Tat IRES modulator of tat mRNA (TIM-TAM): a conserved RNA structure that controls Tat expression and acts as a switch for HIV productive and latent infection

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ABSTRACT

Tat protein is essential to fully activate HIV transcription and processing of viral mRNA, and therefore determines virus expression in productive replication and the establishment and maintenance of latent infection. Here, we used thermodynamic and structure analyses to define a highly conserved sequence-structure in tat mRNA that functions as Tat IRES modulator of tat mRNA (TIM-TAM). By impeding cap-dependent ribosome progression during authentic spliced tat mRNA translation, TIM-TAM stable structure impacts on timing and level of Tat protein hence controlling HIV production and infectivity along with promoting latency. TIM-TAM also adopts a conformation that mediates Tat internal ribosome entry site (IRES)-dependent translation during the early phases of infection before provirus integration. Our results document the critical role of TIM-TAM in Tat expression to facilitate virus reactivation from latency, with implications for HIV treatment and drug development.

INTRODUCTION

Despite effective long-term antiretroviral therapy (ART), HIV-1 persists in a latent state as an integrated provirus in long lived and proliferating CD4+ T cells (1–4). Proviral latency is established early following infection of both resting and activated CD4 T cells (5) and constitutes the main barrier against HIV cure. Only a small fraction of the integrated HIV DNA that persists on ART is intact (6,7) and capable of yielding replication-competent virus leading to viral re-bound after ART interruption (8,9).

One approach being pursued to eliminate latent infection is to activate or reverse latency to allow for production of HIV proteins or virions leading to viral immune mediated cytopathic clearance of infected cells (10).

The transition from latent to productive HIV infection is largely governed by the activities of the HIV Tat regulatory protein (11,12). Both the 72 amino acids (aa) single coding exon and the 101 aa two coding exon forms of Tat release the positive transcription elongation factor-b (P-TEFb) from an inactive complex and recruit it to the viral promoter, where it assists in phosphorylating the carboxy-terminus of RNA polymerase II (Pol II) creating the super-elongation complex (13,14). Tat also promotes the displacement of the negative elongation factors NELF (Negative Elongation Factor) and DSIF (DRB Sensitivity-Inducing Factor), and the recruitment of nucleosome remodelling complexes (SWI/SNF), which leads to a highly functional promoter (15,16). Moreover, Tat assists in post-transcriptional events required for productive replication including RNA capping (17–19) and splicing (20).

Sequencing of HIV DNA from infected individuals on suppressive ART revealed that the majority of viruses that persist on ART have large deletions and stop codons and are defective (7,21). This pool of defective proviruses (HIV\textsubscript{def}) is unable to produce infectious virions but can produce ‘unspliced’ HIV RNA (usHIV-RNA) species in the size range of 0.5–2.6 kb, whilst also retaining appropriate translational open reading frames (ORFs) that potentially code gag, pol,
Indeed, some usHIV-RNAs with internal deletions were predicted to combine parts of Gag and Env, or Gag and Nef in novel HIV chimeric proteins. Over time in individuals on ART, there is an accumulation of defective HIV genomes that retain two flanking long terminal repeats (LTRs), and several key splicing sites that allow ongoing expression of viral protein from aberrant RNAs (24) as well as chimeric host-viral mRNAs (24–27).

The production of aberrant mRNAs that translate virus polypeptides can occur through non-canonical translational pathways such as defective ribosome products (DRiPs) (28,29) or through leaky ribosome scanning (30,31). Control of HIV protein expression is also possible through a tight regulation of mRNA processing and translation by RNA structure and RNA–protein interactions. Several non-canonical translational pathways have been described for HIV, including IREs within the 5’ leader sequence (32,33) and within the Gag ORF (34,35), which ensure successful Gag translation during G2/M phase arrest, oxidative and osmotic stress (36,37). We hypothesised that Tat could be produced at low levels from both intact and HIVdef proviruses through an internal ribosome entry site (IRES), and therefore can influence both the establishment and reversal of HIV latency.

Here, we investigate HIV mRNAs for their capacity to promote a pioneer round of Tat expression required for the reversal of viral latency and promotion of the ensuing phases of productive virus replication. We identify a highly conserved structural element underlying the Tat ORF, named TIM-TAM for a highly conserved structural element underlying the Tat protein, and characterize a mechanism whereby TIM-TAM controls the rate of Tat translation through cap- and IRES-dependent mechanisms, and thus shaping the output of virus reactivation from latency.

**MATERIALS AND METHODS**

**Constructs**

pNL4-3 was obtained from M. Martin (NIH, Bethesda, MD, USA). pDR-ΔEnvNef-EGFP viral clone harbour a deletion that terminates Env (nt 7037–7619, derived from pHIS-HIV-B (38)) and an EGFP coding sequence inserted into KpnI site in Nef ORF, which renders the virus as a single-round EGFP virus once pseudotyped with Env (AD8).

Silaa mut and ΔTIM-TAM viral clones were generated by site directed mutagenesis (Supplementary Table S1) of a sub-clone containing tat1 RNA2D structure analysis of tat2 mRNA by SHAPE were performed as previously described (41). Briefly, 1 pmol of *in vitro* transcribed tat2 RNA was probed in 3× folding buffer (333 mM HEPES-KOH pH 8, 333 mM NaCl, 33.3 mM MgCl2) with 1-methyl-7-nitroisatoic anhydride (1M7, 60 mM in DMSO) or DMSO for 4 min at 37°C, then recovered by ethanol precipitation. Primer extension was conducted as described previously with 0.4 μM fluorescently labelled primer (6-FAM or HEX, Sigma-Aldrich, Supplementary Table S1). The dideoxy sequencing reactions were conducted using unmodified RNA, labeled primers (PET or NED, Applied Biosystems) and 0.5 mM ddGTP. cDNAs were recovered by ethanol precipitation and separated by capillary electrophoresis (ABI 3130) with LIZ500 size standard. Data was processed using the QuShape software (42), and the 2D structure was generated using the normalized SHAPE reactivities as pseudo-free energy in the RNAstructure v5.6 program (b and m parameters were -0.6 and 2.8 kcal/mol, respectively). The maximum allowed distance between paired bases was restrained to less than 300nt. The RNA model was represented using varna.

**SHAPE analysis**

RNA 2D structure analysis of tat2 mRNA by SHAPE were performed as previously described (41). Briefly, 1 pmol of *in vitro* transcribed tat2 RNA was probed in 3× folding buffer (333 mM HEPES-KOH pH 8, 333 mM NaCl, 33.3 mM MgCl2) with 1-methyl-7-nitroisatoic anhydride (1M7, 60 mM in DMSO) or DMSO for 4 min at 37°C, then recovered by ethanol precipitation. Primer extension was conducted as described previously with 0.4 μM fluorescently labelled primer (6-FAM or HEX, Sigma-Aldrich, Supplementary Table S1). The dideoxy sequencing reactions were conducted using unmodified RNA, labeled primers (PET or NED, Applied Biosystems) and 0.5 mM ddGTP. cDNAs were recovered by ethanol precipitation and separated by capillary electrophoresis (ABI 3130) with LIZ500 size standard. Data was processed using the QuShape software (42), and the 2D structure was generated using the normalized SHAPE reactivities as pseudo-free energy in the RNAstructure v5.6 program (b and m parameters were -0.6 and 2.8 kcal/mol, respectively). The maximum allowed distance between paired bases was restrained to less than 300nt. The RNA model was represented using varna.
probing, the RNA mixture was pre-incubated for 20 min at 20°C with sodium cacodylate (50 mM) for efficient DMS and Kethoxal modifications, and with sodium borate (50 mM) for CMCT modification. Probing was performed by treatment with 0.5–0.75 U of T1 RNase, 0.5–0.75 U of T2 RNase, $1 \times 10^{-4}–5 \times 10^{-4}$ U of RNase V1 for 10 min at 30°C and 10–20 μl of CMCT, 1–2 μl of a 1/4 (V/V) DMS/ETOH solution, 2–10 μl of Kethoxal (37 mg/ml). The reactions were stopped as described previously (44) and immediately followed by phenol extraction. Positions of enzymatic and chemical cleavages were identified by primer extension analyses using AMV RT (Q Biogene) and the 5'end labelled primers (Supplementary Table S1). Experiments were repeated several times using different batches of RNA. Stable secondary structures showing the best fit with experimental data were identified using Mfold v3.2 software (45). Probing data were introduced as a constraint in the search.

**Virus production and infectivity assays**

Viral stocks were generated by transfecting the proviral constructs into HEK 293T cells with Lipofectamine 2000 (Invitrogen) in serum free media (Opti-MEM, Gibco). pcMV-EGFP vector was added to each transfection mixture as a normalizer for transfection efficiency. Supernatants were collected after 72 h, filtered through a 0.45μm to clear cell debris, then concentrated using microcon centrifugal filter device (30K, Merck Millipore) and stored at –80°C. Virus titres were quantified by measuring p24CA levels by capture ELISA. Briefly, Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific, Waltham, MA) were pre-coated with anti-p24 sheep antibody D7320 (300 μg/ml). The samples were then incubated for 2 h with anti-p24 mouse antibody BC1071 (EH12E1 clone, 1:2000; Aalto Bio Reagents) in serum free media (Opti-MEM, Gibco). pCMV-Vitrogen) in serum free media (Opti-MEM, Gibco). pCMV-

**Primary cell isolation, culture and infection**

Peripheral blood mononuclear cells (PBMCs) were purified from healthy donor blood packs (Red Cross Blood Bank, Melbourne, Australia) by Ficoll-Plaque PLUS (GE Healthcare) gradient centrifugation. For PBMC infections, cells were stimulated with 2.5 μg/ml PHA (Remel) for 3 days in RPMI 1640 medium (Gibco) supplemented with 10% FBS and IL-2 (10 U/ml, Roche). Infected cells (20 pg p24 per 10^6 cells) were fed every 3–4 days with fresh medium supplemented with 10U/ml IL-2 (47), and virus production was monitored by measuring p24CA titres in the culture fluid and TCID50 in TZM-bl cells. Cells were also harvested at each media change for WB, US (pol) and MS (tat1) RNA quantification by ddPCR. The ddPCR mix consisted of 12 μl 2x ddPCR super mix for probes (no dUTP, Bio-Rad); 900 nM of each primer; 250 nM probe (FAM-MGBNFQ, Applied Biosystems, Supplementary Table S1) and up to 7.2 μl of cDNA (50–140 ng) into 24 μl final volume. Ribonuclease P/MRP 30 kDa (RP30) was used as a reference gene (HEX, Bio-Rad). Following droplets generation (15–18 000 on average), thermal cycling were conducted as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 60 s, followed by 98°C for 10 min (ramp rate 2°C/s for each step) on a C1000 Touch Thermal cycler (Bio-Rad). The droplets were subsequently read by a QX200 droplet-reader (Bio-Rad) and the data were analysed with QuantSoft 1.7.4 software. The limit of detection of our assay was of 0.5 copies/μl. Integrated HIV DNA was also quantified from extracted genomic DNA (AIQamp DNA Mini kit, Qiagen) using a nested real-time Alu-LTR assay, as described previously (48). HIV fragments (nt 5590–6042) were PCR amplified, gel extracted (Marchery-Nagel) and sequenced by BDV3.1 cycle sequencing reactions (AGD, Department of Pathology, UoM) using primers listed in Supplementary Table S1. Competition assays started with equal amount of virus particles. The virus was cultured for 2 weeks then passaged onto fresh activated PBMCs and maintained in cul-
ture until virus-induced syncytia (day 28). The integrated proviral DNA isolated from infected cells (day 28; output) and input (day 1) virus were sequenced, as described above.

A total of 10⁶ Jurkat T cells were infected with 40 and 80 ng of viral particles via spinoculation at 1500 g for 2 h at 23°C, followed by 72 h of incubation at 37°C.

CD4+ T cells were purified from PBMCs by negative selection using CD4+ T cell isolation kit (Miltenyi Biotec), and cultured in RPMI 1640 medium supplemented with 10% FBS and 30 U/ml IL-2. Cells were activated with anti-CD3/CD28 (coated 1 μg/ml OKT3 – soluble 0.5 μg/ml L293, BD) for 48 h and infected by spinoculation at 1200 g using 90 and 180 ng DuoF (+ Env 92HT593.1; #3077, NIH reagents) per 10⁶ cells. At 72 h after infection, cells were washed and stained with Near-IR Live/Dead fixable dead cell staining (Invitrogen), anti-CD4 OKT4 BV650, anti-CD3 UCHT1 A700 according to the manufacturer’s instructions. Cells were fixed with 1% formaldehyde and analysed on a Fortessa (BD) flow cytometer.

Resting CD4+ T cells were isolated by magnetic bead depletion (goat anti-mouse IgG microbeads, MACS) and cell sorting using a cocktail of antibodies (49), with purity >95% as assessed by LSRII flow cytometer using anti-CD4 OKT4 FITC or RPA-T4 PE, CD3 UCHT1 A700, HLA-DR TU36 PE-Cy5, CD69 L78 APC (BD Biosciences) and Live/Dead Near-IR. Purified resting CD4+ T cells were treated on a Fortessa (BD) flow cytometer.

In vitro splicing assays

In vitro splicing assays were performed with HeLa cell nuclear extracts using 10 fmol of uniformly [32P]-labeled L3U1 pre-mRNA per assay with or without the addition of recombinant SRSF2 protein (300 ng), in the conditions previously described (40). Splicing products were fractionated by electrophoresis on a 5% polyacrylamide/8 M urea gels. The experiments were repeated three times using different preparations of pre-mRNA transcript. Splicing efficiencies (MA3/P, amount of Mature/Pre-mRNA) were established by quantifying the radioactivity using a Typhoon 9410 PhosphorImager, the ImageQuant Software Version 5.2 (1999) Molecular Dynamics. The fold activation of splicing by SRSF2 was estimated by dividing the MA3/P ratio in the presence of the recombinant protein by that obtained in its absence.

Translation assays

TZM-bl and HeLa cells were transfected respectively with equimolar amounts of G- or A-capped and polyadenylated tat and tat-lucF RNAs (2.5 μg) using DMRIE-C reagent (1:2.5, Invitrogen). LucRenilla RNAs (1 μg) were co-transfected for normalisation of luciferase activity. Cells were collected 48 h after transfection and divided into two parts for RNA and protein extractions. Translation levels were monitored by a dual-luciferase reporter assay (Promega) using equal amounts of protein lysate (20 μl per condition) on a FLUOstar plate reader. Each assay was carried out in triplicates with a minimum of three independent transfections. For RNA stability assay, cells were treated with actinomycin D (5 μg/ml) for 4 h before collection. Total cellular RNAs were isolated using the TRizol Reagent (Invitrogen) and treated with 2 U of RQ1 RNase-Free DNase (Promega). One μg of total RNA was reverse-transcribed using Omniscript-reverse transcriptase (Qiagen) and d(T)15 primer following the manufacturer’s specifications. Real-time PCR was performed on CFX Connect real-time PCR detection system (Bio-Rad) using FAST SYBR Green Master Mix (Life Technologies) and analysed with CFX Manager 3.0 software. Quantitative PCR (qPCR) analysis of tat transcripts was carried out in duplicates using specific primer pairs (Supplementary Table S1). Each sample was normalised on the basis of GAPDH mRNA content. When mentioned, the luciferase activities (lucF/lucR) were normalized on tat mRNA levels.

Analysis of HIV<sub>def</sub> proviruses

Sequences of defective proviruses were obtained by single-genome amplification and sequencing from four previously published studies (6,7,21,22). GenBank accession numbers for HIV<sub>def</sub> proviruses used in our analysis were reported in (21) (355 sequences, with accession numbers KX505390 to KX505744), (7) (220 sequences, KF526120-KF526339), (22) (208 sequences, KU677989-KU678196 and (6) (448 sequences, KY766193–KY778681 and KY766150–KY766212). Acute, chronic, non-induced, induced, PVL >40 and PVL <40 were defined as previously described in the corresponding studies.

Sequencing reads were first aligned and compared to the reference genome HXB2 using HIVAlign (MAFFT) (52) on the Los Alamos database. Clones that did not align to tat-exon4 (Tat-) were excluded from further analyses. Hypermutations (indels, syn or non-syn, codon stops) were determined using the Tat+ clones and the Los Alamos Highlighter (53) and SNAP v2.1.1 (54) algorithms. Heatmaps were generated using GraphPad Prism 7, while sequence conservation and probability (frequency of each nucleic acid at that position) were generated using WebLogo (55). Secondary structure propensity profiles of RNA sequences at single nucleotide resolution were determined with CROSS (56). A score in the range (0.1) means that the nucleotide is predicted to be paired, while a score in the range (−1.0) indicates that the nucleotide is single stranded.
RESULTS

HIV\textsubscript{def} proviruses harbour intact Tat coding region

Patient derived cells containing HIV intact and defective proviruses transcribe viral pre-mRNAs \textit{in vitro} and \textit{ex vivo} containing active and defective splice donors (SD) and acceptors (SA) whose use during mRNA splicing leads to the production of RNA splice variants (23). We questioned whether aberrant spliced HIV mRNAs could encode the essential HIV Tat protein needed to restore productive viral replication. To examine the potential for HIV\textsubscript{def} to produce Tat protein, we first sought to quantify the level of intact Tat ORF in the HIV proviral DNA from four previous studies of HIV infected individuals using limiting dilution then sequencing of full-length proviruses (6,7,21,22). The proportion of Tat\textsuperscript{+} clones that harbor HIV exon 4 and Tat ORF was low (32.33\%, 398 clones out of 1231, Figure 1A). While the majority of these proviruses presented a mutated Tat start codon or internal stop codons, only a small subset of clones presented intact Tat ORFs and a start codon (20–43.5\%) (Figure 1A). As previously reported, the proportion of hypermutated proviruses (HIV\textsubscript{hypermut}) accumulates rapidly during acute infection (6,7,21,22), simultaneous with the higher frequency detection of insertions-deletions (indels) and pre-mature stop codons (Supplementary Figure S1A, B). In addition, the non-induced clones and patients with low proviral loads (PVL<40 copies per milliliter) presented a higher rate of indels when compared to those from the induced and high PVL (PVL > 40 copies per milliliter) clones. Most indels were detected in the C-terminal region of Tat that has a low impact on Tat trans-activation (57), coinciding with a higher proportion of non-synonymous mutations (non-syn) at the C-terminus (20–43.5\%) (Figure 1A). On the other hand, the N-terminus segment of Tat needed for trans-activation activity of patient provirus had a relative absence of indels and higher frequency of synonymous (syn) mutations (Figure 1B, Supplementary Figure S1C). On the other hand, the N-terminus segment of Tat needed for trans-activation activity of patient provirus had a relative absence of indels and higher frequency of synonymous (syn) mutations (Figure 1B, Supplementary Figure S1D), implying a positive selection of Tat residues encoded by tat-exon4 (Figure 2A, highlighted in gray). These data suggest a relative conservation of the known Tat trans-activation domain within defective proviruses and mRNAs, and potentially any embedded mRNA elements.

Conserved stem–loop structure in Tat coding region

HIV proviruses use alternative splice sites to produce mRNA transcripts that code for essential viral proteins, such as Tat (58). However, HIV 3′ splice sites (3′ss) are suboptimal and are frequently included into stable RNA structures (59). However, while HIV\textsubscript{def} proviruses can be transcribed, it remains unclear whether these could be spliced efficiently. To analyse the potential splicing of tat mRNA from HIV\textsubscript{def} transcripts, we used the approach of computational recognition of secondary structures (CROSS) to profile tat RNA structure compared to HXB2 reference strain at a single nucleotide resolution (56). Despite presenting conserved A3 splice site (SA3) and polypyrimidine sequences compared to HXB2 SA3 consensus (Supplementary Figure S1E), major differences were observed in ribonucleotides pairing into tat mRNA structure overlapping the tat-exon4 (highlighted in grey, Supplementary Figure S1F). These changes were predominantly in the N-terminal region of tat-exon4 that are unique to Tat coding mRNA, but were not apparent in distal HIV coding regions that overlap with rev and nef mRNAs (Figure 2A). This warranted a deeper investigation of the role of potential conserved mRNA structure in the different multiply spliced RNAs.

We compared the relative level of predicted conserved RNA structure (sfc) and nucleotide sequence (nfc) within multiply spliced mRNAs of 34 replication competent HIV strains from different viral subtypes (Figure 2B). This analysis indicates the RNA regions common between 2 kb tat, rev and nef mRNAs share both conserved predicted folded structure and sequence. As expected, the 5′ and 3′ untranslated regions (UTRs) contained a greater proportion of conserved RNA structure than primary sequence. These terminal regions contain conserved RNA structures with known functions, such as trans-activation response (TAR) RNA domain known for Tat-binding. The TAR displays an unpaired RNA motif embedded into stable paired RNA structure in this analysis (Figure 2B). In contrast, the splice donor (D1) and acceptors (A3, A4a and A5) presented a greater proportion of conserved sequences (Figure 2B). This analysis revealed another interesting and unique stem–loop RNA element underlying the N-terminal region of the Tat ORF (nt 5440–5455 HXB2, Figure 2) fulfilling the features for a functional element with highly conserved RNA structure. We called this element TIM-TAM (for Tat IRES Modulator of tat mRNA), as it was included in the 2kb mRNA for tat, but not rev and nef mRNA. However, this element was also present in the previously reported HIV genomic RNA structure (60) (Figure 2C). RNA structure prediction and thermodynamic analysis revealed folding of TIM-TAM into a stem–loop structure displaying an apical tetraloop. Furthermore, comparing 2504 HIV isolates in the Los Alamos HIV sequence database revealed this element had low-level sequence changes within the stem (Figure 2D and Supplementary Figure S2A). Most mutations were detected within the tetraloop and therefore did not have a drastic effect on the predicted thermodynamic stability of this stem–loop (Supplementary Figure S2B, C). Altogether, our results indicate highly conserved stem–loop TIM-TAM structure underlying the Tat ORF, and this is also likely to exist in aberrant mRNAs produced from HIV\textsubscript{def} proviruses that accumulate during ART.

TIM-TAM activates Tat IRES translation, but restricts cap-dependent translation

Due to the close proximity to the A3 3′ss, we first examined the role of this stem–loop in mRNA splicing \textit{in vitro} using the L3U1 construct (40), which harbours tat-exon1 and 4. Deletion or inversion of TIM-TAM in this reporter construct did not significantly affect the splicing at site
A3 (MA3/P 0.2–0.31) or activation by the splicing factor SRSF2, commonly known as SC35 (fold activation 15.6 or 14.5 instead of 16.7, Supplementary Figure S3A). Because RNA structures also play an essential role in modulating protein translation and especially IRES activity, we next examined the ability of this conserved sequence-structure to modulate Tat production through the IRES mechanism. We used in vitro transcribed and ribosome-scanning active G-capped or inactive A-capped polyadenylated tat RNAs to test for IRES translocation activity. RNA transfection of G- and A-capped RNA into TZM-bl cells allowed us to distinguish between cap- and IRES-dependent translation of Tat through expression of Luciferase Firefly (LucF) from an integrated reporter provirus (Figure 3A). Our results confirmed the production of Tat protein through an IRES-mediated mechanism, although at a 30% efficiency of the G-capped mRNA substrate for ribosome scanning. IRES-expressed Tat was functional and transactivated transcription at the HIV 5′LTR (Figure 3A).

To further investigate the role of the TIM-TAM stem–loop in Tat IRES translation, we inserted silent point mutations (Silaa mut) that significantly disrupted the RNA structure as predicted by mfold (ΔG = −2.6 kcal/mol for Silaa mut and +3.1 kcal/mol for ΔTIM-TAM versus −7.7 kcal/mol for the WT sequence, Figure 3B), however did not affect the amino acid sequence of Tat protein. RNA structure mapping of Silaa and ΔTIM-TAM mutants using SHAPE reactivity revealed the introduced changes had a significant impact on tat-exon4 SHAPE reactivity and RNA structure compared to the WT RNA (Figure 3C). These RNA structure studies show a dominant role for the TIM-TAM stem–loop in overall tat mRNA folding.

When in vitro transcribed A-capped and polyadenylated Tat-luciferase Firefly RNA was transfected into reporter cells, a significant reduction in Tat expression was observed for the Silaa mut and ΔTIM-TAM mutants due to a loss in IRES activity, confirming a role of the TIM-TAM stem–loop in Tat IRES-dependent translation (Figure 3D). As both Silaa and ΔTIM-TAM mutations induce drastic changes to tat mRNA structure, it is plausible that these effects on Tat translation might be due to changes in the accessibility of ribosomes to Tat initiation codon. While TIM-TAM conferred a cap independent translation initiation activity, silent point mutations that disrupted the RNA structure induced a paradoxical increase in Tat-Firefly luciferase cap-mediated translation (G-cap + pA, Figure 3D). This effect was not observed when we deleted a distal conserved sequence motif, Δ11 (nt 5373–5383 BRU), located in the vicinity of Tat start codon (Supplementary Figure S3C). We have previously shown Δ11 as a binding motif for various SR and hnRNP proteins, such as SRSF2, SRSF5 and hnRNP A1 proteins (61) that play a key role in splicing (40,61) and translational control of tat1 mRNA (62). These data demonstrate a second role of the stable stem–loop structure in blocking efficient ribosome progression during the cap-dependent translation initiation of tat RNAs. Therefore, TIM-TAM confers moderately efficient cap-independent translation activity for Tat and additionally impedes fully efficient cap-dependent Tat translation.

**TIM-TAM required for an authentic tat mRNA folding**

Due to the strong sequence-structure conservation and role in Tat production, we hypothesised that TIM-TAM may be formed both in unspliced genomic RNA and spliced tat1 RNAs. To test this, we studied in parallel tat1 premRNA and mRNA 2D structure, as the surrounding sequences might influence the folding of TIM-TAM. Very similar profiles of enzymatic digestions by RNase T1, T2 (for single-stranded) and V1 (double-stranded), as well as chemical modifications by DMS, CMCT and Kethoxal (for single-stranded) were observed for both RNA. Representative examples of the primer extension analyses of tat1 mRNA are provided in Supplementary Figure S5. Furthermore, to test if TIM-TAM interacts with neighboring HIV RNA sequences, a smaller tat1 RNA that included only tat-exons1/4 and a larger RNA, tat2, harboring an additional exon upstream of A3 3′ss (exons1/2/4, nt 4494-
Figure 2. Conserved stem–loop structure in Tat coding region. (A) Schematic representation of HIV-1 genome and a sample of the mRNAs produced during early (1.8 kb) and late phases (4 kb) of infection. ORFs are represented by white boxes, while LTRs are in black. HIV-1 genome contains four major donor (D) and 8 acceptor splice sites (A). The cis regulatory element TIM-TAM is highlighted in red. (B) Secondary structure propensity profiles of multiply spliced mRNAs. Structure (sfc) – sequence (nfc) conservation and pairing probabilities (unpaired, paired) of tat1, rev2 and nef2 mRNAs are shown with 8 nt window employed for smoothing. Structure conservation over sequence is highlighted by (sfc.nfc)/nfc2 values above 1, while values below 1 indicate a preferential sequence conservation over structure. Known structured regions in the 5′ and 3′ UTR regions are indicated above. (C) Secondary structure of TIM-TAM within the HIV RNA genome determined by (J. Watts et al., 2009). (D) Sequence conservation of TIM-TAM (nt 5440–5455, BRU) within 2504 HIV strains on the Los Alamos HIV databases. HXB2 strain was used as a reference.
Figure 3. TIM-TAM activates Tat IRES translation. (A) Ability of tat mRNA to induce Tat production in a cap-independent manner. RNA transfection of TZM-bl cells with G- or A-capped and polyadenylated tat mRNAs followed by luminescence assay 48 h post-transfection. Cells were co-transfected with luciferase Renilla RNAs where results are reported as relative luciferase Firefly activity over Renilla (lucF/lucR). Data represent mean ± SEM, n = 6. (B) WT, silent mutant (Silaa mut) and ΔTIM-TAM RNAs used in this study. Silent point mutations introduced in TIM-TAM are represented in red. The free energies (ΔG in kcal/mol) were calculated using MFold webserver (37°C in 1 M NaCl). (C) Effect of TIM-TAM substitution or deletion on tat2 mRNA structure. 2D structure of the stem–loop harbouring Tat start codon and TIM-TAM is represented on top of this panel. The cis regulatory elements; ESS2 (in blue), ESE2 and 3’loop II (in brown), located within this stem–loop are shown. SHAPE reactivities of WT, Silaa mut and ΔTIM-TAM mRNAs are represented as a function of nucleotide position surrounding TIM-TAM over a window of nt 5384 and 5514 (BRU). Nucleotides are colored by their respective shape reactivities, with highly reactive nucleotides report flexible positions (SHAPE reactivity ≥ 0.7 in red and 0.4 ≤ SHAPE reactivity > 0.7 in orange), while low reactive nucleotides are represented in grey (SHAPE reactivity < 0.4). Arcs indicate highly probable helices. Data represents mean ± SEM. Statistical significance was determined using Friedman test. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. (D) Influence of silent point mutations or deletion of TIM-TAM on Tat cap and IRES dependent translation. HeLa cells were co-transfected with Renilla luciferase RNA (G-cap+pA), and results are reported as relative luciferase activity (lucF/lucR) compared to WT RNA, defined as 1. Data represents mean ± SEM, n = 5. Statistical significance was determined using two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
TIM-TAM controls latent infection and virus reactivation

Tat protein exerts an important role during latent infection, as low levels of the protein or nuclear retention of the multiply spliced RNAs that encode Tat contribute in maintaining the cells in a latent state (63). One of the other hand, Tat delivery into latently infected cells can inhibit the establishment of HIV latency (11), and thus induce virus reactivation (64). To investigate the role of TIM-TAM during latency, we used a dual color HIV reporter virus, R7GEmC, that contains an LTR-dependent EGFP and an mCherry constitutively expressed from an EF1α promoter, independently of the HIV 5′LTR activity. This single round HIV reporter distinguishes between productively (eGFP+ mCherry+) and latently (mCherry+) infected cells (65). When infections of Jurkat T cells were carried with the dual colour reporter virus harboring the silent mutation, a drastic reduction in the single mCherry+ population was observed (1.27% for WT versus 0.32% for Silaa mut, Figure 7A), independent of the virus titre used (Supplementary Figure S7F), indicative of a restriction on the establishment of a latent infection in the absence of full TIM-TAM function.

We then tested these viral constructs in primary CD4+ T cells and their ability to generate latent infection. Once
Figure 4. TIM-TAM required for an authentic tat mRNA folding. (A) 2D mRNA structure of full-length tat2 mRNA determined by SHAPE. The SHAPE reactivities were superimposed on the 2D model with highly reactive nucleotides report flexible positions (SHAPE reactivity ≥ 0.7 in red), while low reactive nucleotides represent paired nucleotides (SHAPE reactivity < 0.4 in grey). (B) Secondary structure model of tat2 mRNA in the vicinity of Tat start codon (nt 5377–5514) that we established by SHAPE. Nucleotides are colored as in (a), according to their SHAPE reactivity. (C) Model of tat1 mRNA structure around Tat start codon (nt 5377–5514) that we established by chemical (DMS, CMCT, Kethoxal) and enzymatic (RNase T1, T2, V1) modifications. RNase T1, T2 and V1 cleavages are represented by arrows surmounted by triangles, circles and squares, respectively. Nucleotides modified by DMS, CMCT or Kethoxal are circled. Colours of the arrows and circles indicate the yield of cleavage and modifications: white, green, orange and red for very low, low, medium and strong cleavage or modification, respectively. Positions are numbered in reference to HIV-1 BRU (K02013).
Figure 5. Ability of TIM-TAM to induce Tat translation from full-length genomic RNA. TZM-bl cells were electroporated with capped gRNA (+IN D116N) and luminescence assay performed 24 h post-transfection. The results are reported as relative luciferase activity (lucF/lucR). Bars show mean values and SEM (*n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant (two-tailed Student's t-test).

more, maximal productive infection was impeded by the full TIM-TAM structure (WT 13.2% versus Silaa 15.5% eGFP + mCherry+), while mutations in TIM-TAM led to a significant reduction in latent infection (WT 0.43% versus Silaa 0.30% mCherry+, Figure 7B). A significant reduction of latentproductive infection ratio was also observed when TIM-TAM was mutated (Figure 7C), confirming a role of TIM-TAM in modulating Tat translation during latent infection, and therefore important in the establishment and maintenance of latency.

We next evaluated the extent of virus reactivation from latently infected cells by phorbol myristate acetate and phytohemagglutinin (PMA/PHA) stimulation in the presence WT or mutated TIM-TAM. This was tested in the CCL19 primary model of latency described previously to display the broadest response to reactivation stimuli compared to other latency models (66). The CCL19 model most closely resembles resting CD4+ (rCD4+) T cells from aviraemic patients (66). In this assay, rCD4+ T cells were pre-treated with the CCL19 chemokine and infected with NL4.3 WT or Silaa (MOI 0.1) (49,50). Four days post-infection, rCD4+ T cells were stimulated with PMA/PHA and virus reactivation was quantified by measuring the RT activity in the culture supernatants, as well as the cell associated US RNA levels (Figure 7D). Analysis of RT activity at day 5 post-infection of activated, resting and CCL19 treated cells revealed minimal virus production in CCL19 treated infected CD4+ T cells, consistent with latent infection (data not shown). Interestingly, the lack of cap-independent translation in the case of Silaa mut induced a significant reduction in virus reactivation in the majority of donors tested. Altogether, these results suggest an essential role of TIM-TAM and Tat IRES translation in controlling latent infection and virus reactivation.

DISCUSSION

We have identified a highly conserved RNA structure, TIM-TAM, underlying the ORF in the first coding exon of Tat that reduces the efficiency of Tat protein translation from conventional tat mRNA which pushes the virus towards latency. Our data also support a novel mechanism for translation of Tat from unconventional viral RNAs. TIM-TAM assists in the pioneer round of Tat expression by a cap-independent mechanism from unintegrated 9 kb unspliced genomic RNA promoting establishment of productive viral replication (Figure 8). In addition, this alternative translation mechanism could permit Tat expression from HIV<sup>def</sup> proviruses retaining the TIM-TAM RNA element and the Tat ORF. Furthermore, other aberrant chimeric host-HIV RNAs containing TIM-TAM may facilitate production of viral peptides that impact upon ongoing immune activation and temporal changes in the structure of the latent HIV reservoir during treated HIV disease (24).

The role of TIM-TAM in modulating Tat expression provides a pathway whereby unconventional sources for HIV RNAs may contribute to viral expression, replication and pathogenesis. Our results support a mechanism to prioritize Tat expression from any Tat ORF-containing RNA arising, even from the unintegrated HIV DNA at the earliest stages of infection. The TIM-TAM would permit Tat expression through canonical and/or cap-independent translation, thereby promoting high viral gene expression and productive replication upon completion of provirus integration. These results complement earlier studies that have demonstrated the ability of unintegrated HIV DNA or HIV<sup>def</sup> integrase mutants to serve as a template for antigen production in activated and resting CD4+ T cells (67,68), as well as macrophages for as long as 20 to 30 days (69,70).

TIM-TAM functions may also impact on viral rebound after treatment interruption. For example, in patients un-
Figure 6. TIM-TAM controls productive infection. (A) Virus titres of single-round WT and Silaa mutant proviral clones pDR-ΔEnv Nef-EGFP pseudotyped with AD8 Env. EGFP was used for normalisation of transfection efficiency. Virion production was quantified by p24CA-ELISA of the supernatant 72 h post-transfection (mean ± SEM, n = 5). (B) Viral infectivity of single round EGFP virus was assessed by infection of PHA stimulated PBMCs with equal amounts of viral particles (20 ng per 10⁶ cells). Viral infectivity was then determined by p24CA-ELISA of the supernatant harvested 72 h post-infection (mean ± SEM, n = 8). (C) Western blot quantification of the HIV Tat protein and the endogenous protein GAPDH in the infected PBMCs with single-round infectious clones. (D) Virus kinetics of HIV pNL4–3 WT and silent mutant. Activated PBMCs from four healthy donors were infected with equal amount of pNL4–3 WT and Silaa mut viral particles. Supernatants were collected several days post-infection and assayed for virion production by infection of TZM-bl cells over a six-point series dilution with equal volumes of the supernatant harvested at the different time points. Luciferase activity (RLU) was measured 48 h post-infection of TZM-bl cells. The graph is representative of one kinetic infection of PBMCs. All four independent infections gave similar kinetics. (E) Western blot quantification of the HIV p24, Pr55Gag, Env SUgp120 (αHIV-sera) and Tat proteins, and the endogenous protein GAPDH in the infected PBMCs with pNL4–3 virus several days post-infection (dpi). For panels A, B, statistical significance was determined using two-tailed Student’s t-test, while panel D, One Way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

 Undertaking integrase inhibitor therapy, RNAs arising from episomal unintegrated 1- and 2-LTR circular HIV DNAs (71,72) that abundantly accumulate in macrophages (73), or in infected T cells (74), lymphoid tissues and the brain during therapy (75,76). Tat sequences in rebounding viruses are highly similar to sequences found in unintegrated episomal DNA (77). Unintegrated HIV DNA is gradually silenced into repressive episomal minichromatin, however it remains inducible by HDAC inhibitors and Tat to promote persistent viral DNA transcription in primary macrophages (78). Thus, unintegrated DNA may serve as residual viral reservoir in resting T cells and macrophages. Indeed, when stimulated in the presence of cytokines, low-level viral replication can occur from unintegrated DNA (79–81).

Our data demonstrate a dual mode of action for TIM-TAM with a moderate IRES-dependent translation activity and a strong block of cap-dependent Tat translation due to a stable stem–loop structure (Figure 8). TIM-TAM impedes Tat cap-dependent translation by serving as a thermostable barrier to progressive ribosomal catalysis of amino acids needed for expression of Tat from large levels of 2 and 4 kb HIV mRNAs expressed in the early phase of productive HIV replication.

The TAR stem–loop present in all viral transcripts can also impede translation either by blocking access of translation initiation factors to the 5′-cap structure (82) or by activating the dsRNA-dependent protein kinase R (PKR) (83–85), which results in phosphorylation and inactivation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) to inhibit translation initiation. TAR RNA folds into a stable stem–loop structure that engages with double-stranded RNA binding proteins (dsRBPs), such as PKR (86–88), RNA helicase A (RHA) (89), nuclear factor 90 (NF90) (90,91), TAR RNA-binding pro-
Figure 7. TIM-TAM controls provirus reactivation from latency. (A) Dot plot of mCherry+ population from Jurkat T cells 3 days post-infection with 40 or 80 ng of R7GEmC WT or Silaa virus. (B) Infection profiles of stimulated primary CD4+ T cells infected with R7GEmC WT or Silaa virus. Double-positive (eGFP+mCherry+, actively infected) and single positive (mCherry+, latently infected) cells are shown. Data shown are from a single donor, however are representative of four separate donors. (C) Quantification of productive infection (%eGFP+mCherry+) and ratio of latent to productive infection (%mCherry+/%eGFP+mCherry+) from panel (b). Data represents the mean ± SEM of four donors. * P < 0.05, ** P < 0.01 (two-tailed Student’s t-test). (D) Diagram of the experimental procedure used for infection and re-stimulation of CCL19 treated resting CD4+ T cells. Resting CD4+ T cells were treated for 1 day with CCL19 (30 nM) before infection with WT or Silaa mutant pNL4–3 virus. The infected cells were then cultured with media containing 1 U/ml IL-2 for 4 days before re-stimulation with PMA/PHA (10 μg/ml, 10 nM respectively). PHA activated feeder PBMCs depleted of CD8 T cells were added 24 h after stimulation to enhance detection of virus reactivation. Virus production was quantified by measurement of the RT activity in the culture supernatant at day 10, as well as the cell associated unspliced RNA levels (US RNA copies/μl).

Our sequence and structural analysis revealed that TIM-TAM stem–loop presents an apical tetraloop, with conserved AG nucleotides at position 5446–5447 for subtype B (Supplementary Figure S2). More work is needed to define whether TIM-TAM folds into an AGNN tetraloop and serves as a platform for the binding of dsRBPs such as PKR and thus permitting a common mode of recognition of AGNN tetraloops by proteins with dsRNA binding domains (dsRBD). Moreover, extended RNA helix is required for PKR activation (95,96) and therefore, it would be interesting to assess the potential of TIM-TAM in PKR recruitment and activation through the potential interactions between TIM-TAM and TAR elements that may lead to an extended dsRNA helix structure that is optimal for
Figure 8. Model depicting TIM-TAM roles in controlling Tat expression. (1) At early rounds of infection before provirus integration, TIM-TAM enhances Tat translation from genomic RNA through an IRES-dependent mechanism thus providing an immediate early expression of Tat to pioneer its viral trans-activation functions. (2, 3) By controlling Tat translation, TIM-TAM impacts on timing and level of Tat protein expression at the early and late phase of the virus lifecycle to control HIV production and infectivity. (3a, 4a) Due to the strong structure stability, TIM-TAM impedes fully efficient cap-dependent Tat translation but confers moderately efficient IRES-dependent translation activity from spliced \( \text{tat} \) mRNA. Thus, TIM-TAM acts as a switch for HIV productive and latent infection. (4) Tat IRES enables non-conventional viral mRNA to express HIV peptides or Tat protein to assist reactivation from latency in the presence of latency reversing agents (LRA).

PKR dimerisation and activation for Tat translation inhibition. A similar model of dynamic refolding of interferon-\( \gamma \) (IFN-\( \gamma \)) mRNA enables it to function as a translation template and as a PKR activator preventing cytokine expression (97). Interestingly, the structured TIM-TAM element is located downstream of the authentic Tat AUG initiation site, therefore Tat expression would require the dynamic folding of \( \text{tat} \) mRNA and/or the ‘backward’ scanning of the 40S small ribosomal subunit. This aforementioned phenomenon has been recently reported for HIV-1 (Gag p40-p55) (98) and HIV-2 (Gag p57-p50 and p44) (99) Gag IRES elements.

\textit{In vivo}, the residual provirus remaining on ART varies widely depending on the individual and the reservoir site (26). Latently infected cells are enriched with Tat variants comprising impaired transactivation activity with several of the mutated residues (P10, W11, K/W12, G15) located within TIM-TAM (100). Given that the frequency of HIV infected cells increases over time due to clonal expansion of latently infected CD4+ T cells (3,4,101), and the majority of the HIV-1 proviruses are defective (7,21), an IRES activity embedded within the TIM-TAM motif is an important determinant of whether or not any of these defective proviruses could lead to the production of viral proteins in the setting of prolonged viral suppression. As HIV integration plays an important role in the expansion and persistence of latently infected cells, further studies are needed to determine the role of Tat-IRES mediated translation on the expression of the adjacent cellular genes including \( \text{MKL2, BACH2, STAT5B, and HORMAD2} \) that have been linked to clonal expansion (3,4,101).

Recent studies revealed that HIV\(_{\text{def}}\) proviruses may bypass major splice donor defects to produce novel HIV RNA transcripts that can be translated \textit{in vitro} and \textit{ex vivo} (22,23), and become targets for HIV-specific cytotoxic T lymphocytes (CTLs) (23). Given that Tat is associated with T cell activation, and even if a small number of latently infected cells contained intact Tat sequence and function, Tat expression through IRES-dependent translation may drive the ongoing immune activation and pathogenesis (22,23). Alternatively aberrant mRNAs that capture the TIM-TAM element, such as may occur from deleted proviruses, or aberrantly spliced viral mRNAs, or even aberrant HIV–cellular mRNAs may result in viral peptide expression that enables noise driven events (102) and thus shapes the structure of the latent HIV reservoir (22–24).

Tuning Tat levels could be sufficient to control HIV expression and therefore used to ‘flush out’ the latently infected cells independently of the cell activation state (64,103). On the other hand, inhibition of Tat expression and/or activity could induce a prolonged transcriptional silencing, hence preventing viral rebound and replenishment of the reservoir by maintaining a permanent latent state. Indeed, the use of didehydro-cortistatin A (dCA), a Tat dependent transcription inhibitor, significantly diminished the capacity of virus reactivation from latently infected cells (92% of \( \alpha \text{CD3/CD28} \) and hence the size of the viral reservoir (104,105). Thus, TIM-TAM constitutes a potential drug target for residual Tat expression and latency reversal, which can be targeted by new generation of latency reversing agents such as the 2-acylaminothiazole class that activates Tat expression in HIV-1 latency models (106).
conclusion, Tat IRES-translation may shape the outcome of provirus reactivation from latency.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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