LETTER

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2′–*O* methylation of the viral mRNA cap evades host restriction by IFIT family members

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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[1], the function of 2′-*O* methylation has remained uncertain since its discovery 35 years ago[2–4]. 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 tetratricopeptide 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 restriction of 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) 5′ nucleotides in the nucleus and cytoplasm, respectively[3,5]. Many viral mRNAs also contain Cap 1 and 2 structures, but cap acquisition occurs distinctly among virus families[2,6]. 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* capping or mechanisms to 'snatch' the cap from host cell mRNA[1]. It remains unclear how 2′-*O* methylation contributes to viral infection or cellular mRNA homeostasis[2,3].

Flavivirus is a genus of positive-strand RNA viruses with a 5′ Cap 1 structure that is generated by an MTase in the NS5 protein[7]. Whereas mutations abrogating the N-7 MTase activity abort WNV infection, an E218A substitution that completely abolished the 2′-*O* but not N-7 MTase activity (Supplementary Fig. 1) did not affect replication in permissive BHK cells[8]. Although C57BL/6 mice infected subcutaneously with the parental WNV wild-type (WNV-WT) strain had an approximately 40% mortality rate, recipients of WNV-E218A showed 0% mortality, even at high challenge doses (Fig. 1a, $P < 0.05$, $n = 10$) or after direct intracranial infection (Fig. 1c). Levels of WNV-E218A after subcutaneous inoculation were markedly decreased in the spleen, serum or brain compared with infection by WNV-WT (Fig. 1b).

Because dissemination of WNV-E218A was aborted *in vivo*, we assessed whether 2′-*O* methylation restricted the protective IFN-induced immune response. Mice lacking type I IFN signalling (*Ifnar1*$^{-/-}$) that were infected with WNV-WT showed 100% mortality and a mean time to death of 3.5 days, as seen previously[9] (Fig. 1a). Remarkably, *Ifnar1*$^{-/-}$ mice infected with the WNV-E218A exhibited a similar phenotype with only a slightly delayed mean time to death of 4.5 days. *Ifnar1*$^{-/-}$ mice infected with WNV-E218A at day 3 sustained tissue titres that approached those of WNV-WT (Fig. 1d). Thus 2′-*O* methylation of WNV RNA is required for virulence *in vivo*, and its absence renders the virus sensitive to the IFN response.

Analysis of viral growth in primary mouse embryonic fibroblasts (MEFs) and macrophages (Mϕ), which both produce and respond to type I IFN after WNV infection[10], confirmed attenuation of WNV-E218A in wild-type cells (50- and 151-fold lower at 72 h, $P < 0.05$, $n = 3$ in MEF and Mϕ, respectively) and restored growth in *Ifnar1*$^{-/-}$ cells (Fig. 1e, f). Replication of WNV-E218A was also rescued in *Irf3*$^{-/-}$, *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ or *IPS-1*$^{-/-}$ cells that had altered or abolished IFN-α/β responses[11] (Supplementary Figs 2a–c and 3a–d, respectively), but not in *Irf7*$^{-/-}$ or *Tlr3*$^{-/-}$ cells, which have normal IFN-β or IFN-α and -β responses after WNV infection, respectively[10,12] (Supplementary Fig. 2d, e). These experiments confirmed that rescue of WNV-E218A in primary cells requires attenuation of the IFN response.

Because 2′-*O* methylation rendered WNV-WT less susceptible to the IFN response than WNV-E218A, 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[11], were exposed to IFN-β to

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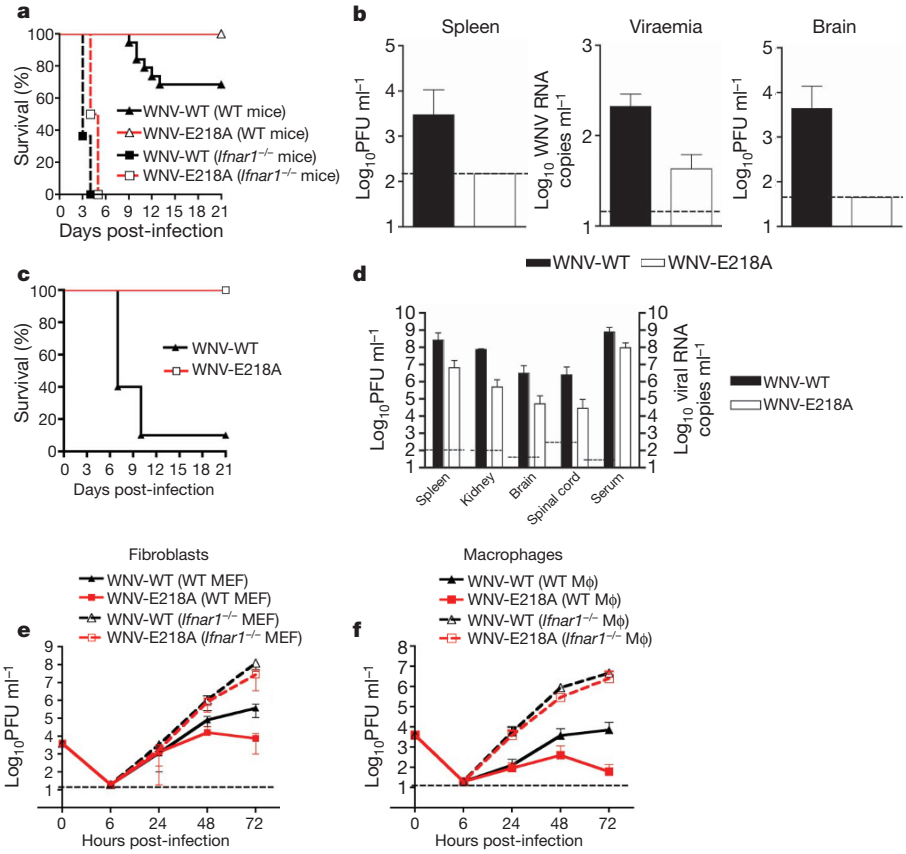
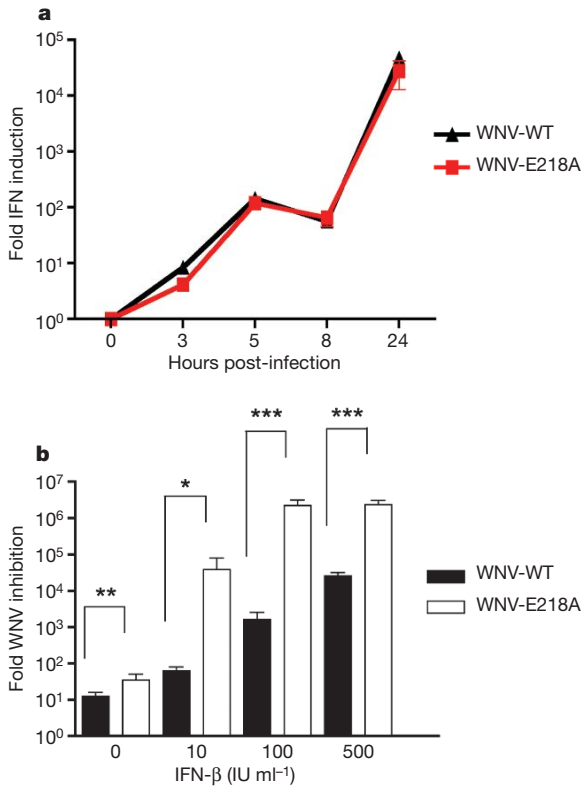


Figure 1 | WNV-E218A is attenuated in wild-type mice and cells but is virulent in *Ifnar1*$^{-/-}$ mice and cells. **a**, Survival curves of wild-type and *Ifnar1*$^{-/-}$ C57BL/6 mice after subcutaneous infection with WNV-WT or WNV-E218A. **b**, Virus replication in wild-type mice in blood (day 4), 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 (10^1) or WNV-E218A (10^5 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.



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 with 500 international units ml^{-1} of IFN-β, respectively) (Fig. 2b).

IFN induces hundreds of ISGs, some of which may have antiviral effector functions[13]. Among these, *Ifit* family members (for example, *Ifit1* and *Ifit2* (also known as *ISG56* and *ISG54*, respectively)) are induced after WNV infection[14], reduced in *Irf3*$^{-/-}$ and *Ifnar1*$^{-/-}$ cells (ref. 15 and Supplementary Fig. 5) and inhibit replication of some viruses[16–18] in part, by interacting with eIF3 and limiting translation of viral mRNA[19,20]. To assess whether differential 2′-*O* methylation of viral RNA might affect suppression by IFIT-1 and/or IFIT-2, we evaluated infection in 3T3 MEFs expressing a murine *Ifit1* or *Ifit2* transgene. As observed in primary cells, WNV-E218A replication in control 3T3 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 3T3–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 *IPS-1*$^{-/-}$ 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.

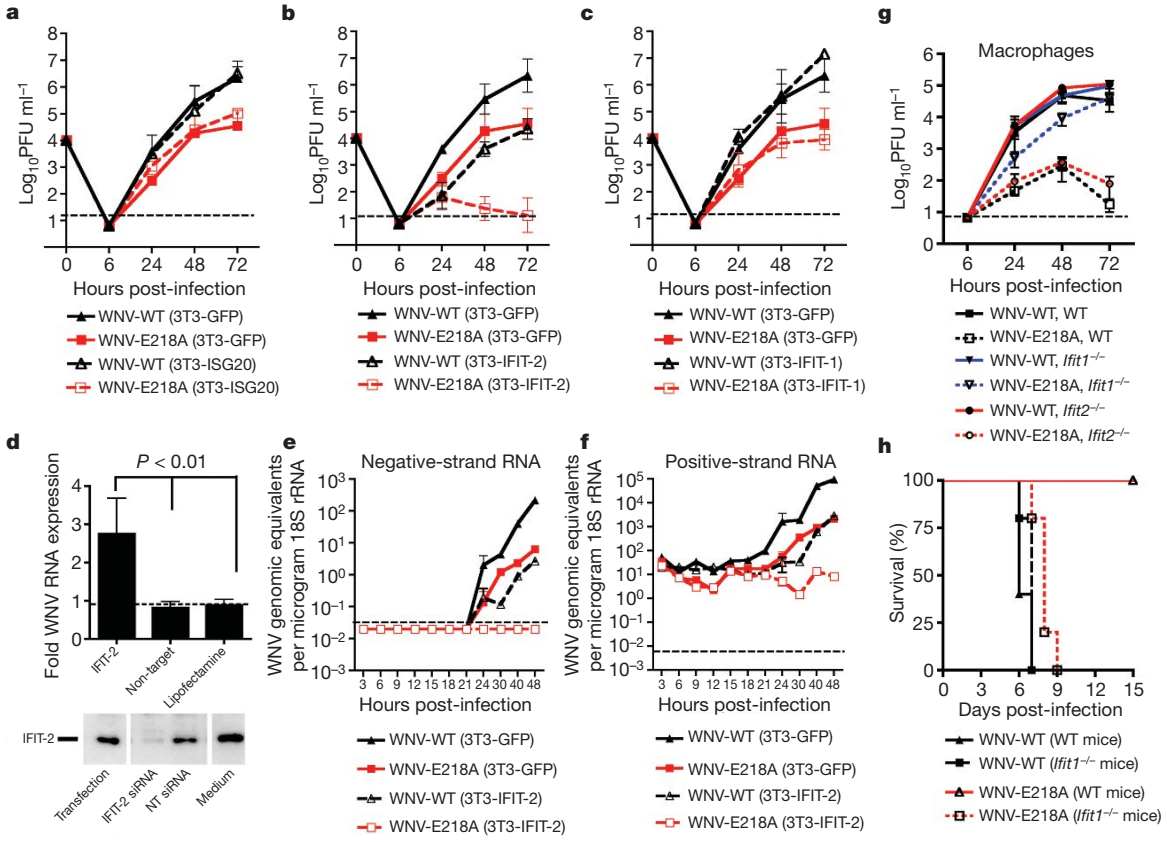


Figure 3 | WNV-E218A is more sensitive to the antiviral actions of *Ifit* genes. **a–c**, Viral replication of WNV-WT or WNV-E218A in 3T3 MEFs transgenically expressing GFP (**a–c**), ISG20 (**a**), IFIT-2 (**b**) or IFIT-1 (**c**). The data are the average of three experiments performed in duplicate. **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*$^{-/-}$ Mϕ but restored in *Ifit1*$^{-/-}$ cells. **h**, Survival curves of wild-type or *Ifit1*$^{-/-}$ mice after intracranial challenge with 10^5 plaque-forming units of WNV-WT or WNV-E218A. Error bars, s.d.; dashed line, limit of sensitivity of the assay.

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 minimized by 2′-*O* methylation of viral RNA.

Although IFIT family orthologues exist over a broad evolutionary time-frame[21], 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.

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[22] but was attenuated in wild-type Mϕ (approximately six- to eightfold reduction at 24–72 h) and fully rescued in *Ifnar1*$^{-/-}$ 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*$^{-/-}$ 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)[23] of mouse hepatitis virus (MHV). MHV-D130A was more sensitive to the effects of IFN-β pretreatment

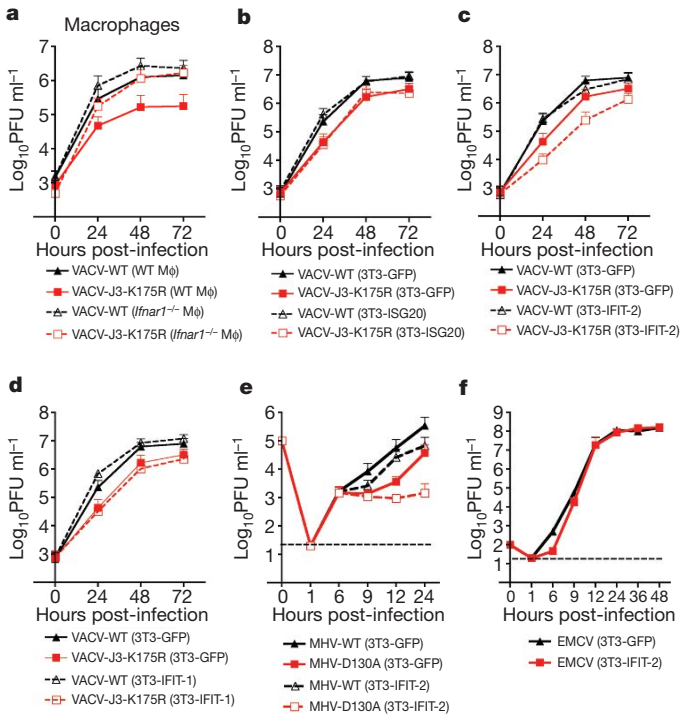


Figure 4 | Poxvirus and coronavirus mutants lacking 2′-*O* methylation are more sensitive to the antiviral effects of murine IFIT-2. a–d, Studies with VACV. **a**, Viral replication of VACV-WT or VACV-J3-K175R in wild-type or *Ifnar1*$^{-/-}$ Mϕ (**a**) or 3T3 MEF expressing GFP (**b**–**d**), ISG20 (**b**), *Ifit2* (**c**) or *Ifit1* (**d**). **e**, Viral replication of MHV-WT or MHV-D130A in 3T3 cells expressing GFP or IFIT-2. **f**, Viral replication of EMCV in 3T3 cells expressing GFP or IFIT-2. Error bars, s.d.; dashed line, limit of sensitivity of the assay.

(Supplementary Fig. 8), attenuated in control 3T3 cells (approximately 6- to 15-fold reduction at 9–24 h, $P < 0.05$, $n = 3$) (Fig. 4e), and sensitive to transgenic expression of mouse IFIT-2 (approximately 8- to 234-fold reduction, $P < 0.05$, $n = 3$) compared with MHV-WT (approximately two- to fivefold decrease at 9–24 h, $P < 0.05$, $n = 3$). Thus, analogous to flaviviruses and poxviruses, the 2′-*O* methylation of coronavirus RNA supports evasion from the antiviral effects of IFIT-2. In contrast, transgenic expression of IFIT-2 did not affect replication of a picornavirus, which lacks a 5′ cap structure (Fig. 4f).

To confirm the role of IFIT proteins in restricting viruses lacking 2′-*O* methylation, growth curves were performed in wild-type, *Ifit1*$^{-/-}$ or *Ifit2*$^{-/-}$ Mϕ. Surprisingly, the infectivity of WNV-E218A was almost completely rescued in IFIT-1$^{-/-}$ Mϕ (2,300-fold increase in titre at 72 h, $P < 0.04$) but not in *Ifit2*$^{-/-}$ Mϕ (Fig. 3g), and the virulence of WNV-E218A was almost entirely restored in *Ifit1*$^{-/-}$ mice (Fig. 3h). Thus, in primary Mϕ and in mice, IFIT-1 plays a dominant role in restricting infection of WNV lacking 2′-*O* methylation.

We demonstrate that among unrelated RNA and DNA viruses that replicate in the cytoplasm and contain 5′ cap structures, 2′-*O* methylation of viral RNA enhances virulence through evasion of intrinsic cellular defence mechanisms. 2′-*O* methylation of cellular RNA may have evolved as a means of distinguishing self from non-self RNA by the host during virus infection. Induction of *Ifit* family genes, several of which attenuate translation[19,20,24], could preferentially recognize viral mRNA lacking 2′-*O* methylation and selectively restrict propagation. Plants, which lack an IFN response network or *Ifit* family member orthologues, and their viruses, accordingly lack 2′-*O*-methylation of mRNA. Given that host 2′-*O* methylation of cellular mRNA largely occurs in the nucleus, pharmacological strategies that disrupt cytoplasmic 2′-*O* MTase activity could represent a novel class of therapy against several globally relevant pathogenic viruses that replicate exclusively in the cytoplasm.

METHODS SUMMARY

Viruses. WNV-WT and WNV-E218A were propagated in BHK21 cells as described[8]. VACV-WT and VACV-J3-K175R[22] (a gift from R. Condit) and encephalomyocarditis virus (EMCV) (strain K) were propagated in HeLa and L929 cells, respectively. Generation of MHV-WT (strain A59) and MHV-D130A recombinant coronaviruses has been described[25].

Mouse experiments. C57BL/6 wild-type and immunodeficient (*Ifnar1*$^{-/-}$, *Ifit1*$^{-/-}$, *Ifit2*$^{-/-}$, *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ 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[11].

Cell culture and viral infection. Bone-marrow-derived Mϕ and MEF were generated as described[11]. 3T3 fibroblasts expressing GFP or ISG were previously described[18]. Cells were infected with WNV, VACV, MHV or EMCV at MOIs of 0.01, 1, 1 and 0.001, respectively. Lysates or supernatants were titred by plaque assay on BHK21-15 cells for WNV and EMCV, BSC-1 cells for VACV and L929 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 quantitative reverse transcriptase PCR as described previously[11].

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 titred by plaque assay.

Strand-specific real-time reverse transcriptase PCR. Quantification of positive- and negative-strand WNV RNA was performed using a T7-tagged primer strategy[9]. 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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