Dynamics of the human and viral m6A RNA methylomes during HIV-1 infection of T cells

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Supplementary Methods

shRNA plasmids and lentiviral transduction

Lentiviral particles for shRNA-mediated gene silencing were produced by transfection of 293T cells with pLKO (shRNA), psPAX2 (packaging), and pMD2.G (envelope) plasmids (1:0.75:0.25 ratio) using Lipofectamine 2000. After 2 days, the supernatant was collected, filtered, mixed with 5 μg/ml Polybrene, and added to MT4 cells overnight. The supernatant was then removed, and the cells were washed in PBS and resuspended in fresh RPMI/10% FBS. Three days after lentiviral transduction, cells were infected with HIV-1 as described above. Cells were used for experiments at 3 days post-infection. pLKO shRNA plasmids were purchased from Sigma (Mission shRNA Library). ShRNA sequences are listed in Supplementary Information Table 3.

Western blot analysis

MT4 cells (106/sample) were lysed in M-PER buffer (Pierce), and samples of 20 μg total protein were resolved by 12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 2% BSA in PBST (PBS/0.1% Tween 20), incubated for 1 h with primary antibodies (1:1,000 dilution in BSA/PBST), washed with PBST, and then incubated for 1 h with secondary antibodies (1:10,000 dilution in BSA/PBST). Antibodies used were: rabbit monoclonal anti-GAPDH (D16H11, Cell Signaling Technology), rabbit polyclonal anti-METTL3 (15073-1-AP, Proteintech Group), rabbit polyclonal anti-METTL14 (HPA038002, Sigma), mouse polyclonal AlkBH5 (SAB1407587, Sigma), rabbit anti-gag p24 antiserum (65-005, BioAcademia), mouse monoclonal anti-Rev antibody (1G7, NIH AIDS Reagent Program), mouse monoclonal anti-FLAG M2 (F3165, Sigma), IRDye 680-conjugated anti-mouse IgG (LiCor), and IRDye 800-conjugated anti-rabbitIgG (LiCor).

Northern blot analysis

5′-amino-modified DNA oligonucleotides specific for HIV-1 RNA were purchased from IDT. The anti-HIV-1 probe sequences were:

anti-gag: CCCGCTTAATACTGACGCTCTCGCACCCA,
anti-vif: ATCCCTAATGATCTTTGCTTTTCTTCTTGGCACTACT,
anti-nef: GCTCAGCTCGTCTCATTCTTTCCCTTACAGTAG.

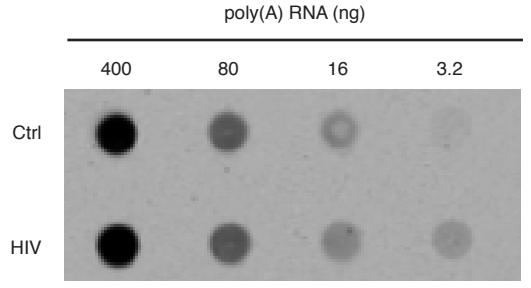
Oligonucleotide conjugation with Alexa Fluor 680 NHS ester was performed in 0.1 M sodium tetraborate, pH 8.5, for 3 h at room temperature. 0.2 mg of NHS ester was dissolved in 30 uL of DMSO and 200 uL of water, mixed with 20 ug of oligo. The conjugation reaction was carried out at rt for 3 hr and then blocked with Tris-buffer, pH 7.5, final concentration of 50 mM followed by precipitation with sodium acetate and ethanol. RNA was extracted with Trizol reagent according the manufacturer's instructions. 5 μg total RNA were loaded for each lane on a 1% agarose/0.4% formaldehyde gels in 20 mM HEPES and 1.5 mM EDTA, pH 7.8. Northern blotting was performed using a NorthernMax kit (Ambion).

RT-qPCR analysis

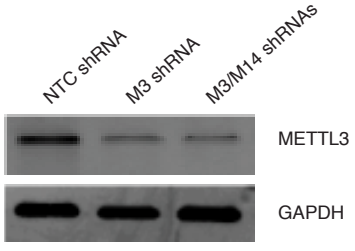
cDNA was prepared from 1 μg total RNA using iScript Supermix (Bio-Rad). qPCR reactions were performed using SSoAdvanced SYBR Green Mix (Bio-Rad) with a LightCycler 480 instrument (Roche). Quantification of the relative fold change in mRNA was performed by the $\Delta\Delta$Cp method. PCR primers are listed in Supplementary Information Table 4.

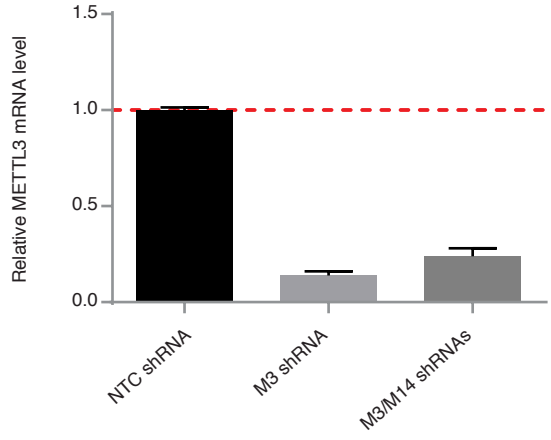
Supplementary Figures

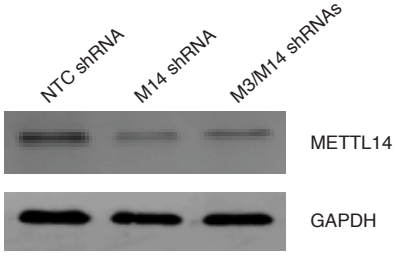
Supplementary Figure 1. m6A content of poly(A)-enriched RNA. Poly(A) RNA was isolated from uninfected (Ctrl) and HIV-1-infected MT4 cells with an oligo(dT)-mRNA isolation kit. Enriched fractions were quantified, serially diluted, and immunoblotted with an antibody against m6A. The data is a representative of five independent experiments.

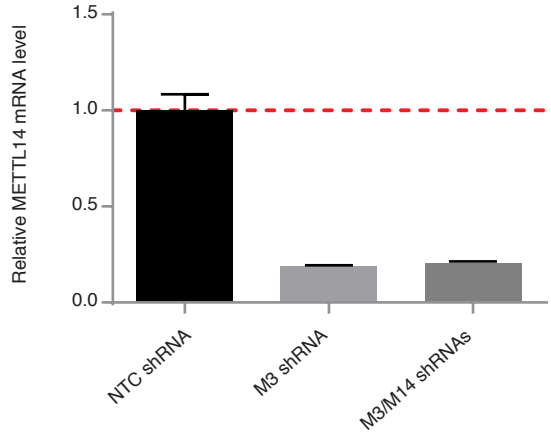


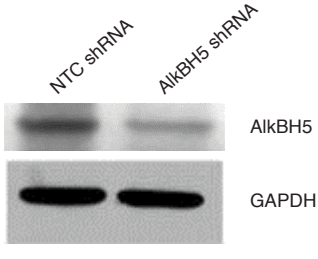
Supplementary Figure 2. METTL3, METTL14, and AlkBH5 knockdown efficiency. MT4 cells were infected with lentiviruses encoding the indicated shRNAs, and 3 days later, knockdown efficiency was assessed by western blot (left) and RT-qPCR (right) analyses. GAPDH served as a loading control for western blotting. The data is a representative of five independent experiments (n=3). RT-qPCR results are the mean ± s.e.m. of 5 independent experiments and are expressed as the mRNA level relative to that in cells expressing nontargeting control (NTC) shRNA. n indicates the number of technical replicates.

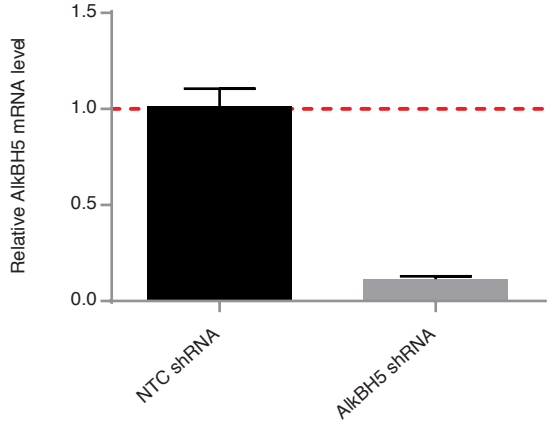




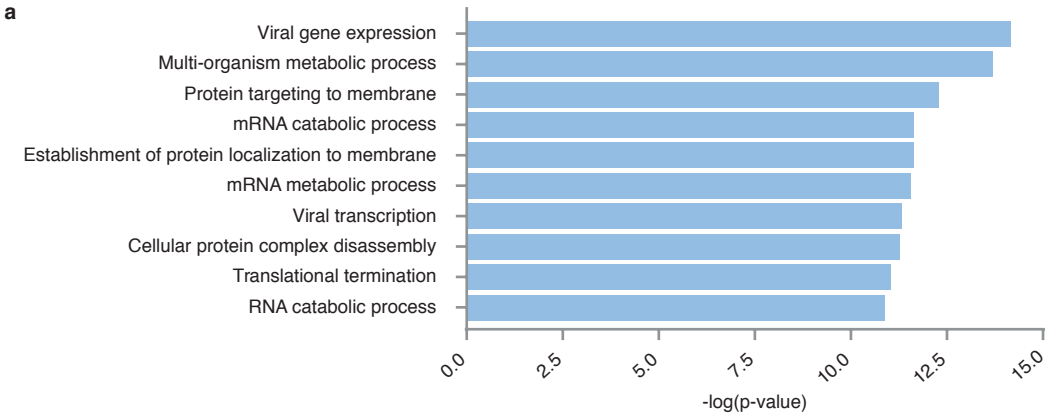




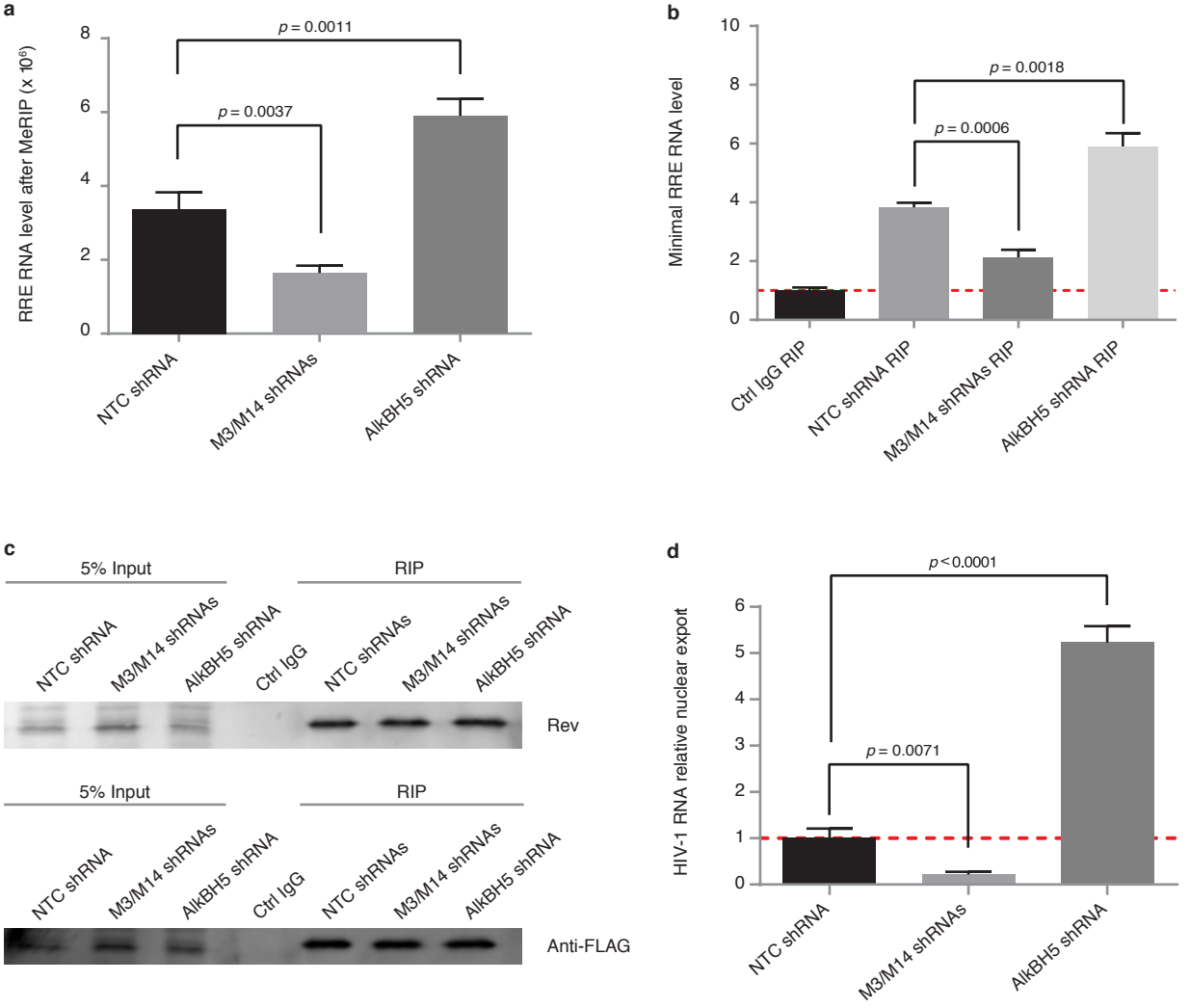




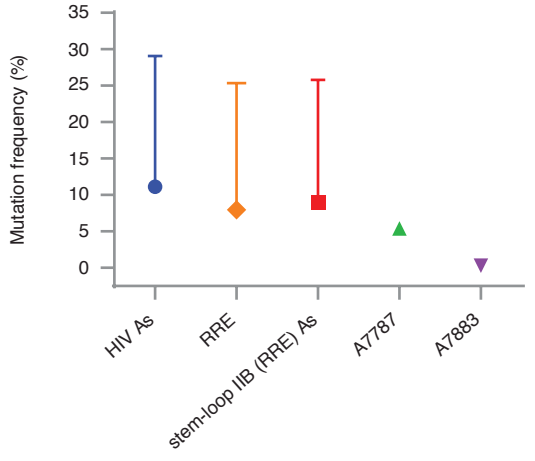
Supplementary Figure 3. 56 genes are uniquely methylated upon HIV-1 infection. a, gene ontology analysis of the uniquely methylated genes during HIV-1 infection. The 10 most enriched categories are shown. Fifty-six genes identified to be uniquely methylated during HIV-1 infection are enriched in virus-related categories. b, Normalized read densities for GTF2I, PSIP1, SIAH2, STARD4, ETS2, DUT and ABHD16A are shown for input and MeRIP in uninfected and HIV-infected samples. The read densities are indicative of 2 technical replicates.



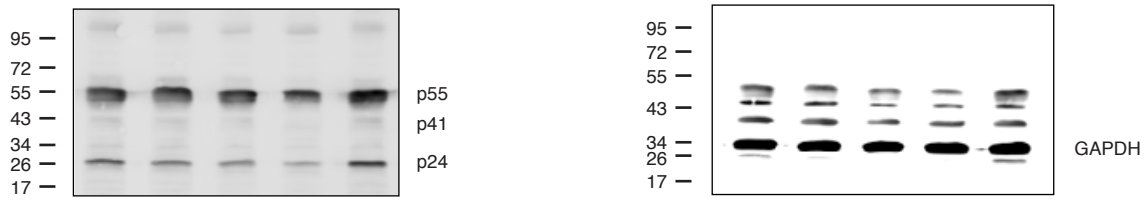
Supplementary Figure 4. RRE methylation is modulated by METTL3, METTL14, and AlkBH5 protein levels and affects nuclear export of viral RNAs. a, RRE RNA methylation level is directly modulated by methyltransferase and demethylase expression. MeRIP/RT-qPCR for RRE RNA was performed in MT4 cells depleted of METTL3/METTL14 or AlkBH5 and subsequently infected with HIV-1 LAI virus. RNA was extracted, poly(A) enriched, fragmented, immunoprecipitated with anti-m6A antibody, and analysed by RT-qPCR. Results are the mean ± s.e.m. of three independent experiments (n=3) and are expressed as the fold enrichment of minimal RRE RNA in anti-FLAG versus control IgG IPs. b, RIP experiments were performed as described in Fig 3b, except that 293T cells were co-transfected with FLAG-Rev and pcDNA3_minRRE, which encodes the minimal RRE. Results are the mean ± s.e.m. of of three independent experiments (n=3) and are expressed as the fold enrichment of minimal RRE RNA in anti-FLAG versus control IgG IPs. c, RIP efficiency was assessed by western blot analysis of input material and immunoprecipitated fractions of poly(A)-enriched RNA from 293T cells. Top: RIP efficiency analysis for the Rev in pLAI2 expression system. Bottom: RIP efficiency analysis for the FLAG-Rev and pcDNA3_minRRE co-expression system. The data is a representative of three independent experiments. d, Cytoplasmic and nuclear fractionation was performed on NTC, M3/M14 and AlkBH5 knock-down 293T cells. Viral RNA distribution in the two compartments is relative to the viral RNA content from total cellular RNA. Results are the mean ± s.e.m. of three independent experiments (n=4). P-values are calculated by unpaired two-tailed Student's t-test. n indicates the number of technical replicates.



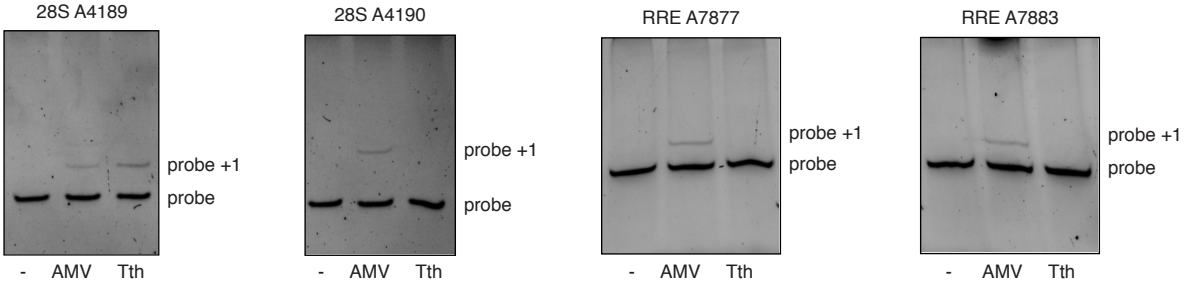
Supplementary Figure 5. Frequency of adenosine mutation in HIV sequences. A total of 2,501 sequences of HIV-1 isolated from infected patients were aligned to the reference HXB2 HIV genome, and the mutation frequency was calculated for all adenosines (As). We downloaded aligned FASTA sequences for 2501 HIV genomes from the HIV database and processed them using a custom Python program. For each position in the reference HXB2 genome, the differences in nucleotides (A, C, T, G and – [gap]) were calculated using the alignment matrix. The position, length, and frequency of insertion/deletion events were also determined using the gaps in the aligned FASTA sequences. Mutation frequencies for total HIV-1, RRE, stem loop IIB in the RRE, and two specific adenosines at positions 7787 and 7883 are shown. Results are the mean + s.d. of 2,501 sequences.



Supplementary Figure 6. Raw data for blots and gels. Raw data for blots and gels included in the manuscript are shown and labeled with molecular weight markers and corresponding figure numbers in the manuscript and supplementary material.

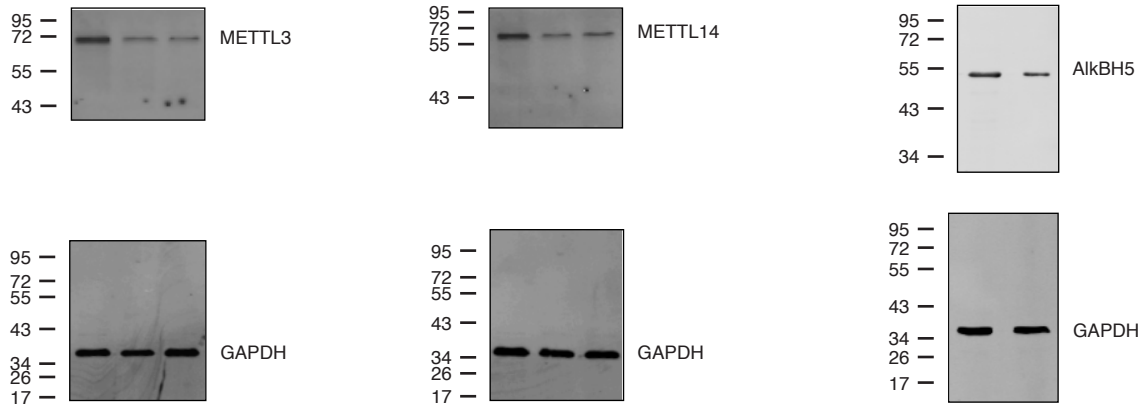


Corresponding to Figure 1c



Corresponding to Figure 3c

Corresponding to Supplementary Figure 4c



Corresponding to Supplementary Figure 2

Supplementary Tables

Supplementary Table 1. HIV-1 and human RNA sequence summary

	Size	Control_1_ Control	Control_1_ MeRIP_2_ Rounds	Control_2_ Control	Control_2_ MeRIP_1_ Round	HIV_1_ Control	HIV_1_ MeRIP_1_ Round	HIV_2_ Control	HIV_2_ MeRIP_1_ Round
chr1	249250621	6832868	3933593	3784733	2680944	2123715	1892236	2179434	2262238
chr2	243199373	6469023	3379318	3243756	2181020	1845529	1530029	1883793	1833678
chr3	198022430	4288317	2504991	2806320	1959027	1722190	1442884	1768307	1733797
chr4	191154276	1924022	1088681	1075035	695596	670717	516272	685510	616162
chr5	180915260	3304806	2053934	2030476	1467805	1294757	1107218	1326916	1327188
chr6	171115067	4426746	2962792	2867309	2089975	1756755	1589860	1803350	1909249
chr7	159138663	3614281	2393964	2404143	1766587	1279686	1136342	1337267	1380211
chr8	146364022	3289331	1779649	1920905	1345071	1112776	958414	1142973	1155069
chr9	141213431	3352335	2230637	2208497	1597142	1273979	1173074	1320443	1410187
chr10	135534747	2203088	1338180	1451100	1029882	849405	722414	878972	872825
chr11	135006516	5493788	4874352	3025129	2599619	1834762	1834245	1866841	2186417
chr12	133851895	4961434	2774654	2894508	1973728	1616271	1399844	1666060	1677010
chr13	115169878	1339693	692360	860444	596699	534065	404220	543055	488834
chr14	107349540	2390202	1504134	1584200	1157848	901800	796801	931435	960097
chr15	102531392	2808404	1809680	1865360	1381717	1032047	915998	1059474	1092762
chr16	90354753	4233523	2663532	2505213	1809370	1523854	1413499	1588488	1700736
chr17	81195210	4993594	3507752	3101901	2387111	1672225	1626389	1728790	1946162
chr18	78077248	666486	350022	429600	285581	270072	212524	276980	258260
chr19	59128983	6015744	4421623	3564544	2716342	1892420	1925482	1967896	2315191
chr20	63025520	1685301	1126442	1124684	829575	632996	586752	655208	706012
chr21	48129895	2495103	872403	804789	508790	454161	377432	458939	449236
chr22	51304566	2289959	1670515	1563640	1147658	832175	809748	870700	965505
chrX	155270560	2457491	1541016	1581668	1163641	869894	780667	896888	941493
chrY	59373566	303054	117952	95046	62774	55584	48807	56244	57087
chrM	16571	3131999	2128883	1730509	1549403	1867543	1155382	1658133	1366022
HIV	11738	40695	28682	12988	15171	29292810	18503288	28420526	23683111
Human	3095693983	84970592	53721059	50523509	36982905	29919378	26356533	30552096	31611428
HIV	11738	40695	28682	12988	15171	29292810	18503288	28420526	23683111
Total		85011287	53749741	50536497	36998076	59212188	44859821	58972622	55294539
Percentage									
Human		99.95%	99.95%	99.97%	99.96%	50.53%	58.75%	51.81%	57.17%
HIV		0.05%	0.05%	0.03%	0.04%	49.47%	41.25%	48.19%	42.83%

Supplementary Table 2. HIV-1-specific m6A transcripts

Gene Symbol	Molecular Function	Previously linked to HIV-1	Reference (PMID)
AKIRIN1	Cytoskeleton organization		
PLK3	Kinase		
PTRHD1	Peptydil-tRNA hydrolase		
B3GNT2	Glucosaminyltransferase		
MOGS	Glucosidase	Yes (+)	25318123
DUSP2	Phosphatase		
MZT2B	Cytoskeleton organization		
TTN-AS1	Not known		
RPL32	Ribosomal protein		
ZBED2	DNA binding		
FAM162A	Apoptosis		
SIAH2	Ubiquitin protein ligase		
MFSD10	Membrane transporter		
GPR125	G-protein coupled receptor		
RPS23	Ribosomal protein		
COX7C	Cytochrome C subunit		
STARD4	Sterol transport		
RPS14	Ribosomal protein		
ABHD16A	abhydrolase		
HSPA1A	Chaperone	Yes (+/-)	21970979
HLA-DPA1	MHC class II protein	Yes	22860026
GTF2I	Transcription factor	Yes (+)	21613400
ZNHIT1	Transcription		
ATP6V1F	ATPase		
RPL30	Ribosomal protein		
PSIP1	DNA binding	Yes (+)	24261564
OSTF1	Stimulating factor		
HNRNPK	Ribonucleoprotein	Yes (+)	18854243
TRAF2	TNF receptor-associated factor	Yes (+)	23774506
RPLP2	Ribosomal protein		
PRKCDBP	PKC binding protein		
ILK	Kinase		
DENND5A	Golgi trafficking		
EIF3M	Translation	Yes	22174317
TUBA1B	Cytoskeleton organization	Yes (+)	19460752
CCT2	Chaperone	Yes (+)	18976975
PABPC3	poly(A)-binding protein	Yes (+)	19460752
HAUS4	Cytoskeleton organization		
SRSF5	Splicing factor	Yes (+)	22933280
C14orf1	Not known		
DUT	deoxyuridine triphosphatase	Yes	22944692
UBL7	Ubiquitin-like protein		
COX5A	Cytochrome C subunit		
NDE1	Not known		
CBFB	HIV Vif function	Yes (+)	22190037
APRT	adenine phosphorybosil transferase		
RNASEK	RNAse		
RNASEK-C17orf49	Not known		
MYL12A	myosin, regulatory	Yes	19794400
MBD2	methyl-CpG binding protein	Yes	19454010
UBA52	Ubiquitin-ribosomal protein	Yes	23874603
RPS16	Ribosomal protein		
RPS5	Ribosomal protein	Yes	22174317
MIR3648-1	Not known		
ETS2	Transcription factor	Yes (+)	18854154
MAGED2	p53 function		

Yes: HIV-related gene, no functional data available.

Yes (+): HIV-related gene, proviral function.

Yes (+/-): HIV-related gene, uncertain function.

Supplementary Table 3. shRNA sequences

shRNA	Sequence
Non targeting control (NTC)	CCGCAGGTATGCACGCGT
METTL3-1	CCGGGCAAGTATGTTCACTATGAAACTCGAGTTTCATAGTGAACATACTTGCTTTTTG
METTL3-2	CCGGGCCAAGGAACAATCCATTGTTCTCGAGAACAATGGATTGTTCCTTGGCTTTTTG
METTL14-1	CCGGCCATGTACTTACAAGCCGATACTCGAGTATCGGCTTGTAAGTACATGGTTTTT
METTL14-2	CCGGGCCGTGGACGAGAAAGAAATACTCGAGTATTTCTTTCTCGTCCACGGCTTTTT
AlkBH5-1	CCGGGAAAGGCTGTTGGCATCAATACTCGAGTATTGATGCCAACAGCCTTTCTTTTTG
AlkBH5-2	CCGGCCACCCAGCTATGCTTCAGATCTCGAGATCTGAAGCATAGCTGGGTGGTTTTTG

Supplementary Table 4. Primer sequences

Primer	Sequence	Use
GAPDH Fwd	TGGCGGGGAAGTCAG	qPCR
GAPDH Rev	CGGAGGAGAAATCGGGC	qPCR
gp120 Fwd	TGAGCCAATTCCCATACATTAT	qPCR
gp120 Rev	CCTGTTCCATTGAACGTCTTAT	qPCR
METTL3 Fwd	GACACGTGGAGCTCTATCCA	qPCR
METTL3 Rev	GGAAGGTTGGAGACAATGCT	qPCR
METTL14 Fwd	TCCCAAATCTAAATCTGACCG	qPCR
METTL14 Rev	CTCTAAAGCCACCTCTTTCTC	qPCR
AlkBH5 Fwd	AGGGACCCTGCTCTGAAAC	qPCR
AlkBH5 Rev	TCCTTGTCCATCTCCAGGAT	qPCR
RRE Fwd	GAGCAGCAGGAAGCACTATG	qPCR
RRE Rev	CCTCAATAGCCCTCAGCAA	qPCR
β-actin Fwd	CACTCTTCCAGCCTTCCTTC	qPCR
β-actin Rev	GGATGTCCACGTCACACTTC	qPCR
28S 4189	AGCTCGCCTTAGGACACCTGCGT	AMV/Tth extension
28S 4190	GAGCTCGCCTTAGGACACCTGCG	AMV/Tth extension
A7877	AGACAATAATTGTCTGGCCTGTACCGTCAGCG	AMV/Tth extension
A7883	TGCTGCACTATATCAGACAATAATTGTCTGGCCTGTACCG	AMV/Tth extension
Mut1 Fwd	ACTATGGGCGCACGGCCAATGGCGCTGACGGTACAGGCCAGA	mutagenesis
Mut1 Rev	TCTGGCCTGTACCGTCAGCGCCATTGGCCGTGCGCCCATAGT	mutagenesis
Mut2 Fwd	GGCGCACGGTCAATGACGCTGGCGGTACAGGCCAGACAATTAT	mutagenesis
Mut2 Rev	ATAATTGTCTGGCCTGTACCGCCAGCGTCATTGACCGTGCGCC	mutagenesis
Mut3 Fwd	CACTATGGGCGCACGGCCAATGGCGCTGGCGGTACAGGCCAGACAATTA	mutagenesis
Mut3 Rev	TAATTGTCTGGCCTGTACCGCCAGCGCCATTGGCCGTGCGCCCATAGTG	mutagenesis