N6-methyladenosine (m6A) is the most prevalent internal modification of mRNAs in most eukaryotes. Here we show that RNAs of human respiratory syncytial virus (RSV) are modified by m6A within discreet regions and that these modifications enhance viral replication and pathogenesis. Knockdown of m6A methyltransferases decreases RSV replication and gene expression whereas knockdown of m6A demethylases has the opposite effect. The G gene transcript contains the most m6A modifications. Recombinant RSV variants expressing G transcripts that lack particular clusters of m6A display reduced replication in A549 cells, primary well differentiated human airway epithelial cultures, and respiratory tracts of cotton rats. One of the m6A-deficient variants is highly attenuated yet retains high immunogenicity in cotton rats. Collectively, our results demonstrate that viral m6A methylation upregulates RSV replication and pathogenesis and identify viral m6A methylation as a target for rational design of live attenuated vaccine candidates for RSV and perhaps other pneumoviruses.
Internal N6-methyladenosine (m6A) is the most prevalent modification in the mRNA of most eukaryotes1,2. Although m6A was discovered in the 1970s3–6, its biological functions remained a mystery for decades. In 2011, m6A demethylases were discovered7,8. It is generally believed that m6A methylation is reversible although studies also suggest that a substantial degree of demethylation did not occur in some mRNA exons9. Transcriptome-wide mapping of m6A by high-throughput sequencing technology was reported subsequently10–12. A series of m6A-related enzymes and proteins were also discovered and characterized in mammalian cells. Recent work has found that m6A regulates RNA metabolism, protein translation, gene expression, and embryonic development in organisms ranging from plants to mice7,13–17.

The m6A modification is installed by the m6A methylation “writer” protein, a multicomponent S-adenosyl-l-methionine (SAM)-dependent methyltransferase complex, composed of two subunits, METTL3 (a catalytic enzyme) and METTL14 (an allosteric activator), and facilitated by the Wilms tumor 1 associated protein (WTAP)18,19. Interestingly, m6A modification can be selectively removed by two mammalian RNA demethylases, FTO (fat mass and obesity-associated protein) and its homolog, ALKBH5, both of which are termed m6A “eraser” proteins7,8. The biological functions of m6A are mediated through m6A “reader” proteins that specifically recognize and bind the methylated adenosine on RNA. The recently characterized m6A binding proteins include the YT521-B homology (YTH) domain family of proteins (YTHDF1, YTHDF2, YTHDF3, and YTHDC1)16,20,21. These m6A binding proteins regulate protein translation and RNA decay22,23.

As obligate intracellular parasites, viruses must synthesize their own genetic material and carry out their reproduction while avoiding innate immune surveillance by mimicking their host. Studies from the early 1970s showed that viral RNAs of several DNA viruses, retroviruses, and influenza virus contained internal m6A modifications24–29. Although it is still controversial whether viral m6A positively or negatively regulates HIV replication30–32, it was shown that m6A promotes gene expression of influenza virus33 and simian virus 4034. In the case of Kaposi’s sarcoma-associated herpesvirus (KSHV), the impact of m6A machinery on viral gene expression is dependent on the cell type35–37. In contrast, m6A negatively regulates the production of hepatitis C virus (HCV) and Zika virus38,39. However, the mechanism(s) by which m6A methylation regulates the virus life cycle remain poorly understood. In addition, the roles of m6A in viral virulence, pathogenesis, and immunity are not clear.

Non-segmented negative-sense (NNS) RNA viruses encompass a wide range of significant human, animal, and plant pathogens. For many of these agents, there are no effective vaccines or antiviral drugs. The replication and gene expression strategy of NNS RNA viruses is unique. During transcription, the polymerase transcribes the linear array of genes in the viral genome into 5–10 mRNAs that are capped and methylated at the 5’ end and polyadenylated at the 3’ end. During replication, the polymerase initiates at the extreme 3’ end of the genome and ignores the gene junctions to synthesize a full-length complementary antigenome which is encapsidated by N protein and subsequently serves as template for the synthesis of full-length progeny genomes that are likewise encapsidated by N protein30,39,40. A good example of NNS RNA viruses is human respiratory syncytial virus (RSV), a member of the Pneumoviridae41. RSV is the most important cause of upper and lower respiratory tract infection of infants, young children, and immunocompromised individuals and second only to influenza virus for the elderly42. Worldwide it is estimated that RSV causes 3.4 million hospitalizations and between 66,000 and 199,000 deaths in children <5 years of age43. Despite major efforts, no vaccine or antiviral drug is yet available for RSV44.

Here, we find that the RSV genome, antigenome, and mRNAs are m6A methylated internally at specific sites which positively regulate RSV replication, gene expression, and virus production in HeLa and A549 cells. Subsequently, the m6A sites in the viral G gene, the most abundant m6A enrichment gene, are mutated in an infectious cDNA clone of RSV. The resultant m6A deficient rgRSVs have significant defects in replication, gene expression, spread, and virus release in A549 cells and primary well differentiated human airway epithelial (HAE) cultures. These m6A mutated rgRSVs are defective in viral replication in the upper and lower respiratory tract of cotton rats and produce less pathology in the lungs. Importantly, we find that one m6A-mutated rgRSV is completely attenuated in cotton rats yet retains a wild-type level of immunogenicity. Collectively, these results reveal that m6A upregulates each step in the RSV replication cycle and viral pathogenesis, and identify m6A as a new target for the rational design of live attenuated vaccine candidates and antiviral drugs for RSV.

Results

The RSV genome and antigenome/mRNAs are m6A methylated. RSV has a NNS RNA genome of 15,222 nucleotides (RSV A2 strain). As is typical for NNS RNA viruses, replication of the viral genomic RNA (vgRNA) produces an exact, positive-sense full-length complementary RNA (cRNA) antigenome44. Both the genome and antigenome are encapsidated by the nucleocapsid (N) protein and both types of nucleocapsids can be packaged into virions, as for many NNS RNA viruses45. To investigate whether RSV RNA contains m6A, RNA was extracted from highly purified virions grown in HeLa cells, and the purity of RNA was examined by real-time RT-PCR to ensure that they were free from any contamination of host RNAs and viral mRNAs (Supplementary Fig. 1). The presence of m6A in viral RNA was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We found that ~0.7% of the A bases were m6A methylated in RSV viral RNAs, a somewhat higher level than the host mRNAs (0.1–0.4%).

To locate the m6A sites on RSV RNA, we sonicated virion RNA and subjected it to m6A-specific antibody immunoprecipitation followed by high-throughput sequencing (m6A-seq), then mapped all the reads onto either the genome or antigenome sequence. Several m6A peaks were identified on both strands of the viral RNA (Supplementary Fig. 2A and Fig. 1a). The RSV antigenomic RNA contained major m6A peaks in the regions complementary to the N, P, G, and F genes and in the regions complementary to the two regulatory elements, the gene end (ge) sequence of N and the intergenic (ig) sequence between the P and M genes in the genome (Fig. 1a, and Supplementary Fig. 2A and Supplementary Table 1). In the genomic RNA, eleven m6A peaks were detected in the NS2, N, P, M, G, and L genes and four regulatory elements including the gene start (gs) of NS2, ig between P and M, ge of M, ig between M and SH, ge of G, and ig between G and F (Fig. 1a and Supplementary Fig. 2A and Supplementary Table 1). Since we used a recombinant RSV harboring GFP between the leader and the NS1 gene (rgRSV), we also searched whether GFP region contains m6A. An m6A peak with a size of 60 nt was detected in GFP gene in genome (Supplementary Table 1). No m6A peak was found in GFP region in antigenome. The G gene regions from both genome and antigenome have the strongest m6A enrichment with peak size of 822 nt and 672 nt, respectively, indicating that there may be multiple adjacent m6A sites in these regions. Together, these results confirm that both RSV genome and antigenome RNAs contain m6A.
We also mapped m\(^6\)A peaks in mRNAs purified from RSV-infected cells. To do this, total RNA was isolated from rgRSV-infected HeLa cells, enriched for mRNA by binding to oligo dT, and subjected to m\(^6\)A-seq. As RSV mRNAs contain poly(A) and are subsequently detected from poly(dT)-enriched m\(^6\)A-seq, we identified 16 m\(^6\)A peaks from RSV mRNAs (Fig. 1b, Supplementary Fig. 2B and Supplementary Table 2) which largely overlapped with those of the antigenome (Fig. 1a, Supplementary Fig. 2A and Supplementary Table 1). Interestingly, the G gene transcript has the strongest m\(^6\)A enrichment with a 846 bp peak size. In addition, no m\(^6\)A peak was detected in GFP mRNA in virus-infected HeLa cells.
We next performed m\textsuperscript{6}A-seq of rgRSV grown in A549 cells, a relevant cell line for RSV infection. Similar to HeLa cells, we found that RSV, genome, antigenome, and mRNAs were m\textsuperscript{6}A methylated in A549 cells (Fig. 1c, d, and Supplementary Fig. 2C, D). For virion RNAs, a total of 9 and 15 m\textsuperscript{6}A peaks were identified in the genome and antigenome, respectively (Fig. 1c and Supplementary Fig. 2C and Supplementary Table 3). Similar to virions grown in HeLa cells, the location of m\textsuperscript{6}A peaks identified from genome and antigenome largely overlap. G gene regions from both genome and antigenome have the strongest m\textsuperscript{6}A enrichment with 696 and 846 bp peak size, respectively. An m\textsuperscript{6}A peak was detected in GFP gene in antigenome but no m\textsuperscript{6}A peak was detected in GFP gene in genome. For the RNAs extracted from virus-infected cells, a total of 18 m\textsuperscript{6}A peaks were identified in RSV mRNAs (Fig. 1d and Supplementary Fig. 2D and Supplementary Table 4). Again, the G gene transcript has the strongest m\textsuperscript{6}A enrichment with 1046 bp peak size. In addition, one m\textsuperscript{6}A peak was found in GFP mRNA in infected A549 cells.

We next analyzed the overlapping regions based on m\textsuperscript{6}A-seq data from HeLa and A549 cells (Supplementary Tables 5 and 6). For virion RNA, six and four overlapping regions were identified in the genome (gs of NS2, NS2, N, P, Ig between P and M, and G) and antigenome (N, M, G, and F), respectively (Supplementary Table 5). For RNAs purified from RSV-infected cells, 11 overlapping m\textsuperscript{6}A peaks were also found in mRNAs, respectively (Supplementary Table 6). Although there are some differences, the majority of m\textsuperscript{6}A peaks are highly conserved between the two cell lines suggesting that RSV utilizes the host m\textsuperscript{6}A machinery to methylate these specific sites.

RSV infection alters the m\textsuperscript{6}A distribution of host RNAs. We next determined the effects of RSV infection on the abundance and distribution of m\textsuperscript{6}A on cellular transcripts. Metagene analysis showed that RSV-infected and mock-infected HeLa cells have m\textsuperscript{6}A peaks enriched near the start and stop codons of open reading frames (Supplementary Fig. 3A), which is consistent with the known distribution of m\textsuperscript{6}A sites on transcripts\textsuperscript{10–12}. Unlike the distribution of m\textsuperscript{6}A peaks on mRNA, the peaks are mostly uniformly distributed on lncRNA with a slightly more enrichment at its 5’ end (Supplementary Fig. 3B). The distribution of m\textsuperscript{6}A peaks in each annotation also recapitulate m\textsuperscript{6}A site distribution\textsuperscript{10–12} with the majority of peaks residing in the CDS and 3’ UTR regions (Supplementary Fig. 3C, D). Differential peak analysis using the count based QNB test\textsuperscript{46} identified 2256 differentially methylated peaks (Supplementary Data 1). Analysis of RNA-seq data from the host cell (HeLa) revealed over 9000 differentially expressed genes at an adjusted P value cutoff of 0.05 (Supplementary Data 2). These data suggest RSV infection significantly altered both the epitranscriptome and the transcriptome of the host cells. Pathway enrichment analysis shows differentially expressed genes are enriched in pathways including cell cycle, metabolism, autophagy, RNA synthesis and transport, and response to viral infection (Supplementary Fig. 3E).

As expected, the distribution of m\textsuperscript{6}A sites was highly conserved between cell lines (Supplementary Fig. 4A–D). RSV infection altered the expression of over 7000 host cell genes in A549 cells (Supplementary Data 3) involved in a series of signal pathways (Supplementary Fig. 4E) but very few m\textsuperscript{6}A peaks were found to be differentially methylated (Supplementary Data 4). We also analyzed the differentially expressed genes which are overlapped in pathways between the HeLa and A549 cells. Many of these overlapped differentially expressed genes are involved cell cycle, metabolism, TNFα signaling, and RNA transport (Supplementary Fig. 4F). Therefore, RSV infection may have widespread effects on host gene expression partially attributed to alteration of the deposition of m\textsuperscript{6}A.

m\textsuperscript{6}A reader proteins positively regulate RSV replication. To begin to explore the role of m\textsuperscript{6}A modification in RSV replication and gene expression, we first took advantage of HeLa cells that stably overexpress m\textsuperscript{6}A “reader” proteins, YTHDF1, YTHDF2, and YTHDF3 (Fig. 2a). As shown in Fig. 2b, stronger GFP expression (more green cells, brighter cells) was observed in HeLa cells overexpressing YTHDF1-3 compared to the vector control. Quantification by flow cytometry showed that significantly more GFP-positive cells and higher GFP density were detected in HeLa cells overexpressing m\textsuperscript{6}A reader proteins than in the vector control (P < 0.05) (Fig. 2c and Supplementary Fig. 5).

We next measured the expression of RSV F, G, and N proteins. As shown in Fig. 2d, more F, G, and N proteins were detected in all three YTHDF-overexpressing HeLa cell lines. Quantitative analysis showed a dramatic increase in viral protein expression during the first 12 h although later time points were not as large (Fig. 2e–g). Next, we measured the release of infectious virus particles in a single-step growth curve. The RSV titer was significantly increased in all three YTHDF-overexpressing cell lines (Fig. 2h) (P < 0.05 or 0.01). Overexpression of YTHDF2 had the most dramatic impact on virus production, increasing RSV titer by 1–2 logs compared to the vector control HeLa cells (P < 0.05 or 0.01) (Fig. 2h).

The upregulating role of m\textsuperscript{6}A reader proteins on RSV replication was also confirmed in HeLa cells transfected with plasmids expressing YTHDF1-3 (Supplementary Fig. 6A). Compared to HeLa cells stably overexpressing YTHDF1-3 (Supplementary Fig. 2d), transient overexpression of YTHDF1-3 led to a more robust enhancement of F and G protein synthesis (Supplementary Fig. 6B) and GFP expression (Supplementary Fig. 6C).
Fig. 2 YTHDF1, 2, 3 (reader) proteins promote RSV replication, gene expression, and progeny virus production. a Detection of YTHDF1, 2, 3 in HeLa cells stably overexpressing YTHDF1-3. Western blot confirmed the overexpression of YTHDF1-3 proteins in HeLa cells using anti-Flag antibody. b YTHDF1, 2, 3 enhance GFP expression in rgRSV-infected cells. HeLa cells stably overexpressing these YTHDF proteins were infected with rgRSV at an MOI of 0.1, and GFP expression was monitored at the indicated times by fluorescence microscopy. c Micrographs with ×10 magnification (scale bar of 100 μm) are shown. c YTHDF1, 2, 3 increase the number of GFP-positive cells quantified by flow cytometry. The P value (Student’s t-test) for YTHDF1 at 12, 18, and 24 h is ***P = 0.00297, **P = 0.00145, and **P = 0.00202, respectively; for YTHDF2 at 12, 18, and 24 h is **P = 0.00318, ***P = 0.000892, and **P = 0.00312, respectively; for YTHDF3 at 12, 18, and 24 h is **P = 0.00119, ***P = 5.19 × 10^{-5}, and **P = 0.000647, respectively. d YTHDF1, 2, 3 enhance RSV protein expression. Total cell extracts were harvested from rgRSV-infected HeLa cells at the indicated times and subjected to western blot using antibody against RSV N, F, or G protein. Western blots shown are the representatives of three independent experiments. RSV F (F0, F1) (e), G (f), and N (g) proteins were quantified by Image J Software. Data are expressed as mean of three independent experiments ± standard deviation. h YTHDF1, 2, 3 increase RSV progeny virus production. The release of infectious RSV particles was monitored by a single-step growth curve. Virus titer was measured by TCID50. The P value (Student’s t-test) for YTHDF1 at 12, 18, and 24 h is ***P = 0.000237, ****P = 3.68 × 10^{-5}, ****P = 5.46 × 10^{-5}, and ***P = 4.96 × 10^{-5}, respectively; for YTHDF2 at 12, 18, and 24 h is ***P = 0.000237, ****P = 1.48 × 10^{-5}, and **P = 0.0184, respectively; for YTHDF3 at 12, 18, and 24 h is ***P = 0.000165, ***P = 0.000128, and **P = 0.0475, respectively. i YTHDF1, 2, 3 enhance RSV genomic RNA replication. Total RNA was purified from rgRSV-infected cells using TRIzol, and genomic RNA was quantified by real-time RT-PCR using specific primers annealing to the RSV leader sequence and GFP gene. The P value (Student’s t-test) for YTHDF1 at 12, 18, and 24 h is ***P = 0.000246, ****P = 5.93 × 10^{-5}, and ****P = 2.39 × 10^{-5}, respectively. j Ratio between mRNA and genomic RNA. The ratio between NS1 mRNA and genomic RNA was calculated for each cell line. All results are from three independent experiments. Flow cytometry data are expressed as mean of three independent experiments ± standard deviation. RNA copy and viral titer are the geometric mean titer (GMT) of three independent experiments ± standard deviation. The P value (Student’s t-test) for YTHDF2 at 12 and 24 h is ***P = 0.000601 and **P = 0.00130, respectively; for YTHDF3 at 24 h is ****P = 0.000599. Data were analyzed using Student’s t-test and statistical differences were indicated as *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001.
We further analyzed viral replication and gene expression in A549 cells. Similar to the observations in HeLa cells, enhanced F, G, and N protein synthesis (Supplementary Fig. 7A) and GFP expression (Supplementary Fig. 7B) was detected when YTHDF1-3 proteins were overexpressed. We also tested RSV replication in Vero cells, the WHO-approved cell line for production of RSV live attenuated vaccine candidates. Similarly, m6A reader protein (YTHDF1) enhanced RSV protein synthesis in Vero cells (Supplementary Fig. 7C). Thus, we observed a pro-viral function for m6A in all three cell lines. It should be noted that overexpression of YTHDF1 protein (a representative of reader proteins) in A549 cells did not significantly affect the growth or survival of the cells (Supplementary Fig. 8A, B).

We measured the RSV genomic RNA (the replication product) and mRNAs (the transcription product) in HeLa cells by real-time RT-PCR. Overexpression of YTHDF1-3 significantly increased both RSV genomic RNA (Fig. 2i) and mRNA synthesis (Fig. 2j). Overexpression of YTHDF1 and 3 did not alter the balance between the synthesis of genomic RNA and mRNA whereas overexpression of YTHDF2 led to a more dramatic increase in replication than transcription (Fig. 2k). It appears that overexpression of YTHDF1-3 enhanced the ability of the RSV polymerase to both replicate and transcribe.

As a complementary approach, we also tested RSV replication and gene expression in HeLa cells when m6A reader proteins were knocked down by siRNA. Counting live cells by flow cytometry showed that siRNA targeting YTHDF1 did not significantly alter cell survival (Supplementary Fig. 8C, D). Knockdown of individual, endogenous YTHDF1-3 proteins (Fig. 3a) significantly reduced viral F and G protein synthesis (Fig. 3b) and GFP expression (Fig. 3c, d) relative to the control siRNA transfected cells. Collectively, these results demonstrate that m6A binding proteins promote RSV genome replication, mRNA transcription, and as a result, viral protein expression, and progeny virus production.

m6A writer proteins positively regulate RSV replication. The internal m6A addition is catalyzed by host methyltransferases termed m6A writer proteins (METTL3 and METTL14)18. We next examined the role of the m6A writer proteins in RSV replication and protein expression. More F and G protein synthesis (Fig. 4a) and GFP expression (Fig. 4b, c) were observed when METTL3 and METTL14 were overexpressed in HeLa cells. In contrast, less F and G proteins were synthesized (Fig. 4d) and less GFP was expressed (Fig. 4e, f) when endogenous METTL3, METTL14, or both, were knocked down. siRNA targeting METTL3 did not significantly alter cell survival (Supplementary Fig. 8C, D). These results suggest that modification of RSV RNA by m6A writers facilitates RSV replication and gene expression.

Next, we determined whether alteration of m6A writer proteins can alter the level of m6A level in viral RNA. As shown in Fig. 4g, h, the m6A contents in viral RNA of virus from METTL3 and METTL14-overexpressed cells were significantly higher (~26% increase) than viral RNA of particles from vector-transfected cells. Knockdown of METTL3 and METTL14 led to 70% of reduction in m6A content in viral RNA (Fig. 4i, j). These experiments suggest that manipulation of m6A writer proteins leads to enhanced or reduced levels of m6A in viral RNAs, which may in turn, affect viral replication and gene expression.
m6A eraser proteins downregulated RSV replication. Internal m6A modifications are reversible and can be removed by m6A eraser proteins. We thus examined the effects of overexpression of eraser proteins ALKBH5 or FTO, or both (Fig. 5). Overexpression of eraser proteins dramatically reduced RSV F and G protein expression by 80- and 20-fold, respectively (Fig. 5a), and number of GFP cells by 1.3-2.7 times (Fig. 5b, c). Next, we knocked down ALKBH5 or FTO, or both, in HeLa cells, followed by rgRSV infection. siRNA targeting ALKBH5 did not significantly alter cell survival (Supplementary Fig. 8C, D). Knockdown of ALKBH5 and FTO enhanced the expression of F protein by 3-fold, G protein by 5-fold (Fig. 5d), and number of GFP cells by 1.2-fold (Fig. 5e, f) compared to the cells transfected with control siRNA. Therefore, overexpression of m6A eraser proteins negatively regulated RSV replication and gene expression. We also determined whether overexpression and knockdown of eraser proteins can affect m6A level of viral RNA. As shown in Fig. 5g, h, the m6A contents in viral RNA of virus from ALKBH5-overexpressed cells led to 46% reduction. Knockdown of ALKBH5 led to 2.12-fold increases in m6A abundance in viral RNA. A549 cells were transfected with siRNA targeting METTL3 and METTL14 or control siRNA, followed by rgRSV infection. RSV particles were harvested and purified. The m6A content in viral RNA was determined as described in Materials and Methods. The P value (Student’s t-test) for METTL3, METTL14, and METTL3 & METTL14 is **P = 0.00441, **P = 0.00458, and ****P = 0.000134, respectively. Overexpression of m6A writer proteins enhances m6A level in viral RNA. Confluent A549 cells were transfected with plasmids encoding METTLE3 and METTLE14 or vector pCAGGS, followed by rgRSV infection. RSV particles were harvested and purified. The m6A content in viral RNA was determined as described in Materials and Methods. The P value (Student’s t-test) is **P = 0.00684. Fold increase in m6A content in viral RNA compared to controls. The P value (Student’s t-test) is ****P = 0.000764. Knockdown of m6A writer proteins reduces m6A level in viral RNA. A549 cells were transfected with siRNA targeting METTL3 and METTL14 or control siRNA, followed by rgRSV infection. RSV particles were harvested and purified. The m6A content in viral RNA was determined as described in Materials and Methods. The P value (Student’s t-test) is ****P = 1.29 × 10−7. Fold reduction in m6A content in viral RNA compared to controls. The P value (Student’s t-test) is ****P = 2.45 × 10−12. Results are from three or four independent experiments.

RSV infection does not alter the translocation of m6A proteins. To directly visualize the locations of the m6A reader, writer, and eraser proteins, mock and rgRSV-infected HeLa cells were stained with antibodies specific to each m6A-related protein and analyzed by confocal microscopy. As shown in Fig. 6a and Supplementary Fig. 9, m6A reader proteins (YTHDF1-3) were distributed in the cytoplasm in both mock and RSV-infected cells. No differences were observed in the locations of the m6A reader, writer, and eraser proteins in mock and RSV-infected HeLa cells.
proteins (METTL3 and METTL14) and eraser protein (ALKBH5) were distributed in the nucleus although a small fraction of these proteins was also found in the cytoplasm (Fig. 6b and Supplementary Figs. 10 and 11). Also, viral N protein was partially co-localized with writer protein (METTL3) (Fig. 6b). Another eraser protein, FTO, was exclusively located in the nucleus (Fig. 6c). Equal amounts of m6A-related proteins were detected in the cytoplasmic and nuclear fractions by Western blot (Fig. 6d, e). Therefore, RSV infection does not significantly alter the distribution pattern of m6A-related proteins in HeLa cells.

m6A reader proteins bind to both RSV genomic RNA and mRNA. Since the biological function of m6A is mediated by m6A binding proteins, we next determined whether YTHDF2 can directly bind to RSV RNAs in virus-infected cells. As expected, YTHDF2 was pulled down by YTHDF2-specific antibody (Supplementary Fig. 12A). Both RSV genomic RNA and N mRNA were efficiently precipitated as complexes, with YTHDF2 (Supplementary Fig. 12B). We further confirmed this result by pulling down HA-tagged YTHDF2 from total cell lysates of HeLa cells overexpressing YTHDF2 with HA antibody (Supplementary Fig. 12C). Similarly, significant amounts of RSV genomic RNA and N mRNA bound to YTHDF2 (Supplementary Fig. 12D). Since G mRNA has the strongest m6A enrichment, we also determined the binding of m6A reader proteins to G mRNA. All reader proteins (YTHDF1, 2, and 3) were capable of binding to G mRNA (Supplementary Fig. 12E).

Abrogation of m6A sites results in attenuation of RSV. Based on m6A-seq, G gene and G mRNA have the most abundant m6A enrichment in both HeLa and A549 cells (Fig. 1). Thus, we decided to mutate the m6A sites in the G gene region which we found to be conserved in the m6A-seq from both HeLa and A549 cells (Supplementary Fig. 13). These mutations were predicted to remove the m6A sites in the G mRNA and in the G gene region of the antigenome without changing the amino acid they encoded. Recombinant RSVs carrying these mutations were recovered by reverse genetics system. First, the potential m6A sites in regions 1, 2, and 3 were mutated and the viral RNA was sequenced to confirm the mutation (Supplementary Fig. 13). The mutations were confirmed by sequence analysis (Supplementary Fig. 13). The mutated RSVs were recovered by reverse genetics system and their RNA sequences were confirmed to contain the predicted mutations (Supplementary Fig. 13). The infected cells were then analyzed for RMN (Fig. 7a and Supplementary Fig. 13). The results showed that the mutated RSVs replicate inefficiently compared to the wild-type RSVs (Fig. 7a and Supplementary Fig. 13). This finding is consistent with the observation that m6A sites are conserved in the m6A-seq from both HeLa and A549 cells (Supplementary Fig. 13). Thus, we conclude that m6A sites are essential for RSV replication in both HeLa and A549 cells.
2, and 3 were mutated individually to produce rgRSV-G1, G2, and G3, respectively. Second, mutations of m6A sites in regions 1 and 2 were combined to produce rgRSV-G12. Third, m6A sites in regions 1, 2, and 3 were combined to produce rgRSV-G123. All m6A-mutated rgRSVs had various degrees of reduction in viral F and G protein synthesis in A549 cells compared to the parental rgRSV (Fig. 7a). Significantly less GFP expression was observed in m6A-mutated rgRSVs compared to rgRSV at 48 h post-infection (Fig. 7b, c, and Supplementary Fig. 14). Single-step growth curves showed that m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication. Real-time RT-PCR results showed that both m6A-mutated rgRSVs had delayed replication kinetics and had 0.5–1.5 log reductions in peak titer compared to rgRSV (Fig. 7d). Overall, mutants rgRSV-G1, G3, G12, and G123 had a moderate defect whereas rgRSV-G2 had a mild defect in replication.

m6A-mutated rgRSVs are defective in replication in HAE. We next tested the replication and spread of m6A-deficient rgRSVs in HAE cultures, a near in vivo model for lower airway infection. These cultures are pseudostratified and polarized, closely resembling the in vivo airway epithelium morphology and function, including mucus production and ciliary motion17. In brief, HAE cultures were infected with 800 TCID50 of each recombinant virus, and viral release and spread was monitored. As in A549 cells, m6A-mutated rgRSVs had a delay in viral gene expression (GFP production) and spread (Fig. 8a, b). At day 4 post-inoculation, m6A-mutated rgRSVs had fewer green cells compared to rgRSV. Although several rgRSV mutants gradually increased at days 6 and 8, the density of green cells remained less than for rgRSV. rgRSV-G1 was delayed in spreading but eventually spread to most susceptible cells at day 8. rgRSV-G2 had a delay at the early time point (day 4) but had wild-type level of spreading at later times. Recombinant rgRSV-G12 was the most defective virus in HAE cultures, displaying a weak GFP signal during the entire experimental period. In addition, m6A-mutated rgRSVs had delays in virus release in HAE culture with 1–2 log defects in virus yield (Fig. 8c). These results demonstrate that m6A-mutated rgRSVs were defective in replication and spread in this near in vivo lung infection model.
4 out of 5 cotton rats had below detection limit level of RSV replication in the nasal turbinate and lung titers, respectively. It should be noted that 4 out of 5 cotton rats had below detection limit level of RSV replication in nasal turbinate (P < 0.05). Mutant rgRSV-G12 had the most dramatic defect in replication, with reductions of 100- and 200-fold in viral titer in nasal turbinate and lung, respectively. It should be noted that 4 out of 5 cotton rats had below detection limit level of RSV replication in nasal turbinate and 3 out of 5 cotton rats had below detection limit level of RSV replication in lungs, suggesting that rgRSV-G12 is highly attenuated in vivo. The rgRSV-G3 had 5-fold reduction in replication in nasal turbinate and lung (P < 0.05). Histologic examination showed that rgRSV caused moderate pulmonary histopathological changes, including interstitial pneumonia and peribronchial lymphoplasmocytic infiltrates (Fig. 9c). In contrast, m6A-deficient rgRSV mutants only had mild and less pulmonary histopathological changes compared to rgRSV (Fig. 9c). These results showed that m6A-deficient rgRSV mutants had significant reductions in viral replication in both the upper and lower respiratory tracts in cotton rats and were less pathogenic.
compared to rgRSV. These results indicate that viral m6A upregulates viral replication and pathogenesis in vivo.

m6A-mutated rgRSVs are highly immunogenic in cotton rats. To determine whether defects in viral m6A methylation impair the immunogenicity of the virus, we evaluated the protection efficacy of a partially attenuated (rgRSV-G1) and highly attenuated (rgRSV-G12) virus in cotton rats. Cotton rats immunized with parental rgRSV or m6A-mutated rgRSVs did not have any detectable infectious virus in either the nasal turbinate or lung tissue after challenge with rgRSV (Fig. 9d). In contrast, unvaccinated challenged controls had average titers of $5.12 \pm 0.28$ and $4.27 \pm 0.07$ log$_{10}$ PFU/g in the lung and nasal turbinate, respectively (Fig. 9d). These results demonstrate that immunization with the rgRSV-G1 and G12 provided complete protection from challenge with rgRSV. Lung histology showed that unvaccinated challenged controls had moderate histologic lesions (Supplementary Fig. 15). However, the vaccinated challenged groups had only mild lesions in lungs. In addition, no enhanced lung damage was observed (Supplementary Fig. 15). The most attenuated m6A-deficient virus (rgRSV-G12) triggered similar levels of neutralizing antibody compared to rgRSV ($P > 0.05$) (Fig. 9e). The rgRSV-G1 had a lower antibody titer at week 3 and 4 compared to rgRSV ($P > 0.05$) (Fig. 9e). The rgRSV-G12 had a lower antibody titer at week 3 and 4 compared to rgRSV ($P > 0.05$) (Fig. 9e). The rgRSV-G12 had a lower antibody titer at week 3 and 4 compared to rgRSV ($P > 0.05$) (Fig. 9e).
observed a reduction in replication and protein expression of the m6A-mutated rgRSVs in ALKBH5 overexpressing cells, but the level of reduction was much less compared to the parental rgRSV. For example, only 17% and 10% reduction in RSV G and F protein synthesis was observed for rgRSV-G12, and 50% and 20% reduction in G and F protein was observed for rgRSV-G1, respectively. We also tested the replication of rgRSV-G123 in m6A writer protein-depleted A549 cells. Knockdown of MELL3 and METTL14 led to 32 and 22% reduction in RSV G and F protein in rgRSV-infected A549 cells whereas only 25% and 8% reduction in G and F in rgRSV-G123-infected A549 cells (Fig. 10b). Thus, these results showed that replication and gene expression of m6A-mutated rgRSVs were less dependent on host m6A machinery, suggesting that the attenuated phenotype of these mutants is likely due to the deficiency in m6A methylation of the viral RNA.

Fig. 9 Pathogenicity and immunogenicity of m6A-mutated rgRSVs in cotton rats. a RSV titer in lungs. Four-week-old SPF cotton rats were inoculated intranasally with 2.0 × 10^5 TCID_{50} of each rgRSV. At day 4 post-infection, the cotton rats were killed, and lungs and nasal turbinates were collected for virus titration by TCID_{50} assay. Viral titers are the geometric mean titer (GMT) of 5 animals ± standard deviation. Detection limit is 2.0 log TCID_{50}/g tissue. The P value (Student’s t-test) for rgRSV-G1, rgRSV-G3, and rgRSV-G12 is **P = 9.99 × 10^{-6}, ****P = 4.06 × 10^{-5}, and ****P = 7.89 × 10^{-7} respectively. b RSV titer in nasal turbinates. The P value (Student’s t-test) for rgRSV-G1, rgRSV-G2, rgRSV-G3, and rgRSV-G12 is ****P = 0.000740, *P = 0.0460, **P = 0.00623, and ****P = 2.88 × 10^{-7} respectively. c m6A mutated rgRSVs had less lung histopathological changes compared to rgRSV. Representative pathological changes from each group are shown. Right lung lobe of each cotton rat was fixed in 4% neutral buffered formaldehyde, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin (HE) for the examination of histological changes by light microscopy. Micrographs with ×20 magnification (scale bar of 500 µm) are shown. d m6A mutated rsgRSV provides complete protection against RSV challenge. Four-week-old SPF cotton rats were inoculated intranasally with 2.0 × 10^5 TCID_{50} of each rgRSV. At week 4 post-immunization, cotton rats were challenged with 2.0 × 10^5 TCID_{50} rgRSV. At day 4 post-challenge, the cotton rats were killed, and lungs and nasal turbinates were collected for virus titration by TCID_{50} assay. Viral titers are the geometric mean titer (GMT) of 5 animals ± standard deviation. The detection limit is 2.0 log TCID_{50}/g tissue. e rgRSV induced a high level of neutralizing antibody. Blood samples were collected from each rat weekly by retro-orbital bleeding. The RSV-neutralizing antibody titer was determined using a plaque reduction neutralization assay, as described in Materials and Methods. The P value (Student’s t-test) for rgRSV-G1 at week 3 and 4 is *P = 0.0166 and *P = 0.0490, respectively.

m6A-mutated rgRSVs are deficient in m6A enrichment. To determine whether m6A sites are indeed missing from the G gene, A549 cells were infected by each m6A-mutated rgRSV, and polyadenylated mRNAs were isolated and subjected to m6A-seq. As shown in Fig. 10c and Supplementary Fig. 16, the enrichment of m6A in the G mRNA of each m6A-mutated rgRSV significantly decreased compared to the G mRNA from the parental rgRSV, confirming that m6A methylation in the G mRNA has indeed been significantly reduced. Our mutagenesis targeted the positive-sense RNA which includes both antigenome and G mRNA. To determine whether antigenome is also defective in m6A methylation, virion RNA was extracted from highly purified virions of rgRSV, rgRSV-G12, rgRSV-G123, and rgRSV-AG (rgRSV with deletion of entire G gene), and m6A content in total RNA was measured. As shown in Fig. 10d, m6A content in virion RNA of rgRSV-G12, rgRSV-G123, and rgRSV-AG was significantly less compared to those in rgRSV (P < 0.05). In addition, there was no significant difference in m6A content between rgRSV-G123, and rgRSV-AG (P > 0.05). Collectively, these results demonstrate that m6A-mutated rgRSVs indeed have defects in m6A methylation.

Discussion
The biological function of m6A methylation in viral RNAs has remained uncertain since its discovery 40 years ago. Recent studies have shown that viral m6A methylation can have pro-viral or antiviral functions dependent on the specific virus species and cell type. Here, we showed that m6A modification positively regulates each step in the RSV replication cycle ranging from genome replication, mRNA transcription, and viral protein synthesis, to progeny infectious particle production. We demonstrated that m6A regulates RSV replication and pathogenesis in an animal model. Furthermore, we provide evidence that m6A could be a target for the development of live attenuated vaccine candidates.

The m6A methylation of RNAs is modulated by writers, erasers, and readers in host cells. It should be noted that m6A
methylation and its reader proteins may play distinct roles in a virus life cycle. In this study, we showed that overexpression of both m6A reader and writer proteins positively regulated RSV replication while knockdown inhibited RSV gene expression and replication. The opposite was true for eraser proteins: overexpression decreased RSV gene expression and replication whereas knockdown increased them. In case of influenza virus, m6A methylation enhanced viral replication as did the reader, YTHDF2, but not YTHDF1 or 3. We found that overexpression of m6A writer proteins led to an increased level of m6A in viral RNA. The impact of m6A-related proteins on RSV replication may result from three aspects: a change in m6A levels in viral RNA which directly affects viral replication, a change in host gene expression which indirectly affect viral replication, or both. However, m6A writer proteins in the HIV life cycle is controversial. Thus, m6A readers have distinct effects on the life cycles of different viruses, as they are multifunctional and play many important biological
roles ranging from RNA stability, decay, and transport, to protein translation.

Our m6A-seq also found that the viral G mRNA has the most abundant m6A peaks among the 10 RSV mRNAs in both HeLa and A549 cells. In addition, the strongest m6A peaks in both the genome and the antigenome are located in the G gene region. Another interesting finding was that the positions of the m6A modifications in the genome and antigenome largely overlapped despite the fact that the sequence of the antigenome is complementary to the genome. However, it is unclear why antigenome and genome are m6A methylated because both of them are completely encapsidated by viral N protein. It was shown that RSV RNA synthesis occurs in cytoplasmic inclusion bodies.

In fact, m6A-related proteins are partially co-localized with viral N protein (Fig. 6a, b), suggesting that host m6A machinery may interact with RSV ribonucleoprotein (RNP) complex. It is possible that m6A methylation occurs prior to N encapsidation, or m6A methylation and encapsidation can occur concurrently.

Since G mRNA has the strongest m6A enrichment, we searched the three peaks in the G sequence for m6A motifs, identifying a total of 18 putative m6A sites. It is known that the G gene is the most genetically diverse RSV gene. However, bioinformatics analysis of 10 RSV strains (Supplementary Fig. 17) found that those 18 m6A sites are highly conserved in the G gene, suggesting that m6A sites in the G gene may provide an evolutionary advantage for virus infection, replication, and spreading. Mutations in these three m6A peaks in the G mRNA showed that peaks 1 and 3 play a major role in regulating RSV replication whereas peak 2 plays a minor role, as recombinant rgRSV mutants in peak 1 and 3 (rgRSV-G1 and G3) had greater deficits in replication compared to mutants in peak 2 (rgRSV-G2).

The G protein is primarily responsible for the attachment of RSV to host cells and plays a role in modulating innate immune responses. Although it is not essential for the production of infectious RSV, RSV G is necessary for full infectivity. The G protein also plays an important role in the assembly of filamentous virions. It is likely that the abundant m6A modifications of the G mRNA enhances its stability, enabling more translation, which may lead to insertion of more G protein into virions and enhanced viral assembly and production of infectious virions. However, a portion of the G protein produced in a cell is released in a soluble form that affects leukocyte migration. Enhanced G protein expression could enhance the production of soluble G protein, thereby affecting the immune response to RSV. It is also possible that m6A modification of viral RNAs facilitates virus escape from the surveillance of host innate immunity to allow for efficient gene expression and virus replication. In this study, we also found that rgRSVs carrying mutations in the m6A sites in G not only decreased G protein expression but also reduced expression of other viral proteins (such as N and F proteins). Since RSV replication and transcription require ongoing protein synthesis, a reduction in viral G protein expression will affect the second round of viral infection, which results in a reduction of overall replication and transcription, and expression of all viral proteins.

Importantly, we found that viral m6A also modulates viral replication and pathogenesis in vivo. However, the degree of attenuation in cell culture did not always match that in vivo. For example, rgRSV-G1 and G12 had similar levels of attenuation in immortalized cells (A549 and HeLa cells). In cotton rats, rgRSV-G1 replication was 7-fold reduced in the lung and nasal turbinate, respectively, whereas rgRSV-G12 had more than 100-fold reductions. Recombinant rgRSV-G2 was only mildly attenuated in cell culture. This recombinant had similar level of replication in lungs (P > 0.05), and only had threefold reduction in nasal turbinates (P < 0.05). Therefore, it appears that m6A sites in peaks 1 and 2 contributed synergistically to the highly attenuated phenotype of rgRSV-G12 in vivo. The phenotype of these mutants in HAE culture seems to correlate better with the phenotype in cotton rats than in HeLa and A549 cells. Thus, HAE culture may be better system to predict virus replication in vivo. Also, m6A-mutated rgRSVs had significantly less histopathology compared to the parental rgRSV. These results demonstrate that m6A not only modulates the virus life cycle in vitro but also regulates viral replication and pathogenesis in vivo.

Recent studies in vesicular stomatitis virus (VSV) found that codon usage also affected genome replication and mRNA transcription in a minigenome assay. Specifically, purine/pyrimidine content in RNA template modulates the stability of the polymerase complex, which results in alteration of the activity of viral RNA synthesis. In this study, we designed mutations in predicted m6A sites to avoid as much as possible alterations to the predicted mRNA secondary structure and to avoid changes in the efficiency of gene expression of the new codon relative to the original codon. We also confirmed the loss of m6A in the predicted region of the G mRNA by m6A sequencing and tested the functional consequences of reducing the m6A modifications. Functional loss of m6A modifications was examined by comparing replication of the mutant rgRSV in A549 cells overexpressing or depleted of m6A-related proteins. The m6A-mutated rgRSVs (G1, G12, and G123) were much less dependent on host m6A enzyme compared to the parental rgRSV, confirming that the attenuated phenotype of m6A-mutated rgRSVs is due to the reduction of m6A sites in G mRNA. Removal of m6A sites in the mRNA also removes them from the antigenome, but not from other viral mRNAs, other locations in the antigenome, or sites in the genome. Therefore, rgRSVs lacking particular m6A peaks in the G gene would be partially but not fully independent of host m6A enzymes.

A potentially important application of this study is in the rational design of live attenuated RSV vaccine candidates by inhibiting m6A addition to the mRNA and antigenome, or perhaps the viral genome. Currently, there is no FDA-approved vaccine for RSV despite the fact that it was first isolated in 1953. A live attenuated vaccine, similar to the effective vaccines for the related measles and mumps viruses, would seem to be one of the most promising methods for protection from RSV disease. However, it has been a challenge to strike the right balance between attenuation and immunogenicity.

Although mutations in individual m6A peaks in the G mRNA were not sufficient to achieve complete attenuation of RSV replication in vivo, the combination of m6A mutations in peaks 1 and 2 resulted in a recombinant virus that was sufficiently attenuated both in vitro and in vivo. Importantly, cotton rats vaccinated with rgRSV-G12 had similar neutralizing antibody response levels compared to parental rgRSV and were completely protected from rgRSV challenge. In addition, no enhanced lung damage was observed. Thus, rgRSV-G12 may be a good live attenuated vaccine candidate for RSV. This study demonstrates that inhibition of m6A methylation may be a novel method for rationally designing live attenuated vaccines. These m6A-mutated rgRSVs would also provide invaluable tools to understand the roles of m6A modification in the innate immune response. In fact, it has been shown that internal m6A modification of in vitro synthesized RNAs prevents recognition of the RNA by the host pattern recognition receptors TLR3 and RIG-I.

This study also provides a novel approach for enhancing viral titers in cell culture, an important consideration in the production of live attenuated vaccines. Attenuated viruses typically grow to lower titers than wild-type virus. In the case of RSV, a relatively large dose of vaccine candidate is required to induce a protective immune response in humans, making vaccine production
One strategy might be to produce live attenuated vaccines in cells overexpressing one or more m^6^A reader or writer proteins, since overexpression of these host m^6^A machinery components enhances virus yield at least 10-fold. Such a boost in the production of a vaccine should greatly enhance its economic feasibility. Unfortunately, this approach would not be useful for m^6^A mutant attenuated viruses, such as the ones described in this report. It should be useful for viruses attenuated by any other method.

In summary, we mapped the internal m^6^A modifications in RSV RNAs and showed that m^6^A enhances RSV replication, gene expression, and virus production. In addition, we provide evidence that m^6^A upregulates RSV pathogenesis and virulence in vivo. These findings highlight viral m^6^A machinery as a possible novel target for rational design of live attenuated vaccines, for enhanced production of live attenuated vaccines, and for broad-spectrum antiviral drug discovery.

Method

Ethics statement

The animal study was conducted in strict accordance with USDA regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council and was approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC; animal protocol no. 2009A00211). The animals were housed within the University Laboratory Animal Resources (ULAR) facilities of The Ohio State University according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). The animal care facilities at The Ohio State University are AAALAC accredited. Every effort was made to minimize potential distress, pain, or discomfort to the animals throughout all experiments.

Cell lines.

HeLa (ATCC CCL-2), A549 (ATCC CCL-185), Vero (ATCC CRL-CCL81), and HEP-2 (ATCC CCL-23) cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% FBS. HeLa cells overexpressing the empty vector (pPB-CAG), YTHDF1, YTHDF2, or YTHDF3 were maintained in DMEM, 10% FBS and 1 µg/ml of puromycin every passage to select for YTHDF1-3 overexpressing cells. Primary, well-differentiated human airway epithelial (HAE) cultures were grown on collagen coated Transwell inserts (Corning Incorporated, Corning, NY) at an air-liquid interface, as previously described.

Upon reaching confluence and forming tight junctions, the apical medium was removed and cultures were maintained at the air–liquid interface for 4–6 weeks to generate well-differentiated, polarized cultures. All cell lines used in this study were free of mycoplasma, as confirmed by the LookOut Mycoplasma PCR Detection Kit (Sigma).

Virus stocks and purification.

Recombinant RSV containing a green fluorescence protein (GFP) gene between the leader sequence and NS1 gene (rgRSV) was propagated and titrated in HeLa cells or A549 cells. To prepare purified rgRSV, 20 T150 flask cultures of HeLa cells or A549 cells were infected by rgRSV at an MOI of 0.1, and cell culture supernatants harvested at 72 h post-infection were clarified by centrifugation at 10,000 × g for 30 min. Virus was concentrated through a 35% (wt/vol) sucrose cushion by centrifugation at 30,000 × g for 2 h at 4 °C in a Ty 50.2 rotor (Beckman). The pellet was resuspended in DMEM with 10% trehalose and further purified through a sucrose gradient (20–55%) by centrifugation at 35,000 × g for 2 h at 4 °C in an SW55 rotor (Beckman). The final pelleted was resuspended in 0.5 ml of DMEM with 10% trehalose.

m^6^A-seq.

High-throughput sequencing of the RSV and host methylome was carried out using m^6^A-seq (MeRP-seq) as described previously.

For m^6^A-seq of the rgRSV genome and antigenome, RNAs were extracted from purified rgRSV virions and purified with the RibonMinus Eukaryote System v2 kit (Thermo Fisher). For m^6^A-seq of host transcripts, total RNAs were extracted from mock or rgRSV-infected HeLa or A549 cells and polyadenylated RNAs were isolated using Dynabeads mRNA DIRECT Purification kit (Thermo Fisher). Purified RNAs were sonicated with Bioruptor Pico (Diagenode) with 30× ON 30× OFF for 30 cycles, mixed with 1 µl of affinity purified anti-m^6^A monoclonal antibody (NEP) in IPP buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.4) and incubated for 2 h at 4°C. Enriched mRNA fragments were purified with RNA Clean & Concentrator kit (Zymo) and used for library generation with TrueSeq Stranded mRNA Library Prep kit (Illumina). Sequencing was carried out on Illumina HiSeq 4000 according to the manufacturer’s instructions. Two replicates of RNA samples from virions, virus-infected cells, and mock-infected cells were subjected to m^6^A-seq.

For data analysis, reads mapping to the adaptors sequence were removed by mapping of reads to human genome (hg38) and rgRSV genome and antigenome by using Hisat2 and peak calling as described.

Metagene analysis was performed by R package

Guitar.

Differential methylation analysis was performed with count based negative binomial model implemented in qnb test.

Quantification of RSV mRNA m^6^A level using LC-MS/MS.

RV RNA (250 mg) was extracted from highly purified rgRSV virions using an RNeasy Mini kit (Qiagen) and purified twice with RibonMinus Eukaryote System v2 kit (Thermo Fisher). To examine the purity of virion RNA, oligo d(T) was used for reverse transcription, followed by qPCR for quantification for β-actin and viral N and G RNAs. Virion RNA which was free of contamination of host RNA and viral mRNAs was used for liquid chromatography-mass spectrometry (LC-MS/MS). m^6^A antibody pulldown assay, and m^6^A-seq. Purified RNA was digested and subjected to a quantitative analysis of the m^6^A level using LC-MS/MS as previously described.

Quantification of RSV RNA m^6^A level using anti-m^6^A antibody.

A549 cells in T75 flasks were transfected with 10 µg of plasmids encoding METTL3 and METTL14, ALKBH5, or vector pCAGGS. For siRNA transfection, A549 cells in T75 flasks were transfected with 150 pmole of siRNA targeting METTL3 and METTL14, ALKBH5, or control siRNA. At 24 h post-transfection, the transfected cells were infected with rgRSV at an MOI of 0.1. At 42 h post-infection, cell culture supernatants (containing RSV particles) were harvested. RSV particles were pelleted and purified through ultra centrifugation. Virion RNA was extracted from purified RSV virions. Antigenome was quantified by real-time RT-PCR. Each amount of antigenome was bound to strip wells using a RNA high binding solution, and m^6^A was detected using a specific capture anti-m^6^A antibody (Abcam ab158912) and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. A standard curve was generated using known m^6^A methylated RNA (range from 0.02 to 1 mg of m^6^A) as a positive control. The m^6^A content was calculated from each RNA samples based on their OD450 values. The percent of changes was calculated by dividing m^6^A contents in viral RNA from the treated group by those from the control group.

Host cell gene differential expression analysis.

Host cell differential gene expression analysis was performed using R package DESeq2 by using Wald-test. The significantly differentially expressed genes were reported at adjusted P value cutoff of 0.05.

GO analysis.

Gene Ontogeny (GO) analysis was performed using the R package clusterProfiler. Specifically, enrichKEGG pathway was called for analysis for enriched pathway and enrichMap function was called to generate network plot of enriched pathway.

Plasmids and site-directed mutagenesis.

The plasmid vector was used to overexpress the readers (YTHDF1, METTL3, METTL14), and erasers (FTO, ALKBH5) as described previously.

Plasmid (RW30) encoding the full-length antigenomic cDNA of RSV strain A2 with GFP inserted between the leader sequence and NS1 gene was propagated and titered in HeLa cells or A549 cells. To prepare purified RSV, A549 cells were infected with rgRSV at an MOI of 0.1. At 42 h post-infection, cell culture supernatants (containing RSV particles) were harvested. RSV particles were pelleted and purified through ultra centrifugation.

Lipofectamine 3000 transfection reagent (Thermo-Fisher) according to the manufacturer’s instructions.

Antibodies and Western blotting.

The antibodies used in this study were anti-YTHDF1 (1:1000, Proteintech, Rosemont, IL), anti-YTHDF2 (1:1000, Abcam, and

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-12504-y | www.nature.com/naturecommunications

15
RNA-immunoprecipitation was amplification and sequenced. At passage 15, the entire genome of each recombinant virus was centrifugation at 1500 × g. Supernatant and cells were harvested by three freeze-thaw cycles, followed by DMEM. Fresh DMEM (supplemented with 2% FBS) was added, and the infected HEp-2 cells were infected with wild-type rgRSV or mutant rgRSV at an MOI of 0.1. After 1 h of incubation, the cell culture supernatant was harvested and used for the next passage in Vero cells. Using this method, each rgRSV mutant was repeatedly inoculated into 6 groups (5 cotton rats per group). Prior to virus inoculation, the cotton rats were anesthetized with isoflurane. The cotton rats in group 1 were inoculated with 2.0 × 10^5 TCID50 of parental rgRSV and served as positive controls. The cotton rats in groups 2–5 were inoculated with 2.0 × 10^5 TCID50 of four m6A deficient rgRSV mutants, rgRSV-G1, G2, G3, and G12. Each cotton rat was intranasally inoculated intranasally with a volume of 100 µl. At day 4 post-infection, the cotton rats were killed via carbon dioxide inhalation. The left lung and nasal turbinates were collected for virus titration and the right lung was collected for histological analysis.

Immunogenicity of rgRSV in cotton rats. For the immunogenicity study, twenty 6-week-old female cotton rats (Envigo) were randomly divided into five groups (5 cotton rats per group). Prior to virus inoculation, the cotton rats were evaluated daily for any possible abnormal reaction and blood samples were collected from each cotton rat weekly by facial vein retro-orbital plexus sampling, and serum was used for detection of neutralizing antibodies. At 4 weeks post-inoculation, the cotton rats in groups 2–5 were challenged with 2.0 × 10^6 TCID50 of parental rgRSV via intranasal route, and evaluated twice daily for the presence of any clinical symptoms. At 4 days post-challenge, all cotton rats were euthanized by CO2 asphyxiation, and their lungs and nasal turbinates were collected for virus titration. The immunogenicity of rgRSV mutants was assessed based on their ability to trigger neutralizing antibody, the ability to prevent rgRSV replication in lungs and nose, and the ability to protect lung from pathological changes.

Pulmonary histology. After killing, the right lung of each animal was removed, inflated, and fixed with 4% neutral buffered formaldehyde. Fixed tissues were embedded in paraffin and a microtome used to generate 5 µm sections. Slides were then stained with hematoxylin-eosin (H&E) for the examination of histological changes by light microscopy. Histopathological changes were evaluated based on the extent of interstitial inflammation, edema, and peribronchial inflammation.

Determination of viral titer in lung and nasal turbinate. The nasal turbinate and the left lung from each cotton rat were removed, weighed, and homogenized in either 3 ml or 2 ml of DMEM. The lung was homogenized using a Precellys 24 tissue homogenizer (Bertin, MD) by following the manufacturer’s recommendations. The nasal turbinates were homogenized by hand with a 15 ml capacity Pycnex® homogenizer (Corning, NY). The presence of infectious virus was determined by TCID50 assay in HEp-2 cells.

Determination of RSV-neutralizing antibody. RSV-specific neutralizing antibody titers were determined using a plaque reduction neutralization assay. In brief, cotton rat sera were collected by retro-orbital plexus sampling weekly until challenge. The serum samples were heat inactivated at 56°C for 30 min. Twofold dilutions of the serum samples were mixed with an equal volume of DMEM containing ~50 TCID50/well rgRSV in a 96-well plate, and the plate was incubated at room temperature for 1 h with constant rotation. The mixtures were transferred to confluent HEp-2 cells in a 96-well plate in triplicate. After 1 h of incubation at 37°C, the virus-serum mixtures were removed and the cells were overlaid with 0.75% methylcellulose in overlay media (1 x MEM, 2% FBS, Sodium bicarbonate, 25 mM HEPES, 1% soybean trypsin, 1% Pen Strep) and incubated for an additional 3 days before counting the fluorescent foci. The numbers of foci at each serum dilution were plotted and the 50% plaque reduction titer was used as the RSV-specific neutralizing antibody titer.

Statistical analysis. Quantitative analysis was performed by either densitometric scanning of autoradiographs or by using a phosphorimager (Typhoon; GE Healthcare, Piscataway, NJ), ImageQuant TL software (GE Healthcare, Piscataway, NJ), and Image J (NIH, Bethesda, MD). Statistical analysis was performed by one-way multiple comparisons using SPSS (version 8.0) statistical analysis software (SPSS Inc., Chicago, IL) or Student’s t-test. A P value of <0.05 was considered statistically significant.

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

Data availability

The authors declare that the data supporting the findings of this study are available with the article and its Supplementary Information files, or are available from the corresponding author upon request. The accession number for the sequencing data obtained from the MeRIP-seq reported in this paper is GEO: GSE125803 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi]. The source data figures and Supplementary Figures are provided as a Source Data file.
Acknowledgements
This study was supported by NIH grant R01 AI090060 to J.L., and P01 AI112524 to M.E.P., S.N., and J.L., and R01 HG008688 and RM1 HG008935 to C.H. We thank Peter Collins for the infectious cDNA clone of RSV. We are grateful to members of the J. Li laboratory for critically reading the manuscript.

Author contributions
M.X., C.H., and J.L. conceived the study. M.X., B.Z., Z.Z., M.L., O.H., P.C., Z.L., A.I., Y.M., Y.X., X.L., J.Z., S.N., M.E.P., C.H., and J.L. performed experiments and data analysis. M.X., B.Z., Z.Z., J.Z., S.N., M.E.P., C.H., and J.L. designed the experiments and interpreted the results. M.X., B.Z., Z.Z., M.E.P., C.H., and J.L. wrote the manuscript, and all other authors edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12504-y.

Correspondence and requests for materials should be addressed to J.L.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work.