N⁶-methyladenosine RNA modification suppresses antiviral innate sensing pathways via reshaping double-stranded RNA

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Double-stranded RNA (dsRNA) is a virus-encoded signature capable of triggering intracellular Rig-like receptors (RLR) to activate antiviral signaling, but whether intercellular dsRNA structural reshaping mediated by the N⁶-methyladenosine (m⁶A) modification modulates this process remains largely unknown. Here, we show that, in response to infection by the RNA virus Vesicular Stomatitis Virus (VSV), the m⁶A methyltransferase METTL3 translocates into the cytoplasm to increase m⁶A modification on virus-derived transcripts and decrease viral dsRNA formation, thereby reducing virus-sensing efficacy by RLRs such as RIG-I and MDA5 and dampening antiviral immune signaling. Meanwhile, the genetic ablation of METTL3 in monocyte or hepatocyte causes enhanced type I IFN expression and accelerates VSV clearance. Our findings thus implicate METTL3-mediated m⁶A RNA modification on viral RNAs as a negative regulator for innate sensing pathways of dsRNA, and also hint METTL3 as a potential therapeutic target for the modulation of anti-viral immunity.
Pathogenic RNA viruses are considered as the primary etiological agents of human emerging pathogens and represent a challenge for global disease control. Therefore, it is critical to know the mechanism and interaction between RNA virus and host innate immunity. The innate immune system, highly conserved among plants and animals, recognizes the invading pathogens through pattern recognition receptors (PRRs) to trigger an effective immune response for defending the pathogens. The virus-encoded molecular signatures, including cytosolic double-stranded RNA (dsRNA) and other distinct RNA species, trigger intracellular nucleic acid sensors, including retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), to recognize these "non-self" RNAs and activate antiviral signaling. Despite RIG-I and MDA5 show different preferences for sensing of 5’ppp/short dsRNA and long dsRNA, respectively, their recognition patterns and functional compensations for sensing foreign RNAs remain largely elusive. On the other hand, viruses also evolve many mechanisms to weaken the host innate immune response through encoding immune response-neutralized proteins or modifications on viral nucleic acids to mimic host "self" components. However, the precise mechanism of N6-methyladenosine (m6A) modification for controlling innate sensing system remains largely unclear.

The RNA modification m6A is one of the most abundant mRNA modifications, which regulates several procedures of mRNA metabolism, especially the mRNA translation and degradation. Accordingly, several m6A machines have been well identified, including m6A "writers" (e.g., METTL3, METTL14, and WTAP), "readers" (e.g., YTHDF1-3 and YTHDC1), and "erasers" (ALKBHF5 and FTO). Based on the diverse functions of m6A, m6A modification has been shown to impact many fundamental biological processes including DNA damage repair, tumorigenesis, inflammation, and T-cell homeostasis. In addition, m6A also functions in modulating the life cycle of various RNA and DNA viruses through m6A-mediated regulation of viral RNA processing. Beyond that, recent studies demonstrated that m6A modification could regulate the innate immune responses via targeting mRNA stability of type I interferons and melanoma differentiation-associated gene 5 (MDA5), to recognize these "non-self" RNAs and activate antiviral signaling. Despite RIG-I and MDA5 show different preferences for sensing of 5’ppp/short dsRNA and long dsRNA, respectively, their recognition patterns and functional compensations for sensing foreign RNAs remain largely elusive. On the other hand, viruses also evolve many mechanisms to weaken the host innate immune response through encoding immune response-neutralized proteins or modifications on viral nucleic acids to mimic host "self" components. However, the precise mechanism of N6-methyladenosine (m6A) modification for controlling innate sensing system remains largely unclear.

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In this study, we identify METTL3 as a negative suppressor for global innate immune signaling cascades in response to infection of RNA virus Vesicular Stomatitis Virus (VSV) in vitro and in vivo. We find that METTL3 translocates to cytoplasm upon VSV infection and catalyzes the methylation of cytosolic viral positive-sense (+) RNAs. Subsequently, METTL3-mediated m6A modification reshapes viral RNA duplex structure and impairs viral RNA sensing efficacy by RIG-I and MDA5. These findings demonstrate the functional significance of METTL3-mediated m6A modification in innate sensing and provide a strong impetus for therapeutic intervention.

Results

METTL3 impedes global innate immune signaling cascades.

To fully investigate the potential roles of m6A modification in innate immunity, we screened the m6A machinery genes with overexpression of m6A "writers" and "erasers" in HeLa cells upon VSV infection, respectively. As shown in Supplementary Fig. 1a, the VSV-induced IFNB1 expression was significantly inhibited by m6A "writers", particularly the methyltransferase METTL3.
RAW264.7 cells (Supplementary Fig. 2a–c). Overall, these results demonstrate that METTL3 could globally interfere with innate immune signaling upon VSV infection.

**Targeting METTL3 enhances innate immune response and viral clearance in vivo.** To fully address the crucial role of METTL3 in innate immunity in vivo, we intravenously infected Mettl3<sup>fl/fl</sup> Lyz2-Cre mice and the littermate control Mettl3<sup>fl/fl</sup> mice with VSV. In line with the in vitro results, the Mettl3<sup>fl/fl</sup> Lyz2-Cre mice were significantly resistant to high dose of VSV-induced lethality compared with control mice (Fig. 2a). When we challenged the mice with a moderate dose of VSV, Mettl3<sup>fl/fl</sup> Lyz2-Cre mice displayed less of the VSV-induced pathologic lesions in the lung and liver at 24 h after infection than control mice (Fig. 2b, c).
and Supplementary Fig. 3a). Consistently, the qRT-PCR and Western blot analysis showed less viral replication, but higher Ifnb1 mRNA in the spleen, liver, and lung of Mettl3−/− Lyz2-Cre mice compared to the control mice (Fig. 2d, e). Accordingly, the IFN-β secretion was significantly increased in the serum of VSV-infected Mettl3−/− Lyz2-Cre mice compared to the controls (Fig. 2f). To examine the in vivo upregulated IFN-β was dependent on enhanced upstream signaling of innate immunity, we collected peritoneal macrophages from infected mice. Consistent with the in vitro results in Fig. 1, Western blot indicated that METTL3 deficiency also promoted phosphorylated Irf3 in vivo (Fig. 2g). These results demonstrate that METTL3 deficiency enhances innate immune response for viral clearance in vivo.

On consideration of other types of cells such as hepatocytes also express PRRs to defend pathogens24, we crossed the Mettl3−/− mice with Alb-Cre mice to generate hepatocyte-specific Mettl3-deficient mice (called it Mettl3−/− Alb-Cre mice hereafter), and confirmed the specific depletion of METTL3 in liver cells through Western blot, qRT-PCR, and dot blot analysis (Fig. 2h and Supplementary Fig. 3b, c). Consistent with Mettl3−/− Lye2-Cre mice, Mettl3−/− Alb-Cre mice decreased VSV-induced lethality compared with control mice after a high dose of VSV infection (Fig. 2i). Upon a moderate dose of VSV, Mettl3−/− Alb-Cre mice also displayed enhanced antiviral ability through upregulating the expression of Ifnb1 and ISGs in lung and liver compared with controls (Fig. 2j, k and Supplementary Fig. 3d, e). Consistently, the secretion of IFN-β was elevated in serum of Mettl3−/− Alb-Cre mice compared with controls post 24 h of VSV infection (Fig. 2l). Taken together, these two kinds of conditional gene editing mice demonstrated that METTL3 acts as a negative regulator in RNA virus-triggered innate immune response in vivo, which suggests METTL3 could be a potential target for antiviral therapy.

**VSV infection induces METTL3 cytoplasmic translocation and dampens type I IFNs.** Based on our observed global interference of METTL3 for innate immune signaling in contrast to current model that m6A-mediated destabilization of IFNB1 mRNA by nuclear METTL3, we hypothesized that viral infection may influence METTL3 expression or subcellular pattern to further regulate upstream of innate sensor signaling cascades. Upon VSV infection, neither Mettl3 mRNA nor protein level was altered in RAW264.7 cells (Fig. 3a and Supplementary Fig. 4a). Interestingly, we found that VSV and SeV infections, but not other treatments including HBV, HSV, and poly(dA:dT) and poly(I:C), robustly enhanced cytoplasmic translocation of METTL3 from nucleus (Fig. 3b–d and Supplementary Fig. 4b), while the nuclear membranes were intact determined by Lamin A/C staining (Fig. 3c), demonstrating that the translocation of METTL3 in the cell was a natural phenomenon in response to infection rather than resulted from the breach of the nuclear membrane. As some studies demonstrated that m6A modification is mediated by METTL3–METTL14 complex25, some investigations reported that these two proteins could function independently13,26. We found that VSV infection cannot regulate the translocation of METTL14 (Supplementary Fig. 4c). Next, we asked whether this cytoplasmic translocation influences METTL3 catalytic activity. To address this question, we mutated the nuclear localization sequence (NLS) of METTL3 (Fig. 3e) and confirmed its cytoplasmic localization by immunofluorescence (Fig. 3f). The dot blot analysis revealed that NLS-mutated METTL3 displayed similar methyltransferase activity with wild-type (WT) METTL3 (Fig. 3g). Furthermore, when we overexpressed WT-METTL3 or NLS-mutated METTL3 in HeLa cells, NLS-mutated METTL3 even more significantly suppressed IFNB1 expression upon VSV infection (Fig. 3h). And the further luciferase assay showed that overexpression of NLS-mutated METTL3 was sufficient to inhibit the upstream of innate immunity upon VSV infection (Fig. 3i). These data demonstrate that VSV induces METTL3 cytoplasmic localization and inhibits type I IFNs activation.

To examine whether the inhibitory effect of METTL3 on innate immunity is dependent on its m6A catalytic activity, we mutated the catalytic domain of METTL3 and confirmed its loss of methyltransferase function by dot blot (Supplementary Fig. 4d, e). The gain-of-function results demonstrated that METTL3-mediated m6A enhanced VSV titer and viral mRNA (Fig. 3j and Supplementary Fig. 4f). Consistently, the suppressed IFN-β expression and secretion by overexpression of METTL3 were eliminated by catalytic domain mutation (Fig. 3k, l). Taken together, VSV induces METTL3 cytoplasmic translocation to suppress type I IFNs and promote viral immune escape through its methyltransferase function.

**METTL3 mediates m6A modification on viral positive-sense RNAs.** Due to the inhibitory effect of METTL3-mediated methylation in multiple upstream cascades of innate immune signaling, we hypothesized that METTL3-mediated m6A modification on viral RNA may modulate the initial innate sensing pathways. To test whether METTL3 mediated viral RNAs methylation, we purified extracellular VSV to extract pure viral RNAs, incubated in vitro with Flag-METTL3 purified by immunoprecipitation (IP) from overexpressed HEK293T cell lysis (Fig. 4a and Supplementary Fig. 5a). The RNA dot blot result showed that the m6A level of VSV RNA was relatively low but was significantly enriched after incubation with purified Flag-METTL3 (Fig. 4b). We next employed the anti-m6A IP-qPCR (MeRIP-qPCR) to analyze the m6A level and
confirmed the enhanced VSV RNA m6A level upon METTL3 overexpression (Fig. 4c). Consistently, RNA-FISH result suggested the co-localization between VSV RNAs and m6A modification in the cytoplasm (Fig. 4d). To further confirm the RNA-binding regions of METTL3 and m6A sites, we carried out photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) sequencing and miCLIP-seq. Consistent with a recent report20, METTL3 and m6A showed specific binding regions on Ifnb1 transcript (Supplementary Fig. 5b–e). Moreover, as VSV RNAs include the positive-sense (+) RNAs and negative-sense (−) genomic RNA, through strand-specific sequencing and analyzing, we found that only VSV (+) RNAs showed abundant METTL3
binding sites (Fig. 4f), which strongly supported our hypothesis that VSV infection triggers METTL3 cytosolic translocation to interact with VSV RNAs. In addition to the METTL3 PAR-CLIP-seq results, miCLIP-seq also identified the accurate m6A modification sites on VSV (+) RNAs while not VSV (−) RNA (Fig. 4e, f). Importantly, the m6A and METTL3 binding sites are coincidentally accumulated at 3′ and 5′ terminals of N, P, M, G viral (+) RNAs (Fig. 4f). To better quantify the m6A abundance of these viral RNA methylated sites in WT and Mettl3-deficient RAW264.7 cells, we performed MeRIP-qPCR assay and identified 18 m6A positions on viral (+) RNAs that were significantly reduced in Mettl3-deficient cells. (Fig. 4g and Supplementary Fig. 5f). Together, these results demonstrate that METTL3 binds to VSV (+) RNAs and mediates m6A modification.

METTL3-mediated m6A modification reshapes viral dsRNA structure. Since recent studies reported that m6A “reader” proteins suppress innate immunity sensing via compete with RLRs to foreign RNA5,22,23, indeed, we found that YTHDF2 could bind to VSV (+) RNA (Supplementary Fig. 6a), but knockdown of YTHDF2 did not entirely rescue the expression of IFNB1 upon transfection with m6A-modified VSV (+) RNA oligo (biotin-m6A2,964) (Supplementary Fig. 6a, b). This suggests that YTHDF2 might interact with m6A at the m6A-correlated positions on the RNA and may affect its recognition by the immune system. To date, it is widely accepted that virus-encoded dsRNA triggers cellular sensors to recognize as foreign distinct RNA signature to launch the innate immune responses27,28. However, it remains unclear whether and how viral dsRNA is modified by host cells. Consistent with previous studies, we also observed abundant dsRNA foci in cytoplasm induced by VSV infection (Fig. 5a). Furthermore, we analyzed the role of METTL3 in dsRNA formation upon VSV infection. Interestingly, dsRNA loads were significantly increased in the METTL3 knockdown cells compared to the control cells (Fig. 5a, b). To investigate the role of endogenous METTL3-mediated RNA modification on VSV infection-derived dsRNA, we purified dsRNA by anti-dsRNA (J2) immunoprecipitation (dsRIP) with confirming the efficiency of the anti-J2 antibody for dsRIP and measured their m6A level by dot blot (Fig. 5c). Importantly, we observed a reduced m6A level in immunoprecipitated dsRNA in METTL3 knockdown HeLa cells (Fig. 5d), revealing that METTL3 influenced dsRNA formation after VSV infection. Based on our identification of the m6A-modified positions of VSV RNAs (Fig. 4f, g), we speculated that m6A modification may influence the formation of dsRNA on these sites. As shown in Fig. 5e, dsRIP-qPCR results depicted an increase of dsRNA level among majority of these identified m6A positions on VSV RNA upon METTL3 ablation (Fig. 5e). Overall, these data suggest that METTL3-mediated m6A modification on VSV RNA impairs the formation of dsRNA.

m6A modification impairs viral RNA sensing efficacy by RLRs. Based on our observation that m6A modification impairs the formation of dsRNA, we next asked whether METTL3-mediated m6A could influence the recognition of RLRs, including Mda5 and Rig-I, for viral RNAs. To identify their RNA binding sites, we firstly performed PAR-CLIP-seq analysis for Mda5 and Rig-I, accompanied with miCLIP-seq to display the m6A peak regions. We found that both Mda5 and Rig-I were less prone to bind on VSV (−) genomic RNA (Supplementary Fig. 7a, b), and showed no peaks on Gapdh mRNA as negative control (Supplementary Fig. 7c). As shown in Fig. 6a, b, we identified abundant binding clusters of Mda5 and Rig-I on VSV (+) RNAs, which displayed high correlations with the m6A regions (Fig. 6a, b). However, hyper-methylation level of m6A positions was matched with poor binding regions of Mda5 or Rig-I, and vice versa, suggesting that m6A modification negatively controls the binding of RLRs to VSV dsRNA. To exclude the impact of METTL3-mediated m6A to RLR expressions, we performed Western blot and observed METTL3 depletion did not influence the basal level of RLRs, including Rig-I and Mda5 (Supplementary Fig. 7d). Consistent with our data in Fig. 1, METTL3 impedes global innate cascades, these data also suggest the potential role of m6A modification in innate sensing efficacy.

To investigate the impact of m6A modification on RNA binding efficacy of RLRs, we synthesized two biotin-labeled viral RNA sequences for RNA pulldown assays at the m6A-correlated RLRs peak regions (from VSV (+) RNA 2912–2974 nt) that, respectively, contained variants including an unmodified nucleotide (biotin-A2,964) and a m6A modified mimetic nucleotide (biotin-m6A2,964) (Supplementary Fig. 7e). Significantly, the biotin-m6A2,964 bound weakly to Rig-I and Mda5 proteins compared with the biotin-A2,964 (Fig. 6c). To demonstrate that m6A modification disrupts viral dsRNA formation and changes the affinity of RLRs on m6A-modified dsRNA, we tested the binding affinity of biotin-A2,964/ biotin-m6A2,964 to J2 antibody by immunoprecipitation. We found m6A modification indeed decreased the binding between J2 antibody and synthesized VSV RNA oligo (Supplementary Fig. 7f), suggesting that m6A modification impairs dsRNA formation on VSV RNA to attenuate RLRs sensing. Additionally, we synthesized biotin-poly(A), the widely used mimic for dsRNA, together with the methylated biotin-poly(m6A:A) to pulldown RLRs (Supplementary Fig. 7g), and observed that m6A modification attenuated the interaction between poly(A:A) and RLRs (Supplementary Fig. 7h). Consistently, methylated poly(m6A:A) failed to induce TBK1-1RIF3-IFNβ1-ISGs signaling activation in sharp contrast to the robust induction by poly(A:A) (Supplementary Fig. 8a–f). These data demonstrate that m6A interferes with the recognition of RLRs on Viral RNAs and abolishes the RLR-mediated innate sensing pathways. To understand the endogenous role of
METTL3, we knocked down METTL3 in HeLa cells to determine the influence on intercellular innate sensing efficacy of RLRs. Compared to the control, METTL3-depleted cells displayed more co-localization of RLRs and dsRNA intracellularly upon VSV infection (Fig. 6d). Consistently, METTL3 depletion significantly promoted the interaction of RLRs with VSV RNAs by RIP-qPCR (Fig. 6e). Taken together, these results strongly support our hypothesis that METTL3-mediated m6A modification plays as an innate immune suppressor to inhibit the sensing of RLRs to viral RNAs directly, which suggests that m6A-modified viral RNA motif acts as cis-acting element to control innate sensing through decreasing dsRNA formation and dsRNA loads (Fig. 7).

Discussion
In this study, we extensively uncover a signature network that METTL3-mediated m6A RNA modification on viral RNA as a
expression which depended on its catalytic activity. Although our activity, but cytosolic METTL3 significantly inhibited interferon expression which depended on its catalytic activity. Although our findings reveal that VSV could recruit cytosolic METTL3 to suppress innate immunity, the intrinsic mechanism on how METTL3 translocated from the nucleus to cytosol upon VSV infection needs further investigation.

Emerging evidence indicates that m6A modification influences various RNA metabolisms, which is largely dependent on different m6A “reader” proteins8,10. In many cases, YTHDF1, YTHDF3, and YTHDC2 promote the translation of m6A-methylated mRNA, while YTHDF2 enhances translation and decay9-11. For the innate immunity sensing, m6A “reader” proteins can function as trans-acting factors to compete with and suppress RLRs to bind to m6A-modified RNAs5,22. However, whether and how m6A-modified RNA motif plays as a cis-acting element to regulate innate sensing remains unclear. On the other hand, m6A modification also displays crucial roles in reshaping the intrinsic RNA secondary structure formation35,36. However, whether foreign viral RNA undergoes a similar mechanism remains largely unknown. Our findings identified the significant increase of dsRNA level in VSV-treated METTL3-depleted cells, which specifically promoted dsRNA formation at the m6A-methylated sites of viral RNA (Fig. 5b, e). The PAR-CLIP-seq data demonstrate that both RIG-I and MDA5 bound to VSV (+) RNAs directly, particularly at the m6A-modified regions. Previous studies reported that RIG-I and MDA5 show different recognition preferences for different dsRNA species4,22. In our study, we demonstrated that RIG-I and MDA5 have highly correlated recognition patterns on VSV (+) RNAs, suggesting the direct sensing and functional compensation between RIG-I and MDA5 to viral single-stranded RNA-folded dsRNA. Moreover, m6A-modified dsRNA poly(m6A:U) and VSV (+) RNA oligo indeed disrupted the recognition and activation of RLRs (Fig. 6c and Supplementary Fig. 7d, e). Thus, our findings highlight a ubiquitously biological phenomenon that viruses and host cells could orchestrate the m6A modification for foreign RNAs to mimic “self” component by reshaping the dsRNA structures.

A recent study demonstrated that m6A modification did not alter the phosphorylation of IRF3 by DNA virus HCMV infection37. In consideration of the differences between DNA virus and RNA virus (Fig. 1a, b), we detected the TKB1-IRF3 signaling in various cell lines after VSV infection. Intriguingly, METTL3 inhibited TKB1-IRF3 activation under VSV treatment. Our explanation is that RNA virus containing more species of RNAs, including genomic, replicative intermediate RNA and transcript RNAs, might be regulated by m6A and sensed by RLRs to induce innate immunity. Notably, a recent paper reported that RNA m6A modification attenuates the sensing of RIG-I dependent on m6A “reader” proteins22, but how these “reader” proteins function for RIG-I sensing remains uncovered. It is worth noting that our miCLIP and PAR-CLIP-seq data revealed that METTL3 mediated m6A modification on Ifnb1, consistent with the reported study20,37. Hence, METTL3 seems to globally suppress "self" marker to suppress innate sensing pathways through decreasing viral dsRNA formation. Beyond the recent reports that m6A modification controls innate immune response via targeting mRNA stability of host type I IFN or OGDH30,31, our study revealed that m6A methylation significantly inhibits upstream signaling of innate immunity in various cell lines. Moreover, METTL3 can be translocated from the nucleus to the cytosol upon VSV infection to directly methylate viral RNAs. Consequently, the m6A modified viral RNAs were reshaped with reduced dsRNA loads to suppress innate sensing efficacy by MDA5 or RIG-I, which inhibits global innate immune signaling pathways. In a murine model, METTL3 depletion in monocyte or hepatocyte protects the mice against VSV infection and enhances type I IFN greatly in vivo, presenting the potential therapeutic applications for viral infectious therapy.

The immune response in human beings undergoes a "butterfly effect", initiated from intracellular innate immune sensing to a global adaptive immune response29. Virus-encoded molecular signatures, such as cytosolic dsRNA or otherwise foreign "non-self" RNA species, trigger cellular antiviral innate immune signaling, which is beneficial for the maintenance of body homeostasis during infections29-32. It is widely accepted that cytosolic RNA sensors RLRs, RIG-I, and MDA5 recognize these "non-self" RNAs and activate signal transduction pathways to induce a status of antiviral defense with expression of type I IFNs27. However, the PRRs-triggered elevation of these antiviral cytokines may also lead to severe adverse effects with autoimmune responses33,34. How the host cells modulate the immune sensor triggered signal activation within a scope of control remains largely obscure. In a dsRNA-dependent manner, our present findings reveal a new negative regulation pathway that METTL3 is translocated to the cytoplasm upon VSV infection to methylate viral RNA, and orchestrates innate signaling homeostasis via m6A modification to decrease dsRNA level. From the virus side, our study also elucidates a strategy for virus immune escape that virus hijacks m6A modification to disrupt dsRNA formation to escape RLR sensing.

Although we identified METTL3 as an innate immune rheostat for a broad spectrum of pathogen triggers, the data suggests that METTL3 is more efficient for RNA virus-induced innate immunity, especially for VSV, a negative-sense RNA virus that widely used for innate immune studies. In contrast to DNA virus and some RNA virus-like Influenza virus, VSV RNAs, including genomic (−) RNA and (+) RNAs, mainly localize in the cytoplasm. Based on our finding that METTL3 globally suppresses innate upstream pathways, we further identified that VSV infection attracts METTL3 from the nucleus to the cytoplasm. Intriguingly, this cytosolic translocation did not influence its methyltransferase activity, but cytosolic METTL3 significantly inhibited interferon expression which depended on its catalytic activity. Although our
Fig. 4 METTL3 mediates m6A modification on VSV RNA. a A schematic representation of the experimental procedure used in b. b Dot blot analysis of VSV RNA m6A level treated with immunoprecipitated (IP)-IgG or IP-METTL3, respectively. Methylene blue staining indicated the loading control. c MeRIP-qPCR analysis of VSV RNA m6A level following treatment with overexpression of WT or Mettl3-mut, respectively. n = 2 biologically independent experiments. d RNA-FISH analysis of the co-localization of VSV RNA and m6A modification in the cytosol. e The conserved sequence motif of m6A residues in CIMS-based miCLIP-seq. f Integrative genomics viewer (IGV) plots of the METTL3-binding regions and m6A modification on VSV negative sense (−) genomic RNA (Upper graph) and VSV positive sense (+) RNAs (Lower graph). m6A sites are indicated by red triangles. g MeRIP-qPCR analysis of specific m6A sites on VSV RNA in WT or Mettl3 KO RAW264.7 cells. n = 2 biologically independent experiments. *p < 0.05, **p < 0.01, as determined by two-tailed unpaired Student’s t test (c, g). Error bars represent mean ± SEM.
**Fig. 5 METTL3-mediated m^6A modification reshapes viral dsRNA.**

**a** Immunofluorescent analysis of dsRNA level after PBS treatment or VSV infection for 12 h in shNC and shMETTL3 HeLa cells. \( n = 10 \) cells examined over 2 independent experiments. **b** Dot blot analysis of dsRNA level after VSV infection for 12 h in HeLa cell. Methylene blue staining indicates equal RNA loading. The bar graph shows the statistics (right). \( n = 3 \) independent experiments.

**c, d** Immunoblot (c) and dot blot (d) shows the immunoprecipitated (IP)-IgG or IP-dsRNA, and the m^6A level in the IP-dsRNA in shScr and shMETTL3 HeLa cells. Methylene blue staining indicates equal RNA loading. The bar graph shows the statistics (right). **e** Anti-dsRNA-RIP-qPCR analysis of the dsRNA level in VSV RNA in shScr and shMETTL3 HeLa cells. The bar graph (Right panel) shows the statistics from the mean value of each m^6A site (Left panel). Data are representative of 2 independent experiments. \( ^* p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001, ^{****} p < 0.0001 \) as determined by two-tailed unpaired Student’s t test (**a, b, d, e**-left) or two-tailed paired Student’s t test (**e**-right). Error bars represent mean ± SEM.
innate immune signaling through both disrupting the sensing of RLRs and impairing type I IFNs translation.

Finally, based on our investigation, targeting cytoplasmic METTL3 or METTL3 activity maybe a new strategy to ameliorate anti-viral innate immune response and cure patients who suffered from viral infections. Besides, innate immunity activation is important for the initiation of anti-tumor immunity, therefore, it might be a potential strategy for targeting METTL3 to enhance the immunogenicity of solid tumors and promote innate immunity-induced T-cell infiltration in tumors.

In summary, our findings reveal a dsRNA structure-dependent pathway of m^6^A modification for controlling innate sensing.
Fig. 6 m^6^A modification impairs viral RNA sensing efficacy by RLRs. a Integrative genomics viewer (IGV) plots the m^6^A sites and RIG-I and MDA5-binding regions on VSV (+) RNA. RNA-seq data were used as input control. b Venn diagram showing the overlap between high-confidence MDAS and RIG-I binding clusters. The number of clusters in each category is shown in parenthesis. c Biotin-labeled RNA pull-down and Western blot analysis of RIG-I and MDA5 binding to RNA oligo with or without single m^6^A modification (Lower graph). The upper graph indicates predicted structure of VSV (+) RNA: 2912–2974. Three times each experiment was repeated independently with similar results. d Immunofluorescence analysis of HeLa cells increased the co-localization between RIG-I/MDA5 and dsRNA induced by VSV infection for 8 h. n = 20 cells examined over 2 independent experiments with similar results. e RIP-qPCR analysis of increase of RIG-I and MDA5 binding to VSV (+) RNA (region: 1129–1329 nt, referred to Fig. 5a PAR-CLIP result) after deficient for METTL3 in HeLa cells. Data are representative of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, as determined by two-tailed unpaired Student’s t test (c–e). Error bars represent mean ± SEM.

Methods

Animals. Mettl3 floxed mice were generated by Beijing Biocytogen Co., Ltd. Alb-Cre mouse strain (The Jackson Lab Stock NO.: 016832) was gifted from Dr. Lijian Hui (Shanghai Institute for Biological Sciences, CAS) and Lyz2-Cre mouse strain (The Jackson Lab Stock NO.: 004781) was gifted from Dr. Xiyun Yan (Institute of Biophysics, CAS). We generated Mettl3^fl^ox/fl^ox Alb-Cre and Mettl3^fl^ox/fl^ox Lyz2-Cre mice by crossing Mettl3^fl^ox/fl^ox mice with Alb-Cre and Lyz2-Cre mice, respectively. These animals were maintained under specific pathogen-free conditions in the Animal Facilities of Institute of Biophysics, Chinese Academy of Sciences. All experimental and control mice were co-housed. And euthanasia by cervical dislocation was performed for all the animals in this study. All investigations involving mice were approved by the Animal Care and Use Committee of Institute of Biophysics, Chinese Academy of Sciences.

Cell lines and cell culture. RAW264.7, Vero, HEK293T, LO2, HeLa, A549, and Huh7 (Supplementary Data 2) were purchased from ATCC. AG12 cell line is gifted from Dr. Wenhui Li (National Institute of Biological Sciences, Beijing). All these cell lines were maintained in high-glucose DMEM (Gibco) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin, 100 μg/mL streptomycin. The cells were incubated at 37 °C in a humidified chamber containing 5% CO₂.
Transfection. 2 μg DNA of each plasmid (Supplementary Data 4) or poly(I:C) (InvivoGen, Cat# thl-pic) or poly(dA·dI) (InvivoGen, Cat# thl-pata) was used to transfect cells with Lipofectamine 2000 (Life Technologies) or GenStar (C1010-01) transfection reagents, according to the manufacturer’s instructions. The gene expression level was analyzed by qRT-PCR or Western blot.

RNA interference. Cells were transfected with 100 nM siMETTL3 or siScramble, respectively, using RNAi Max (Invitrogen) for 48 h according to the manufacturer’s protocols. The gene expression level was analyzed by qRT-PCR or Western blot. The sequence information of siRNAs was provided in the Supplementary Data 3.

CRISPR/Cas9. The guide-RNA oligo, with sequence for its target gene, was designed using crispr.mit.edu website. The sequence was cloned into px58SM-Cas9-2A-EFGP-MCS vector. RAW264.7 cells were transiently transfected with px58SM-Cas9-2A-EFGP-MCS plasmid with sgRNA. Cultured about 2 days, the EFGP labeled mRNAs were sorted into the right sample (1 cell per tube) and the control (1 cell per tube). The cell sorter knocked out efficiency of colonies were detected by Western blot. And the sequence information of sgMettl3 was provided in the Supplementary Data 1.

Western blot. Total proteins were lysed from cells by RIPA buffer (150 mM NaCl, 1%NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail and a phosphatase inhibitor on ice for 30 min. The concentration of proteins was detected with BCA Protein Assay Kit (Pierce, Cat#232235) and samples with loading buffer were boiled at 100 °C for 3 min. Each sample was separated into SDS-PAGE and transferred to PVDF membranes. The PVDF membrane was blocked with 5% non-fat milk and incubated with specific primary antibodies (working concentration of each antibody refers to product specification) overnight. Then the membrane was probed with appropriate secondary antibody, and detected the protein bands with Immobilon™ Western HPR Substrate Luminol Reagent (Merck Millipore, Cat#WBKLS0500). Antibody information (Supplementary Data 1).

RNA extraction and real-time RT-PCR. Total RNAs extracted from cultured cells were used for qRT-PCR analysis. Briefly, cells in culture were lysed in TRizol (Ambion, Cat#15596018) and RNA extraction was according to the manufacturer’s instructions. Total RNA was reversely transcribed into cDNA with PrimeScript TM RT Master Mix (TaKaRa, Cat#RR0364). Reverse transcription was performed at 37 °C for 45 min, followed at 80 °C for 5 s. The cDNA samples were diluted and stored at 4 °C. Quantitative real-time PCR (qRT-PCR) analysis was performed with TB Green™ Premix Taq™ (TaKaRa, Cat#RR420A) on the ViiA 7 Real-Time PCR system. All runs were accompanied by the internal control genes Actin for murine and ACTIN for human. And all the primer sequences were provided in the Supplementary Data 3.

ELISA. The ELISA assay was performed with a Mouse IFN-β ELISA Kit (BioLegend, Cat#439407) (Supplementary Data 5) according to the manufacturer’s instructions.

Immunofluorescence staining. Cells were fixed with 4% parafomaldehyde (PFA) for 30 min at room temperature (RT) and then permeated with 0.1% Triton X-100, 0.5 mM DTT, 50 mM Tris-HCl, pH 7.5, 1% SDS, 0.5% Triton X-100, and 0.1% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 1% NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM PMSF, 2 mM VRC, protease inhibitor cocktail. After sonication on ice with 10 s ON, 10 s OFF for 3 min, cells were collected by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatants were collected and pre-cleared with 15 μl protein A/G beads and 20 μg/ml yeast RNA at 4 °C for 30 min. Afterwards, the supernatants were incubated with the primary antibody and pre-protein-coated protein A/G beads overnight at 4 °C. After incubation, the beads were first washed with washing buffer I (50 mM Tris-HCl pH7.5, 1 mM NaCl, 1% NP40, 1 mM sodium deoxycholate, 2 mM VRC) 5 min for 3 times, then with washing buffer II (50 mM Tris-HCl pH7.5, 1 mM NaCl, 1% NP40, 1% Sodium Deoxycholate, 2 mM VRC, 1 mM urea) 5 min for 3 times, and resuspended with 140 μl elution buffer (100 mM Tris-HCl pH7.5, 0.5 mM EDTA, 10 mM DTT, 1% SDS). 40 μl was saved for protein analyses and 100 μl for RNA extraction. 5 μl of 10 pg/ml proteinase K was added into the RNA sample and incubated at 55 °C for 30 min. Finally, 1 ml TRizol (Ambion) was added for total RNA isolation and detected by qRT-PCR.

Biotin-labeled RNAs to pulldown proteins. Biotin-labeled RNA pulldown assay was described as follows. Biotin-poly(A(U), biotin-poly(μA(U), biotin-A1272, and biotin-μA1272 were artificially synthesized (Sangon Biotech). The sequences information was provided in the Supplementary Data 3. 2 × 10^5 RAW264.7 cells were treated with 200 μl cell lysis (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM DTT, 50 mM Tris-HCl, pH7.5, 1% sodium deoxycholate, 1 mM PMSF, and 2 mM VRC, with protease inhibitor cocktail, Roche and 2.5 μg RNAse ribonuclease inhibitor, Promega) on ice for 30 min. The supernatant was collected and added pre-cleared 40 μl Streptavidin Dynabeads (Invitrogen) was added for 30 min at 4 °C, then 20 μg/ml yeast RNA was added to block unspecific binding and incubated for 20 min at 4 °C. 4 μg biotinylated RNAs were added for incubation 1.5 h at RT and then by addition of 40 μl Streptavidin Dynabeads to incubate for 2 h at RT. Beads were washed 5 min for 4 times with RIP buffer containing 0.5% sodium deoxycholate and then saved 40% for running agarose gel and boiled 60% sample in 2× SDS loading buffer at 100 °C for 5 min. The RNA-protein complex was detected by Western blot with primary antibodies of anti-IgH-A5 and anti-BIG-1.

Viral infection and plaque assay. Mouse peritoneal macrophages or other cells were seeded for 24 h before virus infection. Cells were infected with VSV, HSV-1, HCMV, HCV, or SeV for various times, as indicated in the figures. VSV plaque assay and VSV replication were determined by a standard TCID50 assay on permissive Vero cell monolayers in 96-well plates with a series of tenfold-diluted samples. After 1 h of infection, the plates were incubated for 48 h. The medium was removed and the cells were fixed with 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 30 min before plaque counting.

Viral infection in vivo. For in vivo VSV infection studies, 6-week-old control and monoctye or hepatocyte-specific METTL3-deleted mice were infected with high dose of VSV (1 × 10^8 PFU/mouse) or moderate dose of VSV (2.5 × 10^8) by tail vein injection. High dose of VSV induced mice death in a short time to detect the resistance of mice for VSV infection. For moderate dose of VSV infected mice, 24 h after infection, we collected the blood from the orbital sinus for ELISA and obtained the lungs, spleen, and liver from each mouse for analysis of RNA and protein. The liver and lung were fixed by 4% paraformaldehyde for H&E staining.

RNA-seq and miCLIP-seq. mRNAs were purified from total RNAs using Dyna-beads mRNA purification kit (Life Technologies, 61006), and subjected to generate the CDNA libraries according to TruSeq RNA Sample Prep Kit protocol. All samples were sequenced by Illumina HiSeq X-ten with paired-end 150 bp read length. Single-base resolution high-throughput sequencing was carried out according to previously reported methods with some modifications. Briefly, mRNAs were purified using Dynabeads mRNA Purification Kit (Life Technologies, 61006) and fragmented to a size around of 100 nt using the fragmentation reagent (Life Technologies, AM8740). 10 μg of purified mRNAs were mixed with 25 μg of anti-miR-34a antibody (Ambac, ab151230) in 450 μl immunoprecipitation buffer (300 μl 50 mM Tris, pH 7.4, 100 mM NaCl, 0.05% NP-40) and incubated by rotating at 4 °C for 2 h. The solution was then transferred to a clear flat-bottom 96-well plate (Corning) on ice and irradiated three times with 0.15 J/cm^2 at 254 nm in a CL-1000 Ultraviolet Crosslinker (UVF). The mixture was then immunoprecipitated by immunotaining 3 μl Dynabeads Protein A/G (Life Technologies, Sigma, 1001D) at 4 °C for 2 h. After extensive washing and on-bead end-repair and linker ligation, the bound RNA fragments were eluted from the beads by protease K digestion at 55 °C.
for 1 h. RNAs were isolated from the eluate by phenol-chloroform extraction and ethanol precipitation. Purified RNAs were reversely transcribed with SuperScript III reverse transcriptase (Life Technologies, 18080093) according to the manufacturer’s protocol. First-strand cDNA was size-selected on a 6% TBE-Urea gel (Life Technologies, EC6865BOX), and circularization and re-linearization of cDNA were performed with CircLagasse II (Epitope, CL0021K) and BamHI (NEB, R0136), respectively. Libraries were PCR amplified with Accuprime Supermix 1 enzyme (Life Technologies, 12342010) for 20 cycles and size-selected on an 8% TBE gel (Life Technologies, EC6215BOX). Sequencing was carried out on Illumina HiSeq X-ten platform according to the manufacturer’s instructions.

PAR-CLIP. RAW264.7 cells treated with VSV for 12 h were cultured in medium supplemented with 200 μM 4-thiouridine (4-SU) (Sigma) for 14 h, and then irradiated once with 400 mJ/cm² at 365 nm using the CL-1000 Ultraviolet Crosslinker (UVP). Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP-40, 1 μM NaF, 1× protease inhibitor cocktail (Bimake), 0.04 U/ml RNasin (Beyotime)) and rotated for 30 min at 4 °C. Cell debris was removed by centrifugation at 12,000 × g for 30 min at 4 °C and the supernatant (3–4 mg/ml) was digested by 1 U/ml RNase T1 at 22 °C in a water bath for 8 min and cooled on ice for 5 min. Then the lysates were incubated with indicated antibody overnight at 4 °C. Washed magnetic Protein A beads were added to the mixture and incubated for another 2 h, and the beads were then washed three times with IP wash buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.05% (v/v) NP-40, 1× protease inhibitor cocktail (Bimake), 0.04 U/ml RNasin (Beyotime)). Beads were digested with 10 U/ml RNase T1 again at 22 °C in a water bath for 8 min, cooled on ice for 5 min, then washed three times in high salt wash buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) NP-40, 1× protease inhibitor peptide cocktail (Bimake), 0.04 U/ml RNasin (Beyotime)), resuspended in 100 μl dephosphorylation buffer (50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM MgCl₂), and incubated with 0.05 U/ml intestinal alkaline phosphatase (CLIP, NEB) for 10 min at 37 °C with gentle rotation. Beads were washed twice with Tris-phosphate wash buffer (50 mM Tris-HCl pH 7.5, 20 mM EGTA, 0.5% (v/v) Triton X-100) with 3 min rotation. Beads were resuspended with 200 μl protein kinase K buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, 4 μg/μl protease K (Roche)) and incubated at 55 °C for 2 h. RNA was extracted using Phenol: Chloroform. Sequencing libraries were constructed using the SMARTer smRNA-seq kit (Clontech) according to the manufacturer’s instructions. Sequencing was performed on an Illumina HiSeq X-Ten instrument with paired end 150-bp read length.

RNA-seq analysis. The quality of raw sequencing data was checked by FastQC (v0.11.5) first. Genomic alignment was performed on paired-end reads using hisat2 (v2.0.5) to map to the corresponding genome (Ensembl version 88) and VSV positive-sense (+) RNA (RefSeq, NC_001560), respectively, using bowtie(v1.0.1) with default settings. Only reads with mapping quality score (MAPQ) ≥ 30 were kept for peak calling. Binding regions of RBPs were obtained by PARalyzer (v1.5) with default settings, a software defined the binding cluster based on T-to-C conversions. We filtered the binding region with reads count ≥ 50 for more analysis.

PAR-CLIP-seq analysis. Forward sequencing reads were trimmed by Cutadapt (v1.5) to remove low quality bases and adapters, and then aligned to the mouse reference genome (GRCh38/mm10; Ensembl version 68) and VSV positive-sense (+) RNA (RefSeq, NC_001560), respectively, using bowtie(v1.0.1) with following parameter: -v 2 -m 10 --best -strata44. Only the reads short than 100nt were kept for peak calling. Binding regions of RBPs were obtained by PAalyzer (v1.5) with default settings, a software defined the binding cluster based on T-to-C conversions. We filtered the binding region with reads count ≥ 50 for more analysis.

Statistics. Unless otherwise indicated, data are presented as mean ± SEM of 3 independent experiments. All statistical analyses were performed with Graph Prism 6.0 software, and the statistics were analyzed by unpaired Student’s t test. The correlation between genes expression and survival rate and overall patient survival were analyzed by Kaplan–Meier survival curve. p values were provided as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw data can be accessed from Genome Sequence Archive at the National Genomics Data Center, Beijing Institute of Genomics, CAS/China National Center for Bioinformation as accession CRA002259 [https://bigsdb.big.ac.cn/search/?dbId=ggbig-GRCA002259]. Besides, the original data is also available at Sequence Read Archive with number PRJNA636708 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA636708/]. The raw numbers for charts and graphs are available in the Source Data file whenever possible. All other data supporting the findings of this study are available from the corresponding author on reasonable request. Source data are provided with this paper.

Code availability

The custom R scripts for RPMK calculation is available on GitHub [https://github.com/ndqyhyunbin/VSV-m6A]. Other custom scripts for analyzing data are available upon request.

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