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

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Abstract

Cellular mRNA of higher eukaryotes and many viral RNA 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) signaling. 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 (ISG) implicated in regulation of protein translation. Poxvirus and coronavirus mutants that lacked 2′-O MTase

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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 likely serves as a paradigm 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 mRNA 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.

Flaviviruses are 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-WT strain had a ∼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 to infection by WNV-WT (Fig 1b).

Since dissemination of WNV-E218A was aborted in vivo we assessed whether 2′-O methylation restricted the protective IFN-induced immune response. *IFNαβR*$^{-/-}$ mice infected with WNV-WT showed 100% mortality and a mean time to death (MTD) of 3.5 days, as seen previously 9 (Fig 1a). Remarkably, *IFNαβR*$^{-/-}$ mice infected with the WNV-E218A exhibited a similar phenotype with only a slightly delayed MTD of 4.5 days. *IFNαβR*$^{-/-}$ mice infected with WNV-E218A at day 3 sustained tissue titers 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 a virus sensitive to the IFN response.

Viral growth analysis in primary mouse fibroblasts (MEF) 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-fold lower and 151-fold lower at 72 h, $P < 0.05$, $n = 3$ in MEF and Mφ, respectively) and restored growth in *IFNαβR*$^{-/-}$ cells (Fig 1e and f). Replication of WNV-E218A also was rescued in *IRF-3*$^{-/-}$, *IRF-3*$^{-/-}$ × *IRF-7*$^{-/-}$, or *IPS-1*$^{-/-}$ cells that have altered or abolished IFN-α/β responses 11 (Supplementary Fig 2a-c and 3a-d, respectively), but not in *IRF-7*$^{-/-}$ 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.

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Since 2′-O methylation rendered WNV-WT less susceptible to the IFN response than WNV-E218A, we hypothesized 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, *IFNαβR*$^{-/-}$ MEF, which produce IFN-β without responding to it, were infected at a high 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*$^{-/-}$ MEF, which do not produce type I IFN after WNV infection but can respond to it 11, were exposed to IFN-β to induce ISG, and then infected. WNV-E218A displayed increased sensitivity to IFN-β pretreatment when compared to WNV-WT (2,400,000 and 20,000-fold inhibition with 500 IU/ml 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 (e.g., IFIT-1 and IFIT-2 (also known as ISG56 and ISG54, respectively)) are induced after WNV infection 14, reduced in *IRF-3*$^{-/-}$ and *IFNαβR*$^{-/-}$ cells (15 and Supplementary Fig 5) cells, 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 MEF expressing a murine IFIT-1 or IFIT-2 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 to 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) when compared to replication in 3T3-GFP cells. In comparison, expression of IFIT-2 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, 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 orthologs exist over a broad evolutionary time frame 21, humans have a distinct complement of *IFIT* genes (IFIT-1 (ISG56), IFIT-2 (ISG54), IFIT-3 (ISG60), and IFIT-5 (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), suggesting a species-specificity of IFIT genes in restricting WNV lacking 2′-O methylated RNA.

We assessed the stage of the WNV lifecycle that was restricted by mouse IFIT-2. Using strand-specific qRT-PCR to quantify genomic (positive strand) and replicative intermediate

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(negative strand) viral RNA, we found that in control 3T3 cells each increased by 18 h post infection (Fig 3e and f), whereas the **e**xpression of mouse IFIT-2 delayed production of both by ∼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 prior to 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 lacks 2′O MTase activity, replicates normally in BSC40 cells 22 but was attenuated in wild type Mφ (∼6 to 8-fold reduction at 24-72 h) and fully rescued in *IFNαβR*$^{-/-}$ 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 (∼3 to 5-fold 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 ($P > 0.5$, $n = 3$) suggesting 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 and 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 *IFNαβR*$^{-/-}$ mice, VACV-J3-K175R was virulent as all animals succumbed to infection with similar kinetics compared to 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 (Supplementary Fig 8), attenuated in control 3T3 cells (∼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 (∼8 to 234-fold reduction, $P < 0.05$, $n = 3$) in comparison to MHV-WT (∼2 to 5-fold 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, IFIT-1$^{-/-}$ or IFIT-2$^{-/-}$ Mφ. Surprisingly, the infectivity of WNV-E218A was almost completely rescued in IFIT-1$^{-/-}$ Mφ (2,300-fold increase in titer at 72 h, $P < 0.04$) but not in IFIT-2$^{-/-}$ Mφ (Fig 3g), and the virulence of WNV-E218A was almost entirely restored in IFIT-1$^{-/-}$ 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 defense mechanisms. 2′-O methylation of cellular RNA may

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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 orthologs, 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 a number of 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 (gift of R. Condit) and 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 (*IFNαβR*$^{-/-}$, *IFIT-1*$^{-/-}$, *IRF-3*$^{-/-}$, *IRF-7*$^{-/-}$, *IRF-3*$^{-/-}$ × *IRF-7*$^{-/-}$, and *IPS-1*$^{-/-}$) mice were bred at Washington University. Infection experiments were performed with approval of the Washington University and Saint Louis University Animal Studies Committees. Viral titers 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 MOI of 0.01, 1, 1, and 0.001, respectively. Lysates or supernatants were titered 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

IFN-αβR$^{-/-}$ MEF were infected at an MOI of 10 with WNV-WT or WNV-E218A. Total RNA was isolated, treated with DNAse (Qiagen), and IFN-β mRNA were amplified by qRT-PCR as described previously 11.

IFN-β pretreatment experiment

IPS-1$^{-/-}$ MEF were pre-treated 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 harvested at 48 or 12 h post infection, respectively, and titered by plaque assay.

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Strand-specific real time RT-PCR

Quantitation of positive and negative strand WNV RNA was performed using a T7-tagged primer strategy 26. Fibroblasts expressing GFP or mouse IFIT-2 were infected with WNV-WT or WNV-E218A at an MOI of 1 and total RNA was harvested at indicated time points.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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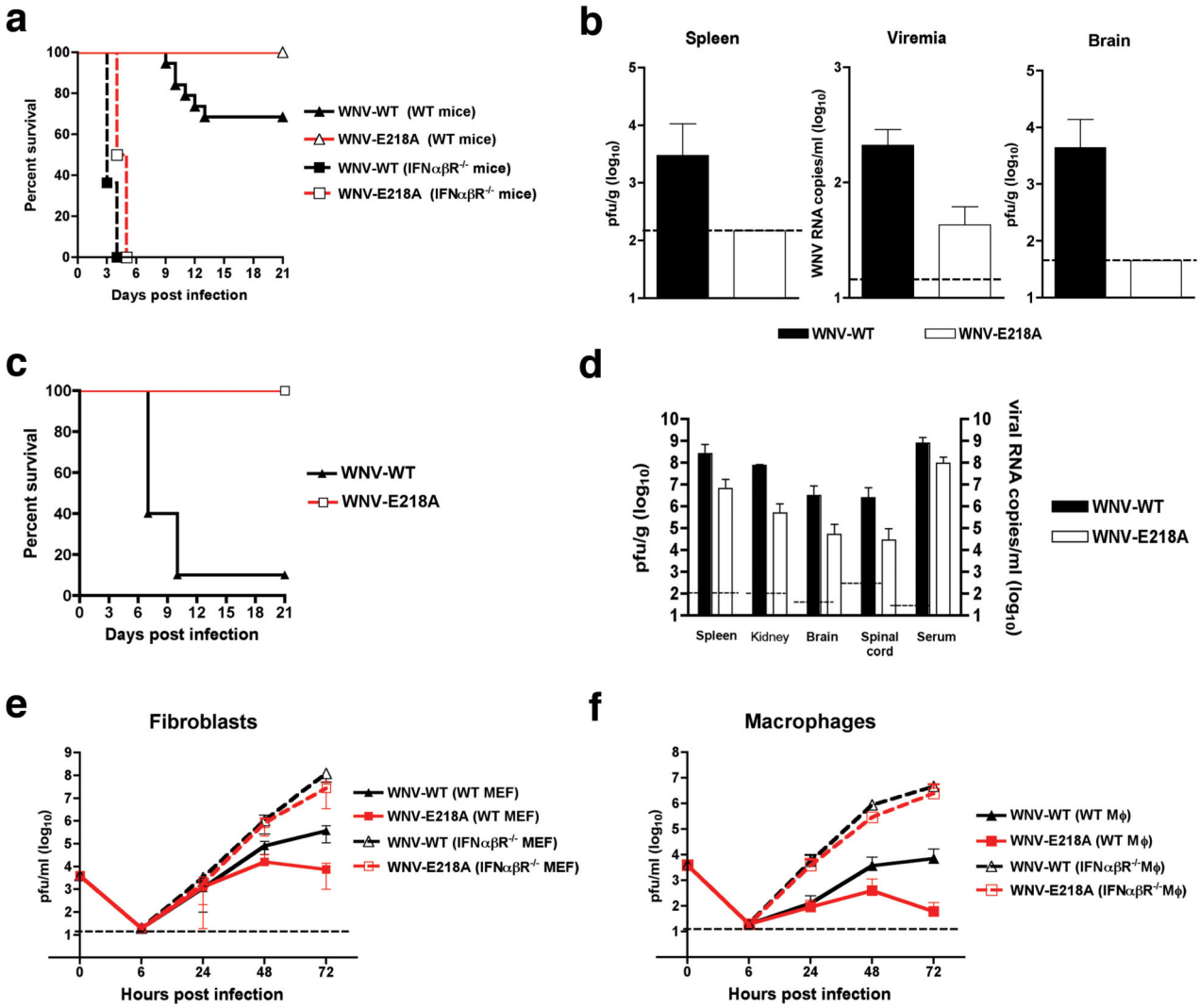

Figure 1.

WNV-E218A is attenuated in wild type mice and cells but is virulent in *IFNαβR*$^{-/-}$ mice and cells. **a**. Survival curves of wild type and *IFNαβR*$^{-/-}$ 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 PFU). **d**. Viral burden in the serum, spleen, kidney, spinal cord, and brain from *IFNαβR*$^{-/-}$ mice at day 3 after infection. **e-f**. Replication of WNV-WT and WNV-E218A in wild type or *IFNαβR*$^{-/-}$ MEF (**e**) or Mφ (**f**). Results are the average of three experiments performed in triplicate. Error bars indicate standard deviations and dashed line indicates the limit of sensitivity of the assay.

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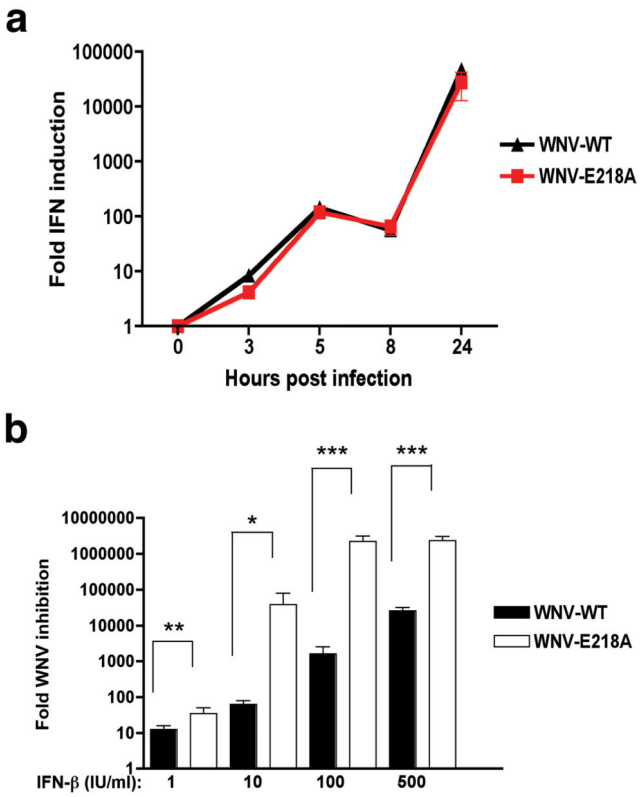

Figure 2.

2′-O methylation of viral RNA alters the sensitivity of WNV to the antiviral effects of IFN. **a**. IFN-β gene induction in *IFNαβR*$^{-/-}$ 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 indicate standard deviations.

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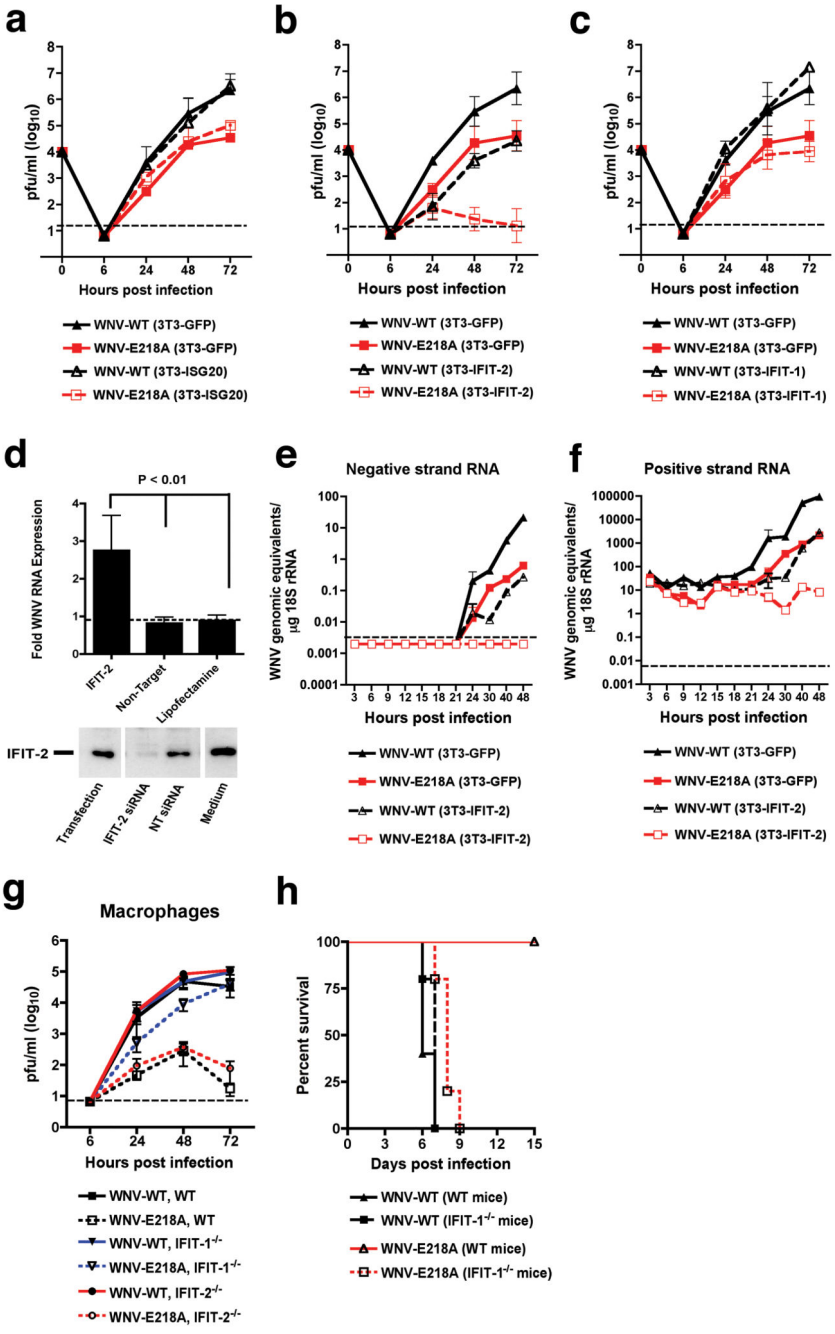

Figure 3.

WNV-E218A is more sensitive to the antiviral actions of IFIT genes. **a-c**. Viral replication of WNV-WT or WNV-E218A in 3T3 MEF transgenically expressing GFP (**a, b**, and **c**), ISG20 (**a**), IFIT-2 (**b**), or IFIT-1 (**c**). The data are the average of three experiments performed in duplicate, and the asterisks indicate statistically significant differences. **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 harvested and (*top*) viral RNA was assayed by qRT-PCR. The data are the average of three experiments performed in duplicate. (*Bottom*) Knockdown

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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 *IFIT-2*$^{-/-}$ Mφ but restored in *IFIT-1*$^{-/-}$ cells. **h**. Survival curves of wild type or *IFIT-1*$^{-/-}$ mice after intracranial challenge with 10^5 PFU of WNV-WT or WNV-E218A. Error bars indicate standard deviations and dashed line indicates the limit of sensitivity of the assay.

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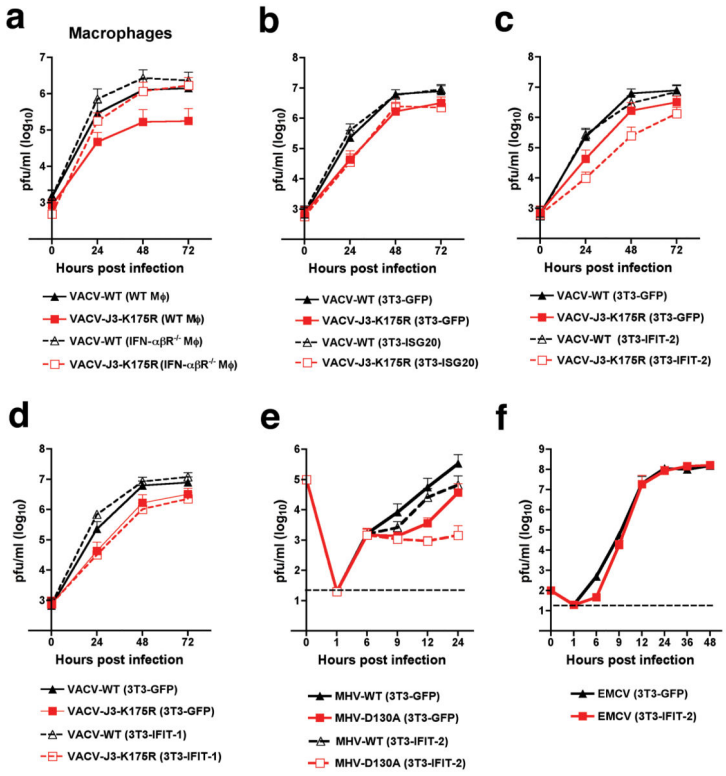

Figure 4.

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 IFNαβR$^{-/-}$ Mφ (**a**) or 3T3 MEF expressing GFP (**b, c**, and **d**), ISG20 (**b**), *IFIT-2* (**c**), or *IFIT-1* (**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 indicate standard deviations and dashed line indicates the limit of sensitivity of the assay.