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RNA N⁶-adenosine methylation (m⁶A) steers epitranscriptomic control of herpesvirus replication

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Abstract

Latency is a hallmark of all herpesviruses, during which the viral genomes are silenced through DNA methylation and suppressive histone modifications. When latent herpesviruses reactivate to undergo productive lytic replication, the suppressive epigenetic marks are replaced with active ones to allow for transcription of viral genes. Interestingly, by using Kaposi's sarcoma-associated herpesvirus (KSHV) as a model, we recently demonstrated that the newly transcribed viral RNAs are also subjected to post-transcriptional N⁶-adenosine methylation (m⁶A). Blockade of this post-transcriptional event abolishes viral protein expression and halts virion production. We found that m⁶A modification controls RNA splicing, stability, and protein translation to regulate viral lytic gene expression and replication. Thus, our finding for the first time reveals a critical role of this epitranscriptomic mechanism in the control of herpesviral replication, which shall shed lights on development of novel strategies for the control of herpesviral infection.

Keywords

RNA m⁶A modification; RTA pre-mRNA splicing; KSHV lytic replication; epitranscriptomics

Kaposi's sarcoma-associated herpesvirus (KSHV) is an oncogenic virus associated with multiple malignancies including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD)^[1-3]. Like all herpesviruses, KSHV enters a latent phase shortly after primary infection. Under immune suppressive conditions, the latent virus reactivates to undergo lytic replication to produce new viruses. Productive lytic replication not only causes *de novo* infection but also plays an essential role in the development of KS and MCD^[4, 5]. Previous studies demonstrate that the switch from latency to lytic replication is primarily controlled at the viral chromatin level through epigenetic mechanisms^[6, 7]. Indeed, the majority of KSHV genome is silenced during

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Author contributions

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latency through DNA methylation, repressive histone modifications, and other negative gene expression regulatory mechanisms^[7–11]. When the latent virus reactivates, prompt epigenetic changes occur, leading to transactivation of the viral genome. However, our recent study discovered that KSHV reactivation stalls if the newly transcribed viral RNAs fail to undergo post-transcriptional N⁶-adenosine methylation (m⁶A)^[12]. Our finding highlights a pivotal role of this epitranscriptomic mechanism in the control of KSHV lytic replication.

RNA N⁶-adenosine methylation (m⁶A) is one of the most abundant types of RNA modifications found in over 25% of RNA species in mammalian cells^[13–15]. A complex of three methyltransferases: methyltransferase like 3 (METTL3), methyltransferase like 14 (METTL14), and Wilms tumor 1 associated protein (WTAP) acts as m⁶A writers and catalyze RNA m⁶A at specific sites with the consensus sequence (G/AGAC)^[16–18]. Two demethylases, fat mass and obesity associated protein (FTO), and AlkB Homolog 5 (ALKBH5), act as m⁶A erasers and reverse this process^[19–21]. Most m⁶A sites are located near the transcription start sites, exonic regions flanking splicing sites, stop codons, and the 3' untranslated region (3'UTR)^[14, 22–24]. The biological functions of m⁶A are mediated by m⁶A readers. In the nucleus, for example, heterogeneous nuclear ribonucleoproteins hn-RNP-C and hn-RNP-A2/B1 selectively bind RNA at m⁶A sites to regulate pre-mRNA processing and alternative splicing^[22, 24–27]. In addition, the YTH domain containing 1 protein (YTHDC1) binds pre-mRNA at m⁶A sites and preferentially recruits the serine/arginine-rich splicing factor 3 (SRSF3) over SRSF10 for exon inclusion splicing^[28–31]. In the cytoplasm, three members of the YTH domain-containing family proteins, YTHDF1, YTHDF2, and YTHDF3, preferentially bind m⁶A-containing mRNAs to regulate RNA stability, protein translation, and RNA decay^[32–35]. In addition, the eIF3, a component of 43S translation pre-initiation complex^[36], directly binds m⁶A sites in the 5' untranslated region (5'UTR) of mRNAs to enhance protein translation^[37]. Therefore, m⁶A represents a very important cellular mechanism for the control of gene expression at the post-transcriptional level. Interestingly, massive increases in m⁶A modification occur in the RNAs of human immunodeficiency virus-1 (HIV-1)^[38, 39]. Blockade of m⁶A effectively abolishes HIV-1 protein expression and virion production, suggesting that this epitranscriptomic mechanism also controls viral gene expression.

Similar to HIV-1, most KSHV transcripts undergo m⁶A modification, and the level of m⁶A-modified mRNA of a given viral transcript increases in parallel with that of total mRNA when latently infected cells are induced by phorbol ester (TPA) or other lytic replication stimuli. Expressional knocking down of the m⁶A writer METTL3 substantially reduces TPA induction of KSHV lytic genes, and blockade of m⁶A reaction literally abolishes expression of all lytic genes examined and halts virion production. In contrast, expressional knocking down or activity inhibition of the m⁶A eraser FTO has the opposite effects.

To understand how RNA methylation controls KSHV replication, we examined the effect of m⁶A on expression of viral regulator of transcription activation (RTA), which, encoded by open reading frame 50 (ORF50), is a key mediator of the switch from latency to lytic gene expression^[40]. Due to differential splicing, the ORF50 (RTA) and ORFK8 loci produce at least three different groups of transcripts, including ORF50 /ORFK8/ORFK8.1 tricistronic

mRNAs, ORFK8/ORFK8.1 bicistronic mRNAs, and monocistronic ORFK8.1 mRNAs^[41]. RTA, which is expressed from the tricistronic mRNAs, consists of two exons and one intron (Fig. 1). Interestingly, blockade of m⁶A substantially reduces the level of TPA-induced RTA mRNA but has much less an effect on the level of RTA pre-mRNA, suggesting that m⁶A controls RTA pre-mRNA splicing. Indeed, multiple m⁶A sites are identified in RTA pre-mRNA. Data from genetic mutation assays demonstrate that the m⁶A sites in the intron near the two splicing sites are critical for RTA expression, and one m⁶A site in Exon2 near the splicing site also plays an important role in RTA pre-mRNA splicing. Data from RNA immuno-precipitation (RIP) assays confirm that these sites are indeed m⁶A modified. In addition, both SRSF3 and SRSF10 are present at the m⁶A sites in the intron near the two splicing sites, and the levels of m⁶A and these splicing factors increase significantly upon TPA treatment. Mutation of these m⁶A sites abolishes the RNA-protein interactions and RTA protein expression, thus suggesting that m⁶A modification of these sites is critical for recruitment of SRSF3 and SRSF10 and exclusion of the intron. In contrast, the m⁶A site in Exon2 near the splicing site is critical for removal of SRSF10 and Exon2 inclusion splicing. Therefore, our data highlight a pivotal role of m⁶A modification in RTA pre-mRNA splicing. Interestingly, when the m⁶A sites in both the intron and Exon2 are simultaneously mutated, the level of RTA pre-mRNA drops dramatically (un-published results), suggesting that m⁶A modification also contributes to stability of RTA pre-mRNA. In addition, similar to host mRNAs and HIV-1 transcripts, m⁶A modification may also promote RTA mRNA stability and protein translation through association with m⁶A readers YTHDF1, YTHDF2, and YTHDF3.

Finally, we also found that expression of RTA protein increases the levels of m⁶A modification and promotes its own pre-mRNA splicing. RTA is known to enhance its own transcription^[42]. Thus, our data for the first time demonstrate that RTA increases its own expression through both transcriptional and post-transcriptional mechanisms. Very interestingly, the KSHV latent protein LANA, which inhibits RTA expression to promote latency^[43], suppresses TPA induction of RNA m⁶A modification and inhibits RTA pre-mRNA splicing (un-published results).

In summary, our results not only demonstrate an essential role of m⁶A in regulating RTA pre-mRNA splicing but also suggest that KSHV has evolved two opposite mechanisms to manipulate the host m⁶A machinery to its advantage in promoting lytic replication and latency respectively. This epitranscriptomic mechanism may be used by other herpesviruses as well. Our findings shall shed light on development of new strategies for the control of herpesviral infection.

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Abbreviations

KSHV	Kaposi's sarcoma-associated herpesvirus
KS	Kaposi's sarcoma
PEL	primary effusion lymphoma
MCD	multicentric Castleman's disease
m⁶A	N ⁶ -adenosine methylation
METTL3	methyltransferase like 3
METTL14	methyltransferase like 14
WTAP	Wilms tumor 1 associated protein
FTO	fat mass and obesity associated protein
ALKBH5	AlkB Homolog 5
3'UTR	3'untranslated region
5'UTR	5'untranslated region
YTHDC1	YTH domain containing 1 protein
HIV-1	human immunodeficiency virus-1
SRSF3	serine/arginine-rich splicing factor 3
SRSF10	serine/arginine-rich splicing factor 10
RIP	RNA immuno-precipitation
TPA	12-O-Tetradecanoylphorbol-13-acetate
RTA	regulator of transcription activation
LANA	<i>latency-associated nuclear antigen</i> .

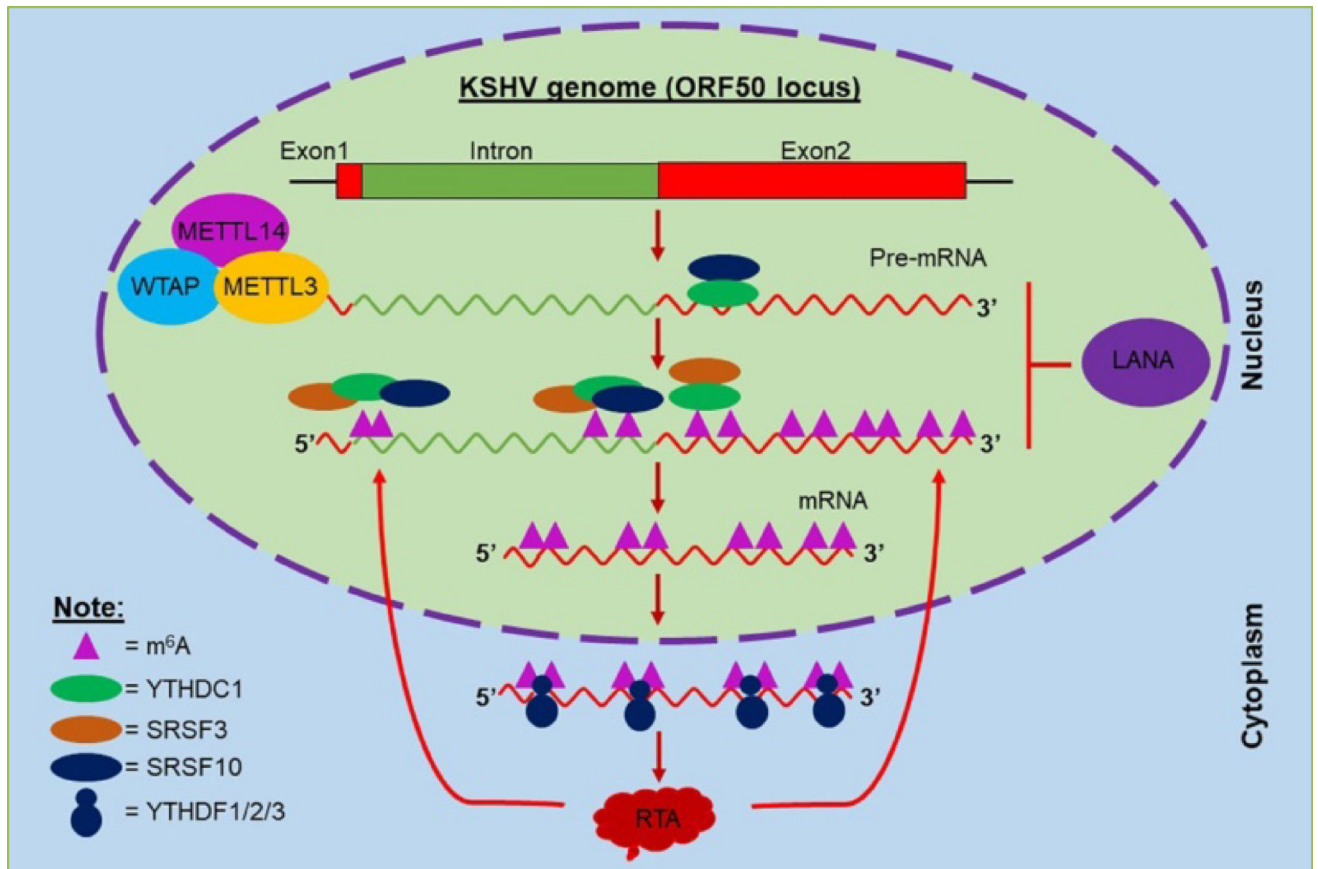


Figure 1. Post-transcriptional m⁶A modification controls KSHV RTA (ORF50) pre-mRNA splicing

Multiple m⁶A sites are found in RTA pre-mRNA, which are methylated by m⁶A writers METTL3, METTL14, and WTAP. The m⁶A sites in the intron near the two splicing sites are critical for YTHDC1 binding and recruitment of splicing factors SRSF3 and SRSF10 while the m⁶A site in Exon2 near the splicing site is important for recruitment of SRSF3 and dissociation of SRSF10. Interactions between the m⁶A-modified RTA pre-mRNA and the different splicing factors ensure exclusion splicing of the intron to generate RTA mRNA. The other m⁶A sites may enhance RTA mRNA export, stability, and translation through interaction with m⁶A readers YTHDF1, YTHDF2, and YTHDF3. The expressed RTA protein enhances the host's m⁶A machinery to increase the levels of m⁶A to promote its own pre-mRNA splicing and KSHV lytic gene expression. In contrast, KSHV latent protein LANA has the opposite effects on m⁶A and RTA pre-mRNA splicing.