information in the mutation library to identify RNA helices directly (**Fig. 5a**). When mutating a nucleotide in a stem structure, the base pairing partner, now unpaired, becomes more reactive to the chemical probe leading to correlated mutations in the sequencing data^{46,58}. This two-dimensional data can directly detect RNA helices (along the diagonal) as well as non-canonical and tertiary interactions that are otherwise impossible to predict from classical one-dimensional RNA structural probing experiments.

Signals for RNA helices were visible in the raw mutational and z-score normalized data along the diagonals (**Fig. 5b, 5e** and **Sup. Files**). These signals were refined by applying convolution and threshold filters to enhance stems as well as tertiary interactions (**Fig. 5b** and **5e**). Finally, high confidence stems were highlighted by applying a helix filter and algorithm to select the 'best' non-conflicting stems with the highest score (**Fig. 5c** and **5f**). Stem signals corresponding to SL1 were systematically present in dimer selected samples and absent in monomer selected samples (**Sup. Fig 8** and **Sup. Files**). In the 1G/2G dimer sample, both SL1 and polyA stem signals were observed. In the 3G monomer, polyA and SL1 stems were replaced with a signal matching the long distance SL1-polyA interaction (compare **Fig. 5b** and **5c** with **5e** and **5f**). Unexpectedly, in the 3G monomer we detected an additional novel interaction between the PBS loop and SL1, as well as a weaker signal between TAR and PBS, both of which were supported by a bootstrapping analysis (**Fig. 4e, 4f** and **Sup. Fig. 8**). In the previous structural prediction these regions in PBS and SL1 had high Shannon entropies and were poorly resolved (**Fig. 4**).

Intriguingly, and uniquely in the 1G/2G structures, the TAR and polyA stem signals in the filtered z-scores were accompanied by punctate signals characteristic of tertiary contacts, alternative folds or non-canonical base-pairings (**Sup. Fig. 6**). Because these contacts were consistently present in the 1G/2G samples and missing from the 3G samples, we speculate that they help to stabilize the 5' end of the HIV-1 transcript to inhibit the translation of 1G/2G transcripts (**Sup. Fig. 7** and **Sup. Files**)¹³. Additionally, in the 1G/2G monomer, the mutually exclusive polyA stem and the polyA-SL1 interaction were both observed, strengthening the idea that 1G/2G samples are preferentially dimeric and that some interconversion occurs even when monomers are isolated (**Sup. Fig. 7**).

273 To obtain enhanced structural models of the dimer and monomer structures we focused on the
274 1G/2G dimer and 3G monomer samples as these were the most structurally uniform. Here, the
275 best stems obtained by multidimensional chemical probing were used as additional hard
276 constraints in RNA structure prediction (**Fig. 5d** and **5g**). The enhanced 1G/2G dimer structure was
277 nearly identical to that obtained without hard constraints, and contained the TAR, PolyA, PBS, SL1
278 and SL3 stem loops, as well as the AUG/U5 interaction as previously predicted (**Fig. 5d**). The
279 enhanced 3G monomer structure contained TAR, polyA-SL1 interaction, SL2, SL3 SL4 and polyA-
280 Gag interaction, as before, but now included a stem loop structure due to base pairing between
281 PBS and SL1 (**Fig. 5g**). A TAR-PBS pseudoknot interaction was added post-hoc, as it was selected
282 by the best stem algorithm and supported by a bootstrapping analysis, although we note that the
283 2d stem score was relatively weak. All in all, multidimensional chemical probing not only provided
284 direct experimental evidence that 1G/2G dimer and 3G monomer fractions are structurally distinct,
285 but also identified structural features that couldn't be predicted by classical RNA structural probing
286 experiments.

287 **SL1 stability is a key element for genome dimerization.** One of the strengths of FARS-seq
288 is the coupling of RNA structural and functional information at single nucleotide resolution. We
289 therefore mapped the K^{dimer} values onto the dimer and monomer structures. In both buffers, the
290 median mutations with the strongest effects mapped to the apical portion of SL1, with mutations to
291 the palindromic loop sequence revealed to be the most destabilizing for dimerization, in agreement
292 with their crucial role in the kissing loop interaction (**Fig. 6a**, see **Extended K^{dimer} Data Tables**).
293 The unpaired adenosine residues flanking the loop sequence were less important for dimerization
294 than the palindromic sequences, in keeping with the observation that they can be individually
295 mutated without disrupting dimerization⁵⁹. Mutations to the stem of SL1 also strongly inhibited
296 dimerization, with apical stem mutations generally having a stronger effect on dimerization
297 compared to the basal stem mutants [$\log_2(K^{\text{dimer}})$ values 0.61-6.85 vs 0.31-3.49] (**Sup. Fig. 7**).
298 Surprisingly, mutations at several positions within the SL1 internal loop (G247, A271, G272, G273)
299 strongly enhanced dimerization upon mutation (**Fig. 6a** and **6b**, **Sup. Fig. 7**, **Extended K^{dimer} Data**
300 **Tables**). Dimer enhancing mutations at these positions presumably stabilize SL1 by closing or

reducing the size of the internal loop, strongly indicating that SL1 stability is a critical parameter for dimerization.

Whilst two-dimensional structural probing identified SL1 as a short stem loop with an apical and basal stem separated by an internal loop (nucleotides 243-277), our data nevertheless reveals structural plasticity in SL1. This realization comes from mapping the functional data to different extended forms of SL1 that have been proposed in the literature: a two-internal loop model (2IL), a three-internal loop model (3IL), and three-way junction (3WJ) model (**Fig. 6b**). Even though these models have mutually exclusive internal loop configurations, mutations that closed or reduced the size of SL1 internal loops were invariably dimerization enhancing (**Fig. 6b**, green arrows). For example, A235C, A235U or G281U strongly enhanced dimerization by converting the A235-G281 internal loop into a base pair in the three-way junction (3WJ) model, even though these mutations would have no effect on SL1 stability on the other structural models (**Fig. 6b**, green arrows). Similarly, G282C and G239C would close the internal loop in the 3 internal loop (3IL) model explaining their dimerization enhancing properties (**Fig. 6b**, green arrows). To confirm the structural plasticity of SL1, we performed in solution DMS-MaPseq analysis of mutants A235C and A239C and showed that they reconfigured the SL1 stem, as predicted (**Sup. Fig. 9**). Interestingly, mutations A242C or A242U reduced the size of an SL1 internal loop in all models but nevertheless disrupted dimerization (**Fig. 6b** and **6c**; red arrows). These functional effects are explained by the fact that A242C or A242U extend the PBS-SL1 interaction to stabilize the monomer structure. Thus, the core dimerization structure in SL1 comprises an apical 7 nt stem and a basal 4 nt stem separated by an internal loop that can be further stabilized by metastable stem extensions or disrupted by a base-pairing interaction with PBS.

Inter-domain interactions regulate dimerization. Outside of SL1, we found several structural domains and inter-domain interactions that affected dimerization (**Fig. 6**). Our data support a role for the AUG-U5 interaction in positively regulating dimerization, as conversion of GU base pairs at U107-G342, G108-U341, G112-U337 to either AU or GC base pairs consistently enhanced dimerization, whereas mutations disrupting the interaction were inhibitory (**Fig. 6a**). SL3 stem mutations weakly inhibited dimerization, most likely because disruption of SL3 would induce

misfolding of the RNA (**Fig. 6a**). Finally, mutations to SL2 were generally dimerization enhancing, and these types of mutations were especially evident in the 3' SL2 stem (**Fig. 6a**).

We also validated the novel short- and long-range interactions between polyA-SL1 and PBS-SL1. Mutations to the base of polyA generally inhibited dimerization, indicating that destabilizing the polyA stem favours the formation of the polyA-SL1 interaction (**Fig. 6a**). On the other hand, mutations to the upper portion of polyA enhanced dimerization by disrupting the polyA-SL1 base pairing (**Fig. 6c**). In the same vein, we found stretches of nucleotides in PBS that strongly enhanced dimerization upon mutation (**Fig. 6c**). Functional profiles in the lower PBS stem were particularly interesting as this stem structure is universally found in contemporary models of the HIV-1 5'UTR and contains the primer activation sequence (PAS) known to be important for efficient reverse transcription⁶⁰. We found that mutation of two nucleotides G217 and C218 in the lower PBS stem very strongly enhanced dimerization, even though mutations to this stem were generally inhibitory (**Fig. 6a**). This can be mechanistically explained because mutation of these nucleotides disrupted a novel base-pairing between PBS and SL1 that stabilizes the monomer structure.

Because these results suggested a functional interaction between primer tRNA binding and dimerization, we next assessed whether disruption of the PBS with tRNA mimic oligos affected dimerization. cPBS₁₈₂₋₁₉₉ annealed to the loop region disrupted the putative TAR-PBS interaction, whereas cPBS₁₉₉₋₂₁₆ disrupted the novel PBS-SL1 stem loop (**Fig. 7a**). Both oligos enhanced dimerization confirming a functional interaction between PBS and dimerization. Surprisingly, annealing the cPBS₁₈₂₋₁₉₉ oligo also led to the formation of a higher, **presumably** tetrameric molecular species. The TAR apical loop contains a 10-nucleotide palindromic sequence that has been proposed to dimerize by a TAR–TAR kissing interaction analogous to the one used by SL1²⁶. We therefore postulate that cPBS₁₈₂₋₁₉₉ disrupts the TAR-PBS interaction detected by multi-dimensional structural probing, allowing TAR to dimerize independently of SL1.

Finally, since genome dimerization is thought to be a pre-requisite for **genome packaging**, we **selected mutations in adjacent nucleotides with divergent effects on dimerization and measured their effects on Pr55^{Gag} binding by microscale thermophoresis (MST) (Fig. 7b)**. Importantly, none

of these mutations resided in the HIV-1 packaging domain (SL1-SL3). In PBS, C218G, which strongly enhanced dimerization had higher affinity (K_d 19 nM) to Pr55^{Gag} compared with WT RNA (K_d 38 nM). In contrast, PBS A220G-G221A, which was unable to dimerize, did not bind Pr55^{Gag} at any of the concentrations tested (K_d n.a.). In polyA, dimerization enhancing mutation C84A-C85C bound Pr55^{Gag} with higher affinity (17 nM) than WT, whereas dimerization disrupting mutation U86G-A89C bound Pr55^{Gag} with lower affinity than WT (110 nM). In solution DMS-MaPseq analysis of these four mutants validated their effects on the polyA-SL1 and PBS-SL1 interactions (Sup. Fig 10). We also introduced these mutations into the full-length HIV-1 genome and assessed their effects on packaging efficiency in competition assays (Fig. 7c). In PBS, dimer promoting mutant C218G was enriched 1.5-fold in virions compared to the monomer promoting mutant A220G-G221A. In polyA, dimer promoting mutant C84A-C85A was enriched 2-fold in virions compared to the monomer promoting mutant U86G-A89C. In a five-way competition assay between wild-type (WT) HIV-1 and the mutants, dimer promoting mutants C218G and C84A-C85A were packaged equivalently or better than WT. Conversely, monomer promoting mutants U86G-A89C and A220G-G221A were deficient in packaging compared to WT. In cell DMS-MaPseq analysis of these four mutants again validated their effects on the polyA-SL1 and PBS-SL1 interactions (Sup. Fig 11). Together, these results provide a clear mechanistic explanation for the link between dimerization, Pr55^{Gag} binding and packaging. Furthermore, we show how changes to the PBS functionally link the tRNA binding region to packaging (Fig. 7d).

Discussion

Accumulating evidence emphasizes dimerization as a key step in HIV-1 life cycle that is regulated, at least in part, through the folding of the HIV-1 genomic RNA^{5,11,62,12–16,27,28,61}. Here, we resolved the structure of the monomeric and dimeric RNAs using a novel approach that integrates information from RNA structural probing with high-throughput functional profiling. This experimental strategy has significant advantages over other chemical probing methods that make ensemble measurements over all possible conformations of the RNA in solution. Such ensemble measurements, unless cautiously interpreted, can lead to false predictions when mapped to a

single structure. We overcome this problem by physically isolating RNA structural conformations with respect to their function, akin to in gel SHAPE which was first developed to resolve structural differences between monomeric and dimeric species of the HIV-1 5'UTR²⁸. Moreover, by performing chemical probing on mutagenic libraries we obtain model-free information on RNA helices in the same way as “mutate and map”⁵⁸ or “M2-seq”⁴⁶. Finally, and most importantly, our approach enables a deep understanding of how RNA structures relate to RNA function by uniquely coupling structural information with a functional read-out.

Taken together, our data recognizes a core dimerization domain of SL1 comprised of a 7 bp apical stem and 4 bp basal stem separated by an internal loop. This core dimerization domain is present in most structural models of SL1, but there is significant disagreement on whether SL1 is further extended^{63–66}. In some structures, extensions to SL1 even lead to the complete disruption of SL2¹². Here, we found no direct evidence that SL1 is in an extended form in dimeric RNA and consistently observe signals for SL2 as a short imperfect stem containing a bulged adenosine. Nevertheless, functional profiling provides strong evidence that mutually exclusive extended forms of SL1 can be readily generated, either directly through stabilizing mutations, or indirectly by destabilizing SL2. The fact that single point mutations could have such dramatic effects on dimerization provides evidence that the 5'UTR is dynamic and metastable. In the context of viral infection this is noteworthy because it provides a mechanism to regulate dimerization through the binding of viral or cellular factors to the genome (**Fig. 7c**).

The metastable nature of SL1 was strikingly revealed in monomeric RNA. In contrast to SL3, which was present in both monomer and dimer structures, SL1 was destructured in monomeric RNA. Instead of a stem-loop, SL1 was reorganized into a short-range interaction with PBS and a long-distance interaction with polyA. These results are in agreement with the prevalent idea that RNA conformational switches regulate HIV-1 replication^{33,67}. The dimer and monomer structural conformations we present here are reminiscent of the branched multiple hairpin (BMH) and long-distance interaction (LDI) models that were proposed as alternative structures that would regulate the dimerization, packaging, splicing and translation of the HIV-1 genome^{5,15,16,27}. The BMH exposes the TAR, polyA, PBS, SL1, SL2 and SL3 structures, and contains the U5-AUG interaction.

411 The LDI model includes the interaction between polyA and SL1, but also includes additional
412 rearrangements that we did not observe, such as an extension of SL3 and a disruption of SL2.
413 Moreover, the LDI model does not include the novel PBS-SL1 interaction. Nevertheless, certain
414 mutants designed to alter the LDI/BHM equilibrium are directly applicable to our structural model.
415 In particular, mutations destabilizing the polyA stem inhibit dimerization and packaging^{15,27},
416 whereas mutations disrupting the polyA-SL1 interaction enhanced dimerization¹⁶. These data are
417 in agreement with our results showing that polyA-SL1 regulates not only dimerization, but also
418 genome packaging. Significantly, recent work has identified the primary Pr55^{Gag} binding site for
419 HIV-1 as SL1^{32,38,39,68} with polyA providing an additional packaging signal in cells^{39,69}. The fact that
420 SL1 and polyA are completely disrupted in the monomer population provides a mechanistic
421 explanation for the long-postulated link between dimerization and packaging.

422 Recently the structure of the 3G capped transcript was solved by NMR revealing the disruption of
423 the polyA stem in 3G transcripts and the formation of a long range interaction between SL1 and
424 U5¹⁴. Thus, our results agree that 3G transcripts are preferentially monomeric, yet disagree with
425 precise structural details, in particular the base pairing partner of SL1. One way to reconcile these
426 data is that the NMR structure was obtained with the Mal isolate, in contrast to the NL43 isolate
427 used in the present study. Mal is a complex recombinant containing a 23-nucleotide duplication in
428 the PBS, strikingly in the same region regulating dimerization in this study. Moreover, this PBS
429 duplication in Mal leads to marked structural differences in the initiation of reverse transcription
430 compared to the prototypic subtype B strain NL43^{52,53}. It is therefore plausible that Mal and NL43
431 isolates use related, yet distinct, structural rearrangements to regulate dimerization. Nonetheless,
432 both the polyA-SL1 and PBS-SL1 interactions are conserved amongst 800 curated sequences in
433 the Los Alamos HIV-1 sequence database indicating regulation of dimerization by polyA and PBS
434 is widespread (Sup. Files).

435 Finally, we identified a novel interaction between PBS and SL1 that acts as negative regulator of
436 dimerization, Pr55^{Gag} binding and packaging. We demonstrated that this negative regulation can
437 be counteracted through the binding of oligos to the primer binding site. Disruption of this negative
438 regulation would mechanistically explain why tRNA annealing enhances dimerization^{31,70}, and also

opens up the possibility that primer binding to the PBS affect other steps of the HIV-1 life-cycle, such as translation, by altering the monomer / dimer equilibrium. It also reveals a general principle by which RNA structural changes induced by host factors can regulate key stages of the HIV-1 life cycle (**Fig. 7d**).

Methods

Plasmid. NL43 sequences were obtained from pDRNL43 ΔEnv plasmid, which is containing full-length NL43 but without flanking cellular sequences⁷¹ and contains a deletion in Env for biosafety.

Protein expression and purification. Expression, purification and characterization of NL4.3 Pr55^{Gag} with an appended C-terminal His₆-tag was performed as described by McKinstry *et al.*⁷²

Mutant library preparation. DNA templates were prepared by PCR using Taq DNA polymerase (NEB) with RNA expression plasmid pDRNL43- ΔEnv and forward primers containing T7 RNA polymerase promoter and 3G/2G/1G at 5'end

AAAGaagacTTggggTAATACGACTCACTATAGGGTCTCTCTGGTTAGACCAG /

AAAGaagacTTggggTAATACGACTCACTATAGGTCTCTCTGGTTAGACCAG /

AAAGaagacTTggggTAATACGACTCACTATAGTCTCTCTGGTTAGACCAG and reverse primer mGmATCTAAGTTCTTCTGATCCTGTCTG. PCR amplifications were performed in 1X reaction buffer, 0.2 mM dNTPs, 250 nM forward primer and reverse primer, 1 ng plasmid as template, 1.25 U Taq DNA polymerase (NEB) using the PCR cycling conditions: 98°C for 30 s, followed by 32 cycles of 98°C for 10 s, 60°C for 30s, and 68°C for 1 min. Products were visualized by electrophoresis on 1% agarose gels in 1X TAE buffer and column purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The purified PCR products were used as template for error prone PCR using the Mutazyme II DNA polymerase (Agilent) and forward primer TAATACGACTCACTATA and reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATCTAAGTTCTTCTGATCCTGTCTG.

The PCR reaction volume was 50 µL and consisted of 2 ng of template DNA, 1X buffer, 200 µM dNTPs, 0.25 mM of each primer, 2.5 U of Mutazyme II DNA polymerase. PCR cycling conditions

were 95 °C for 2 min followed by 35 cycles of 95°C for 30 s, 35-42°C for 30 s and 72°C for 1 min. Products were visualized by electrophoresis on 1% agarose gels in 1X TAE buffer. A final column purification was carried out with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) kit.

RNA preparation. Purified WT and mutated PCR products (900 ng) were used as templates for RNA *in vitro* transcription with a homemade T7 RNA polymerase. Reaction contained 1X reaction buffer (40 mM Tris pH 7.5, 18 mM MgCl₂, 10 mM DDT, 1 mM Spermidine), 5 mM NTPs, 40 U RNasin (Molox), 900 ng DNA template, and 0.05 U of Pyrophosphatase, (NEB) and 5 µl of homemade T7 RNA polymerase. The reaction was incubated at 37°C for 3 h, followed by DNase I treatment for 30 min at 37 °C. RNA was gel purified after electrophoresis on 1% agarose gels in 1X TAE buffer using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer (Macherey-Nagel). Half of the purified RNA was capped with Vaccinia Capping System (NEB). Briefly, 10 µg of RNA was mixed with nuclease-free H₂O in a 1.5 ml microfuge tube to a final volume of 15 µl. The sample was heated at 65°C for 5 min, then placed on ice for 5 min. 2 µl of 10x capping buffer, 1 µl 10 mM GTP, 1 µl 2 mM SAM, and 1 µl of Vaccinia Capping Enzyme were added and the sample was incubated at 37°C for 30 min. Capped RNA was column purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer (Macherey-Nagel).

Native agarose gel electrophoresis. RNA (600 ng) was denatured at 90°C for 2 min followed by chilling on ice for 2 min. RNA was incubated at 37°C for overnight (15-17 h) in high salt buffer (10 mM KH₂PO₄, pH 7.4, 1 mM MgCl₂, 122 mM KCl) or low salt buffer (10 mM NaCl, 10 mM Tris, pH 7.4). Samples were loaded with native loading dye (0.17% Bromophenol Blue and 40% (vol/vol) sucrose) on 1% agarose gel prepared with 1X Tris-Borate Magnesium (TBM) buffer (89 mM Tris base, 89 mM boric acid and 0.2 mM MgCl₂) and fractionated at 100 V for 85 min at room temperature. In some experiments, 12 pmol of oligos cPBS(182-199) GTCCCTGTTCGGGCGCCA and/or cPBS(199-216) TTCCCTTTCGCTTTCAAG were added to the RNA before denaturing to assess the effect of disrupting the PBS on dimerization.

Native polyacrylamide gel electrophoresis. RNA (800 ng) was denatured at 90°C for 2 min followed by chilling on ice for 2 min. RNA was then incubated at 37°C for 30 min in high salt buffer

(50 mM sodium cacodylate, pH 7.5, 300 mM, KCl, and 5 mM MgCl₂) or low salt buffer (50 mM sodium cacodylate, pH 7.5, 40 mM KCl, and 0.1 mM MgCl₂). Samples were loaded with native loading dye (0.17 % Bromophenol Blue and 40 % (vol/vol) sucrose) on 4 % acrylamide non-denaturing gel prepared with 1X Tris-Borate Magnesium (TBM) (89 mM Tris base, 89 mM boric acid and 0.1 mM MgCl₂) and fractionated at 150 V for 4 h at 4°C, including 2 reference samples with SYBR gold (Invitrogen), which could be visualized under blue LED light. The dimer and monomer bands in samples were cut from the gel according to the position of reference samples by scalpel.

In gel DMS probing. Each gel piece from the polyacrylamide gel was divided into 2 parts. Half was soaked in 1X TBM containing 170 mM DMS (dissolved in EtOH), incubated at 37°C for 15 min, followed by quenching with 50% (final) β-mercaptoethanol. The other half was soaked in 1X TBM (89 mM Tris base, 89 mM boric acid and 0.1 mM MgCl₂) containing the equivalent volume of EtOH as the DMS treated sample, and incubated at 37°C for 15 min. Gel slices were crushed into small pieces, soaked in 1X TBM (89 mM Tris base, 89 mM boric acid and 0.1 mM MgCl₂) buffer at 4°C overnight. RNA was extracted using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer (Macherey-Nagel).

Reverse transcription of 35 ng of DMS modified RNA or 25 ng of control RNA was performed with 200 U SuperScript II reverse transcriptase (Invitrogen), 0.1 μM of reverse transcription primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATCTAAGTTCTTCTGATCCTGTCTG, 0.5 mM dNTPs, 50 mM Tris·HCl, pH 8.0, 75 mM KCl, 6 mM MnCl₂, 10 mM DTT in 20 μl reactions. The RT reaction was incubated at 42°C for 3 h.

Library preparation. For the functional probing MIME experiments, reverse transcribed cDNAs were amplified with 250 nM primers Fw-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCTCTCTGGTTAGACC, Rv-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATGGTTGTAGCTGTCCCAG, 200 μM dNTPs, 1X Q5 reaction buffer, Q5 polymerase (NEB) using the PCR cycling conditions: 98°C for 30 s, followed by 32 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s. The PCR products

were visualized by electrophoresis on 1% agarose gels in 1X TAE buffer and column purified (using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). 25 ng purified products were used in the final sequencing library preparation with Nextera DNA Flex Library Prep (Illumina) and Nextera DNA CD Indexes (96 Indexes, 96 Samples, Illumina), according to the manufacturer's instructions. For structural profiling by DMS, we performed amplicon sequencing. PCR reaction volume was 25 μ l, 200 μ M dNTPs, 250 nM primer pair 1 (Fw-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGggtctctctggttagacc and Rv-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCGTACTCACCAGTCGCC) or primer pair 2 (Fw-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGcgaaagtaaagccagaggag and Rv-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCCCTGCTTGCCCATAC), 1X GXL reaction buffer, 0.625 U of PrimeSTAR GXL DNA Polymerase (Takara Bio). Two PCR amplifications were performed using the PCR cycling conditions: 98°C for 30 s, followed by 34 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. Amplified libraries were column purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Paired end PE150 sequencing was carried out on an Illumina Novaseq instrument (Novogene).

Data analysis Sequencing data relating to MIME functional profiling experiments was first preprocessed using automated python scripts. First, sequencing reads were quality trimmed and stripped of adaptors with CutAdapt with the parameters "--nextseq-trim 35 --max-n 0 -A CTGTCTCTTATA -a CTGTCTCTTATA". Second, reads were aligned to the HIV-1 5'UTR using Novoalign with the parameters "-o SAM -o SoftClip". Sam files were then analyzed using MIMEAnTo⁷³ to generate Kdimer, which is a quantitative metric relating the effect of a mutation on dimerization (derivation in **supplementary methods**). Statistical methods used in MIMEAnTo are described in detail elsewhere^{38,73}.

Sequencing data relating to DMS structural probing was first preprocessed with ShapeMapper2 using parameters "--output-parsed-mutations --output-counted-mutations --render-mutation". EtOH treated and DMS treated raw sequencing reads were passed to ShapeMapper2 via the modified and unmodified parameters, respectively⁵¹. DMS reactivities were calculated from ShapeMapper2 mutation rates using 90% Winsoring⁴³. DMS reactivities were saved as XML files for processing

with rf-fold module of the RNA Framework software package^{54,74}. rf-fold was used to calculate Shannon entropies and base pairing probabilities with the parameters “-ow -dp -KT -sh -g”. Initial RNA structure predictions of monomer and dimer conformations, using DMS reactivities as soft constraints, were performed with rf-fold using the RNA folding algorithms in the Vienna RNA 2.0 package⁵⁵. Refined RNA structure predictions using multidimensional probing results as additional hard constraints were performed using RNAfold of the Vienna RNA 2.0 package⁵⁵. Cluster maps of DMS reactivities were generated using the clustermap function of the python Seaborn data visualization library using ‘kendall’ correlation method and ‘average’ cluster method. Principal Component Analysis was carried out using the PCA function of the python scikit-learn library. Variances in DMS reactivities were calculated using the var function from the python NumPy library. Pairwise comparison of DMS reactivities were carried out using a modified deltaSHAPE calculation⁷⁵. This modified deltaSHAPE analysis uses several criteria to identify statistically significant changes in reactivities. First, a z-factor test identifies nucleotides where DMS reactivities change by > 1.96 standard deviations of the DMS errors. Second, a standard score threshold of 1.5 is applied, meaning that delta reactivity values are at least 1.5 standard deviations away from the mean reactivity change. To filter these statistically significant sites for biological meaning, we next applied an absolute and a relative threshold filter. The absolute difference threshold ensures that a minimum reactivity change of 0.2 is needed for the site to be considered biologically relevant. The relative threshold filter was set so that a relative change of at least 0.75-fold was needed to remove false positives where DMS reactivities are high in both conditions such that a large change in reactivity is unlikely to affect RNA structure. RNA structures were visualized using Visualization Applet for RNA (VARNA)⁷⁶.

RNA structural interference by multi-dimensional structural probing was carried out using the M2-seq pipeline⁴⁶. Briefly, data was preprocessed by ShapeMapper into simple files which are string representations of mutations in each read. Simple files were converted into the rich and compact rdat format specific for RNA structure mapping experiments⁷⁷. A two-dimensional matrix containing mutation rates at pairs of nucleotide positions was constructed. Mutation counts were subsequently normalized for total number of mutations along each row to give a true modification frequency. RNA

575 structure signatures were further refined by calculating z-scores. A thresholding of 0 was applied
576 to remove negative values, and a convolution filter was applied to enhance cross diagonal features.
577 RNA helices were finally identified in an unbiased manner by applying a filter for stems of Watson-
578 Crick and G-U wobble base pairs of at least 3 base-pairs in length. Best stems were predicted by
579 eliminating conflicting stems through selecting the highest scoring stem. Bootstrapping analyses
580 were performed using the rna_structure function of the Basic Inference Engine for RNA Structure
581 (Biers) (<https://ribokit.github.io/Biers/>) using the default parameters (100 bootstrapping iterations).

582 **Microscale Thermophoresis.** RNA was labeled at the 3' end using pCp-Cy5 (Cytidine-5'-
583 phosphate-3'-(6-aminoethyl) phosphate) (Jena Biosciences) with T4 RNA ligase (NEB) overnight
584 at 16°C, followed by RNA Clean & Concentrator Kits (ZYMO). 500 nM labeled and purified RNA
585 was denatured at 90°C for 2 min followed by chilling on ice for 2 min. RNA was folded at 37°C for
586 overnight (15-17 h) in high salt buffer (10 mM KH₂PO₄, pH 7.4, 1 mM MgCl₂, 122 mM KCl). For
587 each binding experiment, RNA was diluted to 10 nM in high salt buffer (10 mM KH₂PO₄, pH 7.4, 1
588 mM MgCl₂, 122 mM KCl, 0.01% Triton X-100, 10mM DTT and 0.02% BSA). A series of 16 tubes
589 with Pr55^{Gag} dilutions were prepared in high salt buffer, producing Pr55^{Gag} ligand concentrations
590 ranging from 30 pM to 1 μM. For measurements, each ligand dilution was mixed with one volume
591 of labeled RNA, which led to a final concentration of 5 nM labeled RNA. The reaction was mixed
592 by pipetting, incubated for 30 min at 37°C, followed by 30 min on ice. Samples were then
593 centrifuged at 10,000 x g for 5 min. Capillary forces were used to load the samples into Monolith
594 NT.115 Premium Capillaries (NanoTemper Technologies). Measurements were performed using a
595 Monolith Pico instrument (NanoTemper Technologies) at an ambient temperature of 25°C.
596 Instrument parameters were adjusted to 5% LED power, medium MST power, and MST on-time of
597 1.5 seconds. An initial fluorescence scan was performed across the capillaries to determine the
598 sample quality and afterward, 16 subsequent thermophoresis measurements were performed.
599 Data of three independently pipetted measurements were analyzed for the ΔF_{norm} values, and
600 binding affinities were determined by the MO. Affinity Analysis software (NanoTemper
601 Technologies). Graphs were plotted using GraphPad Prism 8.4.3 software.

In solution DMS-MaPseq (*in vitro*). RNA (300 nM) was denatured at 90°C for 2 min followed by chilling on ice for 2 min. Next, RNA was refolded at 37°C for overnight (15-17 h) in high salt buffer (10 mM KH₂PO₄, pH 7.4, 1 mM MgCl₂, 122 mM KCl). DMS was added to the RNA solution to final concentration 170 mM, incubated at 37 °C for 6 min, followed by quenching with β-mercaptoethanol and purification with ethanol precipitation. The purified DMS probed RNAs followed the same reverse transcription and library preparation process as the in gel DMS probed RNA samples.

In solution DMS-MaPseq (in cells). 24hr prior transfection, 10⁷ HEK293T cells were plated in 10 mL DMEM media containing 10% FBS. 4 ug of plasmids expressing HIV-1 WT or HIV-1 mutants were mixed with 48 uL Polyethylenimine (1mg/mL, Max 40k, Polysciences) and 500 uL DMEM and incubated for 10 min at room temperature before being added dropwise on the cells. 24hr post-transfection the cells were probed by replacing the media with 3 mL DMEM containing 170mM DMS and incubated at 37°C for 6 min. Cells were then washed with 5 mL PBS containing 140 mM β-mercaptoethanol to quench the DMS. 1 mL TRI-reagent (Sigma-Aldrich) is directly added on the cells to extract RNA according to the manufacturer's instructions. DNA contaminants were removed by TurboDNase (Invitrogen) treatment for 30 min at 37°C. RNA was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer (Macherey-Nagel) and eluted in 20 uL of 5 mM Tris-HCl pH 8.0. 7 uL of the purified DMS probed RNAs were used for the reverse transcription following the same reverse transcription and library preparation process as for the in gel DMS probed RNA samples.

Competition Assay. 24hr prior transfection, 7*10⁵ HEK293T cells were plated in 2 mL DMEM media containing 10% FBS. For the co-transfection experiments, equal amount of plasmids expressing WT or mutants (600 ng total) were mixed with 7.2 uL Polyethylenimine (1mg/mL, Max 40k, Polysciences) and 100 uL DMEM and incubated for 10 min at room temperature before being added dropwise on the cells. Cells and viral supernatant were collected at 24 h post-transfection. Cells were washed with 2 mL PBS and RNA was extracted with 1000 uL TRI-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Viral supernatant was first clarified for 2 min at 17000 x g, followed by a filtration step through 0.45 micron filter. The filtrate was then transferred into a new tube and the virus was pelleted for 2 h at 17000 x g. The viral pellet was extracted with

500 μ L TRI-reagent (Sigma-Aldrich) and DNA contaminants were removed by TurboDNase (Invitrogen) treatment for 30 min at 37°C. RNA was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer (Macherey-Nagel) and eluted in 20 μ L of 5 mM Tris-HCl pH 8.0. 10 μ L purified RNA was heat denatured at 65°C for 5 min together with 0.67 μ M reverse primer (GATGGTTGTAGCTGTCCCAGTATTTGCC) and 1.67 mM dNTPs in 15 μ L total volume, then chilled on ice for 2 min. RNA was then reverse transcribed by adding 1X SSIV buffer, 5 mM DTT, 20U RNasin, 100U SSIV in 25 μ L total volume and incubating at 52°C for 1 h. cDNAs were amplified with 250 nM primers Fw-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGgggtctctctggttagacc, Rv-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCGTACTCACCAGTCGCC, 200 μ M dNTPs, 1X Q5 reaction buffer, and 0.02 U/ μ L of Q5 polymerase (NEB) using the PCR cycling conditions: 98 °C for 1 min, followed by 22 cycles of 98 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s. The PCR products were visualized by electrophoresis on 1% agarose gels in 1X TAE buffer and column purified (using the NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel). 40 ng purified products were used in the final indexing PCR using 2.5 μ L of Nextera DNA CD Indexes (96 Indexes, 96 Samples, Illumina) in a 14 μ L reaction (200 μ M dNTPs, 1X Q5 reaction buffer, and 0.02 U/ μ L of Q5 polymerase (NEB)). The PCR cycling conditions were 98 °C for 2 min, followed by 5 cycles of 98 °C for 30 s, 6255 °C for 320 s, and 72 °C for 1 min. Paired end PE150 sequencing was carried out on an Miniseq instrument (Illumina) according to the manufacturer's instructions.

Data availability

The datasets generated during this study are available at the NCBI bioproject id PRJNA771368.

Code availability

Data were analyzed using publicly available tools as outlined in the material and methods.

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Author contributions

RPS, ASG and LY conceived the study. LY, ASG, CB, UBA, SA, and MON performed the experiments. RPS, PB and MS performed the analysis. AK and NC purified Pr55^{Gag} and performed MST measurements. RPS and LY wrote the manuscript with contributions from the other authors.

Competing interests

The authors declare they have no competing interests.

Bibliography

1. Paillart, J.-C., Shehu-Xhilaga, M., Marquet, R. & Mak, J. Dimerization of retroviral RNA genomes: an inseparable pair. *Nat. Rev. Microbiol.* **2**, 461–72 (2004).
2. Dubois, N., Marquet, R., Paillart, J.-C. & Bernacchi, S. Retroviral RNA Dimerization: From Structure to Functions. *Front. Microbiol.* **9**, 527 (2018).
3. Nikolaitchik, O. a *et al.* Probing the HIV-1 Genomic RNA Trafficking Pathway and Dimerization by Genetic Recombination and Single Virion Analyses. *PLoS Pathog.* **5**, e1000627 (2009).
4. Sakuragi, J., Ueda, S., Iwamoto, A. & Shioda, T. Possible role of dimerization in human immunodeficiency virus type 1 genome RNA packaging. *J. Virol.* **77**, 4060–9 (2003).
5. Ooms, M., Huthoff, H., Russell, R., Liang, C. & Berkhout, B. A riboswitch regulates RNA dimerization and packaging in human immunodeficiency virus type 1 virions. *J. Virol.* **78**, 10814–9 (2004).
6. Russell, R. S. *et al.* Sequences downstream of the 5' splice donor site are required for both packaging and dimerization of human immunodeficiency virus type 1 RNA. *J. Virol.* **77**, 84–96 (2003).
7. Nikolaitchik, O. A., Rhodes, T. D., Ott, D. & Hu, W.-S. Effects of mutations in the human immunodeficiency virus type 1 Gag gene on RNA packaging and recombination. *J. Virol.* **80**, 4691–7 (2006).

683 8. Moore, M. D. *et al.* Dimer initiation signal of human immunodeficiency virus type 1: its role
684 in partner selection during RNA copackaging and its effects on recombination. *J. Virol.* **81**, 4002–
685 11 (2007).

686 9. Chen, J. *et al.* High efficiency of HIV-1 genomic RNA packaging and heterozygote formation
687 revealed by single virion analysis. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 13535–40 (2009).

688 10. Smyth, R. P., Davenport, M. P. & Mak, J. The origin of genetic diversity in HIV-1. *Virus Res.*
689 **169**, 415–29 (2012).

690 11. Lu, K. *et al.* NMR detection of structures in the HIV-1 5'-leader RNA that regulate genome
691 packaging. *Science* **334**, 242–5 (2011).

692 12. Keane, S. C. *et al.* RNA structure. Structure of the HIV-1 RNA packaging signal. *Science*
693 **348**, 917–21 (2015).

694 13. Kharytonchyk, S. *et al.* Transcriptional start site heterogeneity modulates the structure and
695 function of the HIV-1 genome. *Proc. Natl. Acad. Sci. U. S. A.* 201616627 (2016)
696 doi:10.1073/pnas.1616627113.

697 14. Brown, J. D. *et al.* Structural basis for transcriptional start site control of HIV-1 RNA fate.
698 *Science* **368**, 413–417 (2020).

699 15. Huthoff, H. & Berkhout, B. Two alternating structures of the HIV-1 leader RNA. *RNA* **7**, 143–
700 57 (2001).

701 16. Abbink, T. E. M., Ooms, M., Haasnoot, P. C. J. & Berkhout, B. The HIV-1 Leader RNA
702 Conformational Switch Regulates RNA Dimerization but Does Not Regulate mRNA Translation †.
703 *Biochemistry* **44**, 9058–9066 (2005).

704 17. Skripkin, E. *et al.* Identification of the primary site of the human immunodeficiency virus type
705 1 RNA dimerization in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 4945–9 (1994).

706 18. Paillart, J. C., Marquet, R., Skripkin, E., Ehresmann, B. & Ehresmann, C. Mutational
707 analysis of the bipartite dimer linkage structure of human immunodeficiency virus type 1 genomic
708 RNA. *J. Biol. Chem.* **269**, 27486–93 (1994).

709 19. Paillart, J. C. *et al.* A dual role of the putative RNA dimerization initiation site of human
710 immunodeficiency virus type 1 in genomic RNA packaging and proviral DNA synthesis. *J. Virol.* **70**,
711 8348–54 (1996).

712 20. Muriaux, D., De Rocquigny, H., Roques, B. P. & Paoletti, J. NCp7 activates HIV-1Lai RNA
713 dimerization by converting a transient loop-loop complex into a stable dimer. *J. Biol. Chem.* **271**,
714 33686–92 (1996).

715 21. Fu, W., Gorelick, R. J. & Rein, A. Characterization of human immunodeficiency virus type
716 1 dimeric RNA from wild-type and protease-defective virions. *J. Virol.* **68**, 5013–5018 (1994).

717 22. Jalalirad, M. & Laughrea, M. Formation of immature and mature genomic RNA dimers in
718 wild-type and protease-inactive HIV-1: differential roles of the Gag polypeptide, nucleocapsid
719 proteins NCp15, NCp9, NCp7, and the dimerization initiation site. *Virology* **407**, 225–36 (2010).

720 23. Russell, R. S., Hu, J., Laughrea, M., Wainberg, M. a. & Liang, C. Deficient dimerization of
721 human immunodeficiency virus type 1 RNA caused by mutations of the u5 RNA sequences.
722 *Virology* **303**, 152–63 (2002).

723 24. Shen, N., Jetté, L., Wainberg, M. A. & Laughrea, M. Role of stem B, loop B, and nucleotides
724 next to the primer binding site and the kissing-loop domain in human immunodeficiency virus type
725 1 replication and genomic-RNA dimerization. *J. Virol.* **75**, 10543–9 (2001).

726 25. Marquet, R., Paillart, J. C., Skripkin, E., Ehresmann, C. & Ehresmann, B. Dimerization of
727 human immunodeficiency virus type 1 RNA involves sequences located upstream of the splice
728 donor site. *Nucleic Acids Res.* **22**, 145–51 (1994).

729 26. Song, R., Kafaie, J. & Laughrea, M. Role of the 5' TAR stem-loop and the U5-AUG duplex
730 in dimerization of HIV-1 genomic RNA. *Biochemistry* **47**, 3283–93 (2008).

731 27. Abbink, T. E. M. & Berkhout, B. A novel long distance base-pairing interaction in human
732 immunodeficiency virus type 1 RNA occludes the Gag start codon. *J. Biol. Chem.* **278**, 11601–11
733 (2003).

734 28. Kenyon, J. C., Prestwood, L. J., Le Grice, S. F. J. & Lever, A. M. L. In-gel probing of
735 individual RNA conformers within a mixed population reveals a dimerization structural switch in the
736 HIV-1 leader. *Nucleic Acids Res.* **41**, e174 (2013).

737 29. Obayashi, C. M., Shinohara, Y., Masuda, T. & Kawai, G. Influence of the 5'-terminal
738 sequences on the 5'-UTR structure of HIV-1 genomic RNA. *Sci. Rep.* **11**, 1–9 (2021).

739 30. Lu, K., Heng, X. & Summers, M. F. Structural determinants and mechanism of HIV-1
740 genome packaging. *J. Mol. Biol.* **410**, 609–633 (2011).

741 31. Brigham, B. S., Kitzrow, J. P., Reyes, J.-P. C., Musier-Forsyth, K. & Munro, J. B. Intrinsic
742 conformational dynamics of the HIV-1 genomic RNA 5'UTR. *Proc. Natl. Acad. Sci.* **116**, 10372–
743 10381 (2019).

744 32. Abd El-Wahab, E. W. *et al.* Specific recognition of the HIV-1 genomic RNA by the Gag
745 precursor. *Nat. Commun.* **5**, 4304 (2014).

746 33. Mailler, E. *et al.* The Life-Cycle of the HIV-1 Gag-RNA Complex. *Viruses* **8**, 248 (2016).

747 34. Freed, E. O. HIV-1 assembly, release and maturation. *Nat. Rev. Microbiol.* **13**, 484–96
748 (2015).

749 35. Sakuragi, J. I. & Panganiban, a T. Human immunodeficiency virus type 1 RNA outside the
750 primary encapsidation and dimer linkage region affects RNA dimer stability in vivo. *J. Virol.* **71**,
751 3250–4 (1997).

752 36. Jones, K. L., Sonza, S. & Mak, J. Primary T-lymphocytes rescue the replication of HIV-1
753 DIS RNA mutants in part by facilitating reverse transcription. *Nucleic Acids Res.* **36**, 1578–88
754 (2008).

755 37. Clever, J. L. & Parslow, T. G. Mutant human immunodeficiency virus type 1 genomes with
756 defects in RNA dimerization or encapsidation. *J. Virol.* **71**, 3407–14 (1997).

757 38. Smyth, R. P. *et al.* Mutational interference mapping experiment (MIME) for studying RNA
758 structure and function. *Nat. Methods* **12**, 866–872 (2015).

759 39. Smyth, R. P. *et al.* In cell mutational interference mapping experiment (in cell MIME)
760 identifies the 5' polyadenylation signal as a dual regulator of HIV-1 genomic RNA production and
761 packaging. *Nucleic Acids Res.* **46**, e57–e57 (2018).

762 40. Pop, C. *et al.* Causal signals between codon bias, mRNA structure, and the efficiency of
763 translation and elongation. *Mol. Syst. Biol.* **10**, 770 (2014).

764 41. Cordero, P., Kladwang, W., Vanlang, C. C. & Das, R. Quantitative dimethyl sulfate mapping
765 for automated RNA secondary structure inference. *Biochemistry* **51**, 7037–7039 (2012).

766 42. Mailler, E., Paillart, J.-C., Marquet, R., Smyth, R. P. & Vivet-Boudou, V. The evolution of
767 RNA structural probing methods: From gels to next-generation sequencing. *Wiley Interdiscip. Rev.*
768 *RNA* **10**, (2019).

769 43. Rouskin, S., Zubradt, M., Washietl, S., Kellis, M. & Weissman, J. S. Genome-wide probing
770 of RNA structure reveals active unfolding of mRNA structures in vivo. *Nature* **505**, 701–5 (2014).

771 44. Zubradt, M. *et al.* DMS-MaPseq for genome-wide or targeted RNA structure probing in vivo.
772 *Nat. Methods* **14**, 75–82 (2017).

773 45. Tian, S. & Das, R. RNA structure through multidimensional chemical mapping. *Q. Rev.*
774 *Biophys.* **49**, e7 (2016).

775 46. Cheng, C. Y., Kladwang, W., Yesselman, J. D. & Das, R. RNA structure inference through
776 chemical mapping after accidental or intentional mutations. *Proc. Natl. Acad. Sci. U. S. A.* **114**,
777 9876–9881 (2017).

778 47. Gavazzi, C. *et al.* A functional sequence-specific interaction between influenza A virus
779 genomic RNA segments. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 16604–9 (2013).

780 48. Gavazzi, C. *et al.* An in vitro network of intermolecular interactions between viral RNA
781 segments of an avian H5N2 influenza A virus: Comparison with a human H3N2 virus. *Nucleic Acids*
782 *Res.* **41**, 1241–1254 (2013).

783 49. Fajardo, T., Sung, P.-Y., Celma, C. C. & Roy, P. Rotavirus Genomic RNA Complex Forms
784 via Specific RNA-RNA Interactions: Disruption of RNA Complex Inhibits Virus Infectivity. *Viruses* **9**,
785 (2017).

786 50. Masuda, T. *et al.* Fate of HIV-1 cDNA intermediates during reverse transcription is dictated
787 by transcription initiation site of virus genomic RNA. *Sci. Rep.* **5**, 1–15 (2015).

788 51. Busan, S. & Weeks, K. M. Accurate detection of chemical modifications in RNA by
789 mutational profiling (MaP) with ShapeMapper 2. *RNA* (2017) doi:10.1261/rna.061945.117.

790 52. Goldschmidt, V. *et al.* Structural variability of the initiation complex of HIV-1 reverse
791 transcription. *J. Biol. Chem.* **279**, 35923–35931 (2004).

792 53. Goldschmidt, V. *et al.* Direct and indirect contributions of RNA secondary structure
793 elements to the initiation of HIV-1 reverse transcription. *J. Biol. Chem.* **277**, 43233–43242 (2002).

794 54. Incarnato, D., Morandi, E., Simon, L. M. & Oliviero, S. RNA Framework: an all-in-one toolkit
795 for the analysis of RNA structures and post-transcriptional modifications. *Nucleic Acids Res.* **46**,
796 e97 (2018).

797 55. Lorenz, R. *et al.* ViennaRNA Package 2.0. *Algorithms Mol. Biol.* **6**, 26 (2011).

798 56. Nikolaitchik, O. A. *et al.* Unpaired Guanosines in the 5' Untranslated Region of HIV-1 RNA
799 Act Synergistically To Mediate Genome Packaging. *J. Virol.* **94**, (2020).

800 57. Deigan, K. E., Li, T. W., Mathews, D. H. & Weeks, K. M. Accurate SHAPE-directed RNA
801 structure determination. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 97–102 (2009).

802 58. Kladwang, W., VanLang, C. C., Cordero, P. & Das, R. A two-dimensional mutate-and-map
803 strategy for non-coding RNA structure. *Nat. Chem.* **3**, 954–62 (2011).

804 59. Clever, J. L., Wong, M. L. & Parslow, T. G. Requirements for kissing-loop-mediated
805 dimerization of human immunodeficiency virus RNA. *J. Virol.* **70**, 5902–5908 (1996).

806 60. Huthoff, H., Bugala, K., Barciszewski, J. & Berkhout, B. On the importance of the primer
807 activation signal for initiation of tRNA(lys3)-primed reverse transcription of the HIV-1 RNA genome.
808 *Nucleic Acids Res.* **31**, 5186–94 (2003).

809 61. Ooms, M., Verhoef, K., Southern, E., Huthoff, H. & Berkhout, B. Probing alternative foldings
810 of the HIV-1 leader RNA by antisense oligonucleotide scanning arrays. *Nucleic Acids Res.* **32**, 819–
811 27 (2004).

812 62. Kasprzak, W., Bindewald, E. & Shapiro, B. a. Structural polymorphism of the HIV-1 leader
813 region explored by computational methods. *Nucleic Acids Res.* **33**, 7151–63 (2005).

814 63. Wilkinson, K. a *et al.* High-throughput SHAPE analysis reveals structures in HIV-1 genomic
815 RNA strongly conserved across distinct biological states. *PLoS Biol.* **6**, e96 (2008).

816 64. Watts, J. M. *et al.* Architecture and secondary structure of an entire HIV-1 RNA genome.
817 *Nature* **460**, 711–6 (2009).

818 65. Siegfried, N. A., Busan, S., Rice, G. M., Nelson, J. A. E. & Weeks, K. M. RNA motif
819 discovery by SHAPE and mutational profiling (SHAPE-MaP). *Nat. Methods* (2014)
820 doi:10.1038/nmeth.3029.

821 66. Paillart, J. C. *et al.* First snapshots of the HIV-1 RNA structure in infected cells and in virions.
822 *J. Biol. Chem.* **279**, 48397–48403 (2004).

823 67. Balvay, L., Lastra, M. L., Sargueil, B., Darlix, J. L. & Ohlmann, T. Translational control of
824 retroviruses. *Nat. Rev. Microbiol.* **5**, 128–140 (2007).

825 68. Bernacchi, S. *et al.* HIV-1 Pr55 Gag binds genomic and spliced RNAs with different affinity
826 and stoichiometry. *RNA Biol.* **14**, 90–103 (2017).

827 69. Houzet, L. *et al.* HIV controls the selective packaging of genomic, spliced viral and cellular
828 RNAs into virions through different mechanisms. *Nucleic Acids Res.* **35**, 2695–704 (2007).

829 70. Seif, E., Niu, M. & Kleiman, L. Annealing to sequences within the primer binding site loop
830 promotes an HIV-1 RNA conformation favoring RNA dimerization and packaging. *RNA* **19**, 1384–
831 93 (2013).

832 71. Gibbs, J. S., Regier, D. A. & Desrosiers, R. C. Construction and In Vitro Properties of HIV-
833 1 Mutants with Deletions in ‘Nonessential’ Genes. *AIDS Res. Hum. Retroviruses* **10**, 343–350
834 (1994).

835 72. McKinstry, W. J. *et al.* Expression and purification of soluble recombinant full length HIV-1
836 Pr55(Gag) protein in Escherichia coli. *Protein Expr. Purif.* **100**, 10–8 (2014).

837 73. Smith, M. R., Smyth, R. P., Marquet, R. & von Kleist, M. MIMEAnTo: profiling functional
838 RNA in mutational interference mapping experiments. *Bioinformatics* **32**, 3369–3370 (2016).

- 839 74. Incarnato, D., Neri, F., Anselmi, F. & Oliviero, S. RNA structure framework: automated
840 transcriptome-wide reconstruction of RNA secondary structures from high-throughput structure
841 probing data. *Bioinformatics* **32**, 459–61 (2016).
- 842 75. Smola, M. J., Calabrese, J. M. & Weeks, K. M. Detection of RNA–Protein Interactions in
843 Living Cells with SHAPE. *Biochemistry* **54**, 6867–6875 (2015).
- 844 76. Darty, K., Denise, A. & Ponty, Y. VARNA: Interactive drawing and editing of the RNA
845 secondary structure. *Bioinformatics* **25**, 1974–5 (2009).
- 846 77. Cordero, P., Lucks, J. B. & Das, R. An RNA Mapping DataBase for curating RNA structure
847 mapping experiments. *Bioinformatics* **28**, 3006–8 (2012).

848

1 **Figure 1**

2 HIV-1 dimerization and its analysis (a) Dimerization is a key step in the HIV-1 life cycle. Monomeric
3 RNA is thought to be preferentially translated, in contrast to dimeric RNA which is a pre-requisite
4 for packaging into virions. Dimeric RNA helps maintain genome integrity through recombination (b)
5 The HIV-1 5'UTR is composed of distinct structural domains linked to different functions in the HIV-
6 1 life cycle. TAR for transcription. PolyA for polyadenylation which is inactive in the 5'UTR. U5 in
7 unique 5 region, PBS, or primer binding site, for annealing of the host tRNA for initiating reverse
8 transcription. SL1-SL3 contain the packaging signal. SL2 contains the splice donor site.
9 Dimerization occurs through a kissing loop interaction at a sequence in SL1. Long distance
10 interactions / alternative folds involving long distance interactions, such as between SL1-U5 and
11 U5-AUG may regulate dimerization. (c) Functional Analysis of RNA Structure (FARS-seq). Mutant
12 RNA sequences are generated by mutagenic PCR and *in vitro* transcription. Mutant populations
13 are physically separated into monomer and dimer fractions and probed with DMS or left untreated.
14 Mutation frequencies are analyzed by next generation sequencing. (d) Functional profiles are
15 obtained by mutational interference. K^{dimer} is a quantitative measure of dimerization based on the
16 ratio of mutations in the dimer selected vs monomer selected population, corrected for mutations
17 introduced during the library preparation and sequencing (e) Structural profiles are obtained by
18 DMS which specifically reacts with unpaired A and C residues. DMS-MaP-seq measures DMS
19 reactivities as mutation rates in DMS treated vs untreated controls (2) Two-dimensional analysis
20 identifies RNA stems through correlations between stem-disrupting mutations and mutations
21 induced by DMS.

a **b** **c** **d** **e** **f**

22 **Figure 2**

23 Functional profiling of sequences involved in dimerization by mutational interference (a) 1G, 2G,
24 and 3G capped and uncapped transcript variants migrate as distinct monomer and dimer bands on
25 native agarose gels in both low salt and high salt buffers (b-h) k^{dimer} is a relative measure of the
26 effects of a mutation on dimerization, calculated as the ratio of mutation frequencies in the
27 monomer vs the dimerized RNA, and corrected for errors introduced during library preparation and
28 sequencing. (b) $\log_2(k^{\text{dimer}})$ values binned according to functional domain in the 5'UTR: TAR, U5,
29 PBS, SL1, SL2, SL3 and SL4. None refers to nucleotide positions that do not fall into any structural
30 domain. (c-d) median $\log_2(k^{\text{dimer}})$ values for each genome position for all three uncapped transcript
31 variants in high and low salt buffers. Lines represent median $\log_2(k^{\text{dimer}})$ values smoothed with a
32 window size of 5 nt. (e-h) $\log_2(k^{\text{dimer}})$ values of the 1G and 3G transcript variants compared to the
33 mean of the 1G, 2G and 3G transcripts in high and low salt buffers.

34 **Figure 3**

35 Structural profiling reveals identifies structural conformations of the HIV-1 5'UTR. (a) Clustering of
36 Kendal rank correlations of DMS reactivities across all positions reveals structural relationships
37 between monomer and dimer isolated populations from uncapped and capped transcript variants
38 in high and low salt buffer. Relationships between sample DMS reactivities was determined by
39 hierarchical clustering using the 'average' linking method (b) Principal component analysis of DMS
40 reactivities identifies structural four structural classes of the HIV-1 5'UTR (c) Variance in DMS
41 reactivities across genome positions from all samples is enriched at SL1 and TAR/polyA boundary
42 (d) Statistical analysis DMS reactivities in 1G/2G and 3G structural classes find significant
43 differences in reactivities are mainly localized to polyA and SL1. A z-factor test identifies
44 nucleotides where DMS reactivities change by > 1.96 standard deviations of the DMS errors. An
45 absolute difference threshold ensures that a minimum reactivity change of 0.2 is needed for the
46 site to be considered biologically relevant. The relative threshold of 0.75-fold was used to remove
47 false positives where DMS reactivities are high in both conditions such that a large change in
48 reactivity is unlikely to affect RNA structure.

49 **Figure 4**

50 Secondary structure model for 1G/2G dimer and 3G monomer populations. **(a, d)** Secondary
51 structure model of dimer and monomer class, respectively. Models were obtained using DMS
52 reactivities as soft constraints for *in silico* folding in the Vienna RNA structure package. For the
53 dimer structure, the U1sRNA binding site within SL2 is shown. **Structures of polyA and SL1 stem**
54 **loops and polyA-SL1 interaction are shown. DMS reactivities from dimer samples were mapped to**
55 **A and C residues of the polyA and SL1 stem loop structures. DMS reactivities from the monomer**
56 **samples were mapped to A and C residues of the polyA-SL1 interaction.** Red signifies highly
57 reactive positions that are unpaired. Pale yellow signifies unreactive positions that are base-paired.
58 **(b, e)** DMS reactivities and Shannon entropies for the 1G/2G dimer and 3G monomer class,
59 respectively. Arc plots show base pairing probabilities (green=70-100%; blue=40-70%; yellow=10-
60 40%; grey=5-10%). Grey bar in **(e)** signifies polyA-SL1 interaction. **(c, d)** Dot plots of RNA base
61 pairing probabilities for the 1G/2G dimer and 3G monomer class, respectively, reveal alternative
62 folding possibilities. RNA stems are shown along the diagonals.

a two-dimensional RNA structural probing

b 1G/2G dimer structure z-score

c stem score

d

e 3G monomer structure z-score

f

g

Figure 5

Two-dimensional mapping of RNA structure in 1G/2G dimer and 3G monomer populations. (a) Mutations disrupting RNA stems lead to increases in DMS reactivity at positions opposite the mutation. Positions of DMS modification are read out as mutations leading to correlated mutations at pairs of nucleotides involved in RNA structure. RNA secondary structures (blue circles) are identified along the diagonals. Punctate signals (purple circles) can signify non-canonical or tertiary interactions. (b, e) z-score analysis of mutation frequencies from 1G/2G dimer and 3G monomer populations, respectively. Raw Z-scores (lower diagonal) reveal pairs of positions enriched with mutations. Filtered z-scores (upper diagonal) enhance stem signals by applying a convolution filter and signal threshold. Insets are zooms of the filtered z-scores for the polyA-SL1, PBS-SL1 and SL1 stems (c, f) Stem detection in 1G/2G dimer and 3G monomer populations, respectively. All stems (lower diagonal) reveal all possible stems of minimum length 3 by applying a filter for Watson-Crick and Wobble base pairs to the filtered Z-score. Best stem (upper diagonal) selects the best non-conflicting stems by removing conflicting stems based on filtered Z-score. Coloured boxes represent regions that are highlighted in enhance RNA secondary structure models (d, g) Enhanced RNA secondary structure models of 1G/2G dimer and 3G monomer populations, respectively. Coloured base pairings were detected in multi-dimensional mapping and used as hard constraints before in RNA secondary structure prediction. Dark blue is TAR. Light blue is polyA. Orange is PBS. Mustard is SL1. Dark green is SL2. Light green is SL3. Red represents the polyA-SL1 interactions, Pink the novel SL1-PBS interaction, and purple the TAR-PBS interaction.

Figure 6

Structure/function analysis of HIV-1 dimerization. Single nucleotide resolution functional profiling data mapped the (a) dimer and (c) monomer structures expressed as $\log_2(K^{\text{dimer}})$ values. Each individual mutant shown as one of three circle in the order A,C,G,U clockwise from upper position (excluding the WT base). Validation of structural models on 3G RNA by point mutagenesis followed by native agarose gel electrophoresis in two different buffer conditions. Red circles show mutations inhibiting dimerization, and blue circles show mutations enhancing dimerization. $\log_2(K^{\text{dimer}})$ values above 2 are capped. (b) Functional profiling data mapped to different structural models of SL1 containing mutually exclusive internal loop configurations. Two internal loop (2IL), three internal loop (3IL), and the three-way junction (3WJ) are mutually exclusive models of SL1 structure based on chemical probing or biophysical measurements. Green arrows show mutations that improve dimerization by closing or reducing the size of internal loops, providing evidence that SL1 is metastable, and that alternative SL1 conformations can form and dimerize. Red arrows show mutations that have complex effects on dimerization because they impact the novel PBS-SL1 interaction.

Figure 7

PBS regulates dimerization and Pr55^{Gag} binding. (a) PBS targeting oligos can trigger dimerization of a 3G RNA. cPBS(182-199) disrupts the TAR-PBS interaction leading to the formation of a higher order RNA structure. cPBS(199-216) disrupts the PAS-antiPAS stem and enhances dimerization. The effects of both oligos are additive (b) Mutations targeting the polyA-SL1 and PBS-SL1 interaction affect Pr55^{Gag} binding as measured by microscale thermophoresis (MST) (c) Competition assays to measure the relative effects of mutations on genome packaging into virions. Two-way competition assays show that dimer promoting mutations C218G and C84A-C85A are enhanced in genome packaging compared to monomer promoting mutations A220G-G221A and U86G A89C. Five-way competition assays between wild-type (WT) HIV-1 and mutants show that dimer promoting mutants are packaged similar or better than WT, whereas monomer promoting mutants are packaged less efficiently than WT. Experiments were performed in duplicate. Statistical test was a one-way ANOVA with Dunnett's correction for multiple comparisons (d) Model showing how the binding of host factors can regulate viral replication, in part, through re-modelling RNA structure.

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