

Reviewed Preprint

v1 • August 21, 2026

Not revised

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Funding: See page 15

Reviewing editor: Melody Man Hing Li, University of California, Los Angeles, United States

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# Macrodomain catalytic activity modulates Chikungunya virus dissemination and transmission potential in *Aedes* mosquitoes

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## eLife Assessment

This **important** study investigates how viral macrodomains in dual-host viruses functionally contribute to their infection in the vector, which was previously understudied. The findings that chikungunya virus (CHIKV) macrodomain mutants have unique impacts on virus replication in mammalian cells and in live mosquitoes are **convincing** and significant; however, the study can be strengthened by further investigating the role of ADP-ribose binding and potential other compensatory mutations. The work will be of interest to virologists and biologists who study macrodomains and ADP-ribosylation.

<https://doi.org/10.7554/eLife.112183.1.sa4>

## Abstract

Viral macrodomains are promising antiviral targets that counteract host ADP-ribosylation-mediated antiviral responses in mammals. However, their role in dual-host viruses within the mosquito vector remains largely unknown. Here, we investigated the role of the Chikungunya virus (CHIKV) nsP3 macrodomain catalytic activity by mutating the active-site asparagine 24 (N24). N24 mutant viruses rapidly selected for compensatory second-site mutations at aspartate 31 (D31) in mammalian Vero cells, while interferon-competent human cells favored reversion at position 24 instead, suggesting adaptive responses to macrodomain functional impairment. Catalytically inactive double-mutant viruses replicated poorly in interferon-competent human cells but comparably to wild-type in mosquito cells. In *Aedes* mosquitoes *in vivo*, macrodomain mutations modulated CHIKV replication and infectivity in a mutation-specific manner: one mutant reduced them while another enhanced them. Biochemical and structural analyses confirmed that these phenotypes arise in the context of a catalytically inactive macrodomain that retains ADP-ribose binding capacity, with D31 mutations lining the substrate exit path. These findings demonstrate that macrodomain catalytic activity modulates CHIKV dissemination and transmission potential in *Aedes* mosquitoes, revealing fundamental differences in host-dependent evolutionary advantage for a catalytically inactive viral enzyme.

## Introduction

The emergence and re-emergence of RNA viruses pose ongoing global public health threats, from pandemic coronaviruses to mosquito-borne arboviruses such as chikungunya, dengue, and Zika. Identifying conserved viral proteins tractable to therapeutic targeting is therefore a priority. Viral macrodomains are conserved enzymatic domains encoded within the nonstructural genes of alphaviruses, coronaviruses, rubella virus, and hepatitis E virus<sup>1,2</sup>, that have emerged as attractive

antiviral candidates<sup>3–6</sup>. These domains counteract host ADP-ribosylation-based antiviral defenses central to the mammalian innate immune response<sup>7–13</sup>, and recent identification of promising small-molecule inhibitors targeting the SARS-CoV-2 macrodomain<sup>14</sup> has provided proof-of-concept for this therapeutic strategy.

Viral macrodomains exhibit ADP-ribosylhydrolase catalytic activity, binding to mono- and poly-ADP-ribosylated proteins and cleaving the ADP-ribose modification, an “eraser” function that directly counters the activity of host polyadenosine diphosphate-ribose polymerases (PARPs)<sup>7–12</sup>. Stimulated by interferon (IFN) signaling, PARPs catalyze the addition of ADP-ribose moieties to viral and host target proteins as part of the antiviral response<sup>15–21</sup>, and viral macrodomains neutralize this defense by reversing these modifications<sup>22–24</sup>. Beyond catalytic erasure, macrodomains can also function as “readers”, binding ADP-ribosylation marks to facilitate protein interactions or to direct modified substrates toward other functional domains<sup>25</sup>. For viral macrodomains, the relative contribution of eraser versus reader activity to viral fitness has not been fully resolved and may critically depend on host context<sup>9</sup>.

This question is particularly relevant for dual-host arboviruses such as chikungunya virus (CHIKV), which must replicate alternately in mammalian vertebrate hosts and *Aedes* mosquito vectors, environments with fundamentally different immune systems and physiological conditions. In mammals, IFN signaling and PARP-mediated ADP-ribosylation are central components of the antiviral response, making macrodomain eraser activity functionally critical<sup>4,7,10–12,23,24,26</sup>. In mosquitoes, antiviral immunity is organized primarily around RNA interference rather than IFN signaling<sup>27–30</sup>, raising the fundamental question of whether macrodomain catalytic activity serves the same function, or any essential function, in the insect host. Despite the importance of this question for both basic virology and antiviral drug development, the role of viral macrodomains in the mosquito vector has remained almost entirely unexplored.

Comparative genomics provides an important clue. The catalytic asparagine at position 24 (N24), essential for ADP-ribosylhydrolase activity<sup>4,12,31–34</sup>, is invariably conserved across dual-host alphaviruses and other human-pathogenic viruses, but is consistently absent in mosquito-specific alphaviruses that are incapable of infecting vertebrates<sup>35,36</sup>. This recurrent loss strongly implies that the selective pressure maintaining macrodomain catalytic activity originates from the vertebrate host, while this pressure is relaxed in viruses replicating exclusively in mosquitoes. These observations raise the question of whether the mosquito host of a dual-host alphavirus such as CHIKV could similarly tolerate loss of macrodomain catalytic activity, and whether the macrodomain’s reader function might be sufficient to support viral replication in the absence of catalysis.

CHIKV is an ideal model to address these questions. A re-emergent human pathogen responsible for millions of cases of febrile arthralgia worldwide<sup>37</sup>, CHIKV is transmitted by *Aedes albopictus* and *Aedes aegypti* mosquitoes and has undergone substantial global expansion in recent decades, with ongoing outbreaks in tropical and subtropical regions. Its positive-sense RNA genome encodes four non-structural proteins (nsP1–nsP4) forming the viral replicase complex, and the structural proteins required for virion assembly<sup>37</sup>. Non-structural protein 3 (nsP3) has been implicated in mosquito vector specificity and neurovirulence<sup>38–45</sup>. Indeed, previous studies have shown that nsP3 as a whole dictates the ability of related alphaviruses to infect specific mosquito species. nsP3 also harbors the catalytically active macrodomain within its N-terminal 160 amino acids. The CHIKV macrodomain has well-characterized ADP-ribosylhydrolase activity<sup>9,11,12,33,34</sup>, however its function in the mosquito has not been examined *in vivo*.

Here, we investigated the role of the CHIKV nsP3 macrodomain during viral replication in mammalian and mosquito hosts. Using CHIKV as a model, we engineered recombinant viruses carrying mutations in the macrodomain that eliminate its catalytic activity. Attempts to propagate these viruses rapidly selected for second-site mutations at residue D31, which did not restore catalytic activity. The infection of mammalian cells and laboratory colonies of *Aedes* mosquitoes

with wild-type and mutant viruses reveals unexpected and host-dependent effects of macrodomain catalytic inactivation on viral fitness and demonstrates that the selective pressures acting on this domain differ fundamentally between mammalian and mosquito hosts.

## Results

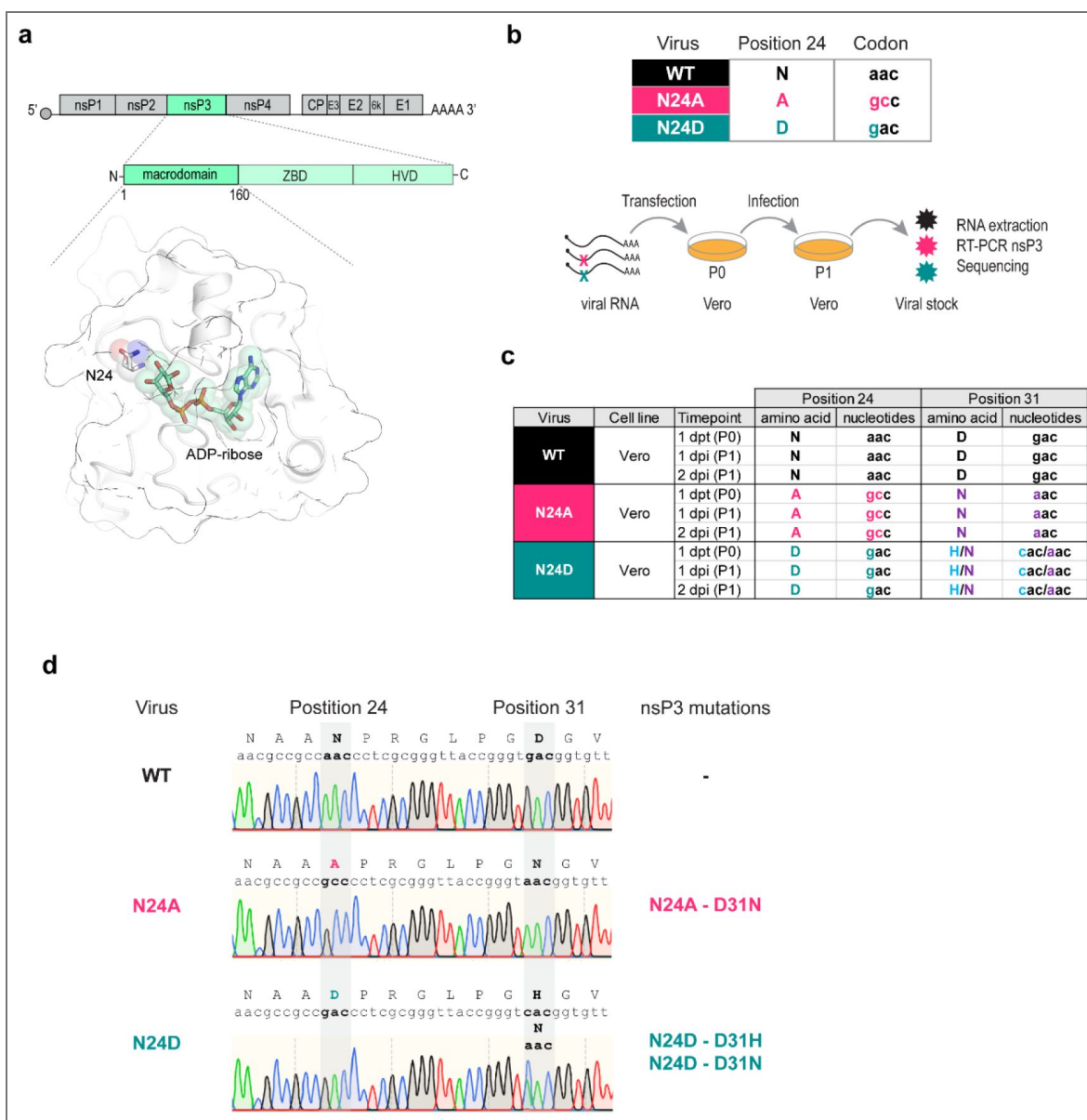
### N24 mutations in the CHIKV macrodomain catalytic site rapidly select for second-site mutations at residue D31

The asparagine at position 24 (N24) of the CHIKV nsP3 macrodomain is located at the active site and makes direct contacts with bound ADP-ribose<sup>31,34</sup> (Fig. 1a [↗](#)). Substitution of the equivalent asparagine in the SARS-CoV-2 macrodomain abolishes ADP-ribosylhydrolase catalytic activity<sup>4,11,33,34</sup>, establishing N24 as essential for catalytic function. To investigate the role of macrodomain catalytic activity in CHIKV replication, we introduced two mutations at this position into the infectious clone of the Caribbean CHIKV strain: N24A (asparagine to alanine) and N24D (asparagine to aspartic acid), both of which abolish macrodomain catalytic activity in the homologous SARS-CoV-2 system<sup>4</sup> (Fig. 1b [↗](#)). We included both mutations because the N24A substitution reduces thermodynamic stability in SARS-CoV-2, whereas N24D abolishes catalytic activity without destabilizing the protein<sup>4</sup>. To produce viral stocks, *in vitro* transcribed RNAs encoding WT, N24A, or N24D viruses were transfected into Vero cells. Cell culture supernatants were collected at one day post-transfection (P0) and used to infect fresh Vero cells. Supernatants from this passage were collected at one and two days post-infection (P1). Total RNA was extracted from supernatants at each timepoint, the nsP3 gene was amplified by RT-PCR, and the products were analyzed by Sanger sequencing (Fig. 1b [↗](#)).

Sequencing of the WT virus revealed no changes in nsP3 at any examined timepoint, confirming genetic stability of the WT sequence under these conditions (Fig. 1c, 1d [↗](#)). In contrast, both N24 mutant viruses rapidly acquired second-site mutations at residue 31 during viral replication, while the introduced mutations at position 24 remained stable. In the N24A mutant, aspartic acid at position 31 was replaced by asparagine (D31N). In the N24D mutant, position 31 acquired either a D31N (GAC to AAC) or D31H (GAC to CAC) substitution, with both variants detected as a mixed population (Fig. 1c, 1d [↗](#)). Consequently, while the N24A mutant yielded a genetically defined double mutant virus (N24A-D31N), the N24D mutant yielded a mixed population of N24D-D31N and N24D-D31H viruses. Because a second-site mutation at the equivalent position has been previously reported in Sindbis virus during replication of a macrodomain double mutant<sup>44</sup>, our results suggest that residue 31 represents a conserved site of genetic compensation across alphaviruses. This raises the question of whether the selective pressure driving D31 second-site mutation emergence operates similarly in immunologically relevant human cells with a competent innate immune response.

### N24 catalytic mutations impair CHIKV replication in interferon-competent human cells but not in mosquito cells

The rapid emergence of D31 second-site mutations during viral stock production in Vero cells suggested that N24 catalytic mutations impose a fitness cost during viral replication. To investigate whether this selective pressure operates in interferon-competent human cells, we transfected N24A and N24D viral RNAs into immune-competent A549 cells and monitored the genetic stability of the introduced mutations over time (Fig. 2a, 2b [↗](#)). For the N24A mutant, pure N24A sequence was detected at days 2 and 3 post-transfection, with no D31 second-site mutations emerging at any timepoint. At passage 1, a mixture of N24A and WT sequence was detected at position 24, indicating partial reversion toward the WT asparagine. For the N24D mutant, pure N24D sequence was detected at all four P0 timepoints, again with no D31 second-site mutations. At passage 1, a mixture of N24D and WT sequence was detected at position 24, consistent with partial reversion. In neither case did D31 second-site mutations emerge, in contrast to the rapid D31 emergence



**Figure 1. N24 mutations in the CHIKV macrodomain catalytic site rapidly select for second-site mutations at residue D31.**

**a**, Schematic of the CHIKV genome highlighting the nsP3 gene. The nsP3 protein comprises three domains: an N-terminal macrodomain (first 160 amino acids), a central zinc-binding domain (ZBD), and a C-terminal hypervariable domain (HVD). Structure of the CHIKV macrodomain (PDB 3GPO) highlighting the catalytic asparagine 24 (N24) and bound ADP-ribose. **b**, Point mutations were introduced into the CHIKV Caribbean infectious clone to generate two mutant viruses: N24A (asparagine to alanine) and N24D (asparagine to aspartic acid). For viral stock production, in vitro transcribed RNAs for WT, N24A, and N24D viruses were transfected into Vero cells. Cell culture supernatant was collected at one day post-transfection (P0) and used to infect fresh Vero cells. Supernatants from this passage were collected at one and two days post-infection (P1) and subjected to RNA extraction, RT-PCR amplification of the nsP3 gene, and Sanger sequencing. **c**, Summary of amino acid and nucleotide identities at residues 24 and 31 in WT, N24A, and N24D viruses at one day post-transfection (P0) and one and two days post-infection (P1) in Vero cells. **d**, Representative Sanger sequencing chromatograms from P1 viral stocks. The WT sequence shows no changes at position 24. N24A and N24D viruses show novel second-site mutations at residue 31 (D31N and D31H/N respectively).

observed in Vero cells. The low viral yields recovered from A549 transfections precluded the production of usable viral stocks. We therefore proceeded with the double mutant stocks produced in Vero cells (N24A-D31N and N24D-D31H/N) for all subsequent experiments.

To assess the replication capacity of the double mutant viruses in mammalian and mosquito cells, we performed growth curve experiments in A549 and U4.4 cells (Fig. 2c). In A549 cells, both N24A-D31N and N24D-D31H/N mutant viruses reached significantly lower viral titers than WT at multiple timepoints across the growth curve, demonstrating that loss of macrodomain catalytic activity impairs CHIKV replication in interferon-competent human cells. In contrast, in U4.4 mosquito cells, both mutant viruses replicated broadly comparably to WT across the time course, except for a significantly lower titer observed for N24D-D31H/N at 32 hours post-infection. The genetic stability of the introduced mutations in both viral stocks was confirmed by Sanger sequencing of nsP3 at 48 hours post-infection in both cell lines (Supplementary Fig. 1). Overall, these results demonstrate a host-dependent difference in the requirement for macrodomain catalytic activity: while it is important for viral replication in interferon-competent mammalian cells, it is largely dispensable in mosquito cells. This raised the question of whether macrodomain catalytic activity is similarly dispensable during CHIKV infection of *Aedes* mosquitoes *in vivo*, and whether the selective pressure driving D31 second-site mutation emergence operates in the mosquito host.

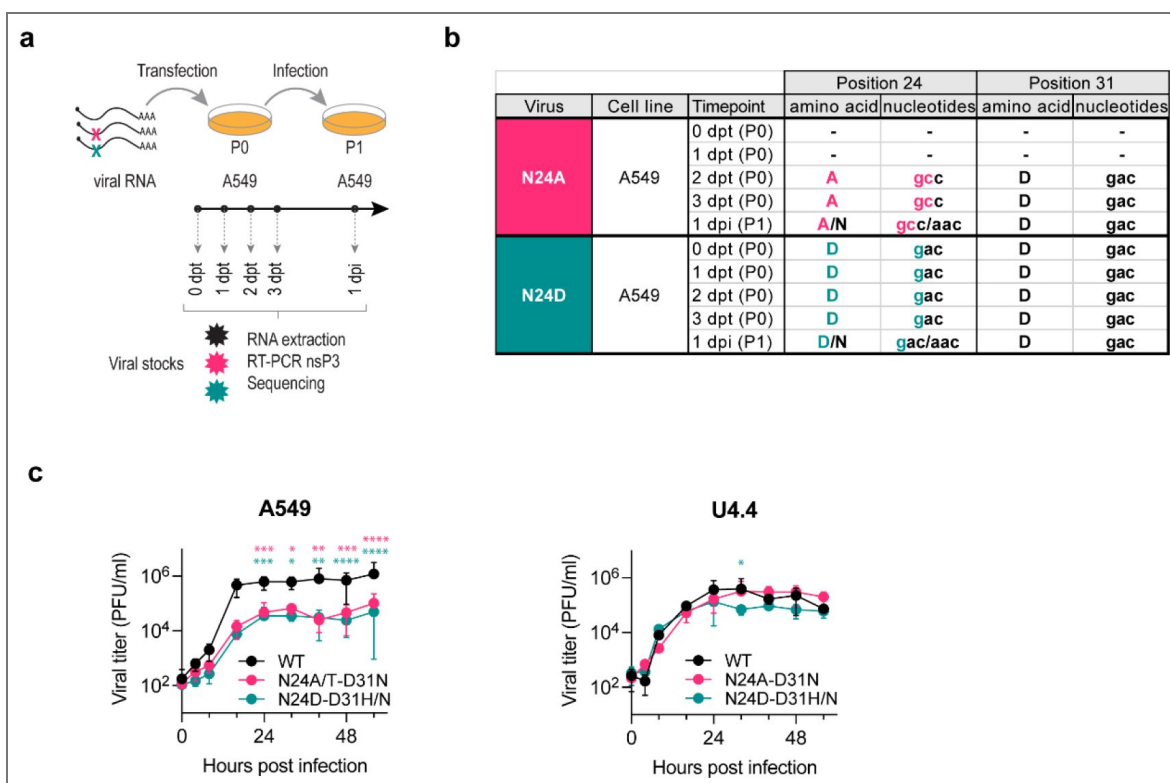
## Loss of macrodomain catalytic activity modulates CHIKV vector competence in *Aedes* mosquitoes

To investigate the role of macrodomain catalytic activity in CHIKV infection of the mosquito host, we exposed laboratory colonies of *Ae. albopictus* and *Ae. aegypti* to an infectious blood meal containing Caribbean WT, N24A-D31N, or N24D-D31H/N viruses. At 2, 5, and 7 days post-infection, individual mosquitoes were dissected into bodies (to study viral dissemination) and heads (to analyze transmission potential). Prevalence of infection and viral titers were determined separately for bodies and heads at each timepoint (Fig. 3a). To assess the genetic stability of the introduced mutations and the selection pressure during mosquito infection, viral RNA from individual infected mosquitoes was extracted and the nsP3 gene was sequenced (Supplementary Table 1).

The N24A-D31N mutant showed reduced dissemination and transmission potential compared to WT virus in both mosquito species. In *Ae. albopictus*, prevalence of infection in bodies was significantly lower for N24A-D31N than for WT at day 7 (56% vs 87%), and no infected heads were detected at this timepoint, compared to 26% for WT (Fig. 3b). In *Ae. aegypti*, viral titers in bodies were significantly lower for N24A-D31N than for WT at day 7, and prevalence of infection in bodies was also significantly reduced at day 7 (41% vs 87%) (Fig. 3c). Head prevalence of infection at day 7 was lower for N24A-D31N than for WT (23% vs 52%), though this difference did not reach statistical significance. Sequencing of nsP3 from individual infected mosquitoes confirmed that both N24A and D31N mutations remained stable throughout the infection time course in both species, with the exception of a single *Ae. albopictus* individual at day 5 showing partial reversion at both positions toward the WT sequence (Supplementary Table 1).

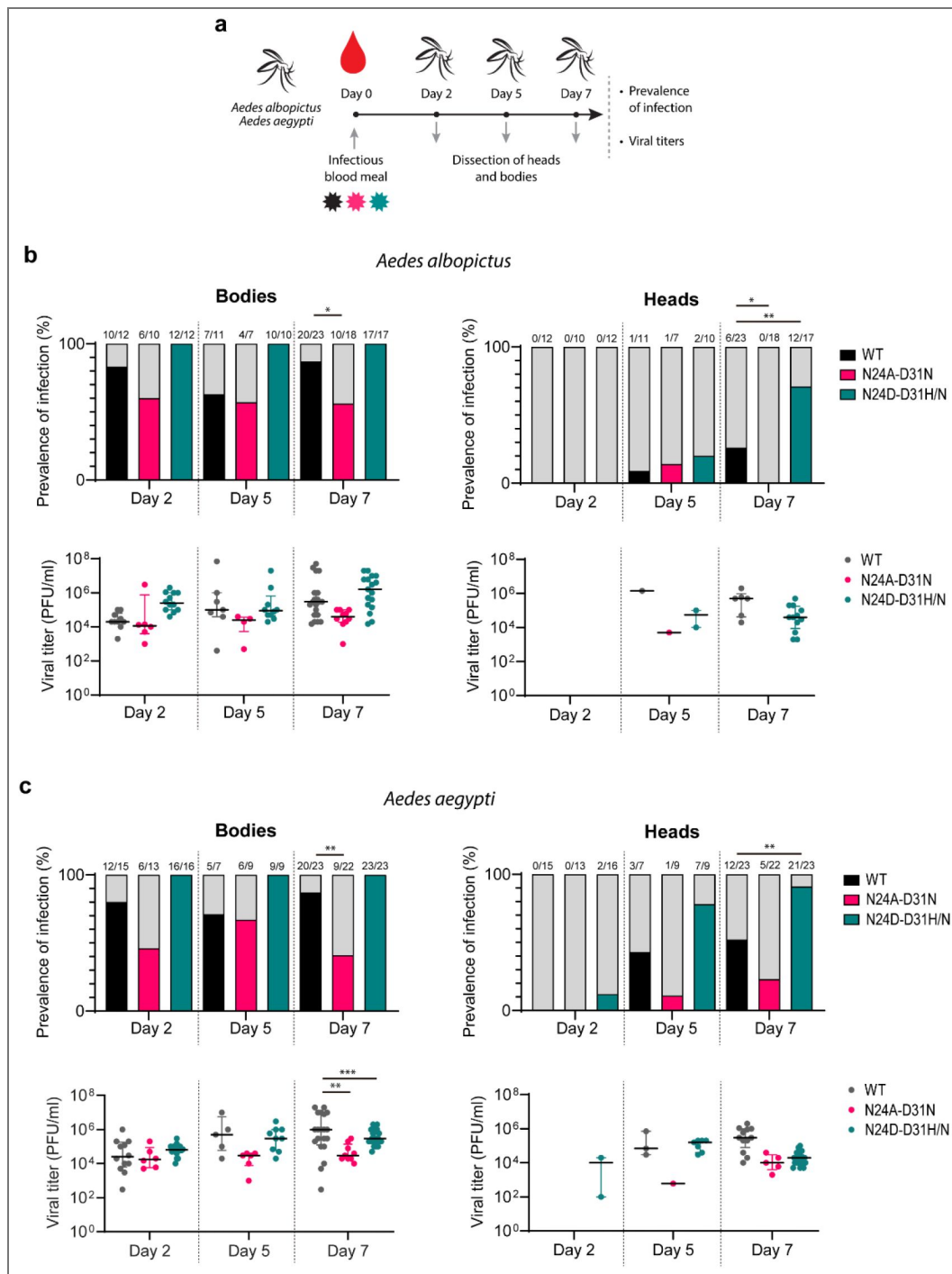
In contrast, the N24D-D31H/N mutant showed enhanced dissemination and transmission potential compared to WT. In *Ae. albopictus*, N24D-D31H/N reached 100% prevalence of infection in bodies at all three timepoints, compared to 83%, 64%, and 87% for WT at days 2, 5, and 7 respectively. Head prevalence of infection at day 7 was significantly higher for N24D-D31H/N than for WT (71% vs 26%), while viral titers in infected heads were comparable between the two viruses (Fig. 3b). In *Ae. aegypti*, N24D-D31H/N again reached 100% prevalence of infection in bodies at all timepoints. Head prevalence of infection at day 7 was significantly higher for N24D-D31H/N than for WT (91% vs 52%), while viral titers in infected heads were comparable. Notably, despite the enhanced dissemination of N24D-D31H/N, viral titers in bodies were significantly lower for this mutant compared to WT at day 7 in *Ae. aegypti*, suggesting that enhanced dissemination is not driven by increased viral replication (Fig. 3c). Sequencing of nsP3 from individual infected mosquitoes revealed that the N24D mutation remained stable throughout the infection time





**Figure 2. N24 catalytic mutations impair CHIKV replication in interferon-competent human cells but not in mosquito cells.**

**a**, Schematic of the experimental design. In vitro transcribed N24A and N24D viral RNAs were transfected into A549 cells. Cell culture supernatants were collected at 0, 1, 2, and 3 days post-transfection (P0) and at 1 day post-infection (P1) and subjected to RNA extraction, RT-PCR amplification of the nsP3 gene, and Sanger sequencing. **b**, Amino acid and nucleotide identities at residues 24 and 31 in N24A and N24D viruses following transfection into A549 cells. Dashes indicate timepoints where insufficient viral RNA was recovered for sequencing. Double peaks indicating coexistence of multiple nucleotides at the same position are denoted by '/'. Amino acid abbreviations: N, asparagine; A, alanine; D, aspartic acid. **c**, Growth kinetics of WT, N24A-D31N, and N24D-D31H/N viruses in A549 and U4.4 cells. Cells were infected at a multiplicity of infection of 0.05. Data are plotted as mean  $\pm$  SD for three independent biological experiments. Data were analyzed using a mixed-effects model with Geisser-Greenhouse correction followed by Tukey's multiple comparison test. Pink and teal asterisks indicate significant differences compared to WT.



**Figure 3. Macrodomain catalytic mutations modulate CHIKV vector competence in *Aedes* mosquitoes.**

**a**, Schematic of the experimental design. Laboratory colonies of *Ae. albopictus* and *Ae. aegypti* were exposed to an infectious blood meal containing WT, N24A-D31N, or N24D-D31H/N viruses. At 2, 5, and 7 days post-infection, individual mosquitoes were dissected into bodies and heads. Prevalence of infection and viral titers were determined by plaque assay. **b**, Prevalence of infection (top) and viral titers (bottom) in bodies and heads of *Ae. albopictus* mosquitoes. **c**, Prevalence of infection (top) and viral titers (bottom) in bodies and heads of *Ae. aegypti* mosquitoes. Numbers above prevalence bars indicate the number of infected individuals

course, except for a single *Ae. albopictus* individual at day 2 showing partial reversion toward the WT sequence at position 24. At position 31, the viral population evolved from a D31H/N mixture at day 2 toward predominantly D31H by day 7 in both species, indicating that the D31H variant is preferentially selected in the mosquito host (Supplementary Table 1 [↗](#)).

To determine whether the enhanced dissemination and transmission potential of the N24D-D31H/N double mutant could be attributed to the D31 mutations independently of N24 catalytic inactivation, we generated single D31H and D31N mutant viruses and assessed their replication in A549 and U4.4 cells and in *Aedes* mosquitoes (Supplementary Fig. 2 [↗](#)). In cell culture, D31H and D31N single mutants replicated broadly comparably to WT in both A549 and U4.4 cells, with only isolated significant differences at individual timepoints. In both mosquito species, D31H and D31N single mutants showed no significant differences in prevalence of infection compared to WT at any timepoint, and viral titers in bodies and heads were broadly comparable to WT. The genetic stability of the introduced D31 mutations was confirmed by Sanger sequencing of nsP3 from individual infected mosquitoes (Supplementary Table 2 [↗](#)). These results demonstrate that D31 mutations alone are not sufficient to enhance virus dissemination and transmission, indicating that the gain-of-function phenotype of the N24D-D31H/N double mutant requires loss of macrodomain catalytic activity at position N24.

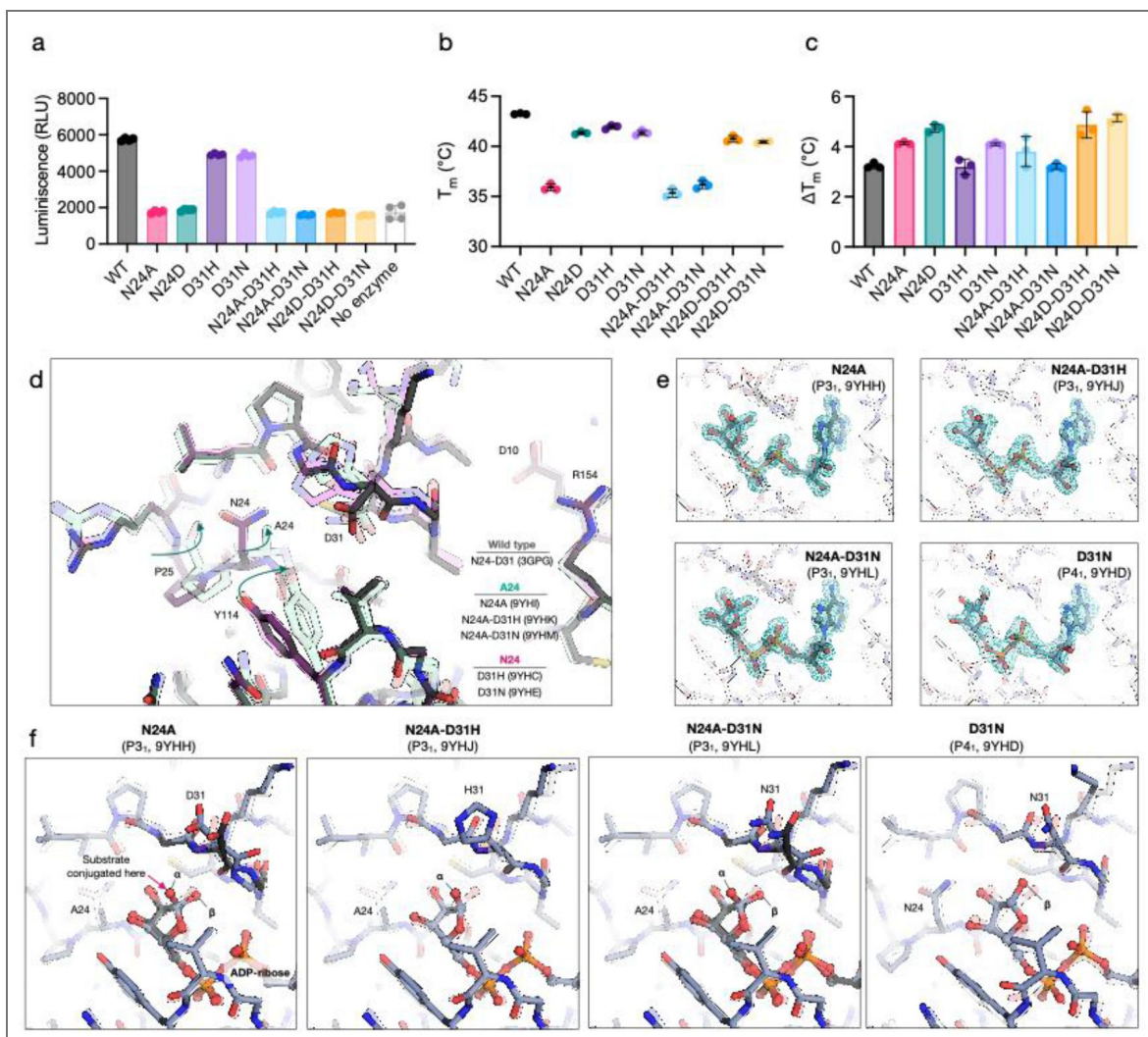
Taken together, these results demonstrate that macrodomain catalytic mutations modulate CHIKV dissemination and transmission potential in a mutation-specific manner that is consistent across both *Aedes* species. The distinct effects are driven primarily by differences in prevalence of infection rather than viral titers in infected individuals, suggesting that macrodomain catalytic activity influences the ability of CHIKV to establish infection and disseminate within the mosquito rather than its replication level once established. The enhanced presence in heads of the N24D-D31H/N mutant in both mosquito species represents a gain-of-function phenotype. Given this finding, and following institutional guidances, we did not repeat experiments with this viral variant that was discarded. over the total number of mosquitoes tested. Horizontal bars represent the median and interquartile range. Individual data points represent single mosquitoes. Prevalence of infection was compared using Fisher's exact test. Viral titers were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. Asterisks indicate statistically significant differences compared to WT.

## The CHIKV macrodomain catalytic activity is abolished by N24 mutations and is not rescued by D31 second-site mutations

Mosquito experiments showed that the N24D-D31H/N mutant, despite carrying a mutation at the catalytic site, enhanced CHIKV dissemination. This raised the question of whether the D31 second-site mutations might rescue catalytic activity, thereby explaining the observed phenotype. To address this, we investigated the structural and functional contributions of N24 and D31 to macrodomain catalytic activity and ADP-ribose binding capacity *in vitro*. We constructed expression plasmids encoding WT and mutant versions of the CHIKV macrodomain and purified the following recombinant macrodomain variants from bacteria: WT, N24A, N24D, D31H, D31N, N24A-D31H, N24A-D31N, N24D-D31H, and N24D-D31N (Supplementary Fig. 3a [↗](#)). First, we assessed the catalytic activity of recombinant macrodomains using an AMP-Glo luciferase assay<sup>4</sup> (Fig. 4a [↗](#)). All N24 mutants (in either single or double combinations with D31) were catalytically inactive, exhibiting equivalent luciferase activity to the non-enzyme control. In contrast, single D31 mutants did not significantly alter catalytic activity compared to WT, demonstrating that the D31 second-site mutations do not rescue catalytic activity and therefore cannot account for the enhanced mosquito infectivity observed for the N24D-D31H/N mutant.

Next, we determined the thermostability and ADP-ribose binding capacity of the mutants using differential scanning fluorimetry (DSF) (Fig. 4b, 4c [↗](#), Supplementary Table 3 [↗](#), Supplementary Fig. 3b [↗](#)). This analysis revealed that the N24A mutant is less stable ( $\Delta T_m$  relative to WT = -7.3°C) than the N24D mutant ( $\Delta T_m$  relative to WT = -1.9°C), paralleling results with the SARS-CoV-2 macrodomain<sup>4</sup>. Both mutants also retained ADP-ribose binding capacity, generating a similar  $T_m$  shift in the presence of 1 mM ADP-ribose to WT (3.3°C for WT, 4.1°C for N24A, 4.7°C for N24D) (Fig.





**Figure 4. Functional and structural characterization of CHIKV nsP3 macrodomain mutants.**

**a**, Assays of the ADP-ribosylhydrolase activity of recombinantly expressed wild-type (WT) and mutant macrodomains. Macrodomains (200 nM) were incubated with auto-MARYlated human PARP10 for 1 hour at room temperature and the production of ADP-ribose was measured using NUDT5 and an AMP-Glo luciferase assay<sup>4</sup>. Data are plotted mean  $\pm$  SD for four technical replicates. **b**, Thermostability of CHIKV nsP3 macrodomain mutants measured by DSF using SYPRO orange. Data are plotted mean  $\pm$  SD for three technical replicates. **c**, Change in CHIKV nsP3 macrodomain thermostability upon incubation with 1 mM ADP-ribose. **d**, Alignment of the crystallographic structures of WT, N24A, N24A-D31N, N24A-D31H, D31H and D31N reveals a peptide flip in P25 and a coupled shift in Y114 in structures containing the N24A mutation. For clarity, only chain A is shown (see Supplementary Fig. 4a [for all chains](#)). **e**, Difference electron density maps ( $F_o - F_c$ , contoured at 3  $\sigma$ ) calculated prior to modeling ADP-ribose. Maps for all chains are shown in Supplementary Fig. 5 [for all chains](#). **f**, Crystal structures of N24A, D31N, N24A-D31H and N24A-D31N bound to ADP-ribose. Chain A is shown for the P3<sub>1</sub> structures and chain D for the P4<sub>1</sub> structure. Although the ADP-ribose binding pose is conserved, there is a rotameric shift at residue 31 that accompanies ADP-ribose binding, and mutations at position 31 change the character of the exit path of the substrate suggesting a role for substrate-specific recognition.

4c [↗](#), Supplementary Table 3 [↗](#)). We note that DSF measures binding capacity at a fixed ligand concentration rather than binding affinity, and we cannot exclude subtle quantitative differences in ADP-ribose affinity among the mutants. The compensatory mutations had minimal effects on both stability and ADP-ribose binding capacity. The D31 mutations were neutral in the WT background and did not rescue stability in the N24-mutant backgrounds. This result suggests that although N24A is destabilizing, the compensatory mutations do not act to restore thermodynamic stability.

To examine how these mutations might alter structure and function, we determined X-ray structures of N24A, N24A-D31H, N24A-D31N, D31H and D31N (Fig. 4d [↗](#), Supplementary Table 4 [↗](#)). The structures reveal a shift in residues 24-25 in the N24A mutants, with a flip in the P25 carbonyl and a correlated shift in nearby Y114 side chain (Fig. 4d [↗](#), Supplementary Fig. 4a, 4b [↗](#)). Given the lack of change in either catalytic or ADP-ribose binding capacity relative to the N24 mutations alone, it is expected that the compensatory residues do not alter the interactions with ADP-ribose. To test this, we soaked ADP-ribose into crystals and determined structures of N24A, N24A-D31H, N24A-D31N and D31N (Fig. 4e [↗](#), Supplementary Table 4 [↗](#), Supplementary Fig. 5 [↗](#)). The structures show that the D31 mutants undergo a similar side chain rotation when ADP-ribose binds (Fig. 4f [↗](#)). We observed both the  $\alpha$  and  $\beta$  anomers of ADP-ribose bound (Fig. 4f [↗](#), Supplementary Fig. 4c-f [↗](#)): the  $\alpha$  anomer is consistent with the substrate bound state, while both anomers are consistent with product bound<sup>46</sup>. The D31 mutations line the “exit” path of the residue modified with ADP-ribose, leaving them poised to influence substrate binding to the macrodomain (Fig. 4f [↗](#)). Collectively, these results confirm that the catalytic activity of the macrodomain is abolished by N24 mutations and is not rescued by the D31 second-site mutations, while ADP-ribose binding capacity is maintained. The structural location of the independently acquired D31 mutations at the substrate exit path raises the possibility that they influence ADP-ribose substrate recognition, potentially tuning the reader activity of the macrodomain toward specific substrates. These findings confirm that the viral phenotypes observed in mosquitoes are not a consequence of restored catalytic activity but arise in the context of a catalytically inactive macrodomain that retains ADP-ribose binding capacity.

## Discussion

Here we investigated the role of macrodomain catalytic activity in CHIKV replication and transmission between vertebrate and invertebrate hosts. While previous studies have established the importance of the alphavirus macrodomain for replication and virulence in mammalian hosts, its specific role in the mosquito vector remained largely unexplored. Our research demonstrates that macrodomain mutations directly impact viral infectivity and dissemination in *Aedes* mosquitoes *in vivo*. This highlights a previously unknown host-specific function of the macrodomain. By introducing N24A and N24D mutations into the CHIKV nsP3 macrodomain, we abolished ADP-ribosylhydrolase activity while preserving ADP-ribose binding capacity. The consistent emergence of D31 mutations specifically in N24 catalytic mutants, combined with the precedent of equivalent compensatory mutations in Sindbis virus<sup>44</sup>, suggests this represents a conserved mechanism for addressing macrodomain functional impairment. However, the specific selective pressures driving D31 emergence - whether catalytic compensation, protein stability effects, or other factors - remain to be definitively established. The structural location of the D31 mutations at the substrate exit path, combined with their progressive selection toward D31H in the mosquito host, raises the possibility that these mutations tune ADP-ribose substrate recognition in a host-specific manner.

The observation that N24 mutations are poorly tolerated in interferon-competent human cells but not in mosquito cells is consistent with the established role of macrodomain catalytic activity in counteracting PARP-mediated antiviral responses in mammals and promoting viral virulence *in vivo*<sup>9,12,47</sup>. It is also consistent with the recurrent loss of N24 in mosquito-specific alphaviruses<sup>35,36</sup>, which replicate exclusively in an environment lacking interferon-driven PARP responses. Our experimental data extend these observations by directly demonstrating, for the first time *in vivo*, that catalytic inactivation of the macrodomain modulates CHIKV dissemination

and transmission potential in *Aedes* mosquitoes in a mutation-specific manner. While the N24A-D31N mutation impaired viral dissemination, the N24D-D31H/N mutation enhanced infectivity and transmission potential in both *Ae. albopictus* and *Ae. aegypti*. Importantly, single D31 mutant viruses did not recapitulate this gain-of-function phenotype, confirming that the enhanced dissemination/transmission phenotype requires loss of catalytic activity at position N24 rather than the D31 changes alone. This differential evolutionary trajectory provides novel insights into how dual-host arboviruses balance conflicting host immune pressures.

The gain-of-function enhancement of mosquito transmission observed for N24D-D31H/N is unexpected and its molecular mechanism remains to be determined. The finding that viral titers in infected mosquito bodies were lower for N24D-D31H/N than for WT despite higher prevalence of infection suggests that this mutant is more efficient at establishing infection across midgut cells rather than replicating to higher levels once infection is established. One possibility is that the catalytically inactive but ADP-ribose binding-competent macrodomain interacts differently with mosquito host proteins, potentially influencing the higher order structures formed by nsP3 that are needed to interact with host factors<sup>48</sup> or organize tubular structures containing viral genomic RNA<sup>49</sup>. The progressive selection of D31H over D31N in the mosquito host further suggests that subtle differences in ADP-ribose substrate recognition may influence viral fitness in the mosquito environment in ways that are not yet understood. Notably, which proteins are ADP-ribosylated under interferon signaling in human cells is only just emerging<sup>50</sup>, and the potential interactome in mosquito cells has not been established. This knowledge gap highlights the challenge in understanding how the D31 mutations redirect macrodomain specificity in a host-dependent manner.

Our findings have important implications for the development of macrodomain-targeted antivirals. While macrodomain inhibitors targeting catalytic activity are promising therapeutic candidates for mammalian infections<sup>14</sup>, our results demonstrate that the function of this domain is fundamentally host-dependent. The unexpected gain-of-function enhancement of virus dissemination and transmission potential in mosquitoes by a catalytically inactive macrodomain variant underscores the importance of evaluating antiviral strategies across the full host range of dual-host viruses. Compounds that inhibit macrodomain catalytic activity may effectively suppress viral replication in mammals while having unpredictable effects on transmission by the mosquito vector. A deeper understanding of macrodomain function across hosts is therefore essential for the rational design of broad-spectrum antivirals targeting this domain.

## Methods

### Construction of recombinant viruses

To introduce the N24A and N24D mutations into the nsP3 gene of the CHIKV-Caribbean infectious clone<sup>51</sup>, 5'-phosphorylated primers were used to amplify the full-length plasmid template via inverse PCR using Q5 High Fidelity DNA Polymerase (New England Biolabs). Forward primers contained 1-2 mismatches relative to the WT CHIKV sequences to incorporate the desired nucleotide changes into the PCR product. N24A mutations were introduced using the forward primer 5'-GACCCTCGCGGGTTACCG-3'. N24D mutations were introduced using the forward primer 5'-GCCCCCTCGCGGGTTACCG-3'. The reverse primer for both mutants was 5'-GGCGGCGTTGACCACGC A-3'. To introduce the D31H and D31N mutations, the same inverse PCR strategy was used. The forward primer 5'-ACCCGGTAACCCGCGAGGG-3' was used for both mutants. The reverse primer for D31H was 5'-CACGGTGTGCAAGGCAG-3' and for D31N was 5'-AACGGTGTGCAAGGCAG-3'. All primers were 5'-phosphorylated. Purified PCR products were blunt-end ligated with T4 DNA ligase and transformed into competent *E. coli* cells. Colonies were minipreped, and plasmid sequences were verified by whole-plasmid sequencing (Eurofins).

The DNAs of the recombinant constructs were linearized by digestion with NotI enzyme and used as templates for transcription by SP6 polymerase in the presence of GpppG cap structure analog, using the mMessage mMachine transcription kit (Thermo Fisher). The RNAs were gel-quantified and used for transfection in cell culture.

## Cells, viral transfections and infections

Mammalian cell lines (Vero and A549) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37°C with 5% CO<sub>2</sub>. Ae. albopictus cell lines (C6/36 and U4.4) were maintained in Leibovitz L-15 medium supplemented with 10% FBS, 1% P/S, 1% non-essential amino acids, and 1% tryptose phosphate broth at 28°C with 5% CO<sub>2</sub>.

For virus stock production, Vero cells were grown to 60-70% confluence in T75 flasks and transfected with 10 µg of in vitro transcribed viral RNAs using Lipofectamine 2000 (Invitrogen). Cell culture supernatants were collected at one day post-transfection and used to infect fresh Vero cells. Viral stocks were harvested from cell culture supernatants at one and two days post-infection and quantified by plaque assay.

## Plaque assays

Viruses from culture supernatants were quantified by plaque assays to determine viral yields. Vero cells ( $1 \times 10^5$  per well) were seeded into 24-well plates and allowed to attach overnight. Viral stocks were serially diluted, and 0.1 mL was added to the cells, followed by a 1-hour incubation. An overlay medium (1× DMEM, 2% FBS, 1% P/S, and 0.8% agarose) was then added to each well. Cells were fixed three days post-infection with 4% paraformaldehyde and stained with crystal violet for plaque visualization.

## Assessment of mutations in viral macrodomains

To assess the genetic stability of N24A and N24D mutations in interferon-competent human cells, A549 cells were transfected in T25 flasks with 3 µg of in vitro transcribed viral RNAs. Cell culture supernatants were harvested at 0, 1, 2, and 3 days post-transfection (P0) for total RNA extraction, nsP3 RT-PCR amplification, and Sanger sequencing. Viruses recovered from supernatants were used to re-infect fresh A549 cells, and cell culture supernatants from this passage (P1) were also harvested for total RNA extraction, nsP3 RT-PCR, and sequencing.

Viral RNAs were extracted from culture supernatants using TRIzol (Invitrogen) and used for reverse transcription (RT) reactions with oligo-dT primers (Maxima H Minus RT, Thermo Fisher Scientific). PCR reactions were performed using primers forward 5'-GCAGTTTGACAATGGCAGAAG-3' and reverse 5'-TGTCTTTCCTCCTGAGTATACAC-3' (DreamTaq DNA Polymerase, Thermo Fisher Scientific). The size and quality of the amplicons were assessed by electrophoresis on a 1.5% agarose gel. The 453-bp amplicons were cleaned up using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) and submitted for Sanger sequencing (Eurofins).

For each PCR sample, individual Sanger chromatograms were visually inspected to assess sequence quality and detect double peaks. All high-quality sequences were aligned against the WT CHIKV nsP3 gene to identify reversions of introduced mutations and detect new mutations.

## Growth curves

Sub-confluent A549 and U4.4 cells in six-well plates were infected with equal amounts of WT or mutant CHIKVs recovered from Vero cells. Infections were performed using a multiplicity of infection (MOI) of 0.05 in 500 µL of phosphate-buffered saline (PBS). One hour post-infection, cells were washed three times with PBS, and 2 mL of growth media were added. At predetermined time points, cell culture supernatants were collected and stored at -80°C. Virus quantification was performed by plaque assay on Vero cells using serial dilutions of the supernatants. Growth curves were performed in three independent biological experiments for double mutant viruses and two independent biological experiments for single D31 mutant viruses. To confirm genetic stability of the introduced mutations, cell supernatants from the 48 hour timepoint were extracted using TRIzol, amplified by RT-PCR, and analyzed by Sanger sequencing as previously described.



## Mosquito rearing

Laboratory colonies of *Ae. aegypti* mosquitoes (17th generation; collected originally in Kamphaeng Phet Province, Thailand) and *Ae. albopictus* (19th generation; collected originally in Phu Hoa, Binh Duong Province, Vietnam) were used. The insectary conditions for mosquito maintenance were 28°C, 70% relative humidity, and a 12-h-light and 12-h-dark cycle. Adults were maintained with permanent access to a 10% sucrose solution. Adult females were offered commercial rabbit blood (BCL, Boisset-Saint-Priest, France) twice a week through a membrane feeding system (Hemotek Ltd.).

## Ethics

Human blood samples to prepare mosquito artificial infectious bloodmeals were supplied by healthy adult volunteers at the ICAREB biobanking platform (BB-0033-00062/ICAREB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the Institut Pasteur in the CoSImmGen and Diagmicoll protocols, which had been approved by the French Ethical Committee Ile-de-France I. The Diagmicoll protocol was declared to the French Research Ministry under reference 343 DC 2008-68 COL 1. All adult subjects provided written informed consent.

## Experimental infections of mosquitoes

Infection assays were performed with 7- to 10-day-old females starved 24 h prior to infection in a biosafety level 3 (BSL-3) laboratory. Mosquitoes were offered the infectious blood meal for 30 min through a membrane feeding system (Hemotek Ltd.) set at 37°C with a piece of desalted pig intestine as the membrane. The blood meal was composed of washed human erythrocytes resuspended in phosphate-buffered saline mixed 2:1 with a prediluted viral stock and supplemented with 10 mM ATP (Sigma-Aldrich). The viral stock was prediluted in Leibovitz L-15 medium with 0.1% sodium bicarbonate (Gibco) to reach an infectious titer ranging from  $1 \times 10^6$  to  $1 \times 10^7$  focus-forming units. Following the blood meal, fully engorged females were selected and incubated at 28°C with 70% relative humidity and under a 12-h-light and 12-h-dark cycle with permanent access to 10% sucrose. At different times post-infection, mosquitoes were cold anesthetized for dissection into bodies and heads. Body parts were homogenized in microtubes containing steel beads (5-mm diameter) and 300 µl of DMEM supplemented with 2% FBS using a TissueLyser II instrument (Qiagen) at 30 shakes/s for 2 min. Homogenates were clarified by centrifugation and stored at -80°C until further processing. Viral titers in individual samples were determined by plaque assay. For the assessment of mutations in the nsP3 macrodomain, RNA extracted with TRIzol from homogenates was used for RT-PCR and Sanger sequencing as previously described.

## Protein purification

Plasmid DNA (pET22b(+)) encoding His<sub>6</sub>-tagged wild type or mutant (N24A, N24D, D31H, D31N, N24A-D31H, N24A-D31N, N24D-D31H, N24D-D31N) macrodomains were transformed into BL21 (DE3) *Escherichia coli*. After overnight growth at 37°C (LB-agar, 100 µg/ml carbenicillin), single colonies were used to inoculate small scale cultures (10 ml LB, 100 µg/ml carbenicillin). Following overnight growth at 37°C, large scale cultures (1 l LB, 100 µg/ml carbenicillin) were inoculated and grown at 37°C until an OD<sub>600</sub> = 0.8, at which point protein expression was induced with 0.2 mM IPTG at 18°C overnight. Cells were harvested by centrifugation (4000 g, 20 min, 4°C) and cell pellets were briefly washed with phosphate-buffered saline before being re-centrifuged (4000 g, 20 min, 4°C). The cell pellets were frozen at -80°C until further purification. Thawed cell pellets were resuspended in 25 ml Lysis buffer (50 mM Tris pH 8.0, 300mM NaCl, 20mM imidazole, 5% glycerol, 0.1% Triton X100) and 1/2 tablet of protease inhibitor (EDTA-free cOmplete, Roche). Cells were lysed by sonication (2 min) and cell debris was collected by centrifugation (30,000 g, 30 min, 4°C). The supernatant was filtered (0.45 µm cellulose acetate) and loaded on a 5 ml HisTrap HP column pre-equilibrated with buffer A (50 mM Tris pH 8.0, 150mM NaCl, 20mM imidazole). Bound protein was eluted via a linear gradient of buffer B (50 mM Tris pH 8.0, 150mM NaCl, 300mM imidazole).



Eluted protein was pooled, concentrated and purified via size exclusion chromatography (HiLoad 16/600 Superdex 75, Cytiva) in 10 mM Tris pH 8.0 and 300 mM NaCl. Relevant fractions were pooled, concentrated and stored at -80°C.

## AMP-Glo assay

The ADP-ribosyl hydrolase activity of CHIKV nsP3 macrodomain wild type and mutants was determined using auto-MARylated human PARP10 as a substrate following the method described previously (PMID: 37651466). ADP-ribose produced by the macrodomain is hydrolyzed by NUDT5 phosphodiesterase to AMP, which was detected using the commercially available AMP-Glo kit (Promega, V5011). Reactions were prepared to a final volume of 8 µl in assay buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>) and contained 100 nM NUDT5, 10 µM MARylated PARP10 and 200 nM CHIKV nsP3 macrodomain or buffer. The AMP concentration was quantified using a BioTek Synergy H1 plate reader after incubation of the reactions at room temperature for 1 hour. Enzyme concentration was calibrated so that no more than 20% of the substrate was consumed during the reaction. All reactions were performed in technical quadruplicates. Data was plotted using GraphPad Prism.

## Crystallography

CHIKV nsP3 macrodomain mutants were crystallized using hanging drop vapor diffusion and MRC 2-well crystallization plates. Crystals grew at room temperature from drops contained 200 nl protein and 200 nl reservoir. Crystallization conditions are reported in [Supplementary Table 2](#)<sup>52</sup>. To obtain structures of mutants bound to ADP-ribose, crystals were soaked in reservoir solutions supplemented with 20 mM ADP-ribose. Crystals were vitrified in liquid nitrogen without additional cryoprotection. Data were collected at 100 K using beam line 8.3.1 of the Advanced Light Source. Data were indexed, integrated and scaled with XDS<sup>52</sup> and merged with Aimless<sup>53</sup>. Phases were obtained by molecular replacement run with Phaser<sup>54</sup> using chain A from PDB 3GPG as a search model. Initial models were improved by iterative cycles of model building using COOT<sup>55</sup> and refinement with phenix.refine<sup>56</sup>. During initial rounds of refinement, waters were automatically added using phenix.refine. During final rounds of refinement, hydrogens were added to protein coordinates using phenix.ready\_set and refined using a riding model. B-factors were refined anisotropically for non-hydrogen atoms except for the structure of the D31N mutant that crystallized in P4<sub>1</sub> (PDB 9YHD). Coordinates and restraints for ADP-ribose were generated with phenix.elbow<sup>57</sup>. Data collection and refinement statistics are summarized in [Supplementary Table 2](#)<sup>52</sup>.

## DSF

Differential scanning fluorimetry (DSF) was performed to determine the thermal stability of the target protein using SYPRO Orange dye (Thermo Fisher Scientific). The protein was diluted to a final concentration of 3 µM in assay buffer consisting of 10 mM Tris pH 8.0 and 150 mM NaCl. SYPRO Orange was used at a final concentration of 5x, prepared from a 5000x stock in DMSO according to the manufacturer's instructions.

Each 20 µl reaction mixture was prepared in a white 96-well PCR plate by combining protein, buffer, dye and ADPr (final concentrations of 0 mM or 1 mM). Samples were mixed gently, sealed with optical film, and briefly centrifuged to eliminate bubbles. All reactions were performed in technical triplicate.

Thermal denaturation was monitored using a CFX96 Real-Time PCR Detection System (Bio-Rad). The temperature was increased from 4°C to 70°C at a rate of 1°C/min, and fluorescence was measured every minute using the FRET channel. The melting temperature (T<sub>m</sub>) was determined by uploading the data to DSF World using fitting model 2. Data was plotted using GraphPad Prism.

## Materials availability

Plasmids generated in this study are available from the lead contact upon reasonable request.

## Data availability

All data reported in this paper is contained within the main text, figures, and supplementary materials. Crystallographic coordinates and structure factors have been deposited in the PDB with the following accessing codes: 9YHC, 9YHD, 9YHE, 9YHF, 9YHG, 9YHH, 9YHI, 9YHJ, 9YHK, 9YHL and 9YHM.

## Acknowledgements

This work was supported by the National Institutes of Health (NIAID Antiviral Drug Discovery (AViDD) grant U19AI171110 to M-C.S. A.A. and J.S.F.; the French Government's Investissement d'Avenir program, Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-10-LABX-62-IBEID), Fondation iXcore - iXlife - iXblue Pour La Recherche and the Explore Donation, MIE project to M-C.S. The authors thank Diego E. Alvarez for critical reading and insight into data interpretation.

## Additional information

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### Funding

| Funder                                               | Grant reference number | Author                                               |
|------------------------------------------------------|------------------------|------------------------------------------------------|
| HHS   National Institutes of Health (NIH)            | U19AI171110            | Alan Ashworth<br>James S Fraser<br>Maria-Carla Saleh |
| LABEX IBEID                                          | ANR-10-LABX-62-IBEID   | Maria-Carla Saleh                                    |
| Fondation iXcore - iXlife - iXblue Pour La Recherche |                        | Maria-Carla Saleh                                    |
| Explore Donation, MIE project                        |                        | Maria-Carla Saleh                                    |

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Competing interests: A.A. is a co-founder of Tango Therapeutics, Azkarra Therapeutics and Kytarro; a member of the board of Cytomx, Ovibio Corporation, Cambridge Science Corporation; a member of the scientific advisory board of Genentech, GLAdiator, Circle, Bluestar/Clearnote Health, Earli, Ambagon, Phoenix Molecular Designs, Yingli/280Bio, Trial Library, ORIC and HAP10; a consultant for ProLynx, Next RNA and Novartis; receives research support from SPARC; and holds patents on the use of PARP inhibitors held jointly with AstraZeneca from which he has benefited financially (and may do so in the future). J.S.F. is a consultant to, shareholder of, and receives sponsored research support from Relay Therapeutics and is a compensated member of the SAB of Vilya Therapeutics. The other authors declare no competing interests.

## Additional files

[Supplementary Materials](#) 

[Supplementary Table 1](#) 

[Supplementary Table 2](#) 

[Supplementary Table 3](#) 

[Supplementary Table 4](#) 

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## Peer reviews

### Reviewer #1 (Public review):

Summary:

This paper from Bardossy et al. explores whether viral macrodomains in dual-host viruses contribute to infection in the mosquito vector. Using the CHIKV Caribbean strain, the authors generated nsP3 macrodomain catalytic site mutants (N24A or N24D) and identified a compensatory mutation site at position 31 during virus propagation in Vero cells. They then assessed the impact of these mutations on viral growth kinetics in A549 (human) and U4.4 (*Ae albopictus* cells), as well as on infectivity and dissemination in vivo in *Ae. aegypti* and *Ae. albopictus*. Biochemical and structural analyses of recombinant macrodomain proteins

(alone or in combination) revealed effects on stability, catalytic activity, and ADP-ribose binding. Overall, the study demonstrates that CHIKV macrodomain catalytic activity plays an important role in virus infectivity and dissemination within the mosquito vector.

#### Strengths:

A complete set of experimental approaches spanning generation of recombinant viruses, in vitro characterization, in vivo studies in mosquitoes, and detailed biochemical and structural characterization.

#### Weaknesses:

(1) The sequence analysis of the generated stocks revealed the emergence of a second-site mutation at position 31 of the nsP3 macrodomain when (N24A or N24D) CHIKV mutants were generated on Vero cells. However, it is not clear from the text or the experimental design how many independent replicates were performed. Based on the current description, it appears this was done only once, which raises the question of whether mutations at position 31 represent a reproducible outcome of infection. This is particularly important because experiments in A549 cells did not reveal emergence of mutations at position 31. To strengthen this finding, the experiment should be performed at least three independent times.

(2) Based on the primer information used to generate amplicons for sequencing, the amplicons evaluated do not span the full nsP3 gene as stated in the text (Line 105). Instead, they cover only the first 119 amino acids of the macrodomain (160 aa long). Thus, the current data do not rule out the emergence of other compensatory mutations elsewhere in the nsP3 macrodomain or in the full-length protein. Additional sequencing is recommended, or the text should clearly state that only a portion of the macrodomain was sequenced.

(3) Another key question is whether this is a specific feature of the Caribbean strain or a feature conserved across different CHIKV lineages.

(4) The use of A549 cells (interferon-competent) to study CHIKV infection is somewhat surprising, as the current literature indicates that this cell line is not efficiently infected by Asian or ECSA lineages of CHIKV (PMID: 17604450) unless the Mxra8 receptor is overexpressed (PMID: 29769725) or IFN signaling is inhibited (PMID: 31682641). The data presented here are compelling and suggest specific features of the Caribbean strain that enable efficient infection of this cell line (Do the authors observe detectable cytopathic effect (CPE) in CHIKV-infected A549 cells?).

However, to further support the authors' claim related to human immunocompetent cells, it would be important to demonstrate the phenotype in an additional interferon-competent cell line that is well-established as highly permissive to CHIKV, such as human fibroblasts.

(5) To fully support the conclusion stated in lines 234- 237, the authors should fully sequence the virus stock used to demonstrate that no additional mutations (beyond N24D-D31H/N) are present that could contribute to the enhanced dissemination phenotype. This is especially important if the experiment was performed with only one stock of virus, given justified gain-of-function concerns.

(6) The authors did not assess transmission but transmission potential (only viral dissemination to heads was measured). The sentence at line 360 should be modified to accurately reflect the data-supported conclusion.

<https://doi.org/10.7554/eLife.112183.1.sa3>

## Reviewer #2 (Public review):

### Summary:

To address how the CHIKV macrodomain contributes to replication dynamics in mammalian and insect hosts, the authors initially created two separate mutations in the highly conserved N24 residue, which is known to be critical for the CHIKV macrodomain's ability to erase ADP-ribose from target proteins. Interestingly, they could not produce a virus with a mutation in this residue without second-site mutations in an aspartic acid residue nearby (D31). However, when tested biochemically, these second-site mutations did not enhance the enzymatic activity of the protein, indicating that other enzyme dynamics, such as substrate binding, may be impacting these mutations. Mutations at this residue allowed the CHIKV to replicate in Vero cells and in mosquito cells, but they replicated poorly in IFN-competent human cells, indicating clear IFN-specific impacts on these viruses. Interestingly, they found unique impacts on virus dissemination and replication in live mosquitoes. While the N24A/D31N virus did poorly in vivo in all accounts, the N24D/D31H/N virus tended to infect both the bodies and heads of the mosquitoes better than the WT virus, though titers were reduced. The authors claimed, based on a DSF assay, that there were no real differences in ADP-ribose binding and thus suggested that these differences could be due to changes in substrate specificity, as the D31 residue resides in the substrate exit path, potentially tuning the virus to unique substrates in different species. The authors also produced crystal structures of the mutants to demonstrate the changes in the binding pocket caused by these mutations.

### Strengths:

The authors have done a rigorous job of evaluating CHIKV macrodomain mutant viruses and the proteins' biochemical activities. The use of live mosquitoes is highly unique and provides important insights into the importance of the macrodomain in different species.

### Weaknesses:

It is not clear if the interpretation of the ADP-ribose binding data is correct. It appears there are notable differences that could explain the results, though the authors chose to minimize the impact that these differences had on the results. The N24D-D31H/N proteins had at least a 1°C degree difference in the thermal shift assay when compared to the N24A/D31N, single D31 mutants, and WT proteins, which is likely significant and could explain the dichotomous results between the two viruses in mosquito cells. Even the single N24D mutant had enhanced binding compared to the WT protein. Furthermore, as this virus has no enzymatic activity, one could hypothesize that enhanced binding to a substrate that is normally cleaved by the protein could certainly lead to alterations in phenotypic effects, whether good or bad. The authors should test the binding activity in a separate assay, such as an ITC assay, to determine if there are, in fact, binding differences or not. Having said this, it is likely that the impacts of these mutations on replication and transmission in human and mosquito cells are multi-factorial and could include both enhanced binding with altered substrate specificity amongst other activities.

Additionally, as both mutants had no detectable enzymatic activity but had quite different phenotypes in mosquitoes, I don't agree with the title stating that catalytic activity modulates dissemination and transmission potential in mosquitoes. It seems more likely that alterations in binding activity or substrate recognition (even suggested by the authors) impact these phenotypes in mosquitoes.

<https://doi.org/10.7554/eLife.112183.1.sa2>

## Reviewer #3 (Public review):

### Summary:

The authors investigated the role of the nsP3 macrodomain catalytic activity in the replication and transmission of CHIKV in mosquito vectors. The conserved dual-host alphavirus catalytic site N24 has previously been shown to be essential for ADP-ribosylhydrolase activity. Despite this, mosquito-specific alphaviruses do not share this catalytic site. To assess whether the macrodomain catalytic activity of a dual-host virus was essential in insect hosts, the authors targeted the N24 site to abolish catalysis while maintaining binding capacity. The loss of ADP-ribosylation led to the emergence of compensatory mutations at site D31 that impact viral infectivity, dissemination, and transmission in *Aedes* sp. mosquitoes in vivo. The conclusions are well supported by the results and provide insight into the importance of nsP3 macrodomain activity in the mosquito vector, which hasn't been explored before.

### Strengths:

The main strength of this study is the use of *Aedes* sp. mosquito models to investigate the selective pressure of macrodomain mutations in vivo. The functional characterization as well as the structural analysis of the mutants provide supporting evidence of a potential role of the compensatory mutations at site D31 in substrate recognition.

### Weaknesses:

A considerable part of this study relies on the use of N24 mutant viral stocks generated in Vero cells, which yields an additional mutation at site 31 and consequently doesn't allow the authors to properly dissect the effect of mutation of N24 and D31 independently. It would be recommended to generate stocks with individual mutations in both A549 and U4.4 cells, pooling and concentrating them if needed. Replication of the N24A mutant in A549 cells does not lead to mutation at residue 31. Yet surprisingly, there is no reversion from N back to D at site 31 when the double mutant Vero stocks are passaged in A549. Since they are double mutants, it isn't possible to assess whether the defects in the growth of mutants N24A/T-D31N and N24D-D31H/N compared to WT are due to site 24 or 31, or both (Figure 2, panel c). Even though the authors emphasize that the compensatory mutation could have additional roles that impact viral infectivity and transmission in mosquito cells, it would strengthen the work to show that these mutations would spontaneously appear in stocks generated directly in mosquito cells. As a corollary, is it known whether insect-specific alphaviruses that lack macrodomain catalytic activity have corresponding mutations at site 31?

Additionally, there is a lack of consistency in the prevalence of WT virus at days 5 and 7 in in vivo experiments with *Ae. albopictus* and *Ae. aegypti* (Figure 3 and Supplementary Figure 2). This raises concern about the reproducibility of these experiments.

The inability to tease apart the roles of N24 and D31 in mosquito hosts partially prevented the authors from fully achieving their aims, but the work is nonetheless of interest to the field and suggests that more work is necessary to fully understand the role of the nsP3 macrodomain and its catalytic activity in the two disparate but obligate hosts for CHIKV and other dual-host alphaviruses.

<https://doi.org/10.7554/eLife.112183.1.sa1>

## Author response:

### Public Reviews:

### Reviewer #1 (Public review):

#### Summary:

*This paper from Bardossy et al. explores whether viral macrodomains in dual-host viruses contribute to infection in the mosquito vector. Using the CHIKV Caribbean strain, the authors generated nsP3 macrodomain catalytic site mutants (N24A or N24D) and identified a compensatory mutation site at position 31 during virus propagation in Vero cells. They then assessed the impact of these mutations on viral growth kinetics in A549 (human) and U4.4 (Ae albopictus cells), as well as on infectivity and dissemination in vivo in Ae. aegypti and Ae. albopictus. Biochemical and structural analyses of recombinant macrodomain proteins (alone or in combination) revealed effects on stability, catalytic activity, and ADP-ribose binding. Overall, the study demonstrates that CHIKV macrodomain catalytic activity plays an important role in virus infectivity and dissemination within the mosquito vector.*

#### Strengths:

*A complete set of experimental approaches spanning generation of recombinant viruses, in vitro characterization, in vivo studies in mosquitoes, and detailed biochemical and structural characterization.*

#### Weaknesses:

*(1) The sequence analysis of the generated stocks revealed the emergence of a second-site mutation at position 31 of the nsP3 macrodomain when (N24A or N24D) CHIKV mutants were generated on Vero cells. However, it is not clear from the text or the experimental design how many independent replicates were performed. Based on the current description, it appears this was done only once, which raises the question of whether mutations at position 31 represent a reproducible outcome of infection. This is particularly important because experiments in A549 cells did not reveal emergence of mutations at position 31. To strengthen this finding, the experiment should be performed at least three independent times.*

We thank the reviewer for raising this important point. The emergence of second-site mutations at residue D31 in Vero cells was actually observed in two independent experiments, each initiated by transfection of viral RNAs encoding either the N24A or N24D mutant. In the second experiment, additional timepoints were sampled for sequencing. Comparison of the two experiments revealed that, in the second replicate, only the D31N variant was recovered, whereas D31H was not detected. We will include the results of both independent experiments in the updated Figure 1 and will modify the text accordingly to improve clarity.

In addition, we will present new experiments performed in other cell types that further confirm the reproducibility of D31 mutation emergence across different cellular contexts. Specifically, we will include results from new viral RNA transfections in BHK-21 mammalian cells and C6/36 mosquito cells, which will be included in the updated Supplementary Figure 1.

*(2) Based on the primer information used to generate amplicons for sequencing, the amplicons evaluated do not span the full nsP3 gene as stated in the text (Line 105). Instead, they cover only the first 119 amino acids of the macrodomain (160 aa long). Thus, the current data do not rule out the emergence of other compensatory mutations elsewhere in the nsP3 macrodomain or in the full-length protein. Additional sequencing is recommended, or the text should clearly state that only a portion of the macrodomain was sequenced.*



We thank the reviewer for this correction. The sequencing was indeed focused on the region of the macrodomain surrounding the introduced mutations, covering the first 119 amino acids of nsP3. This region was selected to confirm the stability of the introduced mutations at position 24 and to monitor the emergence of potential second-site mutations in its immediate vicinity. We acknowledge that this approach does not rule out the emergence of compensatory mutations elsewhere in the macrodomain or in the full-length nsP3 protein. We will correct the text accordingly to accurately reflect the region that was analyzed.

*(3) Another key question is whether this is a specific feature of the Caribbean strain or a feature conserved across different CHIKV lineages.*

This is an excellent point. To address it, we introduced N24A and N24D mutations into an infectious clone of the Indian Ocean strain, a representative of the ECSA lineage, and assessed the emergence of D31 second-site mutations during viral stock production. As observed with the Caribbean strain, the D31N secondary mutation consistently emerged in both N24A and N24D Indian Ocean mutant viruses. These results will be included in the updated Supplementary Figure 1 and in the main text.

*(4) The use of A549 cells (interferon-competent) to study CHIKV infection is somewhat surprising, as the current literature indicates that this cell line is not efficiently infected by Asian or ECSA lineages of CHIKV (PMID: 17604450) unless the Mxra8 receptor is overexpressed (PMID: 29769725) or IFN signaling is inhibited (PMID: 31682641). The data presented here are compelling and suggest specific features of the Caribbean strain that enable efficient infection of this cell line (Do the authors observe detectable cytopathic effect (CPE) in CHIKV-infected A549 cells?).*

*However, to further support the authors' claim related to human immunocompetent cells, it would be important to demonstrate the phenotype in an additional interferon-competent cell line that is well-established as highly permissive to CHIKV, such as human fibroblasts.*

We thank the reviewer for raising this point. We acknowledge that previous studies have reported limited infection of A549 cells by certain CHIKV strains. However, in our hands, with our viral stocks and under our experimental conditions, we observe an increase in viral titers following infection of A549 cells with WT Caribbean strain virus, indicating productive viral replication. We also confirmed that the Indian Ocean strain replicates in A549 cells under the same conditions, and we will include growth curve data for this strain in the updated version of the manuscript. Importantly, we did not observe detectable cytopathic effects in A549 cells infected with either WT or N24 mutant viruses, which is consistent with the notion that CHIKV replicates less efficiently in this cell line compared to other mammalian cell lines. We will include a sentence acknowledging this in the discussion of the revised manuscript.

Regarding the suggestion to use human fibroblasts, we respectfully note that the primary focus of this study is the role of macrodomain catalytic activity in the mosquito host, and the experiments in A549 cells were performed to confirm the known importance of the macrodomain in interferon-competent mammalian cells. We therefore consider the current data in A549 cells sufficient to support this conclusion within the scope of the manuscript.

*(5) To fully support the conclusion stated in lines 234- 237, the authors should fully sequence the virus stock used to demonstrate that no additional mutations (beyond N24D-D31H/N) are present that could contribute to the enhanced dissemination phenotype. This is especially important if the experiment was performed with only one stock of virus, given justified gain-of-function concerns.*

We acknowledge that the full viral genome was not sequenced, and we cannot rule out the presence of additional mutations elsewhere in the genome that could contribute to the

observed phenotype. However, we note that the enhanced dissemination phenotype was also observed in independent experiments performed with the Indian Ocean strain mutant viruses in *Ae. albopictus*, which were generated independently from the Caribbean strain stocks. The consistency of the phenotype across two independently generated sets of mutant viruses from different CHIKV lineages strongly supports the conclusion that the enhanced dissemination phenotype is linked to the macrodomain mutations. These new data will be included in the updated manuscript.

*(6) The authors did not assess transmission but transmission potential (only viral dissemination to heads was measured). The sentence at line 360 should be modified to accurately reflect the data-supported conclusion.*

We thank the reviewer for pointing this out. The text will be modified accordingly to accurately reflect that we assessed transmission potential, based on viral dissemination to heads, rather than actual transmission.

#### **Reviewer #2 (Public review):**

##### *Summary:*

*To address how the CHIKV macrodomain contributes to replication dynamics in mammalian and insect hosts, the authors initially created two separate mutations in the highly conserved N24 residue, which is known to be critical for the CHIKV macrodomain's ability to erase ADP-ribose from target proteins. Interestingly, they could not produce a virus with a mutation in this residue without second-site mutations in an aspartic acid residue nearby (D31). However, when tested biochemically, these second-site mutations did not enhance the enzymatic activity of the protein, indicating that other enzyme dynamics, such as substrate binding, may be impacting these mutations. Mutations at this residue allowed the CHIKV to replicate in Vero cells and in mosquito cells, but they replicated poorly in IFN-competent human cells, indicating clear IFN-specific impacts on these viruses. Interestingly, they found unique impacts on virus dissemination and replication in live mosquitoes. While the N24A/D31N virus did poorly in vivo in all accounts, the N24D/D31H/N virus tended to infect both the bodies and heads of the mosquitoes better than the WT virus, though titers were reduced. The authors claimed, based on a DSF assay, that there were no real differences in ADP-ribose binding and thus suggested that these differences could be due to changes in substrate specificity, as the D31 residue resides in the substrate exit path, potentially tuning the virus to unique substrates in different species. The authors also produced crystal structures of the mutants to demonstrate the changes in the binding pocket caused by these mutations.*

##### *Strengths:*

*The authors have done a rigorous job of evaluating CHIKV macrodomain mutant viruses and the proteins' biochemical activities. The use of live mosquitoes is highly unique and provides important insights into the importance of the macrodomain in different species.*

##### *Weaknesses:*

*It is not clear if the interpretation of the ADP-ribose binding data is correct. It appears there are notable differences that could explain the results, though the authors chose to minimize the impact that these differences had on the results. The N24D-D31H/N proteins had at least a 1°C degree difference in the thermal shift assay when compared to the N24A/D31N, single D31 mutants, and WT proteins, which is likely significant and could explain the dichotomous results between the two viruses in mosquito cells. Even the single N24D mutant had enhanced binding compared to the WT protein. Furthermore, as this virus has no enzymatic activity, one could hypothesize that enhanced binding to a substrate that is normally cleaved by the protein could certainly*

*lead to alterations in phenotypic effects, whether good or bad. The authors should test the binding activity in a separate assay, such as an ITC assay, to determine if there are, in fact, binding differences or not. Having said this, it is likely that the impacts of these mutations on replication and transmission in human and mosquito cells are multi-factorial and could include both enhanced binding with altered substrate specificity amongst other activities.*

We agree that the mutants may indeed have stronger binding for modified substrates than the WT protein; however, given that DSF is not a quantitative measure of binding affinity and that free ADPr is not the relevant ligand (in fact we do not know the relevant ADPr-modified molecule), we have refrained from speculating further than saying in the discussion:

“The progressive selection of D31H over D31N in the mosquito host further suggests that subtle differences in ADP-ribose substrate recognition may influence viral fitness in the mosquito environment in ways that are not yet understood.” (Lines 368-370)

*Additionally, as both mutants had no detectable enzymatic activity but had quite different phenotypes in mosquitoes, I don't agree with the title stating that catalytic activity modulates dissemination and transmission potential in mosquitoes. It seems more likely that alterations in binding activity or substrate recognition (even suggested by the authors) impact these phenotypes in mosquitoes.*

Regarding the title, we agree with the reviewer that it could be misleading, as both mutants lack catalytic activity yet show distinct phenotypes in mosquitoes. We will therefore modify the title to: "Loss of macrodomain catalytic activity modulates Chikungunya virus dissemination and transmission potential in *Aedes* mosquitoes", which more accurately reflects that the observed phenotypes arise as a consequence of the loss of catalytic activity at position N24.

### **Reviewer #3 (Public review):**

#### *Summary:*

*The authors investigated the role of the nsP3 macrodomain catalytic activity in the replication and transmission of CHIKV in mosquito vectors. The conserved dual-host alphavirus catalytic site N24 has previously been shown to be essential for ADP-ribosylhydrolase activity. Despite this, mosquito-specific alphaviruses do not share this catalytic site. To assess whether the macrodomain catalytic activity of a dual-host virus was essential in insect hosts, the authors targeted the N24 site to abolish catalysis while maintaining binding capacity. The loss of ADP-ribosylation led to the emergence of compensatory mutations at site D31 that impact viral infectivity, dissemination, and transmission in *Aedes* sp. mosquitoes in vivo. The conclusions are well supported by the results and provide insight into the importance of nsP3 macrodomain activity in the mosquito vector, which hasn't been explored before.*

#### *Strengths:*

*The main strength of this study is the use of *Aedes* sp. mosquito models to investigate the selective pressure of macrodomain mutations in vivo. The functional characterization as well as the structural analysis of the mutants provide supporting evidence of a potential role of the compensatory mutations at site D31 in substrate recognition.*

#### *Weaknesses:*

*A considerable part of this study relies on the use of N24 mutant viral stocks generated in Vero cells, which yields an additional mutation at site 31 and consequently doesn't allow the authors to properly dissect the effect of mutation of N24 and D31 independently. It*

*would be recommended to generate stocks with individual mutations in both A549 and U4.4 cells, pooling and concentrating them if needed. Replication of the N24A mutant in A549 cells does not lead to mutation at residue 31. Yet surprisingly, there is no reversion from N back to D at site 31 when the double mutant Vero stocks are passaged in A549. Since they are double mutants, it isn't possible to assess whether the defects in the growth of mutants N24A/T-D31N and N24D-D31H/N compared to WT are due to site 24 or 31, or both (Figure 2, panel c). Even though the authors emphasize that the compensatory mutation could have additional roles that impact viral infectivity and transmission in mosquito cells, it would strengthen the work to show that these mutations would spontaneously appear in stocks generated directly in mosquito cells. As a corollary, is it known whether insect-specific alphaviruses that lack macrodomain catalytic activity have corresponding mutations at site 31?*

We thank the reviewer for this important comment. Regarding the generation of viral stocks in A549 cells, transfection of N24A and N24D viral RNAs into A549 cells did not yield sufficient viral titers to produce usable stocks. However, as described in our response to Reviewer #1, we confirmed the reproducible emergence of D31 second-site mutations in C6/36 mosquito cells and BHK-21 mammalian cells, which will be included in the updated Supplementary Figure 1. These results demonstrate that D31 mutations spontaneously emerge in stocks generated directly in mosquito cells, addressing the reviewer's concern.

Regarding the question about insect-specific alphaviruses, we examined the sequence at position 31 using a multiple sequence alignment of 14 alphaviruses with diverse host ranges, including dual-host, insect-specific, and aquatic alphaviruses. We observed that Yada Yada virus (GenBank: QGR15362.1) has an asparagine (N), Tai Forest alphavirus (GenBank: YP\_009333615) has an aspartic acid (D), Mwinilunga alphavirus (GenBank: BBC45634.1) has an aspartic acid (D), Eilat virus (GenBank: QBG67155.1) has an aspartic acid (D), and Agua Salud alphavirus (GenBank: QEV83787.1) has a lysine (K) at this position. These results suggest that insect-specific alphaviruses do not share a conserved residue at position 31, and therefore no clear conclusion can be drawn regarding a direct correspondence with the compensatory mutations observed in our study. This sequence alignment with the corresponding text will be included as supplementary data in the revised manuscript.

*Additionally, there is a lack of consistency in the prevalence of WT virus at days 5 and 7 in in vivo experiments with Ae. albopictus and Ae. aegypti (Figure 3 and Supplementary Figure 2). This raises concern about the reproducibility of these experiments.*

We thank the reviewer for this observation. We acknowledge that the prevalence of WT virus infection shows variability between experiments and timepoints. Based on our experience with infectious blood meal experiments, this variability is sometimes observed between independent experiments even under identical experimental conditions and with the same virus. In this particular case, each set of experiments was performed with independently produced viral stocks. Specifically, for the experiments shown in Figure 3, WT, N24A-D31N, and N24D-D31H/N viral stocks were produced in parallel from transfection of viral RNAs, and the same stocks were used for sequencing, growth curves, and mosquito infections. Subsequently, when we generated single D31H and D31N mutant viruses, a new WT viral stock was produced in parallel with the D31 mutant stocks, and these independently produced stocks were used for the experiments shown in Supplementary Figure 2. Differences in absolute infection rates between experiments are therefore expected, as they reflect both the use of independently produced viral stocks and the inherent variability in the efficiency of midgut infection and dissemination between mosquito cohorts. Importantly, the comparisons between WT and mutant viruses are always made within the same experiment, using stocks produced in parallel.

*The inability to tease apart the roles of N24 and D31 in mosquito hosts partially prevented the authors from fully achieving their aims, but the work is nonetheless of*

*interest to the field and suggests that more work is necessary to fully understand the role of the nsP3 macrodomain and its catalytic activity in the two disparate but obligate hosts for CHIKV and other dual-host alphaviruses.*

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