The HIV-1 capsid-binding host factor CPSF6 is post-transcriptionally regulated by the cellular microRNA miR-125b

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Running Title: CPSF6 is a direct target of miR-125b

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Keywords: cleavage and polyadenylation specificity factor 6 (CPSF6), miR-125b, HIV, 3’UTR, post-transcriptional regulation, epigenetics, RNA-binding protein, host-pathogen interaction, miRNA biogenesis

ABSTRACT

Cleavage and polyadenylation specificity factor 6 (CPSF6) is a cellular protein involved in mRNA processing. Emerging evidence suggests that CPSF6 also plays key roles in HIV-1 infection, specifically during nuclear import and integration targeting. However, the cellular and molecular mechanisms that regulate CPSF6 expression are largely unknown. In this study, we report a post-transcriptional mechanism that regulates CPSF6 via the cellular microRNA miR-125b. An in silico analysis revealed that the 3’ untranslated region (3’UTR) of CPSF6 contains a miR-125b-binding site that is conserved across several mammalian species. Since miRNAs repress protein expression, we tested the effects of miR-125b expression on CPSF6 levels in miR-125b knockdown and over-expression experiments, revealing that miR-125b and CPSF6 levels are inversely correlated. To determine whether miR-125b post-transcriptionally regulates CPSF6, we introduced the 3’UTR of CPSF6 mRNA into a luciferase reporter and found that miR-125b negatively regulates CPSF6 3’UTR-driven luciferase activity. Accordingly, mutations in the miR-125b seed sequence abrogated the regulatory effect of the miRNA on the CPSF6 3’UTR. Finally, pull-down experiments demonstrated that miR-125b physically interacts with CPSF6 3’UTR. Interestingly, HIV-1 infection down-regulated miR-125b expression concurrent with up-regulation of CPSF6. Notably, miR-125b down-regulation in infected cells was not due to reduced pri-miRNA or pre-miRNA levels. However, miR-125b down-regulation depended on HIV-1 reverse transcription but not viral DNA integration. These findings establish a post-transcriptional mechanism that controls CPSF6 expression and highlight a novel function of miR-125b during HIV-host interaction.
CPSF6 is a direct target of miR-125b

Cleavage and polyadenylation specificity factor 6 (CPSF6) functions as part of the cleavage factor I mammalian (CFIm) complex (1,2). CFIm is a RNA binding protein complex and component of a multiprotein cleavage and adenylation complex that regulates mRNA processing and polyadenylation (3). CFIm is a hetero-tetrameric complex consisting of a dimer of CPSF5 and a dimer of either CPSF6 or CPSF7 (1,4,5). CFIm is also a component of paraspeckles, ribonucleoprotein complexes that are involved in regulating gene expression through nuclear retention of adenosine-to-inosine (A-to-I) edited RNA (6,7). A recent study suggested that through its association with paraspeckles, CPSF6 is involved in the regulation of breast cancer cell viability and tumorigenic capacity (8). In recent years, CPSF6 has also garnered a great deal of attention for its emerging role during the early steps of human immunodeficiency virus 1 (HIV-1) infection (9-15).

HIV-1 has infected over 72 million people and has claimed more than 37 million lives worldwide (16). Untreated HIV-1 infection causes progressive CD4+ T-cell loss and a wide range of immunological abnormalities leading to Acquired Immune Deficiency Syndrome (AIDS) (17). Productive HIV-1 infection is dependent on integration of the reverse transcribed viral DNA into the host genome. The virally-encoded integrase (IN) enzyme carries out integration of the viral DNA in the context of a nucleoprotein complex called the preintegration complex (PIC) (18). The PIC, containing the viral DNA and other viral and cellular proteins, is actively transported into the nucleus of the target cell through the nuclear pore complex (NPC) (19). A variety of cellular proteins including cyclophilin A (CypA), nucleoporin (NUP) proteins NUP358 and NUP153, the beta-karyopherin transportin-3 (TNPO3), and CPSF6 have been implicated in PIC nuclear import (10,11,20-23). In the nucleus, HIV-1 DNA preferentially integrates into gene-dense regions of the host genome (24). Accumulating evidence suggests that many of the cellular factors involved in PIC nuclear import also facilitate targeting of the viral DNA integration into gene dense chromosomal regions (25-28). Although the exact mechanism of HIV-1 integration targeting is unclear, recent studies have clarified that CPSF6 is a key player in targeting HIV-1 DNA into the gene dense region of the host chromosomes (29,30).

Despite the established role of CPSF6 in mRNA processing and HIV-1 infection, the cellular and molecular mechanism(s) by which CPSF6 expression is regulated remains largely unknown. In this study, we describe an miRNA mediated post-transcriptional mechanism of CPSF6 regulation. Our bioinformatics analysis revealed that the CPSF6 3’UTR contains a binding site for the cellular miRNA miR-125b. This binding site is strikingly conserved across a number of mammalian species implying a regulatory role of this miRNA. Therefore, we examined miR-125b mediated regulation of CPSF6 by carrying out knockdown and over-expression studies. Results from these genetic experiments revealed that miR-125b levels negatively associate with CPSF6 protein expression. To further pin-point post-transcriptional regulation of CPSF6 by miR-125b, luciferase reporter constructs containing the 3’UTR sequences of CPSF6 mRNA were generated. Luciferase assays revealed that CPSF6 3’UTR activity is negatively regulated by miR-125b expression. Subsequently, physical interaction between the CPSF6 3’UTR and miR-125b was demonstrated by pull-down studies. Interestingly, upon HIV-1 infection, miR-125b levels were reduced concurrent with increased CPSF6 expression. The down-regulation of miR-125b in infected cells was not due to reduced levels of miR-125b precursors such as pre-miRNA or pri-miRNA. Interestingly, our results revealed that miR-125b down-regulation was dependent on reverse transcription of the viral genome but not on viral DNA integration. Finally, over-expression and knockdown of miR-125b in infected cells showed that the miR predominantly affected nuclear entry of HIV-1. Collectively, these studies describe: a) post-transcriptional regulation of CPSF6, b) identify CFSP6 as a direct target of miR-125b, c) establish effects of miR-125b on early steps of HIV-1 infection and d) reveal a potential mechanism by which HIV-1 regulates miR-125b expression during early steps of infection.
RESULTS

CPSF6 3’-UTR contains a highly conserved binding site of miR-125b

CPSF6 is part of the CFIIm complex and is well-established for its role in polyadenylation of cellular mRNAs (3). Emerging evidence also show that CPSF6 plays important roles in HIV-1 infection at the steps of PIC nuclear import (10,11) and integration targeting (29,30). However, the cellular and molecular mechanisms of regulating CPSF6 expression remain largely unknown. In this study, we examined the mechanisms involving post-transcriptional regulation of CPSF6 by initially subjecting its 3’UTR region to in silico analysis. CPSF6 mRNA is ~6.58 kilobase (kb) with 10 exons and the 3’UTR region consists of 4847 nucleotides (Fig. 1A). We used two different algorithms, RNAhybrid 2.1.2 (31,32) and biFold:RNA Structures (33) which employed intramolecular base-pairing of RNA: RNA interactions (RNAhybrid) and unimolecular and bimolecular interactions (biFold:RNA Structures) to enhance in silico prediction accuracy. Interestingly, both platforms predicted a putative miR-125b binding site within the CPSF6 3’UTR (Fig. 1B-C). These studies also showed the formation of a hairpin-loop structure between the two RNA molecules that is essential for miRNA-mediated post-transcriptional regulation (Fig. 1B-C). The 7mer target site of miR-125b was located at the nucleotide positions 2204-2211 of the CPSF6 mRNA within exon 10, corresponding to positions 471-477 of the 3’UTR (Fig. 1A). Importantly, the predicted miR-125b target sequence was highly conserved in the CPSF6 3’UTR of several mammalian species (Fig. 1D), another hallmark of miRNA mediated post-transcriptional regulation (34).

CPSF6 expression inversely correlates with miR-125b levels

To examine the biological relevance of the in silico prediction data described in Fig. 1A-D, we tested whether miR-125b regulates CPSF6 protein expression in knockdown and overexpression studies. We transfected LNA-based miR-125b mimics and anti-miRs into HEK-293T cells and isolated total RNA after 24 h. qPCR analysis of reverse transcribed RNA showed a significant reduction in miR-125b levels in cells transfected with anti-miRs (Fig. 1E) and a marked increase in miR-125b levels in cells transfected with miR-mimics (Fig. 1F). Measurement of CPSF6 protein levels in cellular lysates by Western blot showed that cells with lower levels of miR-125b contained higher CPSF6 protein levels compared to control cells (Fig. 1G). Accordingly, higher miR-125b levels led to a reduced level of CPSF6 protein (Fig. 1G), supporting an inverse association between miR-125b expression and CPSF6 protein levels.

We also examined whether miR-125b expression negatively associates with CPSF6 protein levels in HIV-1 susceptible T and monocytic cell lines. SupT1 and CEM cell lines were specifically chosen as T cell models because SupT1 cells expressed miR-125b endogenously, whereas CEM cells contained very low or undetectable levels of miR-125b (Fig. 1H). Therefore, these two cell lines served as appropriate models for knockdown and overexpression studies, respectively. Knockdown of miR-125b was achieved by electroporation of anti-miRs into SupT1 cells (Fig. 1I) and overexpression of miR-125b was conducted in CEM cells using miR-125b mimics (Fig. 1J). Measurement of CPSF6 levels in SupT1 cells revealed that lower miR-125b levels were associated with higher CPSF6 protein (Fig. 1I). Conversely, increasing miR-125b levels resulted in a significant reduction in CPSF6 protein levels in CEM cells (Fig. 1M), consistent with the data obtained using HEK-293T cells (Fig. 1G). Similar inverse relationship between miR-125b and CPSF6 expression levels was also observed in THP1 monocytic cells (Fig. 1K and N). Collectively, these results provide evidence that miR-125b levels inversely correlate with CPSF6 protein expression and support the in silico prediction of post-transcriptional regulation of CPSF6 by miR-125b.

CPSF6 3’-UTR activity is regulated by miR-125b

miRNAs negatively regulate gene expression by usually binding to the 3’UTR of the
target mRNA (35,36). Therefore, we probed whether miR-125b targets the 3’UTR region of CPSF6 mRNA. To test this, we introduced the CPSF6 3’UTR into a luciferase reporter construct. Since the 3’UTR region of CPSF6 is about 4.9 kb (Fig. 2A), we generated three truncated constructs that retained the miR-125b binding site: 1) 3’UTR-small containing 1-1011 bp from the 5’-end (3’UTR-S), 2) 3’UTR-Medium containing 1-3114 bp (3’UTR-M), and 3) 3’UTR-Large containing 1-4243 bp (3’UTR-L) (Fig. 2A). These 3’UTR reporter constructs or a control plasmid lacking the 3’UTR (ΔUTR) were transfected individually into HEK-293T cells. At 24 h post transfection, cellular lysates were prepared for luciferase activity measurements. The reporter constructs that harbored the miR-125b binding site showed less luciferase activity compared to the control construct that lacked the binding site (Fig. 2B). Since HEK-293T cells express miR-125b (Fig. 1A-B), the reduced luciferase activity observed with the CPSF6 3’UTR constructs is likely due to the effects of the endogenous miRs on the CPSF6 3’-UTR of the reporter constructs.

Each deletion construct supported a similar level of reduced luciferase activity (Fig. 2B), which is consistent with our in silico identification of the miR-125b binding site within the upstream 1011 bp region of the 3’UTR (Fig. 1, 2A). We next co-transfected the CPSF6 3’UTR constructs individually with miR-mimics or anti-miRs. miR-125b expression in co-transfected cells was confirmed by qPCR (data not shown). Alterations in miR-125b levels by knockdown or over-expression minimally affected the luciferase activity of the control reporter that lacked the 3’UTR (ΔUTR) (Fig. 2C). However, in the presence of anti-miRs, a significant increase in luciferase activity was observed in the lysate of cells individually transfected with the three different CPSF6 3’UTR constructs (Fig. 2D-F). Conversely, increasing miR-125b levels resulted in a decrease in the luciferase activity of the three 3’UTR-containing constructs (Fig. 2D-F). The modest decrease in luciferase activity in miR-125b overexpressed cells could be due to competition between the endogenous CPSF6 3’UTR and the 3’UTR of the reporter constructs for binding to the miR-mimics.

To address potential confounding effects from the 3’UTR of the endogenous CPSF6 mRNA, we next carried out co-transfection studies in CPSF6 knockout cells (29). Western blot analysis confirmed lack of CPSF6 protein expression in the knockout HEK-293T (CKO) cells compared to CWT cells that expressed CPSF6 (Fig. 2G). Knockdown and over-expression studies in these cells revealed that altering miR-125b expression levels in CKO cells minimally affected the luciferase activity of the CPSF6 ΔUTR control construct (Fig. 2H), consistent with the data in Fig. 2C. Accordingly, co-transfection of the three CPSF6 3’UTR constructs individually along with anti-miRs also showed significant increases in luciferase activity in CKO cells (Fig. 2I-K) akin to the results with CWT cells (Fig. 2D-F). Conversely, overexpression of miR-125b greatly reduced CPSF6 3’UTR driven luciferase activity in CKO cells compared to the scrambled controls (Fig. 2I-K). Collectively, these results provide further evidence that the CPSF6 3’UTR is negatively regulated by miR-125b expression.

Mutations in the binding site abrogate miR-125b’s effect on CPSF6 3’UTR

Our in silico studies identified that the miR-125b target site is located at nucleotides 2204-2211 of CPSF6 mRNA, corresponding to 471-477 positions within the 3’UTR (Fig. 1A). To determine the role of the genetic interaction between miR-125b and CPSF6 3’UTR in post-transcriptional regulation, we introduced specific mutations within the binding site. First, in silico mutational analyses revealed that introduction of two specific nucleotide substitutions CA>AT (Mut1) and CA>GT (Mut2) in the CPSF6 3’UTR would collapse the secondary structure formed by the interaction between the two RNA molecules (Fig. 3 A-C). Structural models also revealed that these mutations would reduce the stability and binding affinity between miR-125b and the CPSF6 3’UTR as measured by the mean free energy (MFE) (Fig. 3A-C). Therefore, we introduced these two specific substitutions into the CPSF6 3’UTR region of the 3’UTR-S luciferase reporter construct by site-directed mutagenesis. Then, we studied the effects of miR-125b on the mutant 3’UTRs using the
luciferase reporter assay. Transfection of the parental 3’UTR-S or mutant derivative (Mut1 and Mut2) constructs showed that the luciferase activity of the mutants was comparable to that of the CPSF6 ΔUTR control construct (Fig. 3D-E). Importantly, co-transfection of the CPSF6 3’UTR mutant constructs either with the anti-miRs or miR-mimics showed minimal effect on luciferase activity (Fig. 3 G-I) even though the anti-miRs and miR-mimics, as expected, regulated parental 3’UTR-S activity (Fig. 3F). These results indicate that specific genetic interaction(s) between miR-125b and the 3’UTR sequences drive post-transcriptional regulation of CPSF6 expression.

**miR-125b physically interacts with CPSF6 mRNA through complimentary base pairing**

miRNAs negatively regulate gene expression by directly binding to the 3’UTR regions of their target mRNA (35, 36). Our data in Figs. 1-3 provided compelling evidence that miR-125b post-transcriptionally regulates CPSF6 through a direct interaction involving complementary base pairing. To further probe whether miR-125b directly binds to the CPSF6 3’UTR, we employed a pulldown assay using biotinylated miR-mimics (37). The 3’UTR-S and 3’UTR-Mut1 constructs were co-transfected with either biotinylated miR-mimics or scrambled controls. The biotinylated miR-125b-CPSF6 mRNA complex was pulled down with streptavidin-coated magnetic beads. Enrichment of CPSF6 mRNA in the pull-down samples was quantified by qPCR using CPSF6 3’UTR specific primers. PARP-1 was used as a positive control since it is a direct target of miR-125b (37, 38). In addition, β-actin was included as a negative control since the 3’UTR region of its mRNA does not contain the binding site of miR-125b. The data in Fig. 4 show that in the biotinylated miR-125b pull-down complex, CPSF6 mRNA was significantly enriched compared to the scrambled control. As expected, PARP-1 mRNA was also highly enriched in the pull-down complex, whereas there was minimal enrichment of β-actin mRNA (Fig. 6). Finally, analysis of the CPSF6 3’UTR-Mut1 pull-down revealed that the specific mutation in the miR-125b binding site abrogates enrichment of CPSF6 mRNA in the pull-down assay (Fig. 4). Collectively, these data establish that miR-125b physically interacts with the CPSF6 3’-UTR region via complementary base pairing to post-transcriptionally regulate CPSF6 expression.

**HIV-1 infection activates miR-125b/CPSF6 pathway**

miR-125b is a key cellular miRNA that plays important roles in various cellular functions (39-42). miR-125b has also been reported to inhibit HIV-1 replication by reducing viral protein translation (41, 43), which occurs after HIV-1 integration. Conversely, CPSF6 has been identified as an important cellular factor that facilitates HIV-1 nuclear import and integration targeting (10, 11, 29, 30). Given that miR-125b negatively regulates CPSF6 expression (Fig. 1-3), we tested if HIV-1 infection impacts the miR-125b-CPSF6 pathway. HEK-293T cells (Fig. 5A-B), SupT1 cells (Fig. 5C-D), and THP1 cells (Fig. 5E-F) were infected with VSV-G pseudotyped HIV-1 particles that contained the GFP reporter gene. Productive infection of HEK-293T cells was confirmed by expression of HIV-1 capsid protein (p24) in the infected cells by western blot analysis (Fig. 5A). The presence of higher molecular weight protein bands in the infected cell lane represents expected unprocessed HIV-1 Gag protein (p55) and other processing intermediates (44). In SupT1 (Fig. 5C) and THP1 cells (Fig. 5E), infection was probed by measuring GFP expression via flow cytometry. Then, total RNA from infected and uninfected control cells was subjected to qPCR to measure miR-125b levels. Interestingly, miR-125b expression was significantly reduced in HIV-1 infected HEK-293T (Fig. 5B), SupT1 cells (Fig. DF), and THP-1 cells (Fig. 5F) compared to the respective uninfected cells. Furthermore, western blot analysis of the cellular lysates revealed that CPSF6 expression was upregulated in HIV-1 infected cells compared to the uninfected cells (Fig. 5G-J). These results indicate that HIV-1 infection downregulates miR-125b concurrent with the induction of CPSF6 expression.
HIV-1 infection does not affect the levels of pre- and pri-miR-125b levels

miRNAs are derived from primary transcripts termed "pri-miRNAs" that are subsequently processed to precursor miRNA (pre-miRNA) prior to the biogenesis of mature miRNAs [1]. Therefore, to understand a possible mechanism of miR-125b down-regulation by HIV-1 infection, we measured the levels miR-125b precursors in infected cells. HEK-293T cells were infected with VSV-G pseudotyped HIV-1 particles. RNA isolated from the infected and uninfected controls cells were subjected to qPCR using primer sets designed to amplify specific regions of pri-miR-125b, pre-miR-125b and mature miR-125b. Results from these analyses revealed that the levels of both pri-miR-125b (Fig. 6A) and pre-miR-125b (Fig. 6B) were not significantly reduced in the infected cells compared to the expression levels in uninfected controls. As expected, expression of mature miR-125b was significantly reduced in the infected cells relative to the controls (Fig. 6C). These observations strongly suggest that downregulation of miR-125b in HIV-1 infected cells is not due to reduced biogenesis of the miR from its precursors.

Downregulation of miR-125b is dependent on HIV-1 reverse transcription but not on viral DNA integration

HIV-1 replication is dependent on key early steps of infection including reverse transcription, nuclear entry and viral DNA integration (45). To understand whether downregulation of miR-125b is dependent on any of these early steps, we measured miR-125b levels in infected cells in the presence of inhibitors that block HIV-1 reverse transcription (EFV) or integration (RAL). HEK-293T cells were inoculated with VSV-G pseudotyped HIV-1 particles and cultured in the presence of either EFV (100 µM) or RAL (100 µM). At these concentrations of the inhibitor’s HIV-1 infection was dramatically reduced (Fig. 6D). To measure miR-125b levels, RNA from infected and uninfected control cells was subjected to qPCR. As expected, HIV-1 infection resulted in the down-regulation of miR-125b compared to the uninfected cells (Fig. 6E). Interestingly, miR-125b expression was not significantly downregulated in infected cells treated with EFV (Fig. 6E). Notably, miR-125b level in the EFV treated infected cells was comparable to its expression in uninfected control cells (Fig. 6E). Conversely, in infected cells treated with RAL, miR-125b expression remained significantly down-regulated to a level that was comparable to the infected cells cultured without the inhibitors (Fig. 6E). Collectively, these results indicate that reverse transcription of the viral genome is required for the down-regulation of miR-125b in HIV-1 infected cells, however, this effect is not dependent on the integration of the viral DNA into the host genome.

miR-125b expression affects nuclear entry step of HIV-1 infection

Our results in Fig. 5 demonstrated that miR-125b expression was downregulated in HIV-1 infected cells. Therefore, to better understand the interaction between the miR and the virus, we probed the effects of miR-125b expression on the early steps of HIV-1 infection including reverse transcription, nuclear entry and integration. We transfected miR-mimics and anti-miRs into HEK-293T cells and after 24 h, these cells were inoculated with VSV-G pseudotyped HIV-1 particles. DNA isolated from these cells were subjected to qPCR analysis to measure effects of miR-125b expression on accumulation of late reverse transcription products, formation of 2-LTR circles as a surrogate for viral nuclear entry and integration. Results from these analyses revealed that altering miR-125b expression, either through overexpression or knockdown, minimally affected accumulation of reverse transcription products (Fig. 6F). Interestingly, measurement of 2-LTR circles (Fig. 6G) showed that reducing miR-125b expression resulted in an increased in HIV-1 nuclear entry. However, over-expression of miR-125b minimally changed the number of 2-LTR circles (Fig. 6G). Finally, qPCR analysis revealed that over-expression or knockdown of miR-125b did not significantly influence the level of integrated HIV-1 DNA significantly. Collectively, these studies suggest that miR-125b down-regulation enhances HIV-1 nuclear entry presumably due to increased CPSF6 levels.
Discussion

MiRNA’s are small (~17-24 nucleotide) non-coding RNAs that post-transcriptionally regulate gene expression (46,47). miRNAs negatively regulate gene expression by binding to the 3'UTR of the target mRNA that causes translational repression (34,48,49) or mRNA degradation (50,51). Notably, a single miRNA can interact with multiple targets due to redundancy in base-pairing between the two RNAs (48,52,53). Therefore, miRNAs regulate many cellular pathways and play a widespread role in cellular homeostasis including the regulation of host-pathogen interaction (35,36,48).

MiR-125 is a family of highly conserved miRNAs found in species from nematodes to humans (54). In humans, miR-125 consists of three homologs, hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2 that share the same seed sequence (55). miR-125b is transcribed from two loci located on chromosome 11q23 (hsa-miR-125b-1) and 21q21 (hsa-miR-125b-2) (55,56). A body of evidence shows key functions of miR-125b in cell survival, differentiation, and multiple malignancies (39). Additionally, miR-125b regulates genes involved in innate immunity, inflammation and hematopoietic differentiation (39). Interestingly, miR-125b also belongs to a group of cellular miRNAs that suppress HIV-1 replication (41) (42). The anti-HIV activity of these miRNAs was first reported in resting CD4+ T cells through their ability to suppress viral protein translation (41). This study reported that compared to activated CD4+ T-cells, resting CD4+T-cells showed higher levels of anti-HIV miRNAs including miR-125b, miR-28, miR-150, miR-223, and miR-382. Accordingly, in resting CD4+ T-cells that were infected with HIV-1, knockdown of these miRNAs enhanced viral protein production (41). Subsequent studies in T cell lines also demonstrated a negative association between miR-125b levels and HIV-1 protein translation, further supporting the antiviral activity of miR-125b (43). Nevertheless, the mechanism by which miR-125b and other cellular miRNAs confer anti-HIV activity is not clearly understood.

There are two key mechanisms by which miRNAs can confer antiviral activity (57-59). Through a direct-mechanism, miRNAs can inhibit viral protein translation by targeting HIV-1 mRNAs. This is evident from studies showing that miR-125b and other anti-HIV miRNAs (miR-28, miR-150, miR-223, and miR-382) inhibit HIV-1 protein translation by directly targeting the 3' end of HIV-1 RNAs (41). Additionally, miRNAs can indirectly inhibit HIV-1 by regulating expression of cellular factors that positively or negatively affect viral infection (58,59). In this study, we have identified CPSF6 as a direct target of miR-125b. Since CPSF6 is a cellular factor that promotes HIV-1 infection, our data support an indirect mechanism for miR-125b mediated anti-HIV activity. First, our in silico analysis predicted a highly conserved binding site of miR-125b within the CPSF6 3’UTR (Fig. 1D). Significant interspecies conservation of a miRNA binding site in the 3’UTR of a target gene is an indicator of physiological functionality (34). Accordingly, secondary structure predictions supported that miR-125b could mediate post-transcriptional regulation of CPSF6, since a thermodynamically stable stem-loop hairpin structure was observed between miR-125b and the CPSF6 3’UTR (Fig. 1B-C). To validate the in silico predictions and eliminate false positive target identification, knockdown and over-expression studies of miR-125b were carried out. These studies provided experimental evidence that CPSF6 expression negatively correlates with miR-125b levels. This inverse relationship between miR-125b and CPSF6 was observed in cells that are either normally non-permissive (HEK-293T) or permissive (SupT1, CEM, and THP1) to HIV-1 infection (Fig. 1).

Post-transcriptional regulation of gene expression by miRNAs is dependent on the interaction between the 3'UTR of the target mRNA and the miRNA (46,47). Therefore, to probe the interaction between miR-125b and the 3’UTR of CPSF6, we created three different luciferase constructs containing varied lengths of the CPSF6 3’UTR that retained the miR-125b binding site (Fig. 2A). Studies of the reporter constructs in the presence of miR-mimics or anti-miRs demonstrated a negative correlation between miR-125b expression and CPSF6 3’UTR-driven luciferase activity (Fig. 2). Accordingly, the regulatory effects of miR-125b on the CPSF6 3’UTR was abrogated when specific mutations were introduced in the miR-
125b binding site (Fig. 3). These results provided strong evidence that post-transcriptional mechanism of regulation of CPSF6 is driven by specific genetic interaction between miR-125b and the CPSF6 3'UTR. Finally, a physical interaction between miR-125b and CPSF6 mRNA was verified by pull-down studies (Fig. 4). Biotinylated miR-125b mimics effectively pulled-down the wild type CPSF6 3'UTR but failed to enrich the mutant UTR (Fig. 4). These studies underscored that miR-125b physically interacts with the CPSF6 3'UTR for post-transcriptional regulation.

Identification of a post-transcriptional mechanism of CPSF6 regulation by miR-125b is highly significantly since CPSF6 plays key roles in an array of cellular function. CPSF6 is well-established to regulate alternative cleavage and polyadenylation of cellular mRNA (1,4,5,60,61). Recently, an important role for CPSF6 is also described in cancer biology through its association with paraspeckles (8). Most noteworthy is the accumulating evidence showing key roles of CPSF6 during HIV-1 infection. Specifically, CPSF6 is emerging as a key host factor that promotes nuclear entry and integration targeting of HIV-1 (10,11,29,30). Therefore, we attempted to understand the mechanism(s) by which CPSF6 expression is regulated during HIV-1 infection. Our results revealed that HIV-1 infection resulted in the downregulation of miR-125b concurrent with increased CPSF6 protein levels (Fig. 5) suggesting that CPSF6 expression in HIV-1 infected cells is most likely regulated at post-transcriptional level by miR-125b.

To identify the mechanism of miR-125b downregulation in HIV-1 infected cells, we focused on miRNA biogenesis pathway. miRNA biogenesis depends on transcription of the pri-miRNAs from miRNA-encoding genes (34,46-48). The pri-miRNAs contain an RNA hairpin that is cleaved from the pri-miRNA in the nucleus by Drosha, a double-strand-specific ribonuclease (34,46-48). The resulting precursor "pre-miRNA," is then transported to the cytoplasm and cleaved by Dicer to generate single stranded mature miRNA (34,46-48). Interestingly, there is evidence that viruses such as Herpes simplex virus 1 (HSV-1) target miRNA biogenesis to block the synthesis of mature miRNAs from premiRNA (62). Interestingly, neither pri-miR-125b or pre-miR-125b were reduced by HIV-1 infection even though miR-125b levels were significantly downregulated in these cells (Fig. 6A-C). These observations indicated that downregulation of miR-125b in HIV-1 infected cells was not due to reduced transcript levels of the miR but involve an alternative mechanism. HIV-1 could potentially increase the rate of miR-125b turn-over, a hypothesis consistent with observations that other viruses regulate the turnover of cellular miRNAs. For instance, human cytomegalovirus (HCMV) has been shown to bind and degrade the cellular mature miRNAs to promote virus production (63). Further, Herpesvirus saimiri (HVS) has been reported to cause degradation of the cellular mature miR-27 (64).

Our studies with reverse transcriptase and integrase inhibitors provided further insights into the mechanism of miR-125b downregulation in HIV-1 infected cells (Fig. 6E-F). These data showed that HIV infection results in a 2-fold reduction in miR-125b levels. Similarly, HIV infection in the presence of RAL- an inhibitor of HIV integration also resulted in roughly a 2-fold reduction in miR-125b levels (Fig. 6E). However, there was no alteration in miR-125b levels when HIV infection was carried out in the presence of EFV- an inhibitor of reverse transcription (Fig. 6E). These findings suggest that reverse transcription of HIV RNA, but not viral DNA integration, is necessary for HIV-induced downregulation of miR-125b. Since miR-125b negatively regulates CPSF6 protein levels, these observations imply that an optimal level of CPSF6 protein is most likely necessary after reverse transcription of the viral genome. This results are consistent with the predominant role of CPSF6 after the reverse transcription step of HIV-1 infection (10,11). Even though these results do not identify the factors involved in miR-125b down-regulation, we envision that reverse transcription of HIV-1 is most likely coupled with faster turnover of miR-125b levels since biogenesis of the miR was not altered during infection (Fig. 6A-C).
A functional link between HIV capsid integrity and optimal reverse transcription is well-established. Also, the requirement of the functional interaction between capsid and cellular factor Cyclophilin A for optimal reverse transcription is reported by several groups (12,23,25,65). Although a C-terminal truncation variant of CPSF6 has been shown to potently restrict HIV-1 infection at the reverse transcription step (14), a role of endogenous CPSF6 in reverse transcription has not yet been reported. Therefore, CPSF6 seems unlikely to play a role in the HIV reverse transcription-dependent downregulation of miR-125b. It should be noted that an HIV-encoded protein or RNA element has not been shown previously to interfere with cellular miRNA turnover. Therefore, future studies are needed to identify the viral factors involved in the downregulation of miR-125b. Finally, infection studies in miR-125b over-expressing or knockdown cells showed that reducing miR-125b expression increases nuclear entry of the virus without affecting reverse transcription (Fig. 6F-G). However, enhanced nuclear entry in these cells did not result in increased viral DNA integration. It is possible that the upregulated CPSF6 levels (due to the suppression of the endogenous miR-125b by the anti-miRs) leads to increased nuclear entry of PICs, but the excess CPSF6 in these cells has no impact on the efficiency of the integration step. In this scenario there could be an accumulation of unintegrated viral DNA, thus higher levels 2-LTR circles. This hypothesis is consistent with the emerging role of CPSF6 in integration targeting to specific regions of chromatin but lack of a regulatory role of this cellular factor in HIV-1 integration efficiency.

In summary, our studies provide important insights into host miRNA and HIV-1 interaction. Specifically, we have: a) identified CFSP6 as a direct target of the cellular miRNA “miR-125b”, b) uncovered a post-transcriptional mechanism of regulation of CPSF6, c) revealed activation of miR-125b mediated regulation of CPSF6 to promote HIV-1 nuclear entry, and d) suggest a mechanism by which HIV-1 regulates miR-125b expression during infection.

Experimental Procedures

Reagents

Anti-CPSF6 polyclonal antibody and anti-GAPDH monoclonal antibody were purchased from Proteintech (Rosemont, IL, USA). Hsa-miR-125b-5p mimic and inhibitor were obtained from GE Dharmaco (MA, USA). Control siRNA was purchased from Santa-Cruz Biotechnology (USA). Anti-β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Anti-HIV-1 p24 monoclonal antibody (183-H12-5C), Raltegravir (RAL), and efavirenz (EFV) were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Cell Culture

Human T-lymphoblast cell lines (SupT1 and CEM), monocytic cell line (THP1), and the human embryonic kidney epithelial cell line (HEK-293T) were purchased from American Type Culture Collection (ATCC; Manassas, VA). The TZM-bl reporter cell line was obtained from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc., through the NIH AIDS Reagent Program (USA). While SupT1, CEM and THP1 cells were maintained in Roswell Park Memorial Institute medium (RPMI) medium, the HEK-293T and the TZM-bl cells were maintained in Dulbecco’s modified Eagle’s Eagle’s medium (DMEM). Both RPMI and DMEM were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 2 mM glutamine and 1% antibiotics (penicillin–streptomycin) (Gibco, USA), and the cells were maintained at 37 °C in a humidified 5% CO2 atmosphere. The generation and culturing of HEK-293T CPSF6-knockout (CKO) and CRISPR-control variant (CWT) cell lines have been described previously (29).

In silico Analysis

To predict hsa-miR-125b binding sites, the miR-125b sequence and the 3’UTR sequence of the CPSF6 gene (Genbank ascension number: NM_007007.3) were queried on two independent web server platforms: RNAhybrid 2.1.2 (31,32) and RNA Structures-BiFold (33). RNAhybrid predicts secondary structures between two RNA molecules through Minimum Free Energy (MFE) calculations while the RNA Structures-BiFold
algorithm considers intramolecular base pairings involved in secondary structure formation. The putative miR-125b binding site was further analyzed for sequence conservation across mammalian species using Clustal Omega multiple sequence alignment program (66). RNAhybrid 2.1.2 and RNA Structures-BiFold were also used to probe the effects of nucleotide mutations on the secondary structure of miR-125b and the CPSF6 3'UTR. Mutations within the CPSF6 3'UTR included base substitutions and deletions in the predicted seed sequences.

**Knockdown and Overexpression of miR-125b**

HEK-293T cells (5x10⁵ cells) cultured in 6-well culture plates overnight were transfected with 150 picomoles of miR-mimics or anti-miRs or control siRNA using Polyfect transfection reagent (Qiagen, USA). Post-transfection, cells were cultured for 36 h. Thereafter, cells were gently washed with phosphate-buffered saline (PBS), scraped, aliquoted for RNA and protein extraction, and pelleted by centrifugation at 500 g for 5 min. SupT1, CEM, and THP1 cells (2x10⁵ cells) grown in 6-well culture plates were transfected with 150 picomols of anti-miRNAs or mimics or control siRNA using Neon Transfection System (Thermo-Fisher, USA). SupT1 cells were electroporated under conditions: Voltage-1150 V; Width-20 ms; Pulses-2. CEM cells were electroporated under the following conditions: Voltage- 1230 V; Width- 40 ms; Pulses-1. THP1 cells were electroporated under the following conditions: Voltage=1400 V; Width=20 ms; Pulses=2. SupT1, CEM, and THP1 cells were cultured for 24-36 h, pelleted by centrifugation at 500 g for 10 min and subsequently aliquoted for RNA and protein extraction.

**RNA Isolation and qPCR**

For measuring miRNA expression, total RNA was isolated from cells using Quick-RNA Plus kit (Zymo Research, USA) as per manufacturer’s instructions. cDNA was synthesized from the isolated RNA using the miRCURY locked nucleic acid (LNA) miRNA RT kit (Exiqon, USA). SYBR Green-based qPCR was used to quantify miR-125b-5p or 5s ribosomal RNA (5srRNA) in a reaction mixture containing 50 ng of the cDNA as template and 300 nM of LNA-based primers specific for miR-125b and 5srRNA (Exiqon, USA). qPCR assay was performed using the C1000 Touch CFX96 system (Bio-Rad, USA) and executed as per manufacturer’s instructions (Exiqon, USA). The expression levels (Ct values) of miR-125b-5p were normalized to expression levels of 5srRNA as ΔCt values. For multiple samples involving miR-125b mimic and anti-miR along with control scramble siRNA, the relative expression levels of miR-125b were expressed as 2-ΔCt values by comparing the ΔCt values of the control siRNA to the miR-125b mimic or anti-miR samples.

**Western Blot**

To detect protein levels by western blot, cell lysates were prepared using 1x RIPA buffer (Sigma, USA) and total protein concentrations were quantified by BCA protein assay (Pierce, USA). Equal amounts of total protein from cell lysates were electrophoresed on 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes using a semidy blotter (Bio-Rad, USA). Membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline with Tween 20 (TBST; pH 8.0; Chem Cruz). Blots were then probed with the primary antibody in blocking buffer (with dilution of anti-CPSF6 at 1:4,000 and for anti-GAPDH 1:25,000 (v/v)), and subsequently by a secondary antibody conjugated to horseradish peroxidase (anti-rabbit 1:6,000; anti-mouse 1:40,000). All blots were washed in TBST and developed using the enhanced chemiluminescence (ECL) procedure (Bio-rad, USA). Densitometry analysis was performed through LI-COR Image Studio Digits version 5.2 software (LI-COR, USA). Data were normalized to levels of GAPDH or β-actin.

**Construction of Luciferase Reporter Plasmids**

Three luciferase reporter constructs encoding increasing lengths of the CPSF6 3'UTR, all containing the single candidate miR-125b-binding site, were generated by employing a cloning strategy based on PCR and the presence of specific internal restriction enzyme recognition sites within the CPSF6 3'UTR sequence. Total RNA, isolated from SupT1 cells containing 50 ng of the cDNA as template and 300 nM of LNA-based primers specific for miR-125b and 5srRNA (Exiqon, USA), qPCR assay was performed using the C1000 Touch CFX96 system (Bio-Rad, USA) and executed as per manufacturer’s instructions (Exiqon, USA). The expression levels (Ct values) of miR-125b-5p were normalized to expression levels of 5srRNA as ΔCt values. For multiple samples involving miR-125b mimic and anti-miR along with control scramble siRNA, the relative expression levels of miR-125b were expressed as 2-ΔCt values by comparing the ΔCt values of the control siRNA to the miR-125b mimic or anti-miR samples.
using RNeasy mini kit (Qiagen, USA), was used as template in a cDNA synthesis reaction containing oligo(dT) and random primers (iScript Select cDNA Synthesis Kit, Bio-Rad, USA), and the resulting first-strand cDNA was used as template in PCR to amplify CPSF6 3'UTR sequences. All PCRs were performed using the high-fidelity Phusion DNA polymerase (NEB, USA) and custom-made primers. Agarose gel-resolved DNAs were extracted using the QIAquick gel-purification kit (Qiagen, USA), plasmid DNA and PCR amplicons were digested with restriction enzymes from NEB, DNAs were ligated using T4 DNA ligase (NEB, USA), competent DH5α bacterial cells were used for bacterial transformation, plasmid DNAs were isolated using the Zyppy plasmid mini prep kit (Zymo Research, USA), and the recombinant plasmids were verified by restriction enzyme digestion and Sanger DNA sequencing. To construct the luciferase reporter plasmid containing 1011 bp fragment spanning nucleotides 1-1011 of the CPSF6 3'UTR, the corresponding sequence was amplified using forward primer harboring EcoR1 site (5'-TACCAGAATTCAAGCTGAAGGAAGAGGAACAC-3') and reverse primer harboring consecutive HindIII and Mlu1 sites (5'-TTATACGGCTGGATCTTCCCCATTAATACC-3'), and the gel-purified PCR amplicon was digested with HindIII and Mlu1. Second, the PsiI/Mlu1-cut CPSF6.3'UTR-1004-3114 amplicon, which was generated during the construction of p3'UTR-M, was cut at the engineered HindIII site present upstream of the Mlu1 site in the amplicon. Third, the p3'UTR-S plasmid was digested with PsiI and Mlu1 enzymes and gel-purified. Finally, the PsiI/Mlu1-cut p3'UTR-S plasmid, the PsiI/HindIII-cut CPSF6.3'UTR-1004-3114 amplicon, and the HindIII/Mlu1-cut CPSF6.3'UTR-3109-4243 amplicon were all ligated to yield pMirTarget-CPSF6.3'UTR-L (p3'UTR-L). To introduce site specific mutations in the seed sequence in the CPSF6 3'UTR, we used the Q5 Site-Directed Mutagenesis Kit (NEB, USA). The CA>AT (pMut1) and the CA>GT (pMut2) mutations were introduced using forward primers containing the desired mutations (CA>AT: 5'-GTTTACACCTATGGGAAAGTCTTG-3'; CA>GT: 5'-GTTTACACCTGTGGGAAGTCTTG-3') and a reverse primer (5'-ATCAAAATAACTTGAAACAGCTTTAC-3').

**Luciferase Assay**

All transfections were performed using the polyethyleneimine (PEI) transfection reagent (67). To determine if CPSF6 3'UTR activity is regulated by miR-125b, HEK-293T, CKO, and CWT cells (1x10^4 cells/well) cultured separately in 24-well plates overnight were transfected with pMirTarget control vector (termed "pNAUT") or either of the CPSF63'UTR reporter constructs-p3'UTR-S, p3'UTR-M or p3'UTR-L, and were cultured for 24 hours. To determine the effects of mutated seed sequence on CPSF6 3'UTR activity, HEK-293T cells (2.5x10^5 cells/well)
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cultured overnight in 6-well plate were transfected with either the pA-UTR or pUTR-S or CPSF6-3’UTR mutant plasmids- pMut1 or pMut2, independently or, in a miR-125b over-expression or knockdown background. Post-transfection plates were incubated for 48 h. Transfection efficiency was assessed by measuring the expression of the red fluorescent protein (RFP) encoded in the pMirTarget vector. Transfected cells were lysed using 1x GLO lysis buffer (Life Technologies, USA) and luciferase activity was measured using a plate reader (BioTek, USA). Samples were assayed in triplicate and the data are shown as luciferase activity normalized to RFP expression.

Pull-Down Assays

Biotin-tagged LNA oligonucleotides of miR-125b (hsa-miR-125b-5p: 5’-UCCUGAGACCUCUUUGUGA/3’-Bio) and Scrambled control (5’-GAUGGCAUUCGACAGUUCUA/3’Bio) were purchased from Exiqon/Qiagen (USA). For pulldown experiments, HEK-293T cells (1x10⁶ cells per well) cultured overnight were transfected with 100 picomoles of oligonucleotide and the wild type or mutant pCPSF6-3’UTRs and were incubated for 24 h. Cells were harvested by gentle scraping and lysates were prepared as per our published protocol (38). The freshly prepared lysates were incubated with the blocked streptavidin coated magnetic beads on a bench top rotating mixer for 1 h at room temperature. Then the beads were washed using freshly prepared ice-cold pulldown wash buffer. Finally, the beads were resuspended in 100 µL of nuclease free water. Half the volume of this reconstituted complex was processed for on-column DNase digestion (Ambion, USA) followed by column purification and enrichment using Qiagen RNAeasy purification kit. Post purification, the complexes were used as templates for cDNA synthesis using iScript Reverse Transcriptase kit (Bio-Rad, USA).

To detect the target mRNA from the pulldown mixture, we employed a qPCR assay. We designed primers to amplify the 3’-UTR of CPSF6 mRNA. We also included primer sets for detecting PARP-1 mRNA, a known target of miR-125b, as a positive control and Actin mRNA as a non-specific negative control. In the cDNA synthesis reactions, 50 ng of column purified RNA and 1 µl of crude bead eluate were used as templates in separate reactions to perform cDNA synthesis using oligo-dT primers. The cDNA generated from these reactions served as templates for qPCR reactions. All qPCR reactions were performed in triplicate in a BioRad 96 well clear bottom plate in sterile conditions. 9 µL of the reaction mixture from the qPCR master mix was aliquoted into each well of the 96 well PCR plate. Then 1 µL of the cDNA was added to each well to achieve a final volume of 10 µL. Thereafter the PCR plate was sealed using heat resistant PCR plate sealer and loaded into the CFX96 real-time PCR system (Bio-Rad, USA) to perform thermal cycling. The thermal cycling conditions for qPCR analysis included an initial denaturation at 95°C for 10 min followed by amplification and acquisition at 95°C for 10 sec, 56°C for 30 sec and 72°C for 30 sec for 30 cycles, and the thermal profile for melt curve analysis was obtained by holding the sample at 65°C for 31 sec followed by a linear ramp in temperature from 65°C to 95°C with a ramp rate of 0.5°C/sec and acquisition at 0.5°C intervals. The expression level of target genes was analyzed relative to their expression in scrambled controls. Data were plotted as fold change, which was calculated by comparing the expression values of the target genes obtained in 3’ biotinylated-miR-125b-5p pulldown with that of 3’ biotinylated-scrambled control.

Virus Stocks and Infection Assays

Virus stocks were generated using calcium phosphate-based transfection of HEK-293T cells (68,69) with plasmids pNL4.3 GFP, which does not express the HIV-1 envelope but encodes the green fluorescent protein (GFP) in place of the nef gene (70), and pHCMV-G that encodes vesicular stomatitis virus G glycoprotein (VSV-G) (71). Briefly, 2x10⁶ cells were seeded per 10 cm dish and cultured overnight. Next day, cells in each culture dish were transfected with 20 µg of total plasmid DNA (9:1 ratio of pNL4.3 GFP to pHCMV-G). Twelve hours post-transfection, the cells were washed once with PBS, replenished with 6 mL growth medium, and cultured further for 48 h. The virus-containing culture supernatants were harvested, cleared of
cell debris by low-speed centrifugation, filtered through 0.45 μm filters, and treated with DNase I (Calbiochem; 20 μg/mL of supernatant) in the presence of 10 mM magnesium chloride for 1 h at 37°C. The concentration of the virus stocks was quantified by the p24-specific enzyme-linked immunosorbent assay (ELISA) using standard methods (68). Virus infectivity was determined using TZM-bl indicator cells as described (72).

For infection assay, cells (HEK-293T or SupT1 or THP1 cells) seeded in 96-well plates (5x10⁴ cells per well) were spinoculated with pseudotyped HIV-1 particles (equivalent to 15 ng of p24 per well) in the presence of 6 μg/mL polybrene (Sigma, USA) for 2 h at 25°C and then cultured for 48 h. Productive infection of HEK-293T cells was confirmed by western blot analysis probing for HIV-1 p24 protein using anti-CA antibody. Infection of SupT1 and THP1 cells were confirmed by measuring GFP signal intensity using flow cytometry (Millipore GUAVA, USA). For infection studies with HIV-1 inhibitors, cells were infected with VSV-G HIV-GFP virus and concurrently treated with either 1 μM Raltegravir (RAL) or 1 μM Efavirenz (EFV) or DMSO as a control. After 24-36 h, cells were harvested and total RNA was isolated followed by cDNA synthesis and evaluation of miR-125b levels using RT-PCR.

Quantification of Reverse transcription, 2-LTR Circles, and Integration

Reverse transcription products, 2-LTR circles and proviral integration in 100 ng of total DNA from infected and uninfected controls were measured by qPCR and analyzed using our published method (69). Briefly, reverse transcription products were quantified using SYBR green-based qPCR, and 2-LTR circles were quantified using TaqMan probe-based qPCR. To quantify the copy number of chromosomally-integrated proviral DNA in HIV-infected cells, a nested PCR strategy wherein primers designed to amplify only the junctions of the chromosomal-integrated viral DNA but not any unintegrated viral DNA is used in the first round end-point PCR, followed by the second round TaqMan probe-based qPCR with primers that amplify viral LTR-specific sequences present in the first round PCR amplicons (69). A standard curve was generated using 10-fold serial dilutions of known copy numbers of the HIV-1 molecular clone pNL43 (73) or p2LTR plasmid (74) during qPCR in tandem with measurement of sample quantification. Samples were evaluated against appropriate standard curve to generate values for copy number of late RT products and chromosomally integrated proviral DNA (pNL43 standard curve) or 2-LTR circle formation (p2LTR standard curve).

Quantification of Primary (Pri-) and Pre-miR-125b Levels

Primers were selected from the human miR-100-let-7a-2-miR-125b-1 cluster host gene sequence containing primary (pri-), precursor (pre-), and mature miR-125b-5p sequences. A region within 400 nucleotides of the mature sequence was chosen for designing primer sets for the pri-miR-125b sequence. These include: Pri-forward primer = 5’ CTGAGGTATTGAGTATAACCTCTGGG 3’; Pri-reverse primer = 5’ TCCAGGAGCTGCCACTCTCTG 3’. Pre-miR-125b primers were designed to encompass entire 70 bp region of pre-miR-125b sequence: Pre-miR-125b forward primer: 5’ CCTTCAGTCCCTGAGACCTAAC 3’; Pre-miR-125b reverse primer: 5’ GACTCGCAGCTCCAAGAGC 3’.

To measure precursors of miR-125b, 293T cells (5x10⁵ cells/mL) were cultured overnight in six-well plates and subsequently inoculated with VSV-G HIV-GFP virus as previously described. After 24-36 h, cells were harvested and RNA from these cells were isolated. 100 μg of total RNA was used to synthesize cDNA using ABMGood OneScript Plus cDNA synthesis kit (Vancouver, Canada) and random primers as per the manufacturer’s instructions. 2 μL of cDNA were used in a RT-PCR reaction using SYBR Green 2x Supermix (Biorad). Samples were run in triplicates and normalized to 5s rRNA levels.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) obtained from three independent experiments. Significance of differences between control and treated samples
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was determined by Student’s t test. Values of $p < 0.05$ were considered statistically significant.

**Data Availability**

All the data described are contained in the manuscript.

**Conflict of Interest**

The authors declare that they have no conflict of interest with the content of this article.

**Author Contribution**

SD, MB, JP, FV and CD conceptualized the study and designed the experiments. EC, SD, MB, JH and JP performed the experiments. EC, SD, MB, AP, JP, FV and CD analyzed the data. GS and AE provided reagents and assisted in data analysis. GS and AE provided reagents and assisted in data analysis. EC, SD, MB, JP, and CD wrote the manuscript. All other authors reviewed the manuscript.

**FOOTNOTES**

This work was partly supported by the NIH grants R01 AI136740, R56 AI122960, R24 DA036420 and U54 MD007593 to CD, R01 AI052014 and P50 AI150481 to ANE, and T32 AI007245 to GAS. We also acknowledge the Meharry Translational Research Center (MeTRC) grant U54 RR026140 from NCRR/NIH, and Tennessee CFAR grant P30 AI110527 from the NIH.
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CPSF6 is a direct target of miR-125b

Figure 1. miR-125b negatively regulates CPSF6 expression. (A) Schematic representation of CPSF6 mRNA (~6.6 kb) containing the miR-125b seed sequence in the 3’UTR (~4.8 kb). Results from in silico analysis of CPSF6 3’UTR sequences for the presence of miR-125-binding sites and the formation of hairpin structures between the miR and the 3’UTR by using (B) RNAHybrid 2.1.2 and (C) RNAstructure-biFold prediction web servers. mfe = minimum free energy of the predicted secondary structures. (D) Sequence alignment of 3’UTR regions of CPSF6 mRNA from different mammalian species. (E-F) qPCR analyses of miR-125b levels in HEK-293 T cells treated with anti-miRs (E) or miR-mimics (F) of miR-125b or scrambled controls (F). (G) Representative immunoblot showing CPSF6 protein expression in miR-125b-knockdown and overexpressing cells. The graph above the immunoblot shows densitometry analysis of immunoblots from three independent experiments. (H) Endogenous levels of miR-125b in SupT1 and CEM cells as measured by qPCR. Results from qPCR analysis of knockdown of miR-125b levels in SupT1 cells (I) and overexpression of miR-125b via miR-mimics in CEM (J) and THP1 (K) cells. Representative immunoblots showing CPSF6 and GAPDH (loading control) protein expression in miR-125b-knockdown SupT1 cells (L), miR-125b-overexpressing CEM cells (M), and miR-125b-overexpressing THP1 cells (N). The graphs above the blots show densitometry analysis of immunoblots from three independent experiments. Error bars represent SEM. * represents p < 0.05 for the comparison of anti-miR and miR-mimics vs scrambled controls.
Figure 2. miR-125b negatively regulates CPSF6 3’UTR activity. (A) Schematic representation of the three different sized DNA fragments of CPSF6 3’UTR that were cloned downstream of the luciferase stop codon to generate the corresponding reporter vectors: 3’UTR-Small (3’UTR-S), 3’UTR-Medium (3’UTR-M), 3’UTR-Large (3’UTR-L). The location of the miR-125b seed sequence is presented as an inverted black triangle. HEK-293T cells were transfected with either of these 3’UTR luciferase reporter vectors or the control pΔUTR (ΔUTR) vector in the presence or absence of miR-125b mimics or anti-miR-125b. 24 h post transfection, luciferase activity was measured in the cellular lysates and was plotted relative to the controls. (B) Luciferase activity of the CPSF6 3’UTRs and ΔUTR vectors. Luciferase activity of (C) ΔUTR, (D) 3’UTR-S, (E) 3’UTR-M, (F) 3’UTR-L vector in the presence of anti-mRs or miR-mimics or scrambled controls. (G-K) Effects of altered miR-125b levels on CPSF6 3’UTR activity in CPSF6 knockout (CKO) cells. Immunoblots showing CPSF6 expression in wild type HEK-293T (CWT) (G, Top panel) and CKO cells (G, Middle panel) and GAPDH (loading control) expression in CKO cells (G, Bottom panel). Luciferase activity in CKO cells transfected with (H) ΔUTR, (I) 3’UTR-S, (J) 3’UTR-M, (K) 3’UTR-L vector in the presence of anti-mRs or miR-mimics or scrambled controls in CKO cells. Error bars represent SEM, whereas *p < 0.05 stands for the comparison of anti-mR or miR mimic samples versus scrambled controls.
CPSF6 is a direct target of miR-125b

**Figure 3.** Mutations in the miR binding sequence abrogate the regulatory effects of miR-125b on the CPSF6 3’UTR. (A-C) *In silico* analysis of the effects of two different mutations, Mut1 (CA>AT) and Mut2 (CA>GT), introduced at the miR-125b-binding site in CPSF6 3’UTR on their binding stability and affinity (as measured by mfe) and the secondary hairpin loop structure integrity. Shown are the effects of Mut1 (B) and Mut2 (C) in comparison to WT CPSF6 3’UTR (A). (D-E) Effects of endogenous miR-125b on the activity of the CPSF6 3’UTR mutants, Mut1 and Mut2. Shown is the comparison between the luciferase activity in the lysates of HEK-293T cells transfected with Mut1 (D) or Mut2 (E) and the luciferase activity of ΔUTR and 3’UTR-S. (F-I) Effects of miR-125b overexpression or downregulation on the activity of the CPSF6 3’UTR mutants, Mut1 and Mut2. Luciferase activity in lysates prepared from HEK-293T cells transfected with (F) 3’UTR-S, (G) ΔUTR, (H) Mut1, or (I) Mut2 reporter constructs in the presence or absence of anti-miRs or miR-mimics or scrambled controls. Data presented are mean values of three independent experiments with error bars representing SEM. * represents p < 0.05 for the comparison of miR-mimics or anti-miR vs scrambled controls.
4. miR-125b physically interacts with \textit{CPSF6} mRNA. HEK-293T cells were transfected with biotinylated-miR-125b mimics or scrambled controls and the miR-125b:mRNA complexes were pull-downed with streptavidin-coated magnetic beads. Enrichments of mRNAs of interest were quantified by qPCR with primers complementary to their respective 3’UTRs. Data shown are enrichment of \textit{PARP-1} (positive control), actin (negative control) and \textit{CPSF6} 3’UTR levels. Blue bars represent pull-downs with miR-mimics whereas red bars represent pull-downs with scrambled controls. Data presented are mean values of three independent experiments with error bars representing SEM. * represents \( p < 0.05 \) for the comparison of miR-mimics vs scrambled controls.
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Figure 5. HIV-1 infection downregulates miR-125b expression. (A-B) HEK-293T cells were inoculated with pseudotyped HIV-1 GFP reporter virus. (A) Infection was assessed by detecting HIV-1 p24 protein in the cellular lysate by western blot analysis. Purified capsid (CA) protein served as a positive control. (B) RNA isolated from these cells was subjected to qPCR to measure miR-125b levels. (C-D) SupT1 cells and (E-F) THP1 monocyctic cells were inoculated with pseudotyped HIV-1 GFP reporter virus. Productive infection in SupT1 (C) and THP1 (E) cells was assessed via GFP expression. miR-125b expression in uninfected and infected SupT1 (D) and THP1 (F) cells was measured by qPCR. Representative immunoblots showing CPSF6 protein expression in uninfected and infected HEK-293T (G) cells and SupT1 (I) cells. Graphs showing the densitometry analysis of CPSF6 expression in HEK-293T (H) and SupT1 (J) cells from three independent experiments. Error bars represent SEM. * represents p < 0.05 for the comparison of uninfected vs infected cells.
Figure 6. (A-C) HIV-1 infection does not affect miR-125b precursors. (A-C) HEK-293T cells were inoculated with pseudotyped HIV-1 GFP reporter virus and levels of (A) pri-, (B) pre- and (C) mature miR-125b was measured in infected and uninfected cells by qPCR. (D-E) Down-regulation of miR-125b is dependent on HIV-1 reverse transcription. (D) HEK-293T cells were inoculated with pseudotyped HIV-1 GFP virus in the presence of EFV or RAL and productive infection was assessed by GFP expression. (E) RNA from these cells were analyzed for measuring miR-125b levels by qPCR. (F-H) Effects of miR-125b expression on early steps of HIV-1 infection. HEK-293T cells were transfected with either anti-miRs or mimics of miR-125b and after 24 h, inoculated with pseudotyped HIV-1 GFP virus. RNA from these cells was isolated and subjected to qPCR to measure copies of (F) Reverse Transcription, (G) 2-LTR circles, and (H) integration. Data presented are from three independent experiments. Error bars represent SEM. * represents p < 0.05 for the comparison of uninfected vs infected cells.
The HIV-1 capsid–binding host factor CPSF6 is post-transcriptionally regulated by
the cellular microRNA miR-125b

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J. Biol. Chem. published online March 9, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA119.010534

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