Cellular messenger RNA (mRNA) of higher eukaryotes and many viral RNAs are methylated at the N-7 and 2'-O positions of the 5'-guanosine cap by specific nuclear and cytoplasmic methyltransferases (MTases), respectively. Whereas N-7 methylation is essential for RNA translation and stability, the function of 2'-O methylation has remained uncertain since its discovery 35 years ago. Here we show that a West Nile virus (WNV) mutant (E218A) that lacks 2'-O MTase activity was attenuated in wild-type primary cells and mice but was pathogenic in the absence of type I interferon (IFN) signalling. 2'-O methylation of viral RNA did not affect IFN induction in WNV-infected fibroblasts but instead modulated the antiviral effects of IFN-induced proteins with tricopeptide repeats (IFIT), which are interferon-stimulated genes (ISGs) implicated in regulation of protein translation. Poxvirus and coronavirus mutants that lacked 2'-O MTase activity similarly showed enhanced sensitivity to the antiviral actions of IFN and, specifically, IFIT proteins. Our results demonstrate that the 2'-O methylation of the 5' cap of viral RNA functions to subvert innate host antiviral responses through escape of IFIT-mediated suppression, and suggest an evolutionary explanation for 2'-O methylation of cellular mRNA: to distinguish self from non-self RNA. Differential methylation of cytoplasmic RNA probably serves as an example for pattern recognition and propagation of foreign viral RNA in host cells.

Most eukaryotic mRNA contains a 5' Cap 0 (7mGpppN) structure with a methyl group at the N-7 position. In higher eukaryotes, methylation of cellular mRNA occurs additionally at the 2'-O site of the penultimate (7mGpppNm, Cap 1) and antepenultimate (7mGpppNmNm, Cap 2) nucleotides in the nucleus and cytoplasm, respectively. Many viral mRNAs also contain Cap 1 and 2 structures, but cap acquisition occurs distinctly among virus families. RNA and DNA viruses that replicate in the cytoplasm cannot use the host nuclear capping machinery, and thus have evolved MTases to facilitate N-7 and 2'-O methylation of viral mRNA cap to subvert innate host sensing or IFN induction. To address whether 2'-O methylation affects pathogen sensing or IFN induction, we hypothesized that it might directly limit IFN induction by affecting the avidity of viral RNA for the host sensor, RIG-I. However, direct binding assays with recombinant RIG-I and 2'-O unmethylated or methylated WNV RNA (5' untranslated region) showed no change in binding (Supplementary Fig. 4). It remained possible that 2'-O methylation of WNV RNA affected other proteins required for transcriptional activation of the IFN-β gene. To evaluate this idea, Ifnar1−/− MEFs, which produce IFN-β without responding to it, were infected at a high multiplicity of infection (MOI) and IFN-β mRNA was measured. Notably, both WNV-WT and WNV-E218A stimulated IFN-β transcription equivalently after infection (Fig. 2a). Thus a lack of 2'-O methylation does not affect pathogen sensing or IFN induction. To address whether 2'-O methylation of viral RNA serves to antagonize or evade IFN effector functions, IPS-1−/− MEFs, which do not produce type I IFN after WNV infection but can respond to it, were exposed to IFN-β to

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induce ISGs, and then infected. WNV-E218A displayed increased sensitivity to IFN-β pretreatment compared with WNV-WT (2,400,000- and 20,000-fold inhibition in 500 international units ml−1 of IFN-β, respectively) (Fig. 2b).

IFN induces hundreds of ISGs, some of which may have antiviral effectors functions. Among these, Ifit family members (for example, Ifit1 and Ifit2 (also known as ISG56 and ISG54, respectively)) are induced after WNV infection, reduced in Ifit1/−/ mice, viral RNA alters the sensitivity of WNV to 2′-O methylation. WNV replication in 2T3 MEFs expressing a murine Ifit1 or Ifit2 transgene. As observed in primary cells, WNV-E218A replication in control 2T3 cells was reduced (~5- to 60-fold decrease at 24-72 h, P < 0.05, n = 3) compared with WNV-WT, confirming that 2′-O methylation is required for optimal infectivity (Fig. 3a). Transgenic expression of IFIT-2 reduced infection of WNV-WT (~56- to 100-fold decrease at 24-72 h, P < 0.0005, n = 3) (Fig. 3b) compared with replication in 2T3–green fluorescent protein (GFP) cells. In comparison, expression of IFIT-2

Figure 2 | 2′-O methylation of viral RNA alters the sensitivity of WNV to the antiviral effects of IFN. a, IFN-β gene induction in Ifnar1−/− MEF after WNV-WT or WNV-E218A infection. Results are representative of three independent experiments performed in duplicate. b, Viral replication in IPS1−/− MEF after IFN-β pretreatment. The data are the average of two independent experiments performed in triplicate, and the asterisks indicate differences that are statistically significant (**P < 0.0001; ***P < 0.005; **P < 0.05). Error bars, s.d., IU, international units.

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Figure 1 | WNV-E218A is attenuated in wild-type mice and cells but is virulent in Ifnar1−/− mice and cells. a, Survival curves of wild-type and Ifnar1−/− C57BL/6 mice after subcutaneous infection with WNV-WT or WNV-E218A. b, Virus replication in wild-type mice in blood (day 4), spleen (day 4) or brain (day 8) after subcutaneous infection with WNV-WT or WNV-E218A. c, Survival curves of wild-type mice after intracranial infection with WNV-WT (105) or WNV-E218A (105 plaque-forming units (PFU)). d, Viral burden in the serum, spleen, kidney, spinal cord and brain from Ifnar1−/− mice at day 3 after infection. e, f, Replication of WNV-WT and WNV-E218A in wild-type or Ifnar1−/− MEFs (e) or Mφ (f). Results are the average of three experiments performed in triplicate. Error bars, s.d.; dashed line, limit of sensitivity of the assay.
IFIT-2 is an antiviral effector of IFN actions, whose inhibitory activity was assessed by quantitative reverse transcriptase PCR. The data are the average of three experiments performed in duplicate. Top, knockdown of IFIT-2 enhanced replication of WNV-E218A. 3T3 cells were transfected with a non-target (NT) or IFIT-2 siRNA and then infected with WNV-E218A. One day post-infection cells were collected and (top) viral RNA was assayed by quantitative reverse transcriptase PCR. The data are the average of three experiments performed in duplicate. Bottom, knockdown of IFIT-2 protein was confirmed by western blot. e, f, Murine IFIT-2 expression prevents accumulation of negative- and positive-strand viral RNA in WNV-E218A-infected cells. g, Replication of WNV-E218A is attenuated in wild-type and Ifit2<sup>−/−</sup> Mφ but restored in Ifit1<sup>−/−</sup> cells. h, Survival curves of wild-type or Ifit1<sup>−/−</sup> mice after intracranial challenge with 10<sup>5</sup> plaque-forming units of WNV-WT or WNV-E218A. Error bars, s.d.; dashed line, limit of sensitivity of the assay.

As other virus families encode 2′-O MTases, we sought to determine if 2′-O-methylation-dependent evasion of IFIT proteins functions as a more general immune escape mechanism. We obtained a vaccinia virus (VACV) mutant (J3-K175R) that lacked 2′-O MTase activity, replicated normally in BSC40 cells<sup>22</sup> but was attenuated in wild-type Mφ (approximately six- to eightfold reduction at 24–72 h) and fully rescued in Ifnar1<sup>−/−</sup> Mφ (Fig. 4a). Growth curves with VACV-WT and VACV-J3-K175R in 3T3 cells expressing GFP or ISG20 confirmed an essential role of 2′-O methylation in poxvirus infection (approximately three- to fivefold reduction at 24–72 h, P < 0.005, n = 3) (Fig. 4b). Transgenic expression of IFIT-2, however, did not affect replication of VACV-WT (P > 0.5, n = 3), which suggests that IFIT-2 lacks activity against VACV-WT or that the virus efficiently antagonizes its antiviral effect. Expression of mouse IFIT-2 but not IFIT-1 further reduced infection of VACV-J3-K175R (6- to 25-fold decrease, P < 0.01, n = 3) (Fig. 4c, d). Consistent with these findings, wild-type C57BL/6 mice were resistant to lethal challenge with VACV-J3-K175R (0% lethality, n = 6) but sensitive to infection with VACV-WT (100% lethality, n = 13). In contrast, in Ifnar1<sup>−/−</sup> mice, VACV-J3-K175R was virulent as all animals succumbed to infection with similar kinetics compared with those infected with VACV-WT (Supplementary Fig. 6).

We examined the replication of a wild type and 2′-O MTase mutant (D130A in the nsp16 protein)<sup>23</sup> of mouse hepatitis virus (MHV). MHV-D130A was more sensitive to the effects of IFN-β pretreatment virtually abolished replication of WNV-E218A (up to 2,700-fold decrease at 72 h, P < 0.0005, n = 3) (Fig. 3b). Expression of IFIT-1 in 3T3 cells had minimal inhibitory effects on WNV infection (Fig. 3c). To confirm the linkage between IFIT-2 expression and restriction of infection, short interfering RNA (siRNA) knockdown experiments were performed. Transfection of a sequence-specific siRNA that reduced protein expression of IFIT-2 enhanced replication of WNV-E218A (P < 0.01, n = 3) (Fig. 3d). These experiments demonstrate that mouse IFIT-2 is an antiviral effector of IFN actions, whose inhibitory activity is minimally 2′-O methylation of viral RNA.

Although IFIT family orthologues exist over a broad evolutionary time-frame<sup>21</sup>, humans have a distinct complement of Ifit genes (Ifit1 (ISG56), Ifit2 (ISG54), Ifit3 (ISG60) and Ifit5 (ISG58)). Transient transgenic expression of human IFIT-5 but not IFIT-1, IFIT-2 or IFIT-3 in human 293T cells inhibited infection of WNV-E218A (P = 0.003, n = 3) (Supplementary Fig. 6), which suggests a species-specificity of Ifit genes in restricting WNV lacking 2′-O methylated RNA.

We assessed the stage of the WNV life cycle that was restricted by mouse IFIT-2. Using strand-specific quantitative reverse transcriptase PCR to quantify genomic (positive strand) and replicative intermediate (negative strand) viral RNA, we found that in control 3T3 cells each increased by 18 h after infection (Fig. 3e, f), whereas the expression of mouse IFIT-2 delayed production of both by approximately 15 h in the context of WNV-WT infection. In comparison, increases in negative and positive strand RNA were abolished in IFIT-2 transgenic cells infected with WNV-E218A. The levels of WNV-E218A-positive-strand RNA remained essentially constant over the time course, suggesting that the lack of 2′-O methylation did not affect viral RNA stability. Thus mouse IFIT-2 blocks infection of the E218A mutant in fibroblasts at or before negative-strand synthesis.

**Figure 3** | WNV-E218A is more sensitive to the antiviral actions of Ifit genes. a–c, Viral replication of WNV-WT or WNV-E218A in 3T3 MEFs transiently expressing GFP (a–c), ISG20 (a), IFIT-2 (b) or IFIT-1 (c). The data are the average of three experiments performed in duplicate. d, siRNA knockdown of IFIT-2 enhances replication of WNV-E218A. 3T3 cells were transfected with a non-target (NT) or IFIT-2 siRNA and then infected with WNV-E218A. One day post-infection cells were collected and (top) viral RNA was assayed by quantitative reverse transcriptase PCR. The data are the average of three experiments performed in duplicate. Bottom, knockdown of IFIT-2 protein was confirmed by western blot. e, f, Murine IFIT-2 expression prevents accumulation of negative- and positive-strand viral RNA in WNV-E218A-infected cells. g, Replication of WNV-E218A is attenuated in wild-type and Ifit2<sup>−/−</sup> Mφ but restored in Ifit1<sup>−/−</sup> cells. h, Survival curves of wild-type or Ifit1<sup>−/−</sup> mice after intracranial challenge with 10<sup>5</sup> plaque-forming units of WNV-WT or WNV-E218A. Error bars, s.d.; dashed line, limit of sensitivity of the assay.

Consistent with these findings, wild-type C57BL/6 mice were resistant to lethal challenge with VACV-J3-K175R (0% lethality, n = 6) but sensitive to infection with VACV-WT (100% lethality, n = 13). In contrast, in Ifnar1<sup>−/−</sup> mice, VACV-J3-K175R was virulent as all animals succumbed to infection with similar kinetics compared with those infected with VACV-WT (Supplementary Fig. 7).

We examined the replication of a wild type and 2′-O MTase mutant (D130A in the nsp16 protein)<sup>23</sup> of mouse hepatitis virus (MHV). MHV-D130A was more sensitive to the effects of IFN-β pretreatment.
METHODS SUMMARY

Viruses. WNV-WT and WNV-E218A were propagated in BHK21 cells as described, VACV-WT and VACV-J3-K175R (a gift from R. Condit) and encephalomyocarditis virus (EMCV) (strain K) were propagated in HEla and I292 cells, respectively. Generation of MHV-WT (strain A59) and MHV-D130A recombinant coronaviruses has been described.

Mouse experiments. C57Bl/6 wild-type and immunodeficient (Ifnar1−/−, Ifit2−/−, Ifit3−/−, Ifr3−/−, Ifr3−/−×If3−/− and IPS-1−/−) mice were bred at Washington University. Infection experiments were performed with approval of the Washington University and St Louis University Animal Studies Committees. Viral titres in blood and organs were quantified as previously described.

Cell culture and viral infection. Bone-marrow derived Mφ and MEF were generated as described, 3T3 fibroblasts expressing GFP or ISG were previously described. Cells were infected with WNV, VACV, MHV or EMCV at MOIs of 0.01, 1, 1 and 0.001, respectively. Lysates or supernatants were titrated by plaque assay on BHK21-15 cells for WNV and EMCV, BSC-1 cells for VACV and I292 cells for MHV.

Quantification of IFN-β mRNA. Ifnar1−/− MEFs were infected at an MOI of 10 with WNV-WT or WNV-E218A. Total RNA was isolated, treated with DNase (Qiagen), and IFN-β mRNAs were amplified by qualitative reverse transcriptase PCR as described previously.

IFN-β pretreatment experiment. IPS-1−/− MEFs were pretreated with increasing doses of mouse IFN-β (PBL Laboratories) for 24 h and then infected with WNV or MHV at an MOI of 0.1. Supernatants were collected at 48 or 12 h after infection, respectively, and titered by plaque assay.

Strand-specific real-time reverse transcriptase PCR. Quantification of positive- and negative-strand WNV RNA was performed using a Taq-tagged primer strategy. Fibroblasts expressing GFP or mouse IFIT-2 were infected with WNV-WT or WNV-E218A at an MOI of 1 and total RNA was collected at indicated time points.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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