Functional study of N6-methyladenosine (m6A) machinery in HIV infection of primary CD4+ T cells

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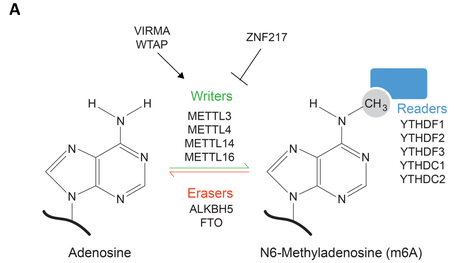
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N6-methyladenosine (m6A) modification

N6-methyladenosine (m6A) modification is the most prevalent post-transcriptional modification of cellular and viral RNA. Previous studies on the role of m6A during Human Immunodeficiency Virus (HIV) infection have failed to reach consensus in the field, particularly in regards to which readers influence HIV replication. Since m6A function varies significantly between cell types, we aimed to characterize the role of m6A during HIV infection using primary CD4+ T cells using a CRISPR-cas9 screen of 46 genes associated with m6a modifications.



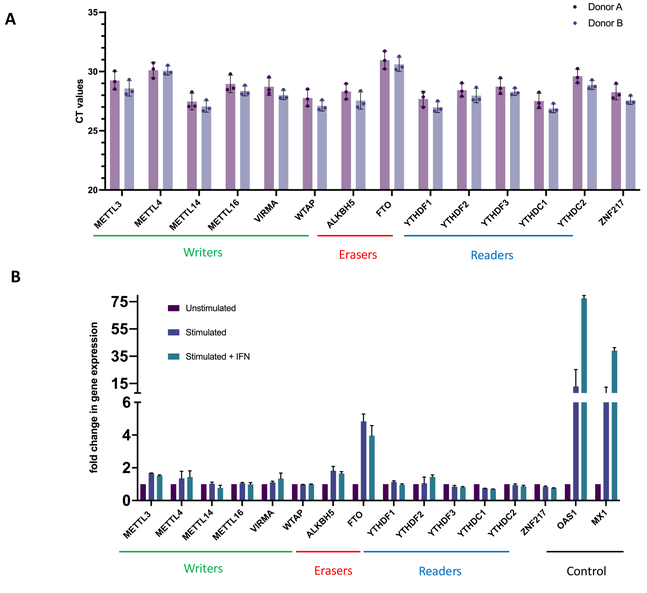
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Additional m6A associated genes included in this screen	
CAPRIN1	NSUN3
CBLL1	NSUN4
ELAVL1	NSUN5
FMR1	NSUN6
GNL3	NSUN7
HNRNPA2B1	RBM15
HNRNPC	RBM15B
HNRNPD	RBM27
IGF2BP1	RTCB
IGF2BP2	SRSF9
IGF2BP3	SRSF10
LARP4B	TRA2A
LIN28A	TRDMT1
MSI2	YWHAG
NOP2	ZC3H7B
NSUN2	

The core m6A machinery (A) Writers deposit the modification on RNA adenosines, erasers remove the modification, and readers recognize the presence of the modification and trigger a response. (B) Additional genes associated with m6A modifications included in the CRSIPR-cas9 targeted screen in CD4+ T cells.

Expression of m6A core machinery in CD4+ T cells

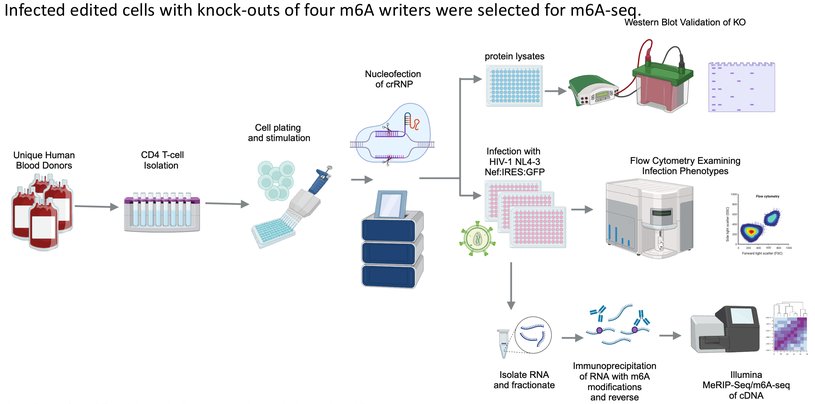
Confirmation that all m6A core machinery is expressed in CD4+ T cells. Primary CD4+ T cells were isolated from two healthy donors; cells remained unstimulated, stimulated with anti-human CD28 and CD3 in the presence or absence of interferon. RNA was extracted from each treatment group and gene expression was measured by quantitative polymerase chain reaction (qPCR).



m6A core machinery expression (A) Expression of mRNA levels of core m6A regulatory proteins measured by qPCR in unstimulated primary CD4+ T cells, normalized to TBP expression, from two independent healthy donors. (B) Expression of mRNA levels of core m6A regulatory proteins measured by qPCR in unstimulated, stimulated and stimulated plus interferon treated primary CD4+ T cells, normalized to TBP expression in unstimulated cells, and averaged across two independent healthy donors. The interferon stimulated genes (ISGs) OAS1 and MX1 are positive controls.

CRISPR-cas9 genome engineering of primary CD4+ T-cells

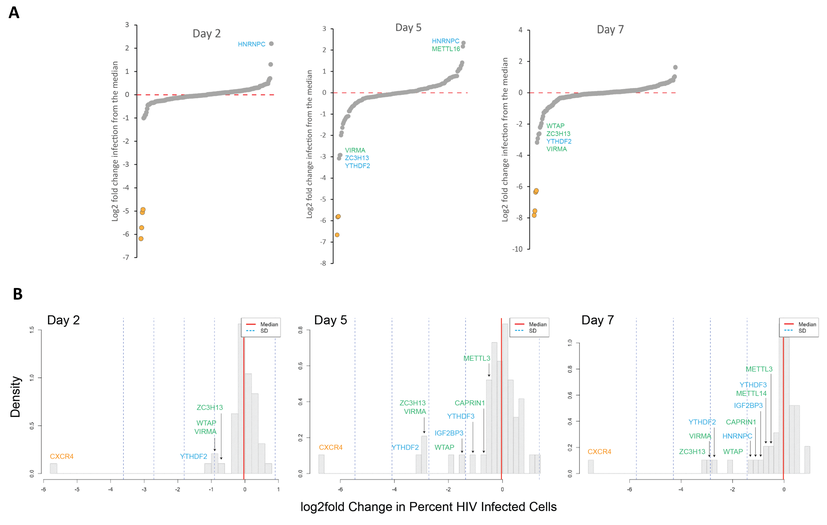
CD4+ T-cells were isolated from healthy donors, followed by nucleofection of CRISPR-Cas9 ribonucleoproteins (crRNPs) to facilitate genome editing. An initial screen systematically targeting 46 genes implicated in m6A regulation was carried out using four independent crRNPs pooled together for each gene target. Cells were subsequently expanded and knock-out efficiency determined by western blot. Edited cells were then assessed for susceptibility to HIV-1 spreading infection using HIV-1 NL4-3 with a GFP reporter measured by flow cytometry. Individual guides for each gene target were then delivered into CD4+ T-cells and the most efficient guides for each gene target were identified and challenged with HIV-1 NL4-3. Infected edited cells with knock-outs of four m6A writers were selected for m6A-seq.



Experimental Workflow Schematic showing experiment design and workflow.

Spreading Infection

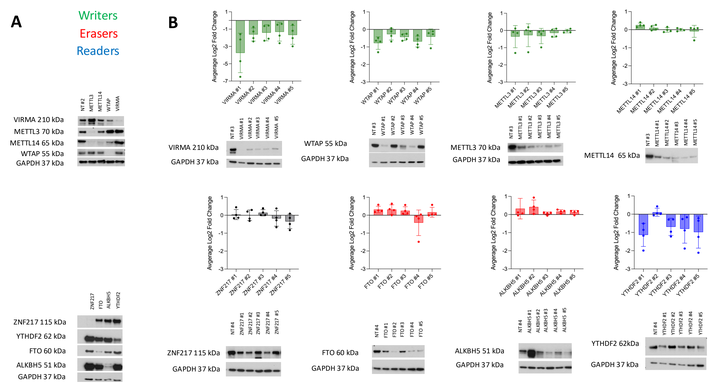
Results from the CRISPR-cas9 screen indicate that m6A writers and readers are dependency factors of HIV infection. Each dot represents one gene that was targeted by four individual guide RNAs delivered simultaneously; a non-targeting (NT) guide RNA was used as a negative control and a CXCR4 guide RNA was used as a positive control.



Spreading Infection in Polyclonal knock-outs (A) S-curve plots of the log2 fold change in infection relative to the plate median for each gene knockout in four independent donors, rank-ordered for each timepoint. The dashed red line indicates median; orange dots represent the positive control CXCR4 guide RNA. (B) Histograms showing the distribution of HIV- 1 infection rates upon knockout of m6A regulatory proteins averaged across donors. Blue dotted lines indicate standard deviations from the median (red line). Infection percent was normalized to cells edited with the non-targeting guide RNA and log transformed.

Infection Phenotypes and Knock-out Validation

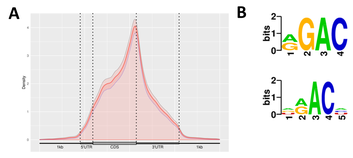
Knock-out validation by western blot for pooled and single guide RNA delivery in primary CD4+ T-cells; coupled with average log2 fold changes in infection five days post infection for single guide delivery. Decrease in protein expression in the pooled guide RNA screen was observed for all targets; in cells where individual guide RNA was delivered, efficient guides for most targets were identified.

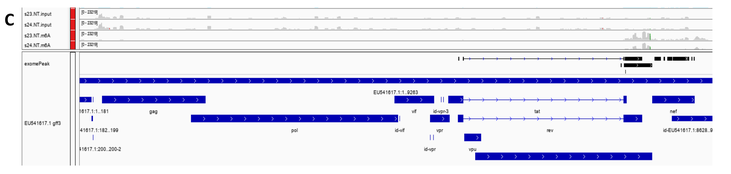


Polyclonal knock-out validation and infection phenotypes (A) Western blots showing polyclonal knock-outs of the pooled CRISPR-cas9 screen in CD4+ T-cells, cell lysates were prepared prior to infection on day 0 of the infection timeline. (B) Log2 fold change in infection averaged across four independent donors and normalized to the average non-targeting guide RNA for each donor. Each infectivity plot is paired with a representative western blot from one donor showing knock-down of each specific guide.

m6A-seq

Primary CD4+ T-cells were edited with single guide RNAs for METTL3, METTL14, Virma, WTAP, and non-targeting; cells were then infected and prepared for m6A- seq. Analysis of m6A modifications in the HIV genome show a majority of the modifications are bias towards the 3' UTR.





m6A-seq for writers involved in m6A modifications (A) Metagene plot of m6A peak density demonstrates 3' UTR enrichment of m6A peaks. (B) The FIRE algorithm (in non-discovery mode) shows the known m6A motif RGAC ([AG]GAC) is enriched among predicted MeRIP-seq peaks relative to randomly generated sequences with similar dinucleotide frequencies. (C) Integrative genomics viewer (ivg) plot of the HIV genome with the non-targeting control input and m6A enrichments.

Conclusions and Future Directions

We found that all core machinery are expressed, and their expression is largely unaffected by interferon stimulation. Of the 46 genes interrogated, only the writer complex, the core erasers, and the reader YTHDF2 significantly altered HIV infection. m6A modifications on the HIV-1 genome were found to be bias towards the 3' UTR, consistent with some of the current literature. In an ongoing effort, we are mapping m6A modifications along the HIV genome for knock-out cells associated with m6A writers.

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