Differential viral RNA methylation contributes to pathogen blocking in *Wolbachia*-colonized arthropods

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Abstract

Arthropod endosymbiont *Wolbachia pipientis* is part of a global biocontrol strategy to reduce the replication of mosquito-borne RNA viruses such as alphaviruses. We previously demonstrated the importance of a host cytosine methyltransferase, DNMT2, in *Drosophila* and viral RNA as a cellular target during pathogen-blocking. Here we report on the role of DNMT2 in *Wolbachia*-induced alphavirus inhibition in *Aedes* species. Expression of DNMT2 in mosquito tissues, including the salivary glands, is elevated upon virus infection. Notably, this is suppressed in *Wolbachia*-colonized animals, coincident with reduced virus replication and decreased infectivity of progeny virus. Ectopic expression of DNMT2 in cultured *Aedes* cells is proviral, increasing progeny virus infectivity, and this effect of DNMT2 on virus replication and infectivity is dependent on its methyltransferase activity. Finally, examining the effects of *Wolbachia* on modifications of viral RNA by LC-MS show a decrease in the amount of 5-methylcytosine modification consistent with the down-regulation of DNMT2 in *Wolbachia* colonized mosquito cells and animals. Collectively, our findings support the conclusion that disruption of 5-methylcytosine modification of viral RNA is a vital mechanism operative in pathogen blocking. These data also emphasize the essential role of epitranscriptomic modifications in regulating fundamental alphavirus replication and transmission processes.

Introduction

Viruses are remarkably adept at using a limited set of viral factors to replicate in vastly different host cell environments. This ability is vital for the success of zoonotic arboviruses, which encounter physiologically and ecologically distinct invertebrate and vertebrate hosts during transmission. As these viruses oscillate between vertebrate and arthropod hosts, the progeny virions reared in one host cell context are primed for the next, predicating successful transmission. However, arbovirus transmission events are influenced by many host-specific biotic and abiotic factors (2-6). Recent studies have identified the vector microbiome as a critical biotic factor influencing arbovirus transmission (5, 6). One notable member of this microbial population is the arthropod endosymbiont *Wolbachia pipientis,* which dramatically impacts the transmission of multiple zoonotic arboviruses, a phenomenon termed "pathogen-blocking" (PB) (7-15). *Wolbachia* is transmitted transovarially and induces a wide range of reproductive manipulations in its host (16, 17). For example, *Wolbachia's* presence results in sperm-egg incompatibility between colonized and non-colonized individuals, mediated by a bacterially-encoded toxin-antitoxin system (17). This phenomenon, known as cytoplasmic incompatibility (CI), allows *Wolbachia* to be inherited at a rate higher than Mendelian inheritance, like a natural gene drive. Over the last

decade, scientists have leveraged this property to deploy *Wolbachia* as a novel vector control agent with the aim of either suppressing or replacing the local mosquito population (18). Recent data suggest that *Wolbachia* release programs significantly reduce the transmission of Dengue virus (DENV) in endemic regions across 11 territories in Asia and Latin America (19, 20). Remarkably, however, despite its success, the underlying cellular mechanism of pathogen-blocking remains unidentified.

We recently showed that viral RNA is a cellular target of *Wolbachia*-mediated inhibition and that loss in progeny virus infectivity occurs at the level of the encapsidated virion RNA, which is compromised in its ability to replicate in naïve vertebrate cells (1). Mosquito-derived viruses, reared in the presence of *Wolbachia,* are less infectious when seeded in either mosquito or vertebrate cells. These results suggest a transgenerational mechanism by which *Wolbachia* limits virus dissemination within the mosquito and subsequent transmission into vertebrates (1). We, therefore, speculated that factor(s) regulating pathogen blocking likely target the viral plus sense RNA genome, a feature shared between all viruses susceptible to *Wolbachia*-mediated inhibition. Notably, the presence of *Wolbachia* reduces the infectivity of the encapsidated virion RNA in mammalian cells (1). This observation alone suggests one or more RNA-targeting factors may be responsible for compromising viral RNA replication in *Wolbachia*-colonized arthropod cells, as well as in mammalian cells, which are devoid of *Wolbachia.* Prior work has implicated mosquito exonuclease in pathogen-blocking, which is in line with the reduced half-life of incoming viral RNAs in *Wolbachia*-colonized cells (1, 21). While faster degradation of viral RNA explains the observed reduction in virus replication in arthropod cells, it does not explain reduced replication in mammalian cells. In light of these findings, we sought to focus our attention on the RNA cytosine methyltransferase DNMT2. Our prior study demonstrated that DNMT2 is essential for pathogen-blocking in fruit flies (9). As an RNA modifying protein, DNMT2 does not directly antagonize viral RNA replication but instead influences the cellular fate of its target(s). Both arthropods and mammals encode proteins capable of interpreting epitranscriptomic signatures on different RNA species, so we hypothesized that DNMT2-mediated modifications to the viral RNA in *Wolbachia*-colonized arthropods impact virus replication in arthropod and mammalian cells.

To test this hypothesis, we investigated whether DNMT2 is essential for *Wolbachia*-mediated pathogen blocking in mosquitoes. Additionally, we ask whether this MTase is functionally crucial to virus regulation in the absence of *Wolbachia.* Given DNMT2's biological role as a cellular RNA cytosine methyltransferase, we further examined the possibility of m5C modification of viral RNA in mosquito cells and whether viral RNA is differentially modified in the presence and absence of *Wolbachia* in mosquito cells. We find that *Wolbachia* and viruses differentially influence MTase expression in mosquitoes. Specifically, the presence of the virus leads to elevated MTase expression, which is proviral in mosquito cells. In contrast, the presence of *Wolbachia* downregulates MTase levels to seemingly disrupt this proviral state, contributing to virus inhibition as well as reduced progeny virus infectivity. Furthermore, the proviral effect is dependent on the catalytic activity of DNMT2. Finally, as a consequence of this downregulation and DNMT2's role as an RNA cytosine MTase, we show that the presence of *Wolbachia* in cells results in a reduced abundance of 5-methylcytosine (m5C) modification of progeny viral RNA. These changes imply that m5C modifications play a role in regulating viral RNA infectivity in mammalian cells. In summary, our findings highlight a previously underappreciated role of RNA methylation in alphavirus replication, with important implications for virus dissemination and transmission. Overall, our results indicate a role of the viral epitranscriptome as regulatory signatures capable of influencing the transmission of other arboviruses.

Results

Virus and *Wolbachia* differentially modulate *Aedes* DNMT2 expression

Wolbachia in *Aedes* mosquitoes is associated with reduced DNMT2 (*AMt2*) expression (22). We, therefore, examined the expression of *AMt2* in *w*AlbB-colonized *Aedes aegypti* mosquitoes. We chose to assess *in vivo AMt2* expression changes in whole mosquitos (which would give us a sense of the *Mt2* environment encountered by disseminating viruses) or in dissected salivary glands (the tissue important for transmission to the vertebrate host).

We measured *AMt2* expression in female *Aedes aegypti* mosquitoes colonized with and without *Wolbachia (w*AlbB) five days post eclosion, forty-eight hours following bloodmeals with and without Sindbis virus (SINV) (Fig 1B). The presence of both endosymbiont and virus was associated with altered *AMt2* expression (Two-way ANOVA, $p < 0.0001$), with a nearly 100-fold increase in *AMt2* levels in *Wolbachia*-free mosquitoes that received an infectious virus-containing blood meal (Fig 1B. W-/V- compared to W-/V+; Two-way ANOVA, $p < 0.0001$). In contrast, we found *Wolbachia* to reduce *AMt2* expression in mock-infected individuals (W+/V-) by approximately 5-fold (Fig 1B, W-/V- compared to W+/V-). Importantly, we also observed low *AMt2* expression in *Wolbachia*-colonized mosquitoes post-infectious (V+) bloodmeal, indicating that *Wolbachia* prevents virus-induced stimulation of *AMt2* expression and that these levels are maintained during infection. Virus replication in *Wolbachia*-colonized mosquitoes, therefore, occurs in a low *AMt2* environment (Fig 1B. W-/V- compared to W+/V+). This pattern of reduced *AMt2* expression was also observed *ex vivo* in cultured *Aedes albopictus*-derived mosquito cells colonized with both a native (*w*AlbB strain in Aa23 cells) and a non-native *Wolbachia* (*w*Mel in RML12 cells) strain (Fig S1).

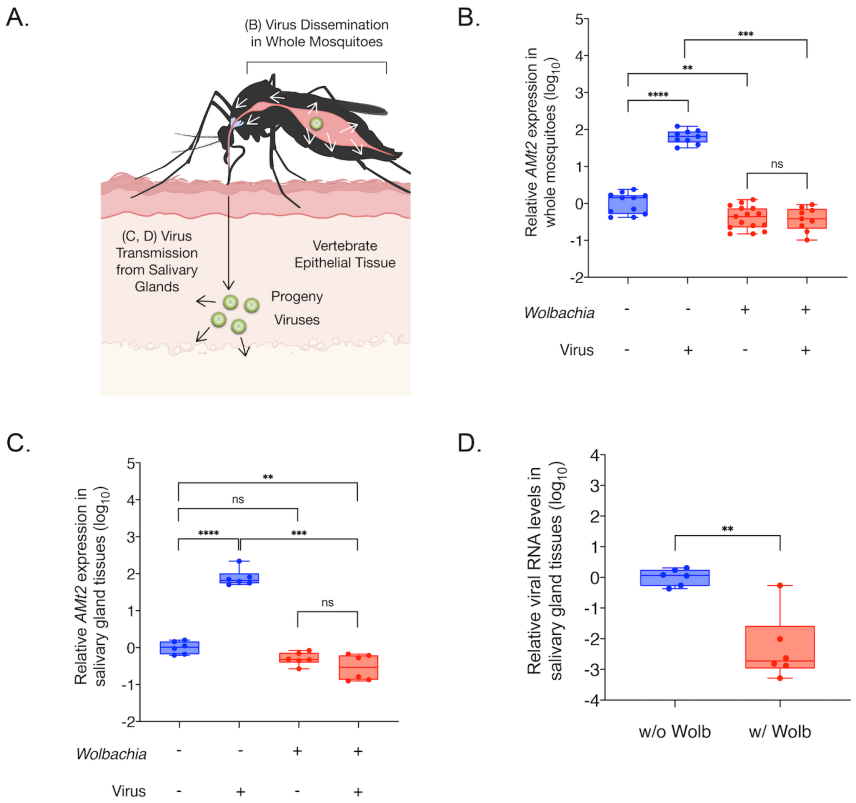

Fig 1. Virus and *Wolbachia* each differentially modulate expression of the RNA methyltransferase gene DNMT2 in mosquitoes. (A) Schematic of virus dissemination (white arrows) within mosquito tissues and transmission into vertebrate host (black arrow). (B) *AMt2* expression was measured 5 days post bloodmeal in whole female mosquitoes using qRT-PCR with and without SINV. Error bars represent standard error of mean (SEM) of biological replicates. Two-way ANOVA with Tukey's post-hoc test of multivariate comparisons. (C) *AMt2* expression measured in dissected salivary gland tissues collected from female mosquitoes with and *Wolbachia*-free 5 days post bloodmeal with or without SINV. Error bars represent standard error of mean (SEM) of biological replicates. Two-way ANOVA with Tukey's post-hoc test of multivariate comparisons. (D) Viral RNA levels were quantified in dissected salivary gland tissues with and *Wolbachia*-free using qRT-PCR at 5 days post infectious blood meal with SINV. Unpaired, student's t-test, error bars represent standard error of mean (SEM) of biological replicates. For all panels: ****P < 0.0001; ***P < 0.001; **P < 0.01; ns = non-significant.

We next quantified *AMt2* expression in isolated salivary gland tissues from five-day-old *Aedes aegypti* mosquitoes colonized with or *Wolbachia*-free (*w*AlbB), post bloodmeal with (V+) or without (V-) SINV (Fig 1C). *AMt2* expression in the salivary gland tissues post-infectious bloodmeal was elevated nearly 100-fold, similar to the increase induced by the virus in whole mosquitoes (Fig 1C. W-/V- compared to W-/V+). As before, the presence of *Wolbachia* alone was associated with lower *AMt2* expression. However, this difference was not statistically significant (Fig 1C. W-/V- compared to W+/V-). Importantly, however, *Wolbachia* did prevent SINV-induced *AMt2* upregulation, reducing it 100-1000 fold (Fig 1C. W-/V+ compared to W+/V+). Under these conditions, we also observed a significant 2 to 3 $\log_{10}$ reduction in viral RNA in the salivary gland

125 tissues (Fig 1D). It should be noted that our observations regarding the effect of *Wolbachia*
126 (*w*AlbB) or SINV on *AMt2* expression are analogous to previous reports that describe differential
127 *AMt2* expression in the presence of the flavivirus DENV-2 and *Wolbachia* (*w*Mel) in *Aedes aegypti*
128 mosquitoes (22).

129 **DNMT2 promotes virus infection in mosquito cells**

130 The positive correlation between *AMt2* expression and SINV genome replication in *Aedes*
131 mosquitoes (Fig 1B-D) led us to examine whether there is a functional consequence of elevated
132 MTase expression on virus infection in these insects. We therefore ectopically expressed *AMt2*
133 and assessed its effect on virus infection in cultured *Aedes albopictus* cells (Fig 2A), using
134 azacytidine-immunoprecipitation (AZA-IP) to determine whether viral RNA in the cell is a direct
135 DNMT2 target. *Wolbachia*-free *Aedes albopictus* (C710) cells were transfected with an epitope-
136 tagged *AMt2* expression vector (FLAG-*AMt2*) or control vector (FLAG-empty) for 48 hours before
137 infection with SINV at an MOI of 10. After 24 hours post-infection, cells were labeled with a
138 cytosine analog, 5-Azacytidine (5-AZAC), for 18 hours to incorporate the label into newly
139 synthesized cellular and viral RNA. We reasoned that if mosquito DNMT2 directly targets viral
140 RNAs for methylation, the presence of 5-AZAC in the RNA should covalently trap the enzyme
141 forming a stable m5C-DNMT2-viral RNA complex, allowing co-immunoprecipitation of the RNA-
142 protein complexes using anti-FLAG antibody (23). Targeted quantitative RT-PCR analyses of total
143 immunoprecipitated RNA revealed enrichment of SINV RNA relative to a control host RNA
144 transcript (GAPDH), confirming that viral RNA is indeed a direct MTase target in these cells (Fig
145 2B).

146 We next assessed the effect of elevated *AMt2* expression on RNA virus infection by measuring
147 the output of infectious progeny viruses following infection of cells expressing FLAG-*AMt2*.
148 Ectopic MTase expression resulted in a four-fold increase in SINV titer, further supporting the
149 positive *in vivo* correlation between *AMt2* expression and virus replication observed previously
150 (Fig 2C). We also observed a concomitant increase in the per-particle infectivity of viruses upon
151 assaying them on vertebrate baby hamster kidney fibroblast cells, as evidenced by higher specific
152 infectivity ratios (Fig 2D). Together these results support the idea of *Aedes* DNMT2 being a
153 proviral factor exploited by the virus to enhance its replication and transmission in the mosquito
154 vector.

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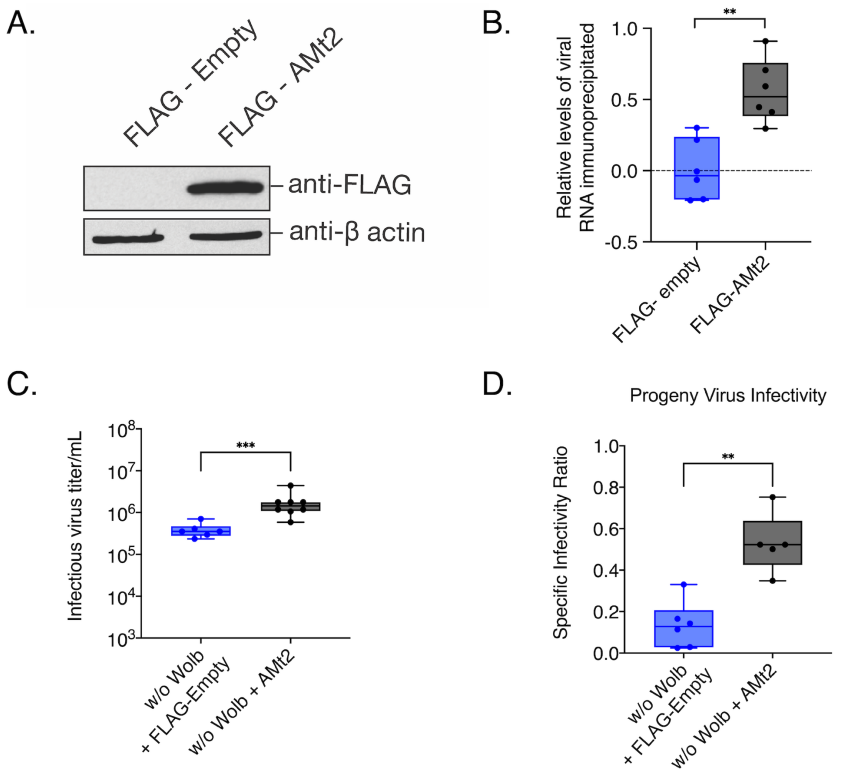

Fig 2. Overexpressing *AMt2* in mosquito cells improve progeny virus infectivity. (A) Western Blot of *Aedes albopictus* (C710) cells transfected with expression vector constructs with (FLAG-AMt2) or without (FLAG-empty) *AMt2.* Cytoplasmic lysates of cells were collected 48 hours post transfection and probed with anti-FLAG and anti-β actin antibodies. (B) Relative levels of SINV RNA recovered following AZA-IP of AMt2 in C710 cells was quantified using qRT-PCR. *Wolbachia*-free C710 mosquito cells were transfected with expression vectors FLAG-empty or FLAG-AMt2 for 48 hours prior to infection with SINV at MOI of 10. Cells were treated for approximately 18h with 5 μM 5-Azacytidine to covalently trap AMt2 with its target cellular RNA prior to RNA immunoprecipitation using anti-FLAG antibody. The horizontal dotted line represents the threshold set at 1 ($\log_{10}$). Unpaired two-tailed t-test with Welch's correction, $p = 0.0004$, $t = 4.216$, $df = 20$ (C) Infectious progeny (PFU/mL) SINV produced from mosquito cells *Wolbachia*-free expressing either FLAG-empty (w/o Wolb) or FLAG-AMt2 (w/o Wolb + AMt2). Cells were transfected 48 hours prior to infection with SINV at MOI of 10. Infectious progeny viruses collected from supernatants 48 hours post-infection were quantified using plaque assays on BHK-21 cells. Unpaired two-tailed t-test with Welch's correction, $p = 0.0002$, $t = 5.404$, $df = 11.81$. (D) Specific Infectivity Ratios of progeny SINV were calculated as described earlier (1). Unpaired two-tailed t-test with Welch's correction, $p = 0.0084$, $t = 3.911$, $df = 5.820$. For all panels error bars represent standard error of mean (SEM) of biological replicates and **$P < 0.01$; ****$P < 0.0001$.

156 *AMt2* expression is reduced in the presence of *Wolbachia.* To test whether virus restriction *in vivo*
157 is a consequence of reduced DNMT2 activity, we measured virus replication in mosquito cells
158 following pharmacological inhibition of DNMT2. Structural homology of DNMT2 to other members
159 of the DNA MTase family has allowed it to retain its DNA binding ability *in vitro.* However, they
160 are canonically known to methylate tRNA molecules (24). Furthermore, DNMT2 is known to
161 methylate RNA substrates by a different mechanism than canonical RNA methyltransferases.
162 This mechanism of action makes DNMT2 susceptible to the action of DNA methyltransferase

163 inhibitors ribo- (5-Azacytidine or 5-AZAC) or deoxyribo- (Deoxy-5-Azacytidine or DAC5) while
164 ensuring that the function of other RNA methyltransferases in the cell remain unperturbed (25,
165 26). We reasoned that pretreatment of mosquito cells with either MTase inhibitor should reduce
166 cellular DNMT2 activity and consequently restrict alphavirus replication. Pretreating *Wolbachia*-
167 free C710 cells with RNA MTase inhibitor 5-AZAC prior to infection reduced SINV RNA replication
168 approximately 5-fold at 24 hours post-infection (Fig 3A). Virus titer was also reduced
169 approximately 10-fold (Fig 3B). Finally, MTase inhibition also negatively influenced SINV per-
170 particle infectivity, as evidenced by a 50-fold reduction in SI ratio (Fig 3C). Similar results were
171 obtained for related alphavirus, Chikungunya virus (CHIKV, Fig S2A,B).

172 Using our previously published live-cell imaging system, we used a fluorescently-tagged CHIKV
173 reporter virus (CHIKV-mKate) to examine the effect of deoxyribo- MTase inhibitor DAC5 on virus
174 replication in *Wolbachia*-free and *Wolbachia*-colonized *Aedes albopictus* cells using the Incucyte
175 live-cell imaging platform (1). As before, fluorescent protein expression was used as a proxy of
176 virus replication in cells with (DAC5) and without (DMSO) inhibitor pretreatment. Virus replication
177 was measured over 50 hours by quantifying mean virus-encoded red fluorescent reporter (mKate)
178 expression observed over four distinct fields of view taken per well every 2-hours (Fig 3D, S2C).

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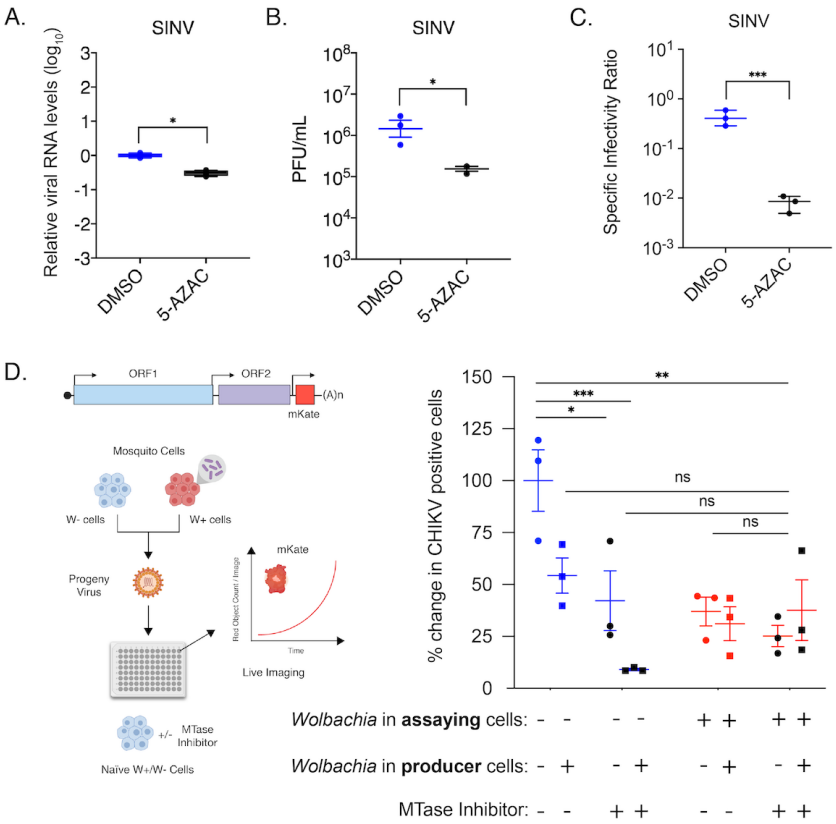

Fig 3. Pharmacological inhibition of mosquito DNMT2 reduces virus replication and per-particle infectivity. Inhibition of mosquito DNMT2 in *Wolbachia*-free *Aedes albopictus* derived C710 cells was carried out using MTase inhibitors 5-Azacytidine (5-AZAC). Dimethyl-sulfoxide (DMSO) was used as the negative control. In each case, cells were pretreated with 5 μM inhibitors overnight prior to infections with SINV at MOI of 10. Cell lysates and supernatants were harvested at 24 hours post infection to quantify cellular viral RNA levels and infectious titer, respectively. (A) Levels of SINV RNA in mosquito cells treated with MTase inhibitor 5-AZAC were determined using quantitative RT-PCR. Unpaired two-tailed t-test with Welch's correction, SINV: p = 0.0012, t = 6.742, df = 4.892. (B) Infectious SINV titers produced from mosquito cells treated with MTase inhibitor 5-AZAC were determined using plaque assays on BHK-21 cells. Unpaired two-tailed t-test with Welch's correction, SINV: p = 0.0339, t = 4.541, df = 2.322. (C) Specific infectivity ratios of progeny SINV was calculated as the ratio of infectious plaque forming units (B) over total viral genome copies present in collected cell supernatants as quantified by qRT-PCR. Unpaired two-tailed t-test with Welch's correction, SINV: p = 0.0002, t = 12.59, df = 3.946. Error bars represent standard error of mean (SEM) of three independent experimental replicates. (D) CHIKV expressing mKate fluorescent reporter protein was grown in C710 *Aedes albopictus* cells in the presence (W+ virus) or absence (W- virus) of *Wolbachia (*strain *w*Stri). These progeny viruses were then used to infect C710 cells without and with *Wolbachia (*strain *w*Stri) pretreated with MTase inhibitor DAC5 (black data points) or DMSO (blue data points for *Wolbachia* free cells, red data points for *Wolbachia* colonized cells) synchronously at a MOI of 1 particle/cell. Virus growth in cells was measured in real time by imaging and quantifying the number of red cells expressing the virus encoded mKate protein forty-eight hours post infection, using live cell imaging. Shape of data points represent the origin of virus used to initiate infection; circles represent viruses derived from W- cells, boxes represent viruses derived from W+ cells. Three-way ANOVA with Tukey's post hoc test for multivariate comparisons. For all panels error bars represent standard error of mean (SEM) of independent experimental replicates (n=3). *P < 0.05; **P < 0.01; ****P < 0.0001, ns = non-significant.

180 In line with previous observations, viruses derived from *Wolbachia*-colonized cells (W+ virus, we
181 will refer to viruses derived from *Wolbachia*-colonized cells as W+ and their counterparts, derived
182 from *Wolbachia*-free cells as W- virus), produced under low *AMt2* conditions, are less infectious
183 on W- cells, limiting their dissemination (1). Notably, this phenotype is particularly pronounced
184 when W+ viruses encounter cells also colonized with the endosymbiont, presumably exhibiting
185 reduced *AMt2* expression (Fig 3D, S2D-E). To further investigate the importance of DNMT2
186 activity in *Wolbachia*-mediated virus inhibition we treated cultured *Aedes albopictus* (C710) cells
187 with DAC5, a DNMT2 inhibitor. We predicted that replication kinetics of W+ viruses in inhibitor-
188 treated *Wolbachia*-free (W-) cells should phenocopy kinetics of W+ virus replication in *Wolbachia*-
189 colonized (W+) cells. Additionally, the kinetics of W- virus replication in inhibitor-treated
190 *Wolbachia*-free (W-) cells should phenocopy W- virus replication in *Wolbachia*-colonized (W+)
191 cells (1). Three-way ANOVA was used to determine the effect of MTase inhibitor (DAC5), progeny
192 virus type (derived from producer cells with or *Wolbachia*-free*),* and/or time on virus replication in
193 recipient cells (assaying cells with or *Wolbachia*-free*)*. In the presence of MTase inhibitor,
194 replication of both W- and W+ viruses was reduced throughout infection, with a greater decrease
195 in the replication of W+ viruses relative to W- viruses phenocopying the replication of W+ viruses
196 in *Wolbachia*-colonized cells. Finally, replication of W- viruses in the presence of inhibitor was
197 comparable to that of W+ viruses in mock-treated *Wolbachia*-free cells. We observed no
198 synergistic effect of virus source and MTase inhibitor on virus replication in *Wolbachia*-colonized
199 cells, likely due to low mean reporter activity. Altogether, these results demonstrate that
200 alphavirus replication is negatively impacted by perturbed DNMT2 activity in either producer (W+
201 virus) or recipient mosquito cells (W- + DAC5 cells or W+ cells) and that the effect is compounded
202 when both co-occur (W+ virus in W- + DAC5 cells or W+ virus in W+ cells).

203 **Ectopic DNMT2 expression rescues alphaviruses from *Wolbachia*-mediated inhibition**

204 Evidence gathered indicates that *AMt2* downregulation is responsible for pathogen blocking in
205 mosquitoes. Therefore, we ectopically overexpressed *AMt2* in *Wolbachia*-colonized mosquito
206 cells to alleviate virus inhibition, including disruption of viral RNA synthesis and progeny virus
207 infectivity (Fig 4A). We observed a significant reduction in viral RNA levels in *Wolbachia*-colonized
208 cells relative to *Wolbachia*-free cells (Fig 4B). Interestingly, expression of FLAG_*Amt2* increased
209 SINV RNA levels 70-fold in *Wolbachia*-colonized cells compared to cells carrying FLAG-empty
210 vector, restoring virus RNA synthesis; One-way ANOVA Holm-Sidak's multiple comparisons test,
211 w/ Wolb vs. w/ Wolb + AMt2, $p < 0.0001$ (Fig 4B). Additionally, we observed a significant
212 improvement in per-particle infectivity of progeny viruses derived from *Wolbachia*-colonized cells
213 ectopically expressing *AMt2* (W+ AMt2+ virus, Fig 4C). Therefore, both phenotypes of pathogen
214 blocking were abrogated upon *AMt2* over-expression. Given that endosymbiont titers can
215 influence the degree of virus inhibition, we checked whether altering *AMt2* levels significantly
216 impacted *Wolbachia* titer in cells. Quantitative PCR was used to measure relative *Wolbachia* titer
217 in cells transfected with FLAG-AMt2 or FLAG-empty. However, no changes in endosymbiont titer
218 were observed following ectopic *AMt2* expression (Fig 4D). Therefore, changes in *Wolbachia* titer
219 do not explain the loss of pathogen blocking.

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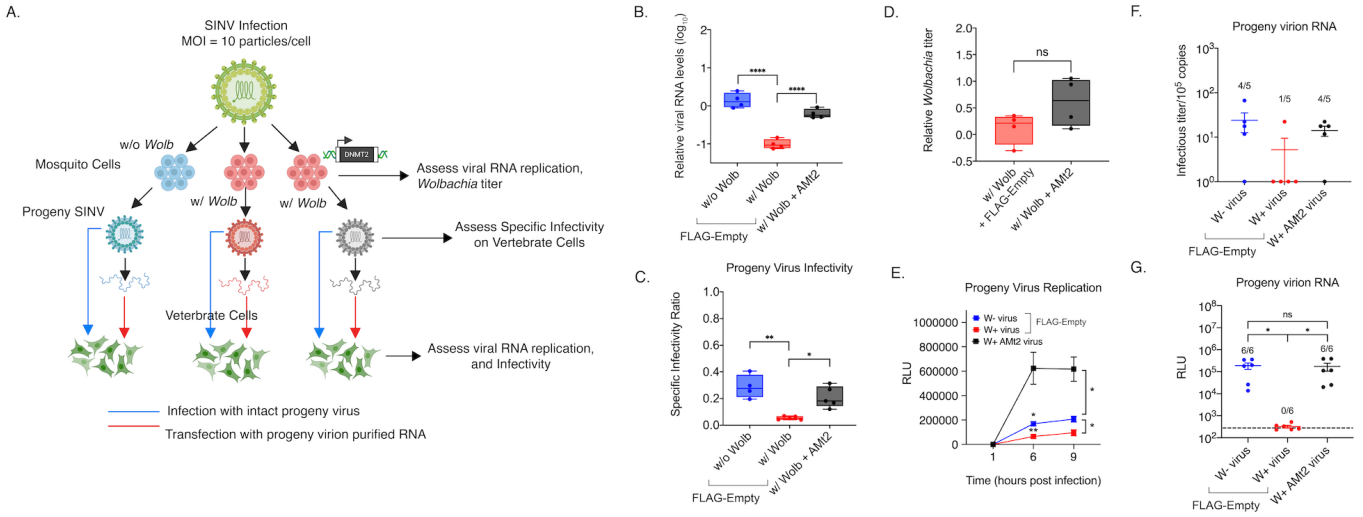

Fig 4. *AMt2* overexpression in *Wolbachia*-colonized cells rescues virus from endosymbiont-mediated inhibition. C710 cells with *Wolbachia* were transfected with expression vectors FLAG-empty (w/ Wolb) or FLAG-AMt2 (w/ Wolb + AMt2) for 48 hours prior to infection with SINV-nLuc at MOI of 10. *Wolbachia* -free cells expressing FLAG-empty (w/o Wolb) were used as a positive control. (A) Schematic of experimental workflow. (B) Viral genome replication in C710 cells was quantified using qRT-PCR using extracted total RNA from infected cell lysates. One-way ANOVA with Tukey's post-hoc test of multivariate comparison. (C) Specific Infectivity Ratios of progeny viruses produced from the aforementioned infection was calculated as described earlier (1). Briefly, infectious progeny viruses collected from supernatants 48 hours post infection were quantified using plaque assays on BHK-21 cells, while total number of progeny virus particles was quantified via qRT-PCR of viral genome copies released into the supernatant. Error bars represent standard error of mean (SEM). One-way ANOVA with Tukey's post-hoc test of multivariate comparison, w/ Wolb vs w/ Wolb + AMt2, $p = 0.0003$, w/o Wolb vs w/ Wolb, $p < 0.0001$. (D) C710 mosquito cells with *Wolbachia* were transfected with expression vectors FLAG-empty (w/ Wolb) or FLAG-AMt2 (w/ Wolb + AMt2) for 48 hours prior to quantification of endosymbiont titer via quantitative PCR using DNA from extracted cell lysates. Error bars represent standard error of mean (SEM). Unpaired, student's t-test, $p = 0.1316$, $t = 1.794$, $df = 5.097$. Statistically non-significant values are indicated by ns. (E) Progeny viruses were used to synchronously infect naïve BHK-21 cells at equivalent MOIs of 5 particles/cell. Cell lysates were collected at indicated times post infection and luciferase activity (RLU), was used as a proxy for viral replication. Two-way ANOVA with Tukey's post-hoc test of multivariate comparison, Time: $p < 0.0001$, *Wolbachia/AMt2*: $p = 0.0003$, Time x *Wolbachia/AMt2*: $p < 0.0001$. (F) Approximately 10^5 copies (determined using qRT-PCR) each of virion encapsidated RNA extracted from the aforementioned W+, W+ AMt2 and W- viruses were transfected into naïve BHK-21 cells and infectious titer was determined by the counting the number of plaques produced after 72 hours post transfection. Numbers above bars refer to the proportion of samples that formed quantifiable plaque-forming units on BHK-21 cells. One-way ANOVA with Tukey's post-hoc test of multivariate comparison. (G) 10^5 copies each of virion encapsidated RNA extracted from the W+, W+ AMt2 and W- viruses were transfected into naïve BHK-21 cells and luciferase activity (RLU) was used as a proxy for viral replication at 9 hours post-transfection. Numbers above bars refer to the proportion of samples that produced luciferase signal above background levels, indicated by the dotted line. One-way ANOVA with Tukey's post-hoc test of multivariate comparison, w/ Wolb vs w/ Wolb + AMt2: $p < 0.00001$, w/o Wolb vs w/ Wolb: $p < 0.0001$; w/o Wolb vs w/ Wolb + AMt2: $p = 0.991$. For all panels error bars represent standard error of mean (SEM). *P < 0.05; **P < 0.01; ****P < 0.0001.

222 Reduced per-particle infectivity of viruses occurring in the presence of *Wolbachia* (W+ viruses) is
223 associated with reduced replication kinetics of these viruses in vertebrate cells and reduced
224 infectivity of the encapsidated W+ virion RNA (1). As *AMt2* overexpression in *Wolbachia*-
225 colonized mosquito cells rescued viral RNA synthesis and progeny virus infectivity, we examined
226 the ability of progeny viruses derived from *Wolbachia*-colonized cells overexpressing *AMt2* (W+
227 derived AMt2+ cells) to replicate in vertebrate cells. We used SINV encoding a luciferase reporter
228 for these experiments, allowing viral replication kinetics to determine the following synchronous
229 infection of three progeny virus types: W- derived virus, W+ derived virus, and W+ derived AMt2+
230 virus. Replication of W+ derived AMt2+ viruses was significantly higher on a per-particle basis
231 relative to W+ derived and, interestingly, W- derived viruses. This could be due to higher ectopic
232 *AMt2* expression relative to what is induced natively during virus infection, implying perhaps a
233 dose-dependent effect (Fig 4E). We then examined whether ectopic *AMt2* expression caused
234 changes in the infectivity of the encapsidated virion RNA itself. Based on results from Fig 3D, we
235 hypothesized that ectopic *AMt2* expression in *Wolbachia*-colonized cells should restore virion
236 RNA infectivity. Indeed, following transfection of virion RNA into vertebrate BHK-21 cells, W+
237 derived virus RNA was largely non-infectious in contrast to RNA derived from viruses derived
238 from W- and W+ AMt2+ cells (Fig 4F). Restored infectivity of W+ AMt2+ derived viral RNA was
239 also validated using the luciferase-based virus replication assay (Fig 4G).

240 As demonstrated in Fig 2B, DNMT2 possesses the ability to bind viral RNA in mosquito cells.
241 However, this alone does not indicate whether its MTase activity is essential for its proviral role.
242 Broadly, DNMT2 comprises a catalytic domain and a target recognition domain responsible for
243 RNA binding (27, 28). It is, therefore, possible that DNMT2's regulatory role is independent of its
244 MTase activity. To determine the importance of catalytic activity, we overexpressed a catalytically-
245 inactive mutant of *AMt2*, replacing the highly conserved cysteine residue (C78) present in the
246 motif IV region with a glycine (*AMt2* C78G, Fig 5A) in *Wolbachia*-colonized mosquito cells and
247 asked whether this allele is capable of relieving pathogen blocking. Our data show *AMt2*-mediated
248 rescue of SINV RNA synthesis and infectivity depends on its MTase activity as expression of the
249 C78G mutant failed to rescue virus from *Wolbachia*-mediated inhibition (Fig 5B). We observed no
250 improvement in SINV infectivity under these conditions (Fig 5C). Similar results were obtained
251 from experiments carried out using CHIKV, where expression of wild-type *AMt2*, but not *AMt2-*
252 *C78G,* resulted in improved virus titer (Fig 5D). However, while expression of *AMt2* increased
253 CHIKV specific infectivity compared to expression of *AMt2-C78G* this increase was not
254 statistically significant (Fig 5E). Based on these results, we conclude that the MTase *AMt2*
255 promotes virus infection in mosquitoes and that lower *AMt2* expression in the presence of
256 *Wolbachia* contributes to virus restriction and that MTase activity of DNMT2 is required for proviral
257 function.

258

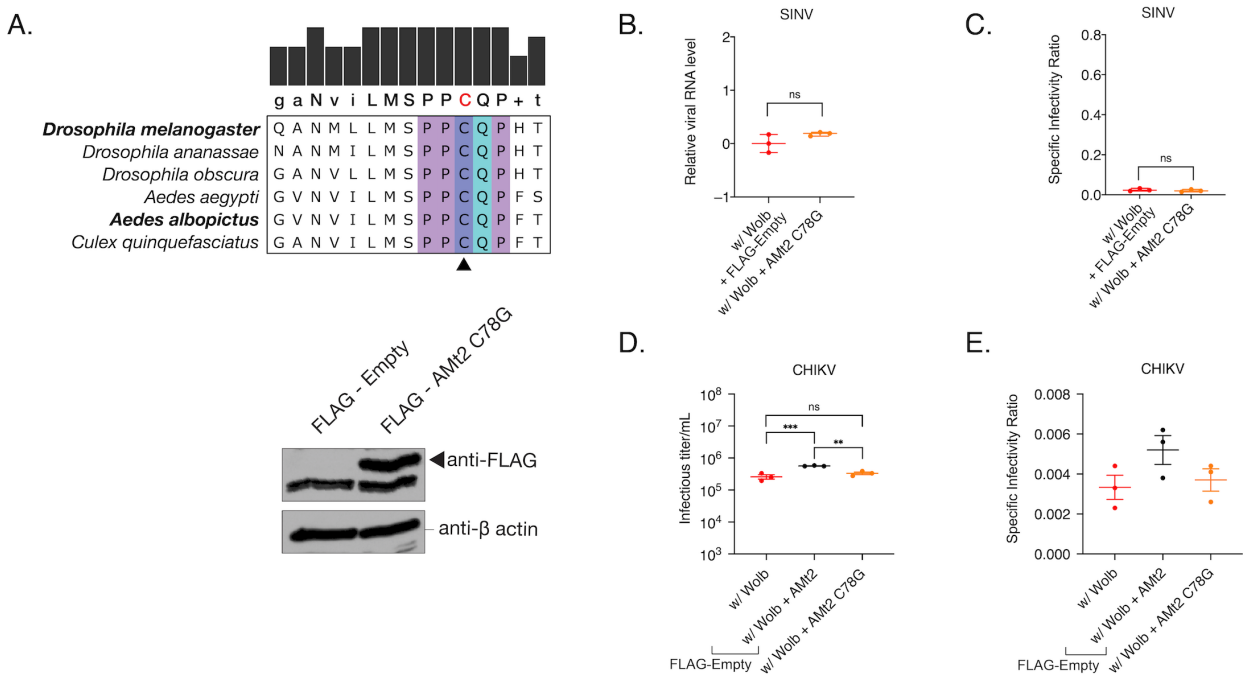

Fig 5: Catalytically inactive DNMT2 is unable to rescue *Wolbachia*-mediated virus inhibition in mosquito cells. (A) Multiple sequence alignment of the Motif IV region of DNMT2 derived from dipterans that are known to be colonized with native or non-native *Wolbachia.* The conserved catalytic cysteine (C) residue, depicted in red on the consensus sequence at the top, was mutated to a glycine (G) to abolish MTase activity of mosquito AMt2. Expression of the catalytic mutant (AMt2 C78G) was determined at 48 hours post transfection using Western Blot. C710 mosquito cells transfected with expression vector constructs with (FLAG-AMt2 C78G) or without (FLAG-empty) *AMt2.* Cytoplasmic lysates of cells were collected 48 hours post transfection and probed with anti-FLAG antibody. The non-specific band appearing below the desired size was used as a loading control. (B and C) C710 mosquito cells with *Wolbachia* were transfected with expression vectors FLAG-empty (w/ Wolb) or FLAG-C78G AMt2 (w/ Wolb + AMt2 C78G) for 48 hours prior to infection with SINV at MOI of 10. Infectious virus and Specific Infectivity (SI) of progeny viruses produced after 72 hours post infection were quantified as before. Unpaired t-test with Welch's correction, $p = 0.1734$, $t = 1.920$, $df = 2.396$ (B), $p = 0.4544$, $t = 0.8291$, $df = 3.937$ (C). (D and E) C710 mosquito cells with *Wolbachia* were transfected with expression vectors FLAG-empty (w/ Wolb), FLAG-AMt2 (w/ Wolb + AMt2) or FLAG-C78G AMt2 (w/ Wolb + AMt2 C78G) for 48 hours prior to infection with CHIKV-mKate at MOI of 10. Infectious virus (D) and Specific Infectivity (E) of progeny viruses produced after 72 hours post infection were quantified as before. One-way ANOVA followed by Tukey's post hoc test for multivariate comparisons for (D), w/ Wolb vs w/ Wolb + AMt2, $p = 0.0009$, w/ Wolb vs w/ Wolb + AMt2 C78G $p = 0.2694$, w/ Wolb + AMt2 vs w/ Wolb + AMt2 C78G $p < 0.0040$. Error bars represent standard error of mean (SEM) of biological replicates. **P < 0.01; ***P < 0.001, ns = non-significant.

DNMT2 orthologs from mosquitoes and fruit flies regulate virus infection differentially in their respective hosts

The proviral role of the *AMt2* is intriguing, given the previously described antiviral role for the corresponding fruit fly ortholog, *Mt2* (9, 29). Interestingly, in our previous study, we observed that knocking down *Mt2* led to increased progeny virus infectivity. Therefore, we reasoned that ectopic

266 expression of *Mt2* should reduce Sindbis virus infectivity (Fig S3). As with mosquito *AMt2,* we
267 asked whether this involved direct targeting of viral RNA. AZA-IP of epitope-tagged Mt2 confirmed
268 direct interactions between viral RNA and fly DNMT2 in *Wolbachia*-free *Drosophila melanogaster*-
269 derived JW18 cells, which showed a 10-fold enrichment in SINV RNA-binding relative to a control
270 host transcript (18S) (Fig S3A, B).

271 In contrast to the proviral effect of mosquito *AMt2*, ectopic *Mt2* expression significantly reduced
272 infectivity of progeny SINV and CHIKV (W- derived Mt2+ virus) relative to those produced from
273 cells expressing the control vector (W- derived virus), confirming our previous findings (Fig S3C).
274 As with *AMt2,* we assessed whether reduced infectivity of W- derived Mt2+ viruses was due to
275 their inability to replicate in vertebrate cells. Indeed, results from our luciferase reporter based
276 viral replication assay revealed significantly reduced replication of W- Mt2+ derived viruses
277 relative to W- derived viruses in vertebrate BHK-21 cells, similar to the behavior observed for W+
278 derived viruses (Fig S3D). Finally, we quantified the infectivity of virion encapsidated RNA from
279 W- derived Mt2+ SINV and CHIKV viruses by measuring the number of plaque-forming units
280 generated following transfection into vertebrate BHK-21 cells. For both SINV and CHIKV, the
281 infectivity of virion encapsidated RNA was reduced for W+ viruses. Notably, this was phenocopied
282 by virion RNA isolated from W- Mt2+ derived SINV and CHIKV (Fig S3E,F).

283 Similar to mosquito *AMt2,* fly *Mt2's* ability to regulate virus fitness also rely on its catalytic activity,
284 as expressing a catalytically inactive mutant (*Mt2* C78A) was unable to restrict the production of
285 infectious virus and per-particle infectivity of SINV (Fig S4). Taken together, these results suggest
286 that progeny virus/virion RNA infectivity is reduced in fly cells under conditions where MTase
287 expression is elevated natively in the presence of *Wolbachia* (W+ virus) or artificially (W- Mt2+
288 virus).

289 **The presence of *Wolbachia* in mosquito cells is associated with altered viral RNA**
290 **methylation**

291 That ectopic *AMt2* expression in *Wolbachia*-colonized *Aedes albopictus* cells can restore the
292 infectivity of SINV progeny virion RNA implies two important things; (i) Sindbis virion RNA carries
293 5-methylcytosine (m5C) modifications, and (ii) that altered *AMt2* expression in the presence of
294 *Wolbachia* is associated with changes in the overall m5C content of the virion RNA. To directly
295 determine if virus RNA is modified differentially in the presence of *Wolbachia*, we subjected virion
296 RNA isolated from progeny SINV produced from *Aedes albopictus* cells colonized with (W+ virus)
297 and without (W- virus) *Wolbachia* to liquid chromatography tandem mass spectrometry (LC-
298 MS/MS) analyses (Fig 6A). We chose to focus our efforts on identifying the presence of 5-
299 methylcytosine (m5C) and 6-methyladenosine (m6A) residues on the viral genome for our present
300 analyses. We examined m6A due to recent reports highlighting the importance of this modification
301 in regulating RNA virus replication (30, 31). A potential complication for these analyses is the
302 presence of residue(s) of similar mass to charge ratio(s) to m5C, such as m3C. However, as
303 shown in Fig S5A, we observed distinct distribution of the individual m3C and m5C peaks in the
304 spectral output, demonstrating our ability to distinguish between these two bases (Fig S5A).

305

306

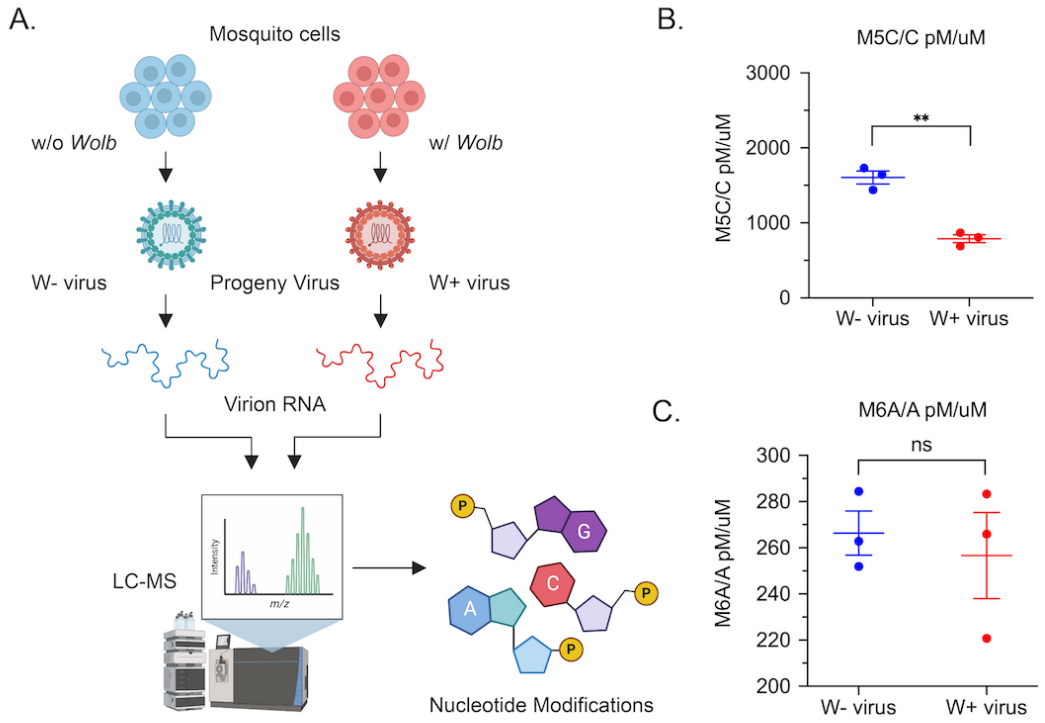

Figure 6: Presence of *Wolbachia* is associated with altered virion RNA methylation. (A) RNA isolated from progeny viruses derived from mosquito cells colonized with (W+ virus) or without (W- virus) *Wolbachia* were subjected to LC-MS/MS analyses to determine their nucleotide content. (B) Normalized 5-methyl cytosine (M5C) content of RNA isolated from W- and W+ viruses represented as a ratio of total unmodified cytosine content. Unpaired two-tailed t-test, $p = 0.0013$, $t = 8.080$, $df = 4$ (C) Normalized 6-methyl adenosine (M6A) content of RNA isolated from W- and W+ viruses represented as a ratio of total unmodified adenosine content. Unpaired two-tailed t-test, $p = 0.666$, $t = 0.4643$, $df = 4$. Error bars represent standard error of mean (SEM) of three independent virus preps from each cell type. **$P < 0.01$; ns = non-significant.

307

308 LC-MS/MS analyses of RNA purified from virion RNA derived from *Wolbachia* free (W-) and
309 *Wolbachia*-colonized (W+) cells demonstrated W+ virion RNA to contain, on average, more than
310 2-fold fewer m5C residues compared to W- virion RNA across three independent virus preps from
311 each cell type (Fig 6B). Notably, both W+ and W- virion RNA was determined to consist of
312 comparable levels of m6A residues across all biological replicates (Figure 6C). In addition, we
313 observed no significant changes in the overall m3C content between W+ and W- virion RNA (Fig
314 S5B). It should be noted that while we did not observe changes in the overall abundance of m6A
315 and m3C residues between W+ and W- virion RNA, it is unclear whether the presence of
316 *Wolbachia* leads to altered distribution of m6A and/or m3C modifications in the context of the
317 overall SINV RNA sequence. Finally, we used LC-MS/MS analyses to quantify viral Type-0 (7-
318 methyl-GpppNp or M7G) cap structures present in W- and W+ virion RNA to estimate relative
319 ratios of capped versus non-capped virus progeny produced in the presence or absence of
320 *Wolbachia*. While there were no statistically significant differences present between the respective

321 W- and W+ sample means (t-test: p=0.999), we found M7G content to vary significantly among
322 W+ virion RNA replicates (F-test: p=0.0088), indicating either that the ratios of capped vs. non-
323 capped viruses vary significantly within virus populations derived from *Wolbachia*-colonized cells,
324 or that viral RNAs produced under these conditions carry varying amounts of internal M7G
325 signatures (Fig S5C).

326 These data are consistent with: (i) DNMT2 being an essential host factor in mosquito cells for
327 efficient virus replication and transmission, (ii) this proviral effect being exert through m5C
328 modification of the viral genomic RNA, and (iii) a mechanism of *Wolbachia*-mediated pathogen
329 blocking being the reduction of DNMT2 expression.

330

331 **Discussion**

332 Virus inhibition in *Wolbachia*-colonized arthropods is associated with two distinct features
333 independent of any particular host-*Wolbachia* strain combination; (i) reduced genome replication
334 of the +ssRNA viruses in *Wolbachia*-colonized cells, and (ii) reduced per-particle infectivity of
335 progeny +ssRNA viruses produced under these conditions (1). While these shared attributes
336 constitute a subset of several virus inhibition phenotypes, it indicates the existence of a conserved
337 cellular mechanism of restriction. In our previous study, we used the prototype alphavirus, Sindbis
338 as our +ssRNA virus model to uncover an essential role of the fruit fly RNA cytosine
339 methyltransferase (MTase) gene *Mt2* (DNMT2) as an essential host determinant of *Wolbachia*-
340 mediated pathogen blocking (9). Furthermore, loss of *Drosophila* DNMT2 is associated with a
341 loss in virus inhibition by *Wolbachia* and increased progeny virus infectivity in mammalian cells,
342 suggesting that DNMT2 might regulate these two aspects of alphavirus replication. These findings
343 thus led us to ask the following question in our present study: Is DNMT2 a conserved host
344 determinant of *Wolbachia*-mediated +ssRNA virus inhibition between fruit flies and mosquitoes?

345 MTase expression in adult *Aedes aegypti* mosquitoes is distinctly altered in the presence of both
346 virus and *Wolbachia* and in opposite directions (Fig 1). DNMT2 expression is elevated in the body
347 of mosquitoes following an infectious bloodmeal. Notably, this pattern is observed in the salivary
348 gland tissues, representing the final site of virus production in the vector prior to transmission to
349 a vertebrate. We show that this is beneficial to the virus, as ectopic MTase expression in cultured,
350 *Wolbachia*-free *Aedes albopictus* mosquito cells promotes virus replication and, importantly,
351 progeny virus infectivity. This has also been reported for DENV-2 infection in *w*Mel-colonized
352 *Aedes aegypti* mosquitoes (22). We observed that baseline MTase activity is required for virus
353 replication and spread in *Aedes* cells (Fig S2). Furthermore, the extent to which virus replication
354 is affected by MTase inhibitors depends on the virus source, with viruses produced from
355 *Wolbachia*-colonized cells (W+ viruses) being more susceptible to MTase inhibition.

356 This outcome phenocopies the scenario in which virus spread is most restricted under conditions
357 where both producer and target mosquito cells are colonized with *Wolbachia* (1). In line with these
358 findings, our data indicate a decrease in MTase expression occurs in the presence of *Wolbachia*
359 (Fig 1). This observation is in line with previous reports (22). Thus, our collective data support a
360 model in which endosymbiont-dependent inhibition of MTase expression and catalytic MTase
361 function contribute to reduced virus replication and per-particle infectivity in mosquitos (Fig 2-4).
362 This consequently limits virus dissemination within the vector and transmission to a vertebrate
363 host (Fig 2-4). Given that our results are coherent with prior reports involving a different RNA virus
364 and *Wolbachia* strain, the interaction between virus, *Wolbachia,* and host MTase expression is
365 likely independent of any particular virus-host-*Wolbachia* combination, representing a conserved
366 feature of pathogen blocking in the native *Aedes* vector.

367 Our data demonstrate an interaction between DNMT2 orthologs from *Aedes albopictus* and
368 *Drosophila melanogaster* and viral RNA (Fig 5B, Fig S4B) (1). However, it remains to be seen
369 whether these interactions are analogous to DNMT2-DCV RNA interactions in *Drosophila,* where
370 the MTase-viral RNA binding occurs specifically at structured viral Internal Ribosomal Entry Sites
371 (IRES) (29). Additionally, it is unclear if DNMT2 interactions in the mosquito cell are specific for
372 viral RNA or whether it extends to host transcripts. Future studies involving PAR-CLIP-sequencing
373 of immunoprecipitated DNMT2-RNA complexes should allow the identification and mapping of
374 distinct DNMT2-binding motifs and/or structural elements within viral and host RNA species.
375 Nevertheless, sites of DNMT2 recruitment to viral RNA, and viral and host proteins are required
376 for recruitment remain unidentified. Assuming that the proviral role of *Aedes* DNMT2 involves the
377 addition of m5C signatures to specific residues on the viral genome, it seems likely that viral co-
378 factor(s) are required for specificity. *Drosophila* DNMT2 is antiviral in fruit flies, however
379 *Drosophila* is not a natural host for alphaviruses, and it is likely that the adaptation of the virus to
380 the natural vector has facilitated an appropriate proviral interaction with *Aedes* DNMT2 or an
381 associated *Aedes*-specific host factors(s) absent in *Drosophila melanogaster* (32).

382 The m5C content of virion RNA produced from *Wolbachia*-colonized cells (W+ viruses) is
383 significantly reduced relative to cells without the symbiont (Fig 6B) (1), consistent with DNMT2s
384 role as a cytosine MTase. Incidentally, this finding follows reports dating back several decades
385 describing the occurrence of m5C residues within intracellular SINV RNA (33). Relative
386 abundance of methylated to total cytosine residues on SINV virion RNA derived from *Wolbachia*-
387 free mosquito cells is 15:10000, accounting for cytosine content in the viral genome we predict
388 there to be 4 to 5 m5C signatures per encapsidated virion RNA genome produced in *Aedes*
389 *albopictus* cells. Our observation supports the involvement of these intracellular m5C signatures
390 in alphavirus genome replication that W+ virion RNA, which are presumably hypomethylated, are
391 less infectious on a per-genome basis (Fig 4E-F) (34). Indeed, based on our data, we can infer
392 that these m5C modifications regulate alphavirus infection across multiple hosts and, thus, by
393 extension, aspects of the virus transmission cycle. It should also be noted that while methylated
394 nucleotide residues like m6A and m5C occur on RNA virus genomes at higher rates than those
395 present in cellular RNA species, our results do not exclude the possibility of other RNA
396 modifications, as well as differential modification of host RNA species and playing a role in
397 regulating virus replication and transmission. This may be of particular consequence given recent
398 evidence of altered m6A modification of specific cellular transcripts during flavivirus infection in
399 vertebrate cells (31, 35, 36).

400 Alphaviruses derived from mosquito cells are more infectious on vertebrate cells on a per-particle
401 basis than vertebrate cell-derived viruses and vice versa (37). This carries the implication that
402 progeny viruses originating from one cell type may possess intrinsic properties that can confer a
403 fitness advantage while infecting a destination host cell type, altering their infectivity on these
404 destination cells on a per-particle level. As to what such properties may represent, current
405 evidence points towards differences in virus structure, such as differential sialation or
406 glycosylation of viral glycoproteins impacting host receptor-binding and/or differences in the
407 encapsidated cargo, e.g., packaging of host ribosomal components (38-40). Furthermore, as our
408 results suggest, another property that might confer unique cell-type-specific advantages to viruses
409 is the differential modification of the virion RNA. Indeed, recent evidence shows that modifications
410 like N^6-methyladenosine (m6A) and 5-methylcytosine (m5C) can regulate viral RNA functionality
411 in the cell (30, 41). Therefore, it is possible that such modifications also influence the infectivity of
412 progeny viruses produced from said cells. However, how these modifications affect virus
413 replication in a cell is still an open question. Indeed, information regarding the functional
414 consequence of m5C or other RNA modifications on viral RNA is limited. Thus, while we may
415 draw certain conclusions based on our current knowledge of known eukaryotic RNA
416 modifications, the potential implications of arbovirus RNA methylation may be broader than we

are currently able to anticipate (42). We can hypothesize that differential viral methylation may alter host responses to infection, in that depending on the host or cell type, as well as the genomic context of methylation, presence or absence of m5C may either allow detection by and/or provide a mechanism of escape from RNA-binding proteins (e.g., Dicer, RIG-I, MDA5, TLRs, APOBEC3) involved in virus restriction or non-self RNA recognition that trigger downstream immune signaling and interferon production (43). Differential modifications of viral RNA may thus also regulate different cytological outcomes of arboviruses infection of arthropod and vertebrate cells i.e. persistence versus cell death.

It remains to be seen whether or not one or more of these situations occur during pathogen blocking and if W- and W+ viruses trigger differential innate immune responses in vertebrate cells. Based on our data, we propose a model in which our current estimates of m5C residues on W- viruses represent the "wild-type" epitranscriptome of mosquito-derived alphavirus. In naïve vertebrate cells, the presence of these signatures allows viruses to replicate efficiently following successful evasion of host innate immunity. In contrast, m5C hypomethylation of W+ viruses renders them more susceptible to host-induced restriction, thus impacting their ability to propagate. Aside from heightened immune susceptibility, the decreased fitness of hypomethylated W+ viruses could also result from reduced incoming viral RNA stability and/or translation.

Given that pharmacological inhibition of MTase activity impacts virus spread in mosquito cells, it is likely that W+ virus hypomethylation also influences dissemination in arthropod cells (1). However, it is also possible that other factors contribute to the reduced fitness of W+ viruses. In particular, our LC-MS/MS analyses suggest increased heterogeneity in m7G moiety abundance on W+ virion RNA, indicating either difference in abundance of internal m7G methylation signatures or a potential imbalance in viral RNA capping in the presence of *Wolbachia.* In addition, past work has shown that SINV populations derived from different hosts vary with regard to the ratios of capped and non-capped SINV RNA (44). Despite being important for alphavirus replication, non-capped SINV RNA alone are compromised in their ability to undergo translation, are more susceptible to RNA decay machineries, and have been shown to induce elevated innate immune response, all of which might contribute to the observed loss in infectivity.

Finally, the data presented here implicating epitranscriptomic regulation of alphaviruses unlocks multiple avenues of investigation, which include, but are not limited to the following. First, it is crucial to determine the genomic context of m5C and other RNA modifications on viral RNA for different hosts, cell types, and infection timeline, which may be achieved by long-read, direct RNA sequencing from virus-infected cells. Doing so would allow sequence-specific mapping of these signatures and help address whether virus infection is regulated solely via targeting viral RNA by cellular MTases. Furthermore, deriving mapping information might inform us whether modifications are directed to specific RNA elements that result in spatiotemporal changes in RNA structure and altered base-pairing, thus regulating virus RNA polymerase fidelity and/or translation in the cell. Additional areas of inquiry involve identifying cellular pathways responsible for determining the fate of W+ viruses and characterizing the functional consequences of abolishing highly conserved m5C residues on the viral RNA. This would allow further exploration into the effect of these signatures on RNA stability, gene expression, and/or packaging across arthropod and vertebrate cells. Lastly, unlike m6A-modifications, little is known regarding how m5C signatures are interpreted, i.e., how they are "read," "maintained," and "erased," in mammalian and, to an even lesser extent, in arthropod cells (42). Promising candidates include m5C-binding "reader" proteins ALYREF and YBX1, which function alongside the known cellular m5C MTase NSUN2 to influence mRNA nuclear transport and stability (45, 46). Following approaches described in recent studies, identification of these RNA-binding proteins, either viral

465 or host-derived, may be achieved via affinity-based immunoprecipitation of viral RNA and form
466 the basis of future studies (47).

467 Like most other RNA viruses, Alphaviruses are limited in their coding capacity and are known to
468 alter their genome structure under various cellular conditions to regulate aspects of their
469 replication as a way to maximize viral genome functionality. Echoing this idea, the findings
470 presented in this study add to our understanding of regulatory mechanisms adopted by these
471 viruses to successfully navigate within and transition between vertebrate and arthropod host
472 species.

473 **Materials and Methods**

474 **Insect and Mammalian Cell Culture**

475 RML12 *Aedes albopictus* cells with and *Wolbachia*-free *w*Mel was grown at 24 ºC in Schneider's
476 insect media (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum
477 (Corning), 1% each of L-Glutamine (Corning), non-essential amino acids (Corning), and penicillin-
478 streptomycin-antimycotic (Corning). C710 *Aedes albopictus* cells with and *Wolbachia*-free were
479 grown at 27 ºC under 5% ambient CO_2 in 1X Minimal Essential Medium (Corning) supplemented
480 with 5% heat-inactivated fetal bovine serum (Corning), 1% each of L-Glutamine (Corning), non-
481 essential amino acids (Corning) and penicillin-streptomycin-antimycotic (Corning). Vertebrate
482 baby hamster kidney fibroblast or BHK-21 cells were grown at 37 ºC under 5% ambient CO_2 in
483 1X Minimal Essential Medium (Corning) supplemented with 10% heat-inactivated fetal bovine
484 serum (Corning), 1% each of L-Glutamine (Corning), non-essential amino acids (Corning) and
485 penicillin-streptomycin-antimycotic (Corning). JW18 *Drosophila melanogaster* cells with and
486 without *Wolbachia w*Mel were grown at 24 ºC in Shields, and Sang M3 insect media (Sigma-
487 Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 1% each of L-Glutamine
488 (Corning), non-essential amino acids (Corning), and penicillin-streptomycin-antimycotic
489 (Corning).

490 **Mosquito rearing and blood meals**

491 *Aedes aegypti* mosquitoes either -infected and -uninfected with *Wolbachia* (*w*AlbB strain)
492 (generously provided by Dr. Zhiyong Xi, Michigan State University, USA), were reared in an insect
493 incubator (Percival Model I-36VL, Perry, IA, USA) at 28 °C and 75% humidity with 12 h light/dark
494 cycle. Four to six-day-old, mated female mosquitoes were allowed to feed for one hour on
495 approximately 10^8 PFUs of SINV (TE12-untagged) containing citrated rabbit blood (Fisher
496 Scientific DRB030) supplemented with 1mM ATP (VWR), and 10% sucrose using a Hemotek
497 artificial blood-feeding system (Hemotek, UK) maintained under a constant temperature of 37 °C.
498 Engorged mosquitoes were then isolated and reared at 28 °C in the presence of male mosquitoes.
499 For harvesting whole tissues, mosquitoes were harvested 5-7 days post blood meal before being
500 snap-frozen in liquid nitrogen and stored at −80 °C before further processing. For salivary gland
501 dissections, mosquitoes were kept immobilized on ice before dissection. Collected salivary gland
502 tissues were washed three times in a cold, sterile saline solution (1XPBS) before being snap-
503 frozen in liquid nitrogen and stored at −80 °C before further processing. Three salivary glands
504 were pooled to create each biological replicate. Samples for qPCR and qRT-PCR were
505 homogenized in TRiZOL (Sigma Aldrich) reagent and further processed for nucleic acid
506 extractions using manufacturer's protocols.

507 **Virion RNA extraction and transfection**

508 Virion encapsidated RNA was extracted from viruses (SINV-nLuc) were purified over a 27%
509 sucrose cushion using TRiZOL reagent (Sigma Aldrich) using the manufacturer's protocol. Post
510 extraction, RNAs were DNase (RQ1 RNase-free DNase, NEB) treated according to the
511 manufacturer's protocol to remove cellular contaminants, and viral RNA copies were quantified

512 via quantitative RT-PCR using primers probing for SINV nsP1 and E1 genomic regions (Table
513 S1) and a standard curve comprised of linearized SINV infectious clone containing the full-length
514 viral genome. To determine infectivity or replication kinetics of Sindbis virion RNA, equal copies
515 of virion isolated RNA (10^5 copies), quantified using qRT-PCR, were transfected into BHK-21 cells
516 in serum-free Opti-MEM (Gibco). Transfection was carried out for 6 hours before the transfection
517 inoculum was removed, and overlay was applied. Cells were fixed post-transfection using 10%
518 (v/v) formaldehyde and stained with crystal violet to visualize plaques. To maximize the production
519 of infectious units, equal mass (1 µg) of virion (SINV-nLuc) isolated RNA derived from JW18 fly
520 cells was transfected into BHK-21 cells. Transfection was carried out for 6 hours before the
521 transfection inoculum was removed, and overlay was applied. Cells were fixed 48 (SINV) or 72
522 (CHIKV) hours post-transfection using 10% (v/v) formaldehyde and stained with crystal violet to
523 visualize plaque-forming units.

524 **Viral replication assays**

525 The viral genome and sub-genome translation were quantified using cellular lysates following
526 synchronized infections with reporter viruses (SINV-nLuc) or transfections with virion-derived
527 RNA from the aforementioned viruses. At indicated times post-infection, samples were collected
528 and homogenized in 1X Cell Culture Lysis Reagent (Promega). In addition, samples were mixed
529 with NanoGlo luciferase reagent (Promega), incubated at room temperature for three minutes
530 before luminescence was recorded using a Synergy H1 microplate reader (BioTech instruments).

531 **Virus infection in cells and progeny virus production**

532 Virus stocks were generated from RML12, C710, or JW18 cells, either with or without *Wolbachia*
533 or overexpressing DNMT2 orthologs by infecting naïve cells with the virus at an MOI of 10. In all
534 cases, serum-free media was used for downstream virus purification. Media containing virus was
535 collected five days post-infection for alphaviruses SINV (SINV-nLuc, TE12-untagged, TE3'2J-
536 GFP, and TE3'2J-mCherry) and CHIKV (CHIKV18125-capsid-mKate). Virus stocks were
537 subsequently purified and concentrated by ultracentrifugation (43K for 2.5 h) over a 27% (w/v)
538 sucrose cushion dissolved in HNE buffer. Viral pellets were stored and aliquoted in HNE buffer
539 before being used for all subsequent experiments.

540 **DNMT2 overexpression in arthropod cells**

541 *Aedes albopictus AMt2* coding region was subcloned into PCR 2.1 TOPO vector (Invitrogen) by
542 PCR amplification of cDNA generated using reverse transcribed from total cellular RNA isolated
543 from C636 *Aedes albopictus* cells using Protoscript II RT (NEB) and oligo-dT primers (IDT). The
544 coding region was validated via sequencing before being cloned into the pAFW expression vector
545 (1111) (Gateway Vector Resources, DGRC), downstream of and in-frame with the 3X FLAG tag
546 using the native restriction sites *AgeI* and *NheI* (NEB). Expression of both FLAG-tagged
547 AaDNMT2 in mosquito cells was confirmed using qRT-PCR and Western Blots using an anti-
548 FLAG monoclonal antibody (SAB4301135 - Sigma-Aldrich, 1:3000 dilution in 2% Milk in 1X TBS
549 + 1% Tween-20) (Fig 4A). In addition, catalytic MTase mutant of *AMt2* (*AMt2*-C78G*)* was
550 generated via site-directed mutagenesis (NEB, Q5 Site-Directed Mutagenesis Kit), using primers
551 listed in the primer table (Table S1). *Drosophila Mt2* (FBgn0028707) cDNA clone (GM14972)
552 obtained from DGRC (https://dgrc.bio.indiana.edu/) was cloned into the pAFW expression vector
553 (1111) with an engineered *SalI* site (Gateway Vector Resources, DGRC) downstream of and in-
554 frame with the 3X FLAG tag using Gibson assembly (HiFi DNA assembly mix, NEB). Expression
555 of FLAG-tagged DNMT2 in fly cells was confirmed using qRT-PCR and Western Blots using an
556 anti-FLAG monoclonal antibody (SAB4301135 - Sigma-Aldrich, 1:3000 dilution in 2% Milk in 1X
557 TBS + 1% Tween-20). Catalytically inactive *Mt2* (*Mt2* C78A) variant was generated via site-
558 directed mutagenesis (NEB, Q5 Site-Directed Mutagenesis Kit) using primers listed in the primer
559 table (Table S1).

560 **Immunoprecipitation of DNMT2-viral RNA complexes**

561 JW18 fly cells and C710 mosquito cells were transfected with expression vectors FLAG-Mt2 and
562 FLAG-AMt2 respectively for 48 hours before infection with SINV at MOI of 10. Control cells were
563 transfected with the empty vector plasmid FLAG-empty. In addition, cells were treated for
564 approximately 18h with 5 μM 5-Azacytidine to covalently trap Mt2 or AMt2 with its target cellular
565 RNA before RNA immunoprecipitation using an anti-FLAG antibody following manufacturer's
566 protocols (SAB4301135 - Sigma-Aldrich, 1:100 dilution) (23).

567 **Real-time quantitative RT-PCR analyses**

568 Following total RNA extraction using TRiZOL reagent, cDNA was synthesized using MMuLV
569 Reverse Transcriptase (NEB) with random hexamer primers (Integrated DNA Technologies).
570 Negative (no RT) controls were performed for each target. Quantitative RT-PCR analyses were
571 performed using Brilliant III SYBR Green QPCR master mix (Bioline) with gene-specific primers
572 according to the manufacturer's protocol and the Applied Bioscience StepOnePlus qRT-PCR
573 machine (Life Technologies). The expression levels were normalized to the endogenous 18S
574 rRNA expression using the delta-delta comparative threshold method (ΔΔCT). Fold changes were
575 determined using the comparative threshold cycle (CT) method (Table S1). Efficiencies for primer
576 sets used in this study have been validated in our previous study (1).

577 **DNMT2 inhibition in mosquito cells**

578 Inhibition of *Aedes* DNMT2 activity in C710 cells was achieved using RNA and DNA cytosine
579 methyltransferase inhibitors, 5-aza-cytidine (5-AZAC, Sigma-Aldrich) and 5-deoxy-azacytidine
580 (DAC-5, Sigma-Aldrich). In each case, *Aedes albopictus* C710 cells were treated overnight with
581 media containing either 5μM inhibitor diluted in Dimethyl sulfoxide (DMSO) or DMSO alone. Due
582 to the poor stability of 5-AZAC, media containing fresh inhibitors was added every day post-
583 infection (48).

584 **Live cell imaging**

585 Live-cell imaging experiments were carried out using a setup similar to our previous study (1).
586 The growth of fluorescent reporter viruses in *Aedes albopictus* (C710) cells was monitored using
587 Incucyte live-cell analysis system (Essen Biosciences, USA). *Aedes albopictus* C710 cells were
588 grown under standard conditions as described earlier under 5% ambient CO_2 at 27 ºC. Cells were
589 plated to 75-80% confluency in 96-well plates to separate adjacent cells and preserve cell shape
590 for optimal automated cell counting. To synchronously infect cells virus was adsorbed at 4ºC. Post
591 adsorption, cell monolayers were extensively washed with cold 1XPBS to remove any unbound
592 virus particles, followed by the addition of warm media (37ºC) to initialize virus internalization and
593 infection. Cells per well were imaged and averaged across four distinct fields of view, each placed
594 in one-quarter of the well every 2 hours throughout the infection. Total fluorescence generated by
595 cells expressing the red fluorescent reporter mKate was calculated and normalized by the cell
596 number for every sample. A manual threshold was set to minimize background signal via
597 automated background correction at the time of data collection. Following the acquisition, data
598 were analyzed in real-time using the native Incucyte ® Base Analysis Software.

599 **Quantification of RNA modification by LC-MS/MS**

600 Total RNA (3-7μg) was digested by nuclease P1 (10 Units) at 50°C for 16 hr. Additional Tris pH
601 7.5 was then added to a final concentration of 100 mM to adjust, followed by the addition of calf
602 intestinal alkaline phosphatase (CIP, NEB, 2Units). The mixture was incubated at 37°C for 1 hour
603 to convert nucleotide 5'-monophosphates to their respective nucleosides. Next, 10μl of RNA
604 samples were diluted to 30 μL and filtered (0.22 μm pore size). 10μL of the sample was used for
605 LC-MS/MS. Briefly, nucleosides were separated on a C18 column (Zorbax Eclipse Plus C18

column, 2.1 x 50mm, 1.8 Micron) paired with an Agilent 6490 QQQ triple-quadrupole LC mass spectrometer using multiple-reaction monitoring in positive-ion mode. The nucleosides were quantified using the retention time of the pure standards and the nucleoside to base ion mass transitions of 268.1 to 136 (A), 244.1 to 112 (C), 284.2 to 152 (G), 258 to 126 (m3C and m5C), 282.1 to 150 (m6A), 298 to 166 (m7G). Standard calibration curves were generated for each nucleoside by fitting the signal intensities against concentrations of pure-nucleoside preparations. The curves were used to determine the concentration of the respective nucleoside in the sample. The A, G, and C standards were purchased from ACROS ORGANICS; m5C was purchased from BioVision; m7G, m1G, and m3C were purchased from Carbosynth, m6G and m6A were purchased from Berry's Associates, and m1A was from Cayman Chemical Company. The modification level on the nucleosides was calculated as the ratio of modified: unmodified.

Data availability

The full Incucyte dataset is available in the form of an excel sheet labeled Dataset S1.

Statistical analyses of experimental data

All statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA).

Graphics

Graphical assets made in © BioRender - biorender.com.

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