Figures and figure supplements

N6-methyladenosine of HIV-1 RNA regulates viral infection and HIV-1 Gag protein expression

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Figure 1. HIV-1 RNA contains m^6A modifications and YTHDF1–3 proteins bind to m^6A-modified HIV-1 RNA. (A–B) The distribution of m^6A reads from m^6A-seq mapped to HIV-1 genome (red line) in HIV-1 infected Jurkat cells (A) or primary CD4^+ T-cells (B). Baseline signal from the RNA-seq of input samples is shown as a black line. A schematic diagram of HIV-1\text{NL4-3} genome is shown above. TAR, transacting response element; RRE, Rev response element. Jurkat cells (A) or primary CD4^+ T-cells (B) were infected with HIV-1\text{NL4-3} and total RNA was extracted for m^6A-seq at 72 or 96 hr post-infection. Figure 1 continued on next page.
(hpil), respectively. (C) YTHDF1-3 proteins bind to the HIV-1 gRNA. HeLa/CD4 cells overexpressing FLAG-tagged YTHDF1-3 proteins were infected with HIV-1_{NL4-3} (MOI= 5) for 72 hr and used in CLIP-seq assay to identify their binding sites on HIV-1 gRNA. The distribution of mapped reads (>16 nt) with corresponding nucleotide positions are shown, forming peaks as putative binding positions. Asterisks mark the peak clusters overlapping with identified m^6A peaks, indicating high-confident YTHDFs binding sites. Read density was normalized to the total number of mapped reads in each sample (YTHDF1: 28438, YTHDF2: 232568, YHTDF3: 124915). The data presented are representative of results from two independent experiments (n=2). DOI: 10.7554/eLife.15528.003
HIV-1 RNA contains m\(^6\)A modifications. HEK293 T cells were transfected with a proviral DNA-containing plasmid (pNL4-3). Total RNA was extracted at 48 hr post-transfection and immunoprecipitated with an m\(^6\)A-specific antibody. Enriched RNA was subjected to next generation sequencing. Peaks show the relative abundance of m\(^6\)A sites on the HIV-1 genome. The distribution of m\(^6\)A reads from m\(^6\)A-seq mapped to HIV-1 genome (red line). Baseline signal from the RNA-seq of input samples is shown as a black line. A schematic diagram of HIV-1 NL4-3 genome features is shown above. TAR, transacting response element; RRE, Rev response element. The data presented are representative of two independent experiments.

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Figure 1—figure supplement 2. Quantification of HIV-1 RNA m^6A level using liquid chromatography-mass spectrometry. HIV-1 RNA (250 ng) was isolated from highly purified HIV-1MN virions (total 600 µg of p24 capsid) and subjected to quantitative analysis of the m^6A level using LC-MS/MS (n=3 of each sample). The results are presented are from representative of two independent experiments.

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A  Un-infected Jurkat cells  
- 3' UTR: 7.9%
- CDS: 3.6%
- 5' UTR: 30.6%
- Noncoding: 58.0%

HIV-1 Infected Jurkat cells  
- 3' UTR: 7.7%
- CDS: 3.5%
- 5' UTR: 30.6%
- Noncoding: 58.2%

B  Un-infected primary CD4+ T-cells  
- 3' UTR: 8.8%
- CDS: 3.3%
- 5' UTR: 28.6%
- Noncoding: 59.3%

HIV-1 infected primary CD4+ T-cells  
- 3' UTR: 8.0%
- CDS: 3.5%
- 5' UTR: 28.3%
- Noncoding: 60.2%

C  
RRACH frequency in m^5A peaks (%)  
- Uninfected  
- HIV-1 Infected  

D  
GGACU frequency in m^5A peaks (%)  
- Uninfected  
- HIV-1 Infected
Figure 1—figure supplement 3. Distribution of m\(^6\)A in cellular RNAs and the frequency of m\(^6\)A motifs in HIV-1-infected cells (A–B) Pie charts show the distribution of m\(^6\)A peaks in the 5' UTR, coding DNA sequence (CDS), 3' UTR, and noncoding regions of transcripts from uninfected and HIV-1-infected Jurkat T-cells (A) or primary CD4\(^+\) T-cells (B). The m\(^6\)A peak distribution in HIV-1-specific RNAs is also shown. (C–D) Frequency of the RRACH motif (C) and the GGACU motif (D) within the m\(^6\)A peaks in cellular RNAs from the uninfected control and HIV-1-infected cells. Data presented are the average results of duplicated samples (n=2). DOI: 10.7554/eLife.15528.006
Figure 1—figure supplement 4. Gene ontology (GO) analysis of m^6A-modified cellular genes in HIV-1 infected cells. (A and B) GO terms specific to virus related pathways and corresponding p values, clustered from methylated genes detected in Jurkat cells (A) or primary CD4^+ T cells (B) infected.
with HIV-1. (C and D) GO graphs showing functional clusters from genes with unique m^6A peaks identified in HIV-1-infected Jurkat cells (C) or primary CD4^+ T-cells (D) when compared to uninfected cells. Data presented are the average results of duplicated samples (n=2).

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Figure 2. YTHDF1–3 proteins negatively regulate post-entry HIV-1 infection in HeLa cells. (A–B) Overexpression of YTHDF1–3 proteins in HeLa cells significantly inhibits HIV-1 infection compared to vector control cells. (A) Overexpression of YTHDF1–3 proteins in HeLa cells was confirmed by immunoblotting. (B) HeLa cells overexpressing YTHDF1–3 proteins were infected with HIV-1 Luc/VSV-G at an MOI of 0.5 and viral infection was measured by luciferase activity at 24 hpi. (C) Overexpression of YTHDF1–3 proteins inhibits HIV-1 Gag protein synthesis in infected cells. HeLa cells overexpressing individual YTHDF1–3 proteins or the vector control cells were infected by HIV-1-Luc/VSV-G at an MOI of 0.5. At 24 hpi, the expression of HIV-1 Gag and YTHDF1–3 proteins (FLAG-tagged) was determined using immunoblotting. GAPDH was used as a loading control and mock-infected vector control cells were used as a negative control. (D and E) Individual knockdown of endogenous YTHDF1–3 proteins in HeLa cells significantly increases HIV-1 infection compared to vector control cells. HIV-1 infection assays were performed as described for panel B. *p<0.05, **p<0.005, and ***p<0.0005, compared to vector control without AZT treatment. All results are shown as mean ± SD (n=3) and data presented are representative of at least three independent experiments.

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Figure 3. YTHDF1–3 proteins negatively regulate post-entry HIV-1 infection in CD4+ T-cells. (A) Individual knockdown of endogenous YTHDF1–3 proteins in Jurkat CD4+ T cells was confirmed by immunoblotting. (B) Knockdown of YTHDF1–3 proteins does not affect proliferation of Jurkat cells. (C) Relative HIV-1 infection (%) in Jurkat cells. (D) Relative HIV-1 infection (%) in primary CD4+ T cells. (E) Relative HIV-1 infection (%) in primary CD4+ T cells. Figure 3 continued on next page.
Jurkat cells ($2 \times 10^4$) were seeded and cultured for 3 days. At the times indicated, cell proliferation was measured using the MTS assay. (C) Knockdown of YTHDF1–3 proteins significantly increases HIV-1 infection compared to vector control cells. (D) Individual knockdown of YTHDF1–3 proteins in activated primary CD4+ T-cells from a healthy donor. (E) Knockdown of YTHDF1–3 proteins significantly increases HIV-1 infection compared to vector control cells. (A and D) GAPDH was used as a loading control. (C and E) The vector controls without AZT were set as 100%. The reverse transcriptase inhibitor AZT treated cells were used as positive control for productive HIV-1 infection. *p<0.05, **p<0.005, and ***p<0.0005, compared to vector control without AZT treatment. All results are shown as mean ± SD (n=3) and data presented are representative of at least three independent experiments.

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Figure 4. YTHDF1–3 proteins inhibit post-entry HIV-1 infection by blocking viral reverse transcription. HeLa cells over-expressing or knocking-down (shRNA) individual YTHDF1–3 proteins were infected with HIV-1-Luc/VSV-G at an MOI of 0.5. (A, B and D) Genomic DNA was isolated from the cells 24 hr post-infection and HIV-1 late reverse transcription (RT) products were quantified by qPCR. (C) YTHDF family proteins reduce the formation of HIV-1 2-LTR circles in infected HeLa cells. At 24 hr post-infection, DNA was isolated from the cells and the 2-LTR circles were analyzed by qPCR and normalized to GAPDH levels. AZT treated vector control cells were used as a negative control for HIV-1 inhibition. *p < 0.05 compared to the vector control without AZT treatment. All results are shown as mean ± SD (n=3) and data presented are representative of at least three independent experiments.

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YTHDF1–3 proteins negatively regulate HIV-1 gag mRNA expression. Specific shRNAs or scrambled shRNA vector-treated cells were infected with HIV-1 Luc/VSV-G at an MOI of 0.5. Total RNA was isolated from the cells 24 hr post-infection and HIV-1 gag mRNA levels were quantified using qRT-PCR. (A and B) HIV-1 gag mRNA levels in the infected HeLa cells with overexpression (A) or knockdown (B, shRNA) of YTHDF1–3 proteins. (C) HIV-1 gag mRNA levels in the HIV-1 infected Jurkat cells after knockdown of YTHDF1–3 proteins. AZT treated vector control cells were used as a negative control of HIV-1 infection (A–C). The vector controls without AZT were set as 100%. *p<0.05, **p<0.005, and ***p<0.0005, compared to vector control without AZT treatment. All results are shown as mean ± SD (n=3) and data presented are representative of at least three independent experiments.

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Figure 5. YTHDF1–3 proteins bind to HIV-1 gRNA in infected cells. (A) Immunoblotting of YTHDF1–3 proteins in the input and immunoprecipitation (IP) samples from HIV-1-Luc/VSV-G infected HeLa cells. FLAG antibodies were used to immunoprecipitate FLAG-tagged YTHDF1–3 proteins overexpressed in HeLa cells after HIV-1 infection. A short and long exposure of the immunoblot is shown. (B) HIV-1 gRNA is bound by YTHDF1–3 proteins expressed in HeLa cells. HeLa cells stably overexpressing FLAG-tagged YTHDF1–3 proteins or empty vector control cells (Ctrl) were infected with HIV-1-Luc/VSV-G at an MOI of 5 for 3 hr. Cell lysates were immunoprecipitated with anti-FLAG, RNA was extracted and HIV-1 gag RNA levels were measured. **p<0.005 compared to the vector control cells. (C) YTHDF1–3 affect HIV-1 gag RNA kinetics. HIV-1 gag RNA levels in YTHDF1–3-expressing HeLa cells were quantified by qRT-PCR. The relative levels of gag RNA in infected cells were normalized to that of the vector control cells at 6 hr post-infection (hpi). ***p<0.0005, compared to the control cells at 6 hpi (set as 100%). All results are shown as mean ± SD (n=3) and data presented are representative of at least three independent experiments.

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Figure 6. The m^6A writers and erasers affect HIV-1 Gag expression in virus-producing cells. (A and B) Individual or combined knockdown of endogenous METTL3 and METTL14 inhibits HIV-1 Gag protein expression. HEK293T cells were transfected with indicated siRNA, and then with an HIV-1 proviral DNA plasmid (pNL4-3). Cells and supernatants were collected for analyses at 36 hr post-transfection. (A) Expression of METTL3, METTL14 and HIV-1 Gag proteins in the transfected HEK293T cells was detected by immunoblotting. (C and D) Knockdown of endogenous AlkBH5, FTO, or both promotes HIV-1 Gag protein expression. HEK293T cells were transfected with indicated siRNA, and then with pNL4-3. Cells and supernatants were collected at 36 hr post-transfection. (C) Expression of AlkBH5, FTO and HIV-1 Gag proteins in the cells was detected by immunoblotting. (A and C) GAPDH was used as a loading control. Relative levels of Gag expression were normalized to GAPDH levels. (B and D) HIV-1 capsid p24 levels in supernatants were measured by ELISA. The relative levels (%) are also shown. *p<0.05 compared to the siRNA control. The results are shown as mean ± SD (n=3) and data presented are representative of three independent experiments.

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Figure 7. Proposed mechanisms and dynamics of m<sup>6</sup>A modification of HIV-1 RNA in regulating viral infection in cells. In the nucleus, the m<sup>6</sup>A writers (METTL3 and METTL14) add the m<sup>6</sup>A marker to HIV-1 genomic RNA (gRNA) or mRNA, and the m<sup>6</sup>A erasers (FTO and AlkBH5) remove the m<sup>6</sup>A modifications of HIV-1 RNA. The m<sup>6</sup>A modification of HIV-1 RNA can promote viral protein translation in cells. In contrast, cytoplasmic m<sup>6</sup>A readers (YTHDF1–3) bind to m<sup>6</sup>A-modified HIV-1 gRNA, which can result in inhibition of HIV-1 reverse transcription (RT), viral mRNA expression, and thereby HIV-1 infection in cells.

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