Manuscript Number: NMICROBIOL-25030831-T

Original Title: The mosquito let-7 microRNA inhibits flavivirus replication by targeting the viral genome and has antiviral therapeutic potential

New Title: Infection-induced mosquito let-7 targets flaviviral RNA to suppress translation and enable miRNA-based intervention

We sincerely thank all reviewers for their thoughtful and constructive comments, which have significantly improved our manuscript. We have carefully revised the manuscript taking into account of all the reviewers' comments. Below, we address each comment in detail. The reviewers' comments are quoted in bold and our responses follow in plain text.

Replies to Reviewer #1:

1. Cui et al. identify miRNA, let-7, as a repressor of flavivirus infection in mosquitos. They uncover that it functions by directly targeting and degrading DENV2, DENV4 and ZIKV genomes. They show that mosquitos that overexpress let-7 upon a blood meal (use of a blood-induced let-7 is nice!), had significantly reduced viral loads and prevented viral transmission to mice. Last, when they provided a let-7 agomir to mice they saw reduced Dengue virus infection in mice.

Response: We thank the reviewer for the positive evaluation and for recognizing the novelty and significance of our study.

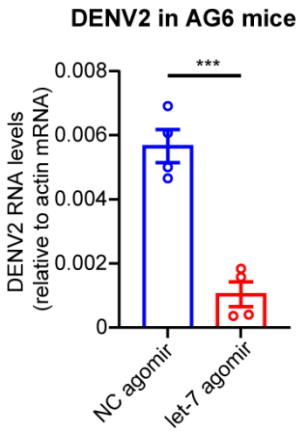
2. The model for let-7 is nice, but the data used to support the model is weak-to-moderate and requires higher rigor with more appropriate data presentation. As example, all imaging has a single image presented. There should be quantification from multiple images representative of multiple replicates in multiple experiments.

Response: We apologize that this was not clearly described in our original submission, which may have led to this misunderstanding. To address the reviewer's concern, we emphasize that the images shown in new **Fig. 1e**, **1g**, and **2i** are representative examples selected from three

independent experimental repeats with similar results. For each experiment, multiple images were taken, and the images shown are intended as representative visual examples to qualitatively illustrate the observed experimental effects. In the revised manuscript, we have added explicit statements in the figure legends to clarify the number of biological replicates and the number of experimental repeats. The quantification of viral load and infection intensity shown in **Figs. 1h**, **1i**, and others was not derived from image-based analysis but was independently measured using RT-qPCR from RNA extracted from cultured cells. We have revised the figure legends and Methods section to explicitly describe this, to avoid any misunderstanding about how quantitative data were obtained. We hope this clarification addresses the reviewer's concern regarding data rigor and reproducibility.

3. The axes for graphs are drawn in a way to exaggerate the data- Figure 5b is comparing 0.00006 and 0.00001 for RT-qPCR. This is within error range for RT-PCR data. Further, it only has 3 replicates, which is very underpowered for a mouse experiment. Mouse experiments should be experimentally repeated multiple times with multiple replicates during each experiment to account for biological variability (especially during viral infection). DENV2 RNA detection is extremely low for a AG6 mouse infection, but as graphed it is hard to compare to other studies. Typically the field uses standard curves of PFU equivalents to allow for a better assessment across studies.

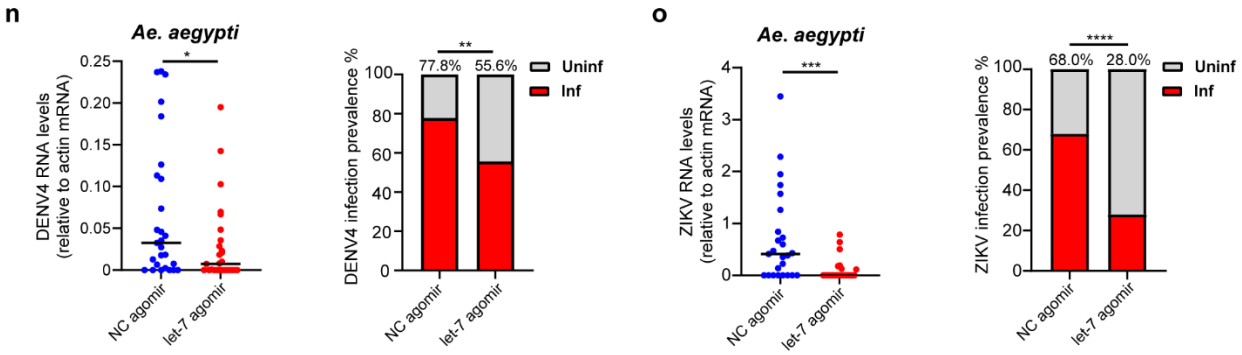
Response: We apologize for not presenting this data clearly enough in the original submission. To address the reviewer's concern regarding biological variability and low viral RNA levels in **Fig. 5b**, we have repeated the mouse infection experiment, with each experiment including at least four to five mice per group to ensure sufficient biological replicates. For improved clarity, we have now revised **Fig. 5b** using representative data from an independent experiment with higher DENV2 titers, which more clearly demonstrate the inhibitory effect of let-7 treatment (see below Figure or **Fig. 5b**).

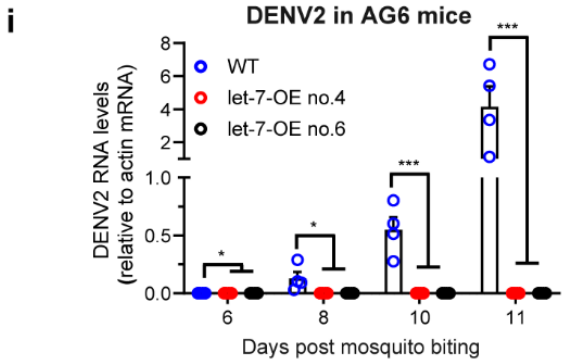


Data are now presented as mean ± s.e.m. Recent studies have also used RT-qPCR for sensitive quantification of DENV RNA in mouse models (e.g. Zhang et al., ***Cell***, 2022; 185(14):2510-2522.e16. Chen et al., ***EMBO Rep***, 2022; 23(11):e55671.), following similar approaches for viral RNA quantification. We have also updated the figure legend and Methods section to include this clarification. We hope these revisions substantially clarify the data presentation and rigorously address the reviewer's valid concerns about both data presentation and experimental rigor.

4. The axes for most graphs are extremely misleading to emphasize small differences (i.e. Figure 1H and 1J 2E and 2F, amongst many others, Figure 3F and 3G). Statistics are not provided on all graphs (i.e. Figure 2H and 2I, Figure 3I).

Response: We thank the reviewer for highlighting these important points. We have now included statistical analysis for Fig. 2h, 2i (new **Fig. 2n** and **2o**), and 3i, with significance values clearly indicated in the revised figures and legends (see below Figures or **Fig. 2n**, **2o** and **3i**).





Regarding the y-axis scaling, we used scientific notation (power of 10) in Fig. 1h, 1i, 2h, and 2k to present viral RNA levels in a more concise and readable format, following conventions used in recent high-impact publications (e.g. Zhang et al., ***Science***, 2024; 384(6693):eadn9524). We would like to emphasize that the observed differences in these figures represent biologically meaningful and statistically significant reductions in viral loads, ranging from ~70% to over 99% relative to controls, as reflected by both the RT-qPCR quantification and indicated by the descending arrow markers in the figures.

5. Number of replicates and number of times experiment completed are not reported. Number of mosquitos in survival and infection prevalence experiments are not reported. Again, all image analysis, especially when using as an orthogonal measurement of viral load, should be accompanied with quantification that represents multiple replicates and experiments.

Response: We thank the reviewer for pointing out the missing reports of experiments times and mosquito numbers. In the revised manuscript, we have added details regarding the number of biological replicates, the number of independent experimental repeats, and the number of mosquitoes used in each survival and infection prevalence experiment. This information is now included in the corresponding figure legend (e.g., lines 798, 801, 824, 829, 869, 876, 896, 905, 907, 908, 913, 933, 946, and 958).

Additionally, for all imaging-based assays used as orthogonal measures of viral load, we now

explicitly state the number of experimental replicates in the figure legend. We would also like to clarify that certain images (e.g., **Fig. 1e**, **1f**, and **1g**) are intended as representative visual examples to qualitatively illustrate the observed experimental effects. The quantification of viral load and infection intensity shown in **Figs. 1h**, **1i**, and others was not derived from image-based analysis but was independently measured using RT-qPCR from RNA extracted from cultured cells. We have revised the figure legends and Methods section to explicitly describe this, to avoid any misunderstanding about how quantitative data were obtained.

6. Further explanation of data is also exaggerated, words like "markedly reduced" are used to describe a 1-day survival advantage. The survival data (Figure 1J) in light of lifespan (Figure 3M) is not convincing.

Response: We have removed the word "markedly" from line 28, 127 in the revised manuscript to avoid overstating the survival data. To clarify, **Fig 3m** shows the baseline lifespan of WT and let-7-OE mosquitoes maintained on sugar meals without viral infection. This experiment was specifically designed to evaluate potential fitness costs associated with let-7 overexpression under normal physiological conditions. In contrast, **Fig. 1j** addresses the role of let-7 in protecting mosquitoes from DENV2-induced mortality following infection. These two survival datasets serve distinct experimental purposes—one assessing fitness under non-infected conditions, and the other measuring survival following viral challenge—and are therefore not contradictory. We have revised the manuscript to make these distinctions clearer.

7. Minor but viruses do not proliferate, they replicate.

Response: We carefully revised our manuscript to replace "proliferate" with "replicate" or "replication" consistently throughout the text (lines 103, 108, 116, 125, 130, 165, 233, 241, 270, 318, 803, 818 and 855).

8. Suggestion to reorder Figure 4 to be in order of discussion (switch c and d).

Response: Thanks for reviewer's suggestion. We have reordered panels 4c and 4d in new Fig. 4.

Replies to Reviewer #2:

1. The manuscript titled 'The mosquito let-7 microRNA inhibits flavivirus replication by targeting the viral genome and has antiviral therapeutic potential' by Cui et al addresses a possible role of a microRNA in regulating viral infection in mosquitoes.

The manuscript starts with the identification of microRNAs induced by DENV infection in the midgut of Aedes aegypti mosquitoes 24h after blood feeding. They then tested whether blocking any of the top 5 up-regulated miRNAs would affect DENV infection. They then went on to perform experiments suggesting direct targeting of the viral genome by binding to a complementary region in the viral genome. Finally, they demonstrate that overexpression of let-7 or treatment with the miRNA mimic can decrease DENV levels in mosquitoes and mice.

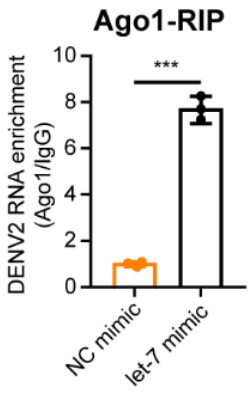
The authors perform an incredible amount of well-crafted experiments and the story is appealing at first sight. However, I think the major hypothesis in the manuscript that a miRNA can directly target a virus has irresoluble flaws.

Response: We thank the reviewer for the careful evaluation of our study and for raising this important conceptual concern. We agree that the claim that a host miRNA directly targets a viral genome requires particularly rigorous evidence. In revising the manuscript, we therefore carried out several additional experiments to strengthen the mechanistic basis of our conclusion

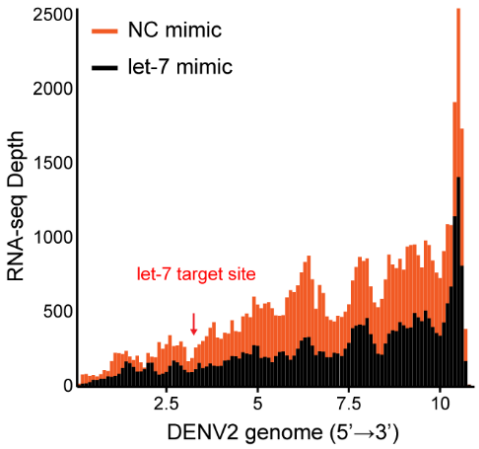
First, our data consistently support direct and sequence-specific recognition of viral RNA by let-7. In the original submission, luciferase reporter assays showed that let-7 represses reporters containing the predicted target sequences from the DENV2 NS1, DENV4 NS4B, and ZIKV NS3 coding regions, whereas mutation of these target sites abolished the repression (**Fig. 2c and 2d**). These data support sequence-specific interaction between let-7 and the viral RNA.

Second, as suggested by the reviewer, we further tested whether viral RNA is associated with the mosquito miRNA effector machinery. We performed Ago1-mediated RNA immunoprecipitation (RIP) using HEK293T cells expressing *Aedes aegypti* Ago1. In the presence of let-7 mimic, DENV2 RNA was significantly enriched in Ago1 immunoprecipitates compared

with the negative-control mimic (see below Figure or new **Fig. 2e**), providing additional evidence that let-7 guides Ago1 to viral RNA.

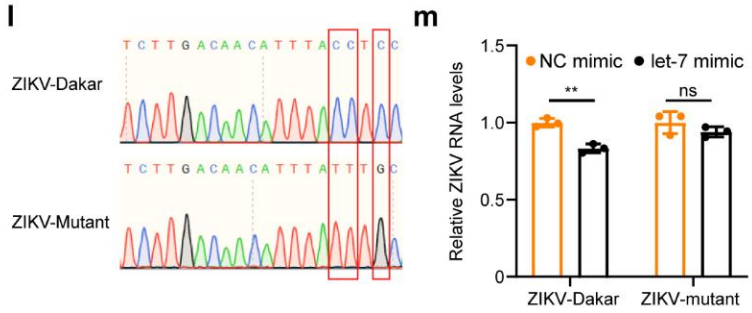


Third, we re-examined the downstream mechanism of repression. In our original manuscript, we inferred that let-7 might promote viral RNA degradation. To test this more directly, we performed RNA ligase-mediated 5′ RACE, but Sanger sequencing failed to detect DENV2-derived cleavage products. We then carried out degradome sequencing using RNA from C6/36 cells transfected with let-7 mimic. Reads mapped to the DENV2 genome were enriched mainly in the viral 3′ UTR and showed no accumulation around the predicted let-7 target sites (see below Figure or new **Fig. 2f**). Consistent with this, let-7 did not alter the transcript abundance of a Renilla reporter fused to the NS1 target sequence. Together, these new data indicate that let-7 does not measurably promote cleavage of viral RNA, but instead represses infection primarily by inhibiting viral RNA translation. We have revised the manuscript accordingly.



Fourth, to further establish that the antiviral effect depends on **base complementarity between let-7 and the viral genome**, we generated a mutant virus by introducing synonymous substitutions into the let-7 target site in a previously described ZIKV-Dakar infectious clone. In C6/36 cells, let-

7 mimic inhibited replication of wild-type ZIKV-Dakar, but failed to inhibit the mutant virus (see Figure below or new **Fig. 2l and 2m**). These results provide strong genetic evidence that let-7 inhibit virus replication through direct interaction with the predicted target sites.



In addition, the antiviral effect of let-7 was reproducibly observed across multiple experimental systems, including mosquito midguts, mosquito cell lines (C6/36 and Aag2), human liver cell lines (Huh7 and HepG2), and AG6 mice. This consistency across independent biological contexts argues against the possibility that the observed effects are coincidental or due solely to indirect off-target effects.

Finally, we expanded our conservation analysis across multiple DENV2, DENV4, and ZIKV strains/isolates (new Extended Data Fig. 4), showing that the predicted let-7 target sites are highly conserved. This strengthens the biological plausibility and potential functional relevance of direct viral targeting by let-7.

Taken together, the revised manuscript now provides substantially stronger evidence that mosquito let-7 directly recognizes flaviviral RNA in a sequence-specific manner and restricts infection primarily through **Ago1-associated translational repression**. We hope these new data and the corresponding revisions address the reviewer's concern.

2. Here are some major points in this regard:

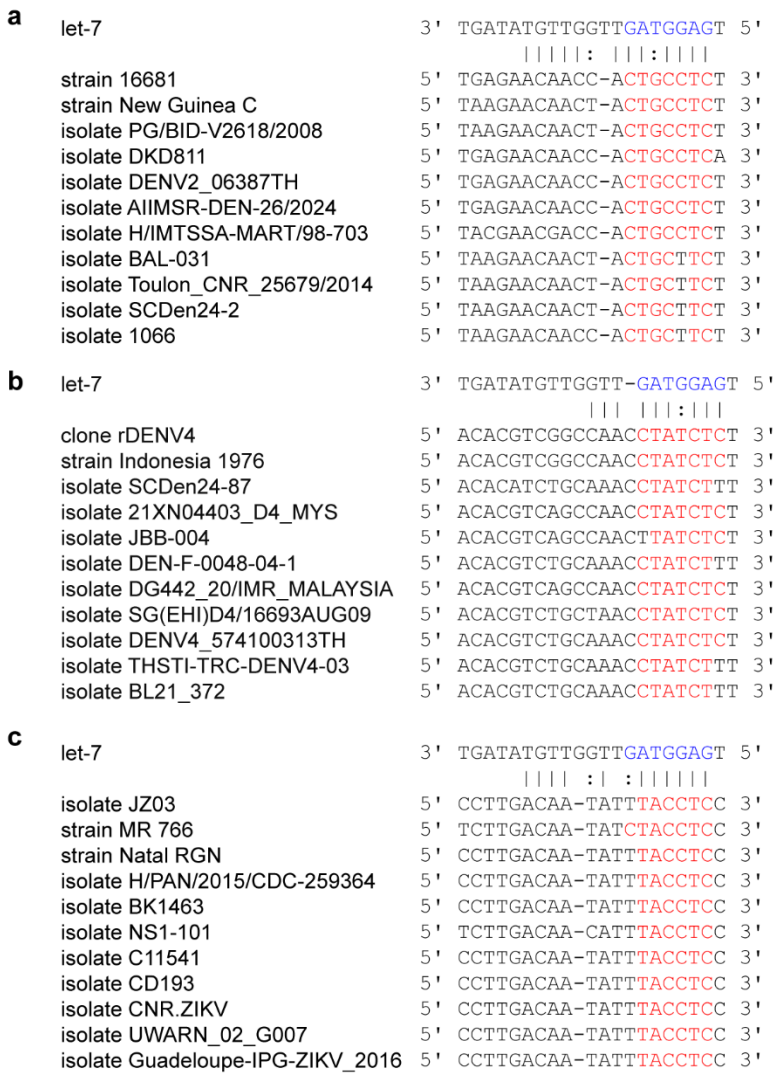
1) A single nucleotide substitution on a 10 nt stretch could render the viral RNA immune to a single miRNA sequence. In addition, the manuscript suggests that targeting is achieved in DENV2, DENV4 and ZIKV in different regions of the genome. This is strange since it would be difficult to think about a miRNA selected to target a sequence commonly found in different viruses. The limited data on the conservation of these regions is weak since it does

not show a complete picture. O good analysis of strains of DENV within each genotypes should be shown as well as across the 4 types. Therefore, there is no indication that this site could not easily mutate and escape miRNA silencing. Therefore, the observed targeting is likely a coincidence and not a real mechanism of defense utilized by the mosquito.

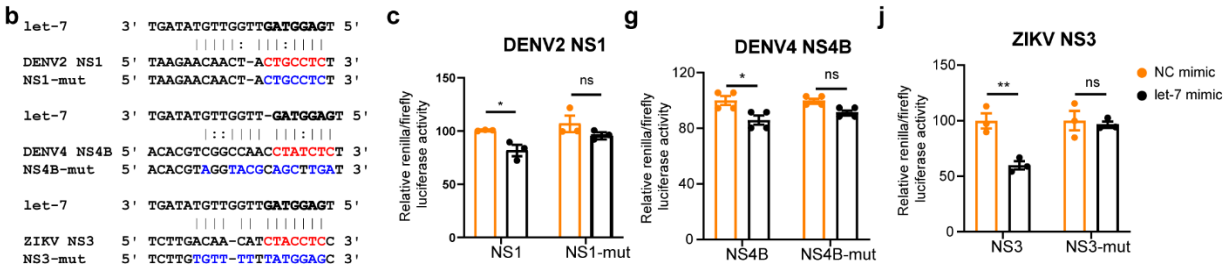
Response: We thank the reviewer for this important comment regarding viral sequence variability and the potential for escape from miRNA-mediated targeting. We agree that viral genomes can evolve to evade sequence-specific repression, and we do not claim that let-7-mediated restriction is evolutionarily escape-proof. Rather, our conclusion is that mosquito let-7 recognizes specific flaviviral RNA sequences in a sequence-dependent manner and thereby contributes to antiviral defense under the conditions examined here.

First, we respectfully note that animal miRNAs typically do not require perfect complementarity across the full target site. Instead, target recognition is driven primarily by the seed region, with some tolerance for mismatches outside this core. Thus, a single nucleotide substitution does not necessarily abolish miRNA-mediated repression, particularly if seed pairing is maintained. In addition, our data do not suggest that let-7 was selected to bind one universally shared sequence across all flaviviruses. Rather, let-7 recognizes **distinct target sites in different viral genomes** (DENV2, DENV4, and ZIKV), consistent with the well-established principle that one miRNA can regulate multiple unrelated RNAs through partial complementarity.

Second, in response to the reviewer's request, we substantially expanded our sequence analysis. In the revised manuscript, we analyzed a larger collection of DENV2, DENV4, and ZIKV strains/isolates from diverse geographic origins and time points. These analyses show that the predicted let-7 target sites in the **DENV2 NS1**, **DENV4 NS4B**, and **ZIKV NS3** regions are highly conserved among the strains examined within each virus group (see below Figure or new **Extended Data Fig. 4**). We agree that conservation alone cannot exclude the possibility of future escape, but it does argue strongly against the idea that the identified sites are random or isolated coincidences.



Third, the functional relevance of these target sites is supported by multiple independent experiments. In our dual-luciferase reporter assays, let-7 repressed constructs carrying the predicted viral target sequences, whereas mutation of these sites abolished repression (see below Figure or new **Fig. 2b, 2c, 2g and 2j**). This is a widely accepted method to validate direct miRNA-RNA targeting interactions. We further showed by **Ago1-RIP** that DENV2 RNA is enriched in *Aedes* Ago1 complexes in the presence of let-7 mimic (new **Fig. 2e**), supporting direct association of viral RNA with the mosquito miRNA effector machinery. In addition, using a **ZIKV-Dakar infectious clone**, we introduced synonymous substitutions into the predicted let-7 target site and found that let-7 inhibited the wild-type virus but failed to inhibit the mutant virus (new **Fig. 2l,m**). These results provide direct genetic evidence that the antiviral effect depends on base complementarity between let-7 and the viral RNA.



Fourth, the antiviral phenotype is itself sequence selective rather than nonspecific. let-7 inhibited **DENV2, DENV4, and ZIKV**, but not **DENV1 or DENV3**, in both mosquito and cell-based assays. This selectivity is consistent with the presence or absence of predicted let-7 target sites in the corresponding viral genomes, and is difficult to reconcile with a purely coincidental or indirect effect.

Taken together, we respectfully disagree that the observed targeting is likely to be a coincidence. Although sequence-specific viral escape is in principle possible, the revised manuscript now provides stronger evidence that let-7 recognizes conserved sites in multiple flaviviruses and suppresses infection through a bona fide, sequence-dependent mechanism.

3. 2) Second, on the general concept of miRNA targeting a viral sequence, there is also a limitation due to the mechanism of silencing. In contrast to siRNAs whose silencing complex can catalyze multiple rounds of RNA cleavage, the miRNA silencing complex requires stoichiometric amounts of miRNA and target. This means that the amount of miRNAs should be compatible with the levels of viral RNAs increase logarithmically in infected cells. The up-regulation observed in the midguts exposed to DENV in about 4-fold, which would not significantly impact the virus. This would also lead to multiple off targets effects due to the sequestering of let-7 by viral RNAs thus releasing endogenous targets.

Response: We thank the reviewer for this thoughtful comment. We agree that the quantitative relationship between miRNA abundance and viral RNA burden is an important consideration when evaluating the biological plausibility of direct antiviral targeting.

First, we respectfully note that our revised data indicate that let-7 restricts flavivirus infection primarily through **Ago1-associated translational repression**, rather than through endonucleolytic

cleavage of viral RNA. We therefore do not argue that let-7 must match viral RNA copy number in a strictly stoichiometric manner to be effective. Instead, even a modest increase in let-7 abundance may be sufficient to reduce the efficiency of early viral protein production, thereby attenuating downstream replication. In this context, timing is likely critical: the induction of let-7 occurs during the early phase of infection in the mosquito midgut, when viral replication is being established, and interference at this stage can have amplified consequences for subsequent viral expansion. Importantly, previous studies have shown that miRNA-loaded RISC complexes (miRISC) can mediate multiple-turnover cleavage of target RNAs (Gregory et al., ***Cell***, 2005;123(4):631–640). This suggests that miRNAs can, in some contexts, exert effects beyond simple stoichiometric limitations.

Second, although let-7 is induced by approximately 4-fold in wild-type mosquito midguts following DENV infection (**Fig. 1b**),our gain- and loss-of-function experiments (agomir, antagomir, and transgenic overexpression) show that this change is biologically meaningful. Inhibition of let-7 consistently increased viral burden, whereas let-7 overexpression or mimic delivery reduced viral replication across multiple experimental settings, including mosquito midguts, mosquito cell lines, human liver cells, and AG6 mice (**Figs. 1-4**).

Finally, we thank the reviewer for raising the concern about off targets effects due to the sequestering of let-7 by viral RNAs thus releasing endogenous targets . To test whether the antiviral effect of let-7 might arise indirectly through altered mosquito immune signaling or broader changes in cellular homeostasis, we analyzed the transcription of key components of the Toll, Imd, and JAK–STAT pathways after let-7 repression and found no significant changes (**Extended Data Fig. 2**). In addition, simultaneous RNAi knockdown of *Rel1A*, *Rel2*, and *STAT* did not impair let-7-mediated antiviral activity in Aag2 cells (**Extended Data Fig. 3a–c**). These results argue that the antiviral effect of let-7 is not mediated through canonical mosquito immune pathways. Our data further argue against the antiviral phenotype being explained primarily by nonspecific physiological disruption. Midgut-specific overexpression of let-7 in transgenic mosquitoes did not produce detectable effects on survival, fecundity, or fertility (**Fig. 3k–m**), and transfection of let-

7 mimic did not reduce cell viability in C6/36 cells (**Extended Data Fig. 1c**), indicating that let-7 manipulation does not cause overt toxicity or major physiological impairment under our experimental conditions. Importantly, we have demonstrated that the antiviral effect of let-7 is strongly sequence dependent. Mutation of the predicted let-7 target sites abolished repression in reporter assays (**Fig. 2b**, **2c**, **2g** and **2j**), and synonymous mutation of the let-7 target site in the ZIKV infectious clone rendered the virus refractory to let-7-mediated inhibition (new **Fig. 2l** and **2m**). Together with the Ago1-RIP result showing enrichment of DENV2 RNA in *Aedes* Ago1 complexes in the presence of let-7 (new **Fig. 2e**), these findings support a direct interaction between let-7 and viral RNA, rather than an antiviral effect arising solely from indirect derepression of endogenous targets.

However, because the antiviral effect is abolished by disruption of the predicted viral target site, indirect effects caused by sequestration of let-7 cannot by themselves account for the observed phenotype. Taken together, our data support the conclusion that infection-induced let-7 exerts a biologically meaningful antiviral effect.

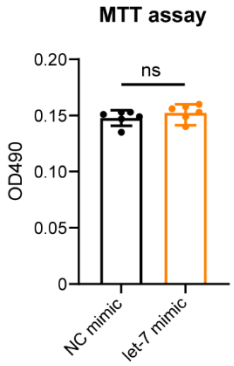
We have added clarification regarding the multiple-turnover potential of miRISC and the biological relevance of modest miRNA induction levels in the revised Discussion section (lines 326-328).

4. 3) In regard to the endogenous targets, this was never addressed by the authors. No measurement of cytotoxicity is made to ascertain that the antiviral effect is due to direct targeting to the virus and not a generic effect on the cell. Even without an effect on survival, the transgenic mosquito line may have a completely different environment in the gut. Indeed, the authors failed to show that there is direct targeting of the viral RNA by showing cleavage products or direct binding of Argonaute proteins to the viral genome mediated by let-7.

Response: We thank the reviewer for raising this important point. In the revised manuscript, we have added new experiments and clarified several points to address this issue.

First, regarding the possibility that the antiviral effect reflects nonspecific cytotoxicity rather

than sequence-specific antiviral activity, we performed an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in *Aedes*-derived C6/36 cells after transfection with 100 nM let-7 mimic. These experiments showed **no detectable reduction in cell viability** relative to the negative-control mimic (see below Figure or **Extended Data Fig. 1c**), arguing against a general cytotoxic effect.



In addition, in vivo overexpression of let-7 in the mosquito midgut did not measurably affect survival, fecundity, or fertility (**Fig. 3k-m**), demonstrating that blood meal-induced midgut-specific elevation of let-7 does not impose an overt fitness cost or broadly compromise host physiology under our experimental conditions.

Second, we examined whether the antiviral phenotype might arise indirectly through altered mosquito immune signaling rather than direct action on viral RNA. We found that repression of let-7 did not significantly alter the transcription of key components of the **Toll, Imd, or JAK–STAT pathways** (**Extended Data Fig. 2**). Moreover, simultaneous knockdown of ***Rel1A***, ***Rel2***, **and *STAT*** in Aag2 cells did not diminish let-7–mediated antiviral activity (**Extended Data Fig. 3a-c**). These results argue that the antiviral effect of let-7 is not simply a consequence of secondary activation of canonical mosquito immune pathways.

Third, in response to the reviewer's request for more direct mechanistic evidence, we performed several new experiments:

(1) We conducted *Aedes* Ago1-mediated RNA immunoprecipitation (RIP) and found that DENV2 RNA was significantly enriched in Ago1 immunoprecipitates in the presence of let-7 mimic (see new **Fig. 2e**), supporting direct association of viral RNA with the mosquito miRNA effector complex.

(2) We further tested whether let-7 induces cleavage of viral RNA. **RNA ligase-mediated 5′ RACE** did not recover DENV2-derived cleavage products, and **degradome sequencing** showed that reads mapping to the DENV2 genome were concentrated mainly in the viral 3′ UTR, without enrichment around the predicted let-7 target sites (see new **Fig. 2f**). These data indicate that let-7 does not measurably promote cleavage of viral RNA. Instead, consistent with our reporter assays, let-7 primarily acts through **translational repression**.

(3) To test whether antiviral activity depends on direct base pairing between let-7 and the viral genome, we introduced **synonymous substitutions** into the predicted let-7 target site in a previously described **ZIKV-Dakar infectious clone**. In C6/36 cells, let-7 mimic inhibited the replication of wild-type ZIKV-Dakar but failed to inhibit the mutant virus (see new **Fig. 2l and 2m**). These results provide direct genetic evidence that the antiviral effect depends on base complementarity between let-7 and the viral RNA.

Finally, the antiviral effect of let-7 was reproducibly observed across multiple independent systems, including ***Ae. aegypti*** **mosquitoes, Aag2 cells, C6/36 cells, human HepG2 and Huh7 cells, and AG6 mice**. Together with the absence of detectable cytotoxicity and the new Ago1-RIP and mutant-virus data, these results strongly support a **specific, direct, sequence-dependent antiviral mechanism**, rather than a generic effect on cellular health or gut homeostasis.

We hope these additional data and clarifications satisfactorily address the reviewer's concern.

5. Therefore, I think the results in the first part of the paper do not represent a meaningful biological response against the virus but are rather a spurious mechanism arising from the random presence of complementary site to let-7 in the genomes of some DENV and ZIKV strains.

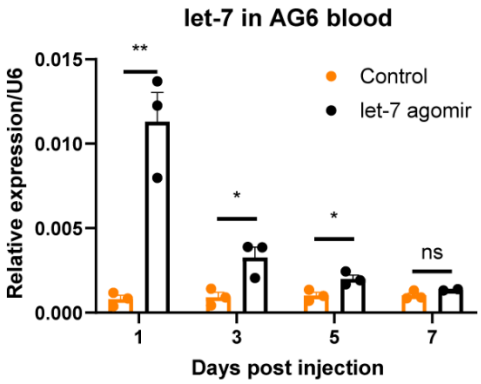
On the second part about the potential use of let-7 to target DENV and ZIKV, although it has potential, there are also major flaws. let-7 is a highly expressed miRNAs with important endogenous functions and its use as an artificial antiviral strategy would be risky. Unwanted off target effects would be very likely due to the interference with an important regulator of cellular processes. Even if this is not an issue, the use of a single miRNA would soon lead to the appearance of mutations on the target site in the virus as mentioned above.

Hence, taking all these points together, I have to recommend the rejection of the manuscript.

Response: We thank the reviewer for the evaluation and for articulating these concerns clearly. However, after performing several additional experiments in response to the reviewer's concerns, we respectfully disagree that the antiviral effect of let-7 is simply a spurious consequence of random complementarity. Rather, the revised data support the conclusion that let-7 constitutes a biologically meaningful host antiviral response.

First, our revised data support the conclusion that let-7-mediated restriction is a bona fide, sequence-dependent antiviral mechanism. The antiviral effect is not broadly nonspecific, but selective for viruses carrying predicted let-7 target sites: let-7 inhibited DENV2, DENV4, and ZIKV, but not DENV1 or DENV3, in both mosquito and cell-based assays. Moreover, reporter assays showed that mutation of the predicted target sites abolished repression, and synonymous mutation of the let-7 target site in the ZIKV infectious clone rendered the virus refractory to let-7-mediated inhibition (new **Fig. 2l** and **2m**). Together with the Ago1-RIP data showing enrichment of DENV2 RNA in *Aedes* Ago1 complexes in the presence of let-7 (new **Fig. 2e**), these results strongly support **a direct and sequence-specific interaction between let-7 and viral RNA**. We agree that sequence-specific antiviral mechanisms are, in principle, vulnerable to viral escape, but this does not negate their biological relevance. Rather, it places let-7 within a class of authentic host restriction mechanisms that act on specific viral sequences.

Second, regarding potential off-target or cytotoxic effects of let-7 agomir in mammalian models, we carefully monitored the physiological status of AG6 mice injected with *Aedes* let-7 agomir. Throughout the experimental period, we observed no adverse effects on diet, behavior, or body weight compared to control groups (**Extended Data Fig. 7**). In addition, the injected let-7 agomir in mice exhibited a transient presence, with detectable levels declining and markedly degrading 3 days post-injection (see below **Figure**), thus minimizing concerns about long-term persistence or unintended sustained effects in vivo. These data show that the let-7 antiviral effect in mice was achieved within a short experimental window, supporting the feasibility of transient delivery rather than sustained long-term exposure.



Third, we agree that therapeutic use of any endogenous miRNA requires careful evaluation of safety. However, the field of miRNA-based therapeutics is advancing rapidly, and several miRNA-based treatments have entered preclinical and clinical development for diverse diseases, including hepatitis C virus infection, vascular diseases, cardiac fibrosis, and various cancers. Notably, let-7 itself has been explored as a therapeutic candidate in oncology, with studies demonstrating its safety and efficacy when combined with drugs such as Erlotinib for treating non-small cell lung cancer (Stahlhut et al., ***Cell Cycle***, 2015;14(13):2171-80). These findings suggest that with appropriate dosing and delivery strategies, therapeutic manipulation of let-7 may be both feasible and safe.

Fourth, with respect to the concern that a single miRNA-based antiviral strategy may select for escape mutants, we agree that this is an important consideration. However, the predicted let-7 target sites in DENV2, DENV4, and ZIKV are highly conserved across the strains/isolates analyzed (**Extended Data Fig. 4**), supporting the current biological relevance of these interactions. More broadly, we view our study as establishing the principle that a mosquito miRNA can directly target flaviviral RNA and that this interaction can be harnessed experimentally to reduce infection and transmission. Future translational development would likely require optimized delivery, safety evaluation, and potentially combination strategies to minimize viral escape.

Taken together, we believe the revised manuscript supports two conclusions. First, infection-induced let-7 represents a biologically meaningful antiviral response in mosquitoes rather than a coincidental interaction. Second, exogenous let-7 delivery provides an initial proof-of-concept that this endogenous antiviral mechanism may be experimentally harnessed, while also highlighting

important challenges that will need to be addressed in future translational work. We have revised the text throughout to better reflect this balance.