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Chapter Six

Tat-dependent production of an HIV-1 TAR-encoded miRNA by a non-canonical miRNA pathway

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Abstract
Evidence is accumulating that retroviruses produce microRNAs (miRNAs). To prevent cleavage of their RNA genome, retroviruses have to use an alternative source as miRNA precursor. The HIV-1 transacting responsive (TAR) RNA element has been suggested as a possible source for miRNAs, but how these miRNAs are produced without impeding virus replication remained unclear. We used deep sequencing analysis of AGO2-bound HIV-1 RNAs to demonstrate that the 3’ side of the TAR hairpin is processed into a miRNA. We demonstrate that non-processive transcription from the HIV-1 LTR promoter and subsequent RNA polymerase II pausing results in the production of short TAR RNAs that serve as precursor for the miRNA. Tat binding to TAR, which induces a conformational change in the hairpin structure, is required for further processing into miRNA by Dicer. This non-canonical pathway for miRNA biogenesis allows HIV-1 to produce the TAR miRNA without cleavage of the RNA genome.

Significance statement
MicroRNAs (miRNAs) are ~21-nt RNAs that have important regulatory roles by targeting mRNAs for cleavage or translational repression in the RNA interference (RNAi) pathway. These miRNAs are derived from short stem-loop RNA structures within long primary transcripts that are usually recognized and cleaved by microprocessor and Dicer. Several DNA viruses produce miRNAs, but for retroviruses like HIV-1 it has remained controversial whether they encode miRNAs (1). The fact that miRNA production would result in degradation of the viral RNA genome and impede HIV-1 replication, was long considered a strong argument against HIV-1 encoded miRNAs. However, in this study we demonstrate how HIV-1 can produce a functional miRNA via a novel pathway without hampering virus replication.
Introduction

MicroRNAs (miRNAs) are small RNAs (~21 nt in size) that have important post-transcriptional regulatory roles by targeting messenger RNAs (mRNAs) for cleavage or translational repression (1). These miRNAs are derived from stem-loop RNA structures within long primary miRNA transcripts (pri-miRNAs). In the canonical pathway, recognition and cleavage of the pri-miRNA into a precursor miRNA (pre-miRNA) hairpin is mediated by the nuclear microprocessor complex that consists of at least two subunits: the ribonuclease (RNAse) III Drosha and the double-stranded RNA (dsRNA)-binding protein DGCR8 (DiGeorge syndrome critical region protein 8) (2-6). The stem-loop structure in the pri-miRNA is recognized by DGCR8 and Drosha cleaves both strands at ~11 basepairs (bp) from the base of the stem (7). The pre-miRNA is exported to the cytoplasm and further processed by the RNase III Dicer (8, 9). Dicer cleaves both strands of the pre-miRNA at approximately 2 helical turns (~21 nt) from the Drosha cleavage sites (1, 10). The guide strand of the double-stranded miRNA is loaded into the Argonaute2 (AGO2) protein, which is part of the RNA-induced silencing complex (RISC) to target a complementary mRNA, while the passenger strand is degraded (11-18).

Several viruses encode miRNAs, including DNA viruses of the Herpesviridae (19-21), Polyomaviridae (22-24) and Adenoviridae (25, 26). For retroviruses like human immunodeficiency virus type-1 (HIV-1), it has remained controversial whether they encode miRNAs. Retroviruses are RNA viruses with a DNA replication intermediate that is transcribed by RNA polymerase II (RNAP II) in the nucleus. Microprocessor and subsequently Dicer will be able to access and process the viral RNAs. However, this canonical miRNA processing will lead to RNA genome cleavage and inhibition of virus replication (27). Recent studies revealed that bovine leukemia virus (BLV) (28, 29) and bovine foamy virus (BFV) use a non-canonical pathway to produce miRNAs. These retroviruses encode sub-genomic RNA polymerase III (RNAP III) transcripts that serve as pre-miRNAs, thus avoiding degradation of their RNA genome. Several studies that focused on the identification of HIV-1 encoded miRNAs did however not consistently detect miRNAs (30-38).

The 57-nt transacting responsive (TAR) element present at the 5’ and 3’ end of all HIV-1 RNA transcripts folds a stable hairpin structure that has been implicated in miRNA production (Fig. 1). The best-studied function of the 5’ TAR element is its essential role in the activation of transcription from the long terminal repeat (LTR) promoter in the proviral genome (39). In the absence of the viral Tat transactivator protein, RNAP II initiates transcription but the nascent TAR RNA structure causes pausing and premature termination of transcription (40). This non-processive transcription results in the synthesis of short TAR transcripts (41-43). Upon binding of Tat and the cellular positive transcription elongation factor b (P-TEFb) complex to TAR RNA, RNAP II is phosphorylated and activated for productive elongation, which allows production of full-length viral RNAs (41). Several studies suggested that the HIV-1 TAR region also encodes miRNAs (30, 34, 36, 37, 44). These TAR miRNAs may prevent apoptosis of the infected cell for the purpose of increased virus production (31, 45). However, the described miRNAs varied in size and position. Other studies failed to detect TAR-derived miRNAs or doubted their relevance (32, 35, 38). Furthermore, none of the studies could explain how HIV-1 was able to produce miRNAs without cleavage of the RNA genome and impeding virus replication.
MiRNAs are loaded in the AGO2/RISC complex to exert their regulatory function. We used this important characteristic to identify HIV-1 encoded miRNAs by deep sequencing of AGO2-bound RNAs in virus producing cells. This analysis revealed that the TAR element encodes a miRNA that originates from the 3’ side of the hairpin. We analyzed production of this miR-TAR-3p by wild-type HIV-1 and TAR- and Tat-mutated virus variants to investigate how this miRNA is produced and the role of the Tat protein. We demonstrate that HIV-1 uses a non-canonical miRNA production pathway to produce miR-TAR-3p.

Results

Analysis of small HIV-1 RNAs bound by AGO2/RISC

To characterize HIV-1 encoded miRNAs, 293T cells were transfected with plasmids expressing FLAG-tagged AGO2 (46) and either the wild-type (wt) HIV-1 DNA genome or a doxycycline (dox)-dependent HIV-1 variant (HIV-rtTA; Fig. 1A). As a control, the pBluescript vector was co-transfected instead of an HIV-1 plasmid. 293T cells are efficiently transfected and support a high level of virus production. These cells do not express the CD4 receptor needed for HIV-1 entry, which prevents re-infection and subsequent rounds of virus replication. In HIV-rtTA, the Tat-TAR transcription mechanism is inactivated through mutations in TAR (TARmut; Fig. 1B (47, 48)) and functionally replaced by the dox-inducible Tet-On system. This HIV-rtTA variant allows deletion of TAR without affecting viral gene expression (49). Two days after transfection, AGO2-bound RNAs were isolated by immune precipitation. Small RNAs (~15 to 55 nt) were purified and analyzed by SOLiD deep sequencing (Fig. 1C). To remove non-viral reads, the data set was filtered for sequences from cellular sources. Moreover, reads that were also observed for pBluescript-transfected control cells were excluded from the analysis. Alignment of the viral reads to the reference HIV sequences revealed a prominent peak in the TAR region (Fig. 2A and B). Whereas 54% of the HIV-1 reads (13,945 of 25,599 reads) corresponded to the TAR region, a significant larger number of TAR reads, 92%, was observed for HIV-rtTA (57,721 of 62,772 reads). Nearly all TAR reads corresponded to the 3’ half of the TAR region (Fig. 2C). Analysis of the size distribution of these TAR reads demonstrated that their size varied slightly, with a major peak at 21 nt, which is in agreement with the general miRNA size (Fig. 2D) (1, 50). These results indicate that the 3’ side of TAR is processed into a miRNA, named miR-TAR-3p, that is loaded into AGO2/RISC. No reads were observed corresponding to the 5’ side of the TAR stem in the HIV-1 and HIV-rtTA samples, which suggests that this 5’ strand is the passenger strand that is not loaded into AGO2/RISC.

Alignment of all TAR reads demonstrates that not a single miR-TAR-3p species is produced, but rather a miRNA population with slightly variable 5’ and 3’ ends (Fig. 2E). Most of the miR-TAR-3p reads started at U⁺⁴¹ (65% and 71% of the HIV-1 and HIV-rtTA TAR reads, respectively) and ended at U⁺⁶¹ (53% and 63%, respectively). The most abundant miR-TAR-3p started and ended at these dominant positions (Fig. 2E). This miRNA is 21 nt in size and represented 36% of all HIV-1 TAR reads and 41% of all HIV-rtTA TAR reads. The identical
pattern observed for HIV-1 (with a wt TAR element) and HIV-rtTA (with a mutated TAR element) indicates that mutations in the TAR bulge and loop sequences do not affect cleavage site selection during miRNA processing.

TAR and miR-TAR RNAs are produced upon non-processive transcription
Northern blot analysis of small RNAs confirmed the higher abundance of miR-TAR-3p in HIV-rtTA expressing versus HIV-1 expressing cells (Fig. 3A; compare lanes 2 and 3), even though virus production (as reflected by the CA-p24 level; Fig. 3B) was higher for wt HIV-1. In addition, larger RNA fragments of ~60 nt were detected that correspond to short TAR RNAs resulting from non-processive transcription starting at the 5' LTR promoter (40, 42). Notably, the level of these TAR RNAs is much higher for the HIV-rtTA sample (Fig. 3A), which correlates with the increased level of miR-TAR-3p RNAs and indicates that these TAR RNAs are the
Figure 2. Deep sequencing analysis of small AGO2-bound HIV RNAs. (A-B) AGO2-bound RNAs isolated from 293T cells expressing HIV-1 (A) or HIV-rtTA (B) and FLAG-tagged AGO2 were analyzed by SOLiD deep sequencing. The virus-encoded reads were aligned with the HIV-1 and HIV-rtTA RNA genome. The number of reads per nucleotide are shown. The reads corresponding to the repeat (R) region (including TAR) are shown at their 5’ position. (C) Alignment of the HIV-1 and HIV-rtTA derived RNAs to the TAR sequence. The wt sequence is shown with the bulge and loop nucleotides mutated in TAR boxed in grey. (D) The size distribution of the TAR-derived reads is shown for the HIV-1 (TAR\textsuperscript{wt}; open bars) and HIV-rtTA (TAR\textsuperscript{mut}; closed bars) samples. The total number of TAR reads (13,945 TAR\textsuperscript{wt} reads; 57,721 TAR\textsuperscript{mut} reads) was set at 100% for each sample and the percentage of reads is shown for the 15 to 27 nt size window. (E) The TAR\textsuperscript{wt} and TAR\textsuperscript{mut} structure is shown with the position of the prevailing 5’ (open triangle) and 3’ (closed triangle) ends of the TAR-derived reads indicated. The percentage of TAR-derived reads that start or end at the indicated position is shown (cut off set at 5%). The vertical lines indicate the 5 most frequent miR-TAR-3p RNAs with their frequency.
Figure 3. TAR and mir-TAR RNAs are produced by non-processive transcription. (A-B) 293T cells were transfected with the HIV-1 and HIV-rtTA constructs and cultured for 48 h without dox (HIV-1 and HIV-rtTA) or with dox (HIV-rtTA). (A) Intracellular small RNAs were isolated and analyzed by northern blotting. An LNA probe complementary to the miR-TAR-3p sequence was used to identify the position of miR-TAR-3p and TAR RNAs. The position of the size markers is shown on the left. Ethidium bromide staining of small 5S rRNA (5S) and tRNAs is shown as loading control below the blot. (B) The CA-p24 level in the culture supernatant was measured to quantify virus production. The mean ±SD for 3 experiments is shown. (C) A set of HIV-rtTA variants was constructed in which the 5', 3' or both TAR elements were deleted (TAR³', TAR⁵' and no TAR, respectively). In addition, the 3' U3 promoter was inactivated through the mutation of the three Sp1 sites in the ΔTAR⁵'+ΔU3³' variant. The possible TAR-containing RNA transcripts are shown for each construct. (D) 293T cells were transfected with the HIV-rtTA variants and after culturing with dox for 48 h, the CA-p24 was measured in the culture supernatant. The mean ±SD for 3 experiments is shown. (E) Small RNAs were isolated from the transfected cells and analyzed by northern blotting using a probe complementary to miR-TAR-3p. The positions of the miR-TAR-3p and TAR RNAs are indicated. (F) Analysis of luciferase production from LTR-promoter/luciferase-reporter constructs with the wt, TAR-deleted (ΔTAR) or Sp1-mutated (ΔU3) HIV-rtTA promoter configuration. Whereas deletion of TAR did not affect luciferase production, the Sp1 mutations inactivated the LTR promoter and prevented luciferase expression.
precursor for the miRNAs. HIV-rtTA production was low in the absence of dox, which is in agreement with the dox-control of viral transcription (Fig. 3B). Production of the short TAR and miR-TAR-3p RNAs did not depend on dox administration (Fig. 3A; compare lanes 3 and 4), which confirms that non-processive transcription is sufficient for the production of the TAR RNAs that are subsequently processed into miRNAs.

TAR is present at both the 5’ and 3’ end of full-length HIV-1 transcripts (Fig. 3C). To exclude the possibility that TAR and miR-TAR RNAs are produced from the 3’ TAR region, we analyzed small RNAs produced by HIV-rtTA variants with only the 5’ TAR, only the 3’ TAR or no TAR element (Fig. 3C). As previously observed (49), deletion of the 5’ TAR element did not reduce virus production, while deletion of the 3’ TAR element reduced virus production approximately 2-fold (Fig. 3D). As expected, deletion of both TAR elements abolished TAR and miR-TAR-3p RNA production (Fig. 3E; lane 5). Deletion of the 3’ TAR did not affect TAR and miR-TAR production (lane 4), which is in agreement with their 5’ TAR origin. Surprisingly, deletion of the 5’ TAR element did also not significantly reduce TAR and miR-TAR-3p RNA production (lane 2). Because transcriptional promoter activity of the 3’ LTR region could have resulted in the production of short TAR transcripts, we included an HIV-rtTA-TAR³'+ΔU3³' variant in which the Sp1-binding sites in the 3’ LTR region were mutated (Fig. 3C, construct 3). These Sp1 mutations completely inactivate 3’ LTR promoter activity (Fig. 3F). As a result, TAR sequences can only be transcribed as 3’ portion of extended HIV-rtTA-TAR³'+ΔU3³' transcripts and not as short TAR transcripts initiated at the 3’ LTR. In contrast to the TAR³' construct, this TAR³'+ΔU3³' variant did not produce TAR and miR-TAR-3p RNAs (Fig. 3E; compare lanes 2 and 3). This result indicates that the TAR and miR-TAR RNAs cannot be produced from the 3’ TAR region present in full-length transcripts that are initiated at the 5’ promoter, but are exclusively derived from short transcripts resulting from non-processive transcription.

Tat is required for miR-TAR production
The experiments with dox-controlled HIV-rtTA variants demonstrated that non-processive transcription from the LTR promoter results in production of short TAR RNAs and miRNAs. The bulge and loop sequence of the TAR element in these viruses had been mutated (TAR\textsuperscript{mut}) to prevent Tat binding. Because these mutations may influence miRNA processing, we next tested whether non-processive transcription from the wild-type HIV-1 LTR promoter, with a wild-type TAR sequence (TAR\textsuperscript{wt}), is also sufficient for production of both RNA species. For this, we analyzed TAR and miR-TAR production of a Tat-negative HIV-1 variant (HIV-Tat\textsuperscript{stop}) that was constructed by introducing two translation stop codons in the Tat open reading frame (51). As expected, HIV-Tat\textsuperscript{stop} gene expression is low and we measured a reduced level of virus production upon transfection of the construct in 293T cells (Fig. 4A). The level of transcription and virus production could be restored by addition of Tat in trans through co-transfection of a Tat-expressing plasmid (pTat). Northern blot analysis of the intracellular small RNAs revealed an increase in TAR RNA upon Tat inactivation, but no miR-TAR-3p was detectable (Fig. 4B; lane 2). Trans-complementation with Tat reduced TAR RNA and increased miR-TAR-3p production to the wt levels. These results demonstrate that the TAR RNAs are...
produced by Tat-independent, non-processive transcription from the HIV-1 LTR promoter. Furthermore, these data indicate that Tat does not only activate processive transcription but also stimulates processing of the TAR^\text{mRNA} RNAs into miRNAs. However, it cannot be excluded that these processes are coupled and that elongated transcripts produced upon Tat activation are required for miRNA processing.

Figure 4. Tat binding to TAR induces processing into miRNA. (A) 293T cells were transfected with the HIV-1 plasmid or with the Tat-deficient HIV-Tat^\text{mRNA} construct and a varying amount of Tat plasmid (0; 50; 500 ng). CA-p24 was measured in the culture supernatant after 48h. The mean ±SD for 3 experiments is shown. (B) Small RNAs were isolated and analyzed by northern blotting using the miR-TAR-3p probe, as described for Fig. 3A. (C) Tat-deficient HIV-rTA constructs with a wild-type (TAR^\text{wt}) or mutated TAR element (TAR^\text{mut}) were transfected into 293T cells. Cells were co-transfected with 500 ng pTat or the empty pcDNA3 plasmid (+ and – Tat, respectively) and cultured in the presence or absence of dox (+ and – dox, respectively) for 48 hr. CA-p24 was measured in the culture supernatant. The mean ±SD for 3 experiments is shown. (D) Small RNA isolated from the transfected cells was analyzed by northern blotting using the miR-TAR-3p probe as described for Fig. 3A.
To demonstrate that Tat binding activates processing of TAR\textsuperscript{wt} RNA into miRNA in a direct way, and not in an indirect way by activating processive transcription, we next analyzed TAR and miR-TAR production from an HIV-rtTA variant with wt TAR sequences and inactivated Tat gene (HIV-rtTA-TAR\textsuperscript{wt}-Tat\textsuperscript{stop}). Processive transcription of this TAR\textsuperscript{wt}-Tat\textsuperscript{stop} virus can be activated by the administration of dox (via dox-rtTA/tetO axis) or Tat (via Tat-TAR axis). Without dox and Tat, transcription is non-processive and we did indeed measure a low level of virus production in transfected cells (Fig. 4C). Addition of dox or Tat activated processive transcription and increased virus production, and highest virus production was measured when both dox and Tat were present (Fig. 4C). Analysis of the small RNAs revealed that, without dox and Tat, this virus produced a high level of TAR RNA but no miR-TAR-3p RNA (Fig. 4D; lane 1). Dox administration did not influence the TAR RNA and miR-TAR levels (Fig. 4D; lane 3), which indicates that processive transcription does not activate TAR\textsuperscript{wt} processing. In contrast, Tat administration reduced the TAR RNA level and increased the miR-TAR-3p level (Fig. 4D; lane 2). These results confirm that non-processive transcription is sufficient for the production of TAR RNA and that Tat activates processing of TAR RNA into miR-TAR.

Tat binds the bulge region in TAR and this interaction is required to exert its transcription activating function (52, 53). To test whether the stimulatory effect of Tat on TAR miRNA processing does depend on binding to the TAR bulge, we constructed an HIV-rtTA variant in which the TAR\textsuperscript{mut} mutations that prevent Tat binding (Fig. 1C) and the Tat\textsuperscript{stop} mutations were combined. Processive transcription of this HIV-rtTA-TAR\textsuperscript{mut}-Tat\textsuperscript{stop} variant is activated by dox but not by Tat. We did indeed measure low virus production in the absence of dox, both without and with Tat, and high virus production with dox (Fig. 4C). The highest virus level was observed when both dox and Tat were administered, which is due to the TAR-independent stimulatory effect of Tat on dox-rtTA activated transcription through an interaction with U3 promoter sequences (51). Northern blot analysis revealed that HIV-rtTA-TAR\textsuperscript{mut}-Tat\textsuperscript{stop} produced a high level of TAR RNA and low level of miR-TAR-3p in the absence of dox and Tat. As observed for TAR\textsuperscript{wt}-virus, the TAR and miR-TAR levels did not change upon dox administration (Fig. 4D; compare lanes 5 and 7). Whereas - as described above - Tat administration to the TAR\textsuperscript{wt} virus stimulated processing of the TAR RNAs into miRNAs, Tat addition to the TAR\textsuperscript{mut} virus did not influence TAR and miR-TAR levels (Fig. 4D; compare lanes 5 and 6 and lanes 7 and 8). These results indicate that binding of Tat to the TAR bulge is required for the activation of miRNA processing. Notably, the TAR\textsuperscript{mut} variant produced a detectable amount of miR-TAR-3p in the absence of Tat, whereas the TAR\textsuperscript{wt} virus did not, which demonstrates that the TAR\textsuperscript{mut} RNAs are partly processed independent of Tat binding.

Changes in the TAR RNA structure activate processing into miRNA

Previous studies demonstrated that the 3-nt bulge causes a bend in the α-helical structure of the TAR\textsuperscript{wt} RNA hairpin (54-56). Furthermore, Tat binding was shown to lead to straightening of TAR (56-58). Possibly, the short TAR RNAs require this Tat-induced straightened conformation for processing into miRNAs. To investigate the effect of such a conformational change on TAR RNA processing, we straightened the TAR structure by deleting the 3-nt bulge.
in HIV-Tatstop (TARΔbulge in Fig. 5A) and compared production of small TAR RNAs by the TARwt and TARΔbulge variants (Fig. 5B). Whereas HIV-Tatstop-TARwt showed an accumulation of TAR RNA and no miRNA production (Fig. 5B; lane 2), the TARΔbulge variant demonstrated efficient TAR RNA processing, resulting in a high miR-TAR-3p level (Fig. 5B; lane 3). This result suggests that the conformation of free TAR hampers processing into miRNAs and this restriction is lifted by straightening of the RNA structure upon Tat binding. As described above, the TARmut virus demonstrated partial processing independent of Tat (Fig. 4D), possibly because the bulge and loop mutations in this variant affect the hairpin structure.

Figure 5. TAR bulge prevents processing of TAR RNA into miRNA. (A) In the HIV-1 TARΔbulge mutants, the 3-nt TAR bulge (nt +22 to +24) was deleted. (B) 293T cells were transfected with wt HIV-1 and HIV-Tatwt variants with wt or bulge-deleted TAR elements (TARwt and TARΔbulge, respectively). Small RNA was isolated after 48h and analyzed by northern blot blotting using the miR-TAR-3p probe as described for Fig. 3A.

TAR RNA is processed by Dicer

The role of Dicer in TAR miRNA production was investigated by analyzing the production of short TAR RNAs and miR-TAR-3p in HCT-116 cells expressing either wt (Dicerwt) or exon 5-disrupted Dicer (Dicerex5). This ex5 mutation in the helicase domain of Dicer reduces Dicer activity (59). When cells were transfected with an LTR-promoter construct that expresses TARmut-containing transcripts, both the TAR RNA precursor and miR-TAR-3p were detected in the Dicerwt cells, whereas an increased TAR RNA and reduced miR-TAR-3p level was observed in Dicerex5 cells (Fig. 6A). These results indicate that Dicer is involved in the processing of short TAR RNAs.

To demonstrate that HIV-1 TARwt RNAs are recognized and bound by Dicer, we transfected 293T cells with a plasmid expressing FLAG-tagged Dicer (60) and the HIV-Tatstop construct that produces a high level of TARwt RNA (Fig. 4B). At 48 h after transfection, the Dicer-bound RNAs were isolated by immunoprecipitation and TAR-containing RNA was analyzed by reverse-transcription PCR using a TAR-specific primer, followed by cloning and sequencing of the cDNA fragments (Fig. 6B). We identified several Dicer-bound TAR RNA fragments with a slightly variable 3’ end (ranging from +59 to + 61; Fig. 6C). This 3’ end of the TAR RNAs corresponds with the 3’ end of the miR-TAR-3p RNAs as identified in the SOLiD deep sequencing analysis (Fig. 2E). These combined results demonstrate that TAR RNA is a substrate for Dicer to produce miR-TAR-3p.
Figure 6. Dicer processes TAR RNA into miR-TAR-3p. (A) HCT-116 cells expressing wild-type (wt) or exon5-disrupted Dicer (ex5) were transfected with the plasmid pLTR²ΔmRNA-luc (49) in which expression of TAR-containing RNAs is driven by the HIV-rtTA U3 promoter. The RNA was analyzed after 48h by northern blotting using the miR-TAR-3p probe. (B) Experimental design for the isolation and characterization of Dicer-bound TAR RNAs. (C) 10 TA-cloned fragments were sequenced to determine the 3’ end of the TAR RNAs (indicated with closed triangle).

Discussion

The debate about whether HIV-1 encodes miRNAs is ongoing for 10 years. The fact that miRNA production via the canonical miRNA pathway, involving subsequent microprocessor and Dicer cleavage of the primary transcript, would result in degradation of the viral RNA genome and impede HIV-1 replication, was long considered a strong argument against HIV-1 encoded miRNAs. However, in this study we demonstrate how HIV-1 can produce a functional miRNA via a non-canonical pathway without hampering virus replication. First, we show that HIV-1 produces a miRNA from the 3’ side of the TAR hairpin, miR-TAR-3p, that is loaded into the AGO2/RISC complex. Second, we demonstrate that short TAR RNAs produced upon non-processive transcription from the LTR promoter function as pre-miRNA and are processed into miRNA by Dicer. MiR-TAR-3p is thus not generated via the canonical miRNA pathway in which a pre-miRNA is formed by microprocessor cleavage of the primary transcript, but via a novel pathway in which the pre-miRNA is produced by pausing and premature termination of RNAP II transcription. By selectively using these short TAR RNAs as miRNA source, HIV-1 can produce the miRNA without inducing cleavage of its RNA genome. Third, we demonstrate that the viral Tat protein activates processing of the TAR RNA into miRNA.

The 21-nt miR-TAR-3p that we identified overlaps with earlier suggested 3’ TAR-derived miRNAs (30, 34, 36-38, 44), but differs in size and position (Fig. 7A). Schopman et al. observed
the same miR-TAR-3p read upon deep-sequencing analysis of HIV-rtTA expressing cells, but not in cells expressing wt HIV-1 (36). HIV-rtTA indeed produces significantly more miR-TAR-3p than wt HIV-1 (Fig. 3A). The differences between our and previous results are likely due to technical differences. The earlier studies used in silico analysis (30) or traditional cloning and sequencing (31, 44), RNAse protection (34) or deep sequencing analysis (36-38) of total intracellular RNA pools, without any selection for functional miRNAs. We combined AGO2-pull down with deep sequencing to specifically analyze functional miRNA molecules that are loaded into the AGO2/RISC complex.

Figure 7. Production of TAR-derived miRNA. (A) Alignment of the TAR-derived small RNAs identified in different studies. All studies (except the in silico analysis) characterized the TAR RNAs in HIV-1 expressing cells, but different techniques were used: SOLiD deep sequencing of AGO2-bound HIV-1 and HIV-rtTA RNAs (this study), Illumina deep sequencing of HIV-1 RNAs (38), SOLiD deep sequencing of small HIV-1 (Schopman 1) (Wagschal) and HIV-rtTA RNAs (Schopman 2) (36, 37), RNAse protection analysis of HIV-1 RNAs (RPA; (34)), cloning and sequencing of small HIV-1 RNAs (31, 44) and in silico analysis (30). In the upper line the HIV-1 LTR sequence is shown with the TAR region (+1 to +57) boxed in grey and the transcription start site indicated (+1). The dominant sequence found in each study is shown for both the 5p and 3p strand (if applicable). (B) Model for HIV-1 miRNA and full-length RNA production. See text for details.
The identical miR-TAR-3p was produced by HIV-1 and HIV-rtTA (Fig. 2E), but the level was much higher in the HIV-rtTA sample. We measured a concomitant increase in TAR transcripts (Fig. 3A), which is in agreement with TAR RNA being the precursor for the miRNAs. The increased production of TAR RNA and TAR miRNAs by HIV-rtTA may have been caused by the modified tetO-LTR promoter region in this virus (61). Possibly, this new configuration increased the non-induced activity of the promoter, resulting in a higher level of non-processive transcription. Alternatively, the mutations introduced in the bulge and loop region of TAR may have changed the local RNA structure and enhanced transcription pausing and termination.

The viral Tat protein, which is known to bind the 3-nt bulge in TAR and straighten the molecule, was found to activate processing of TAR RNA into miRNA. Thus, Tat not only activates processive transcription from the LTR promoter, but also stimulates processing of TAR RNAs that are formed during non-processive transcription. The observed simultaneous production of full-length HIV-1 RNAs (reflected by CA-p24 level; Fig. 4A) and miR-TAR-3p (Fig. 4B) upon Tat induction indicates that activation of processive transcription does not switch off non-processive transcription. A similar conclusion can be drawn from the observation that dox administration to the HIV-rtTA-TAR\textsuperscript{wt}-Tat\textsuperscript{stop} variant, which activates processive transcription of this virus (increased CA-p24 level; Fig. 4C), did not reduce non-processive transcription and production of TAR RNA (Fig. 4D).

Our result demonstrate that Dicer can bind the TAR RNA in the absence of Tat (Fig. 6C), but the binding does not trigger processing into miRNA (Fig. 4B). Possibly, TAR processing is controlled by the hairpin conformation and Tat-binding results in a cleavage-prone structure. In this scenario, the bend in the α-helical stem structure caused by the 3-nt bulge (54-56) may hinder the processive centers of Dicer to access the TAR stem. Deletion of the bulge nucleotides did indeed result in TAR processing in the absence of Tat. Binding of Tat to the TAR bulge straightens the hairpin (56-58) and may similarly result in a cleavage-prone RNA conformation. Mutations in TAR may also affect the bended TAR structure which can explain partial processing of the TAR\textsuperscript{mut} RNAs. Alternatively, the 3-nt bulge may bind a protein factor that prevents Dicer cleavage. Tat binding may displace this inhibitory factor and activate Dicer processing. Bulge deletion or mutation will also prevent binding of the inhibitory factor and activate processing.

Cellular miRNAs are typically produced by subsequent microprocessor and Dicer cleavage. Our results demonstrate that Dicer is involved in miR-TAR-3p production, but the role of microprocessor is less clear. It was recently shown that binding of microprocessor to nascent TAR-containing transcripts initiates RNAP II pausing and premature termination (37). The same mechanism was found to modulate transcription of cellular genes (37, 62, 63) and cleavage by Drosha is dispensable for this microprocessor function (62, 63). It seems likely that microprocessor cleavage is also not involved in miR-TAR production. First, TAR RNA may not be a good substrate because the TAR stem consists of only 23 bp, whereas microprocessor requires longer base-paired regions of ~33 bp for efficient binding (7, 38). Furthermore, microprocessor normally cleaves a pri-miRNA stem at ~11 bp from its base, while the 3’ end of miR-TAR-3p corresponds with the strong transcription pause site that was previously identified in in vitro LTR transcription studies (40) and with the 3’ end of short HIV-1 transcripts.
produced in latently infected U1 cells (64). Our results demonstrate that the TAR RNAs that are produced by non-processive transcription are the precursor for miR-TAR-3p. Taken together, these results suggest that the 3′ end of TAR and miR-TAR-3p is created by pausing, and subsequent premature termination, of transcription and not by microprocessor cleavage. Xie et al. recently documented cellular microprocessor-independent pre-miRNAs, whose capped 5′ ends coincide with transcription start sites and 3′ ends are most likely generated by transcription termination (65). Production and processing of TAR is thus very similar to that of these cellular pre-miRNAs, as the 5′ end of TAR is also determined by the transcription start site and the viral transcripts are capped (66, 67). Notably, Xie et al. demonstrated that after Dicer cleavage of the capped pre-miRNAs, the capped 5p strand is excluded from loading onto AGO2 and only the 3p strand is efficiently loaded, which can explain why we did not observe miR-TAR-5p in the AGO2-bound RNA fraction.

The retroviruses BLV and BFV were recently shown to produce sub-genomic RNAP III transcribed hairpin RNAs that are further processed into miRNAs (28, 68). Similar to what we describe for the HIV-1 TAR pre-miRNAs, the 3′ end of the BLV and BFV pre-miRNAs is determined by transcription termination (in these cases driven by RNAP III termination signals) and not by microprocessor cleavage. Similar to HIV-1, the full-length BLV and BFV transcripts do contain the complete miRNA sequences, but these are not processed because the short stem of the pre-miRNA hairpin structure does not allow efficient microprocessor cleavage. Avoiding the microprocessor cleavage requirement for miRNA production seems to be a general strategy for retroviruses to allow virus-encoded miRNAs without interfering with genomic and messenger RNA production.

We propose a new model for miR-TAR-3p production in Fig. 7B. This model explains how HIV-1 can produce a miRNA without blocking its own replication. Upon formation of non-processive RNAP II complexes at the LTR promoter, transcription is initiated, but frequently pauses when TAR is formed. Subsequent premature termination of transcription results in the production of short 5′ capped TAR RNAs that function as pre-miRNA. These TAR RNAs are recognized by Dicer, but the bended TAR stem structure (or the binding of another factor to TAR) prevents Dicer cleavage. Binding of Tat changes the TAR RNA conformation (or displaces the Dicer-blocking factor), resulting in processing of the TAR RNA and production of miR-TAR-5p and miR-TAR-3p. MiR-TAR-3p is loaded onto the AGO2/RISC complex, whereas loading of miR-TAR-5p is blocked by the 5′ cap. Binding of Tat also recruits the pTEFb complex, which results in phosphorylation of the RNAP II complex and activation of processive transcription. Because the TAR stem in the extended transcripts is too short for efficient microprocessor cleavage, full-length RNAs can be produced and virus replication will be allowed.
Methods

See supplementary experimental procedures for details about cell culture and plasmid construction.

Small RNA library preparation and SOLiD deep sequencing

293T cells (5 x 10⁶ cells per 25 cm² flask) were co-transfected with 5 µg AGO2-FLAG plasmid (46) and 20 µg HIV-1 (69), HIV-rtTA-Tat™ (51) or pBluescript plasmid (Stratagene), and cultured for 48 h. HIV-rtTA transfected cells were cultured with 1 µg/ml dox (Sigma; D-9891). AGO2-bound small RNAs were isolated by immunoprecipitation using anti-FLAG M2 affinity gel (Sigma; A2220) as previously described (70). 5 µg RNA was loaded on a denaturing 15% PAGE gel for size fractionation and the 15-55 nt RNA fragments were isolated using a spin column (Ambion). The quality of the RNA was assayed on a Bioanalyzer 2100 (Agilent) using a small RNA chip. The SOLiD Small RNA Library Preparation protocol (Applied biosystems) was used to prepare an RNA library that was subsequently analyzed using the SOLiD Wildfire system (Applied biosystems).

Bioinformatics

Analysis of the SOLiD colorspace reads was performed with LifeScope Genomic Analysis Software version 2.5 (Applied biosystems) using the small RNA pipeline. First, the libraries were mapped against the human genome filter-sequences (supplied with LifeScope) and miRBase (version 21; http://www.mirbase.org) to eliminate reads generated from cellular or irrelevant sources. The remaining reads were mapped to the reference sequence of HIV-1 or HIV-rtTA, allowing no mismatches. Reads that were also observed with the pBluescript-transfected 293T cells were occluded.

Northern blot analysis

293T cells cultured in 25 cm² flasks were transfected with 5 µg HIV-1 or HIV-rtTA plasmid and cultured for 48 h. When indicated, dox (1 µg/ml) was added to the culture medium. Small intracellular RNA was isolated with the mirVana miRNA isolation kit (Ambion). Isolated RNA (2 µg HIV-rtTA or 4 µg LAI RNA sample) was analyzed by Northern blotting as previously described (36). To check for equal sample loading, the 5S rRNA and tRNA bands were visualized by ethidium bromide staining. The LNA oligonucleotide 5’- AAGCAGTGGGTCCCTAGTAG-3’ (LNA-positions underlined) complementary to the 3’ end of the TAR hairpin was used as probe to detect miR-TAR-3p and TAR-containing RNAs.
Characterization of Dicer bound RNAs
293T cells (5 x 10⁶ cells per 25 cm² flask) were transfected with 1 µg FLAG-tagged Dicer plasmid (Addgene plasmid 19881 (60)) and 4 µg HIV-Tat<sup>stop</sup> plasmid. At 48 h post-transfection, Dicer-bound small RNAs were isolated by immunoprecipitation using anti-FLAG M2 affinity gel (Sigma; A2220) and analyzed in a similar way as previously described for AGO2-binding RNAs (71). Briefly, RNAs were polyadenylated using the Poly(A) Polymerase Tailing Kit (Epicentre Biotechnologies) and subsequently reverse transcribed into cDNA using an oligo-dT₃₀-adaptor primer. To detect the TAR-containing RNAs, the cDNA product was amplified by PCR using a forward primer (TGCGGATCCCCGGTCTCTCTGGTTAGACCAGA; TAR +1 - +21 nt sequence underlined) and a reverse adaptor primer. PCR products were ligated into a TA cloning vector (TOPO-TA cloning kit, Life Technologies) and sequenced.

Author Contributions
AH, BB and ATD designed the research. AH performed the experiments. AH, BB and ATD wrote the manuscript. AJ and AHK analyzed the deep sequencing data.

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References

Supplemental Methods

Cell culture and transfection
Human Embryonic Kidney 293T, HCT-116 and HCT-116-Ex5 cells (1) were cultured as monolayer in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO₂. For the RNA isolations, cells (5 x 10⁶ cells) were cultured in 25 cm² flasks and transfected with 5 μg HIV plasmid DNA using lipofectamin2000 (Life technologies). When indicated, cells were cultured with 1 μg/ml dox (Sigma D-9891). Virus production in culture medium samples was measured using CA-p24 ELISA (2). Luciferase production was analyzed as previously described (3).

Plasmid constructs
The HIV-1 molecular clone pLAI (4) and the dox-dependent derivatives HIV-rtTA-Tat⁷⁶ (3), HIV-rtTA-TAR⁵'⁺³'⁻Tat (3), and HIV-rtTA-ΔTAR⁵'⁻³' are previously described. HIV-rtTA-ΔTAR⁵' was constructed by replacing the 3' BamHI – BglI fragment of HIV-rtTA-ΔTAR⁵'⁺³' with the corresponding fragment of HIV-rtTA-Tat⁷⁶. HIV-rtTA-ΔTAR⁵' was constructed by replacing the 3' BamHI – BglI fragment of HIV-rtTA-Tat⁷⁶ with the corresponding fragment of HIV-rtTA-ΔTAR⁵'⁺³'.

For mutation of the Sp1 sites in the HIV-rtTA LTR promoter, the LTR region was PCR-amplified with primers mutSp1 (GAAAAGTCGACAGAATTCGCGCTGGCTGTTCTCGAGGAATTCTCGAGGCCTCAGATGCTGCA; mutated nucleotides underlined; SalI site in italics and bold) and C(N1) (antisense primer annealing to the U5 region) (6), and with pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO, which includes Env, rtTA and 3' LTR sequences of the HIV-rtTA genome (7), as template. The PCR product was digested with SalI and HindIII, and used to replace the corresponding 3' LTR fragment in pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO. The BamHI-BglI fragment of the resulting shuttle plasmid pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO-mutSp1 was used to replace the corresponding Env-rtTA-3' LTR sequences in HIV-rtTA-ΔTAR⁵'⁺³', which resulted in plasmid HIV-rtTA-ΔTAR⁵'⁻³'.

HIV-Tat⁷⁶ was constructed by replacing the NcoI-BamHI fragment of HIV-1 pLAI with the NcoI-BamHI fragment from HIV-rtTA-TAR⁷⁶-Tat⁷⁶. For the construction of HIV-rtTA-TAR⁷⁶-Tat⁷⁶, mutagenesis PCR was performed using primers tTA3 (3) and wt-TAR CAGAGGCTCCAGGGCTCAAATCTGGT; mutated nucleotides underlined; SacI site in italics and bold) and HIV-rtTA as template. The resulting fragment was digested with SacI and NdeI, and used to replace the corresponding fragment in pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO. The BamHI-BglI Env-rtTA-3' LTR fragment of the resulting plasmid pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO-Wt was used to replace the corresponding sequences in HIV-rtTA-Tat⁷⁶, which resulted in HIV-rtTA-3'TAR⁷⁶-Tat⁷⁶. Subsequently, the LTR region in pBlue3'LTRext-ΔU3-rtTAF₈₆Y A₂₀₉T-2ΔtetO-Wt was PCR amplified using the primers U3-Xba-Not (5) and U5-Nar (5), and the produced fragment was used to replace the corresponding NotI-NarI fragment in HIV-rtTA-3'TAR⁷⁶-Wt, which resulted in HIV-rtTA-TAR⁷⁶-ΔTcp⁷⁶.
A three-step mutagenesis PCR protocol (8) was used for the construction of HIV-TARΔbulge- Tat\textsuperscript{stop}. PCR 1 was performed using the primers tTA3 and TAR-noBulge-rev (GAGAGCTCCCAGGCTCTTGCTAAACCAGA), and pLAI as template. PCR 2 was performed using the primers TAR-noBulge-fw (CTCTCTGTTAGACCAGAGCCTGGGAGCTC) and pLAI3’seq (5), and the pLAI template. The PCR 1 and PCR 2 products were purified and combined to function as template in PCR 3, using the outer primers tTA3 and pLAI3’seq. The resulting LTR-encoding fragment (with the TARΔbulge sequence) was used to replace the XhoI-AatII 3’ LTR fragment in HIV-Tat\textsuperscript{stop}, resulting in HIV-TAR\textsuperscript{3’Δbulge-Tat\textsuperscript{stop}}. The PCR 3 fragment was also used as template in a PCR with primers U3-Xba-Not and U5-Nar. The product was used to replace the Xbal-NarI 5’ LTR fragment in HIV-TAR\textsuperscript{3’Δbulge-Tat\textsuperscript{stop}}, which resulted in the HIV-TARΔbulge-Tat\textsuperscript{stop} mutant with the TARΔbulge mutation in both the 5’ and 3’ LTR.

The plasmid pLTR-2ΔtetO-luc\textsuperscript{ff}, in which the LTR promoter region from HIV-rtTA is coupled to the firefly luciferase gene, was previously described (5). The SalI-HindIII U3-R fragment of this plasmid was replaced by the corresponding fragment from HIV-rtTA-ER2 (5) and pBlue3’LTRext-ΔU3-rTAFA\textsubscript{86}Y A\textsubscript{209}T-2ΔtetO-mutSp1, which resulted in pLTR-2ΔtetO-ΔTAR-luc\textsuperscript{ff} and pLTR-2ΔtetO-ΔU3-luc\textsuperscript{ff}, respectively.

References