Arbovirus high fidelity variant loses fitness in mosquitoes and mice

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The error rate of RNA-dependent RNA polymerases (RdRp) affects the mutation frequency in a population of viral RNAs. Using chikungunya virus (CHIKV), we describe a unique arbovirus fidelity variant with a single C483Y amino acid change in the nsP4 RdRp that increases replication fidelity and generates populations with reduced genetic diversity. In mosquitoes, high fidelity CHIKV presents lower infection and dissemination titers than wild type. In newborn mice, high fidelity CHIKV produces truncated viremias and lower organ titers. These results indicate that increased replication fidelity and reduced genetic diversity negatively impact arbovirus fitness in invertebrate and vertebrate hosts.

Studies with the first RNA-dependent RNA polymerase (RdRp) fidelity variant demonstrated that poliovirus RdRp error rate is a major contributor to population mutation frequency (1) and that reduced genetic diversity negatively impacts dissemination and pathogenesis in vivo (refs. 2–4; although the implications of these findings were limited by a relatively artificial model). The explanation for this reduced fitness is based on the idea that a diverse RNA virus population contains, by chance, more variants with potentially advantageous adaptive mutations, whereas a less diverse population is not as likely to possess such variants. Based on these observations, we hypothesized that RdRp fidelity of other RNA viruses can be modulated with similar fitness costs.

Nucleoside analogs including ribavirin and 5-fluorouracil (5-FU) are mutagenic compounds that are misincorporated into viral genomes during RNA synthesis, resulting in deleterious mutations by mispairing in the following replication cycle (5–7). The consequent increase in the number of mutations places more genomic RNAs beyond a hypothetical error threshold: a mutation frequency at which the majority of the population harbors low fitness or lethal mutations (5, 8). In this theoretical context, RNA virus populations lie close to this threshold—even moderate increases in mutation frequency could severely diminish infectivity. Increasing polymerase fidelity would potentially create a population farther from this error threshold that could better tolerate mutational input. Solid evidence supports this logic: DNA polymerase (9, 10) and reverse transcriptase (11–13) virus variants with antimutator phenotypes are linked to mutations that increase fidelity and reduce base analog incorporation. These observations present an interesting paradox: If point mutations in RNA viruses can increase RdRp fidelity, thereby decreasing replication mistakes, why is higher fidelity in RNA viruses not selected in nature? Is there a tradeoff between being error-prone but adaptable or being less adaptable but possessing higher fidelity? Does higher fidelity necessarily incur a phenotypic disadvantage in hosts, where a reduced ability to produce adaptive mutations impairs fitness, as was suggested by poliovirus studies?

Arthropod borne (arbo-) viruses are unique among RNA viruses in that they obligately cycle between vertebrate and invertebrate hosts, a pattern that imposes additional selective constraints compared with single-host RNA viruses (14–22). In general, arbovirus fitness constraints differ in vertebrates and insects and may be virus-specific, yet fitness is not impaired by alternating hosts. However, studies where one host was artificially removed from alternate cycling reveal that host alternation renders arboviruses less adaptable than single-host viruses because selection is focused on maintaining replication competence in both hosts by favoring generalist genomes whose fitness is a compromise for each organism (16, 20, 21). An arbovirus RdRp fidelity variant is therefore a unique tool for understanding how restricted genetic diversity influences phenotype in invertebrate and vertebrate hosts where selection pressures differ.

Here, we use chikungunya virus (CHIKV; family *Togaviridae*, genus *Alphavirus*) to isolate and describe a unique arbovirus RdRp fidelity variant. CHIKV is a mosquito-borne virus that has caused outbreaks in humans since the 18th century and, since 2004, in Africa, Indian Ocean islands, Southeast Asia, Italy (reviewed in ref. 23), and France (24). By serial passage of CHIKV in ribavirin or fluorouracil (5-FU), we selected a mutagen-resistant variant with a single amino acid change (C483Y) in the nsP4 RdRp gene that increases replication fidelity. In a mosquito host, *Aedes aegypti*, high fidelity virus presents lower infection and dissemination titers than wild type. Higher fidelity CHIKV also produces truncated viremias and lower organ titers compared with wild type in newborn mice. These results suggest that increased replication fidelity and reduced genetic diversity impose a fitness cost in invertebrate and vertebrate arbovirus hosts.

Results

Generation of RNA Mutagen-Resistant CHIKV Populations. To select for a high fidelity (antimutator) variant of CHIKV, we sought to generate resistance to mutagenic agents by using previously described optimized conditions (25). Wild-type (WT) CHIKV rescued from an infectious clone was serially passaged 20 times in the presence or absence of 100 μM ribavirin or 10 μg/mL 5-FU. After one passage (p1), mean ribavirin- and 5-FU–treated CHIKV titers were reduced by ≈2.0 logs ($P < 0.001$) compared with CHIKV titers for untreated replicates (Fig. 1*A*). By p5, the mutagen-treated CHIKV resisted treatment with both compounds: No significant reduction in titer was observed for ribavirin-treated CHIKV, and 5-FU–treated CHIKV achieved significantly higher titers than untreated CHIKV ($P < 0.007$). This phenotype was observed through the series to p20, indicating that mutagen resistance had been generated.

Single Amino Acid Change in the nsP4 Polymerase, C483Y, Confers Resistance to RNA Mutagens. To identify the mutation(s) conferring resistance to ribavirin and 5-FU, the CHIKV nsP4 polymerase gene was amplified and sequenced from selected passage replicates. nsP4 was chosen because polymerase point mutations

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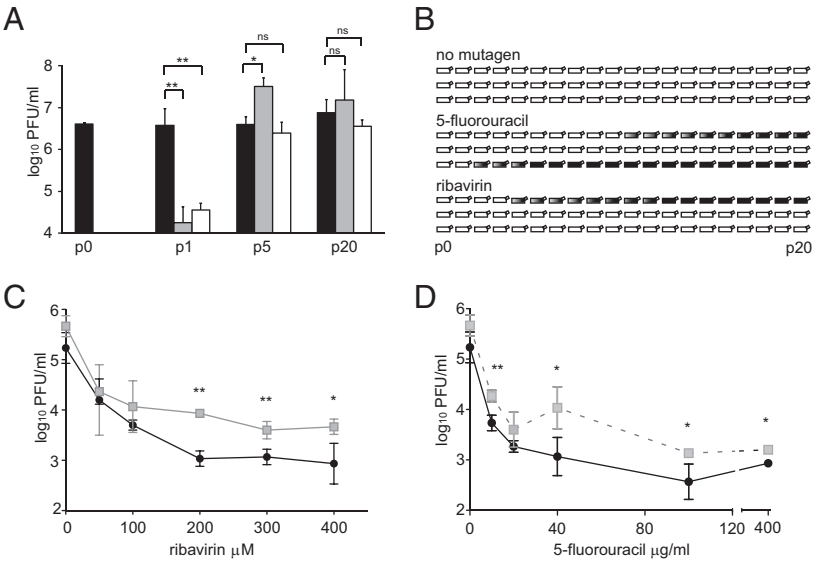

Fig. 1. (*A*) Generation of RNA mutagen-resistant CHIKV. WT CHIKV was serially passaged in 100 μM ribavirin (white), 10 μg/mL 5-FU (gray), or no compound (black) at 0.1 pfu/cell. p0 shows the initial WT stock titer. Mean titers of harvests ± SD are shown (Student's *t* test, $n = 3$, $*P < 0.05$, $**P < 0.001$). (*B*) Schematic of mutagen passage series showing appearance of nsP4 C483Y mutation. Selected replicates from the 20 triplicate passages were sequenced by flanking CHIKV nsP4 483. WT CHIKV is shown in white, the emergence of Y is represented by black/white flasks, and replacement of WT by C483Y is shown in black. (*C* and *D*) Resistance of CHIKV WT and C483Y to mutagens. Matched titers of WT (black line) and C483Y (gray line) were inoculated in triplicate in HeLa cells with increasing concentrations of ribavirin (*C*) or 5-FU (*D*). Mean titers ± SD are shown (Student's *t* test, $n = 3$, $*P < 0.05$, $**P < 0.001$).

were identified as mediators of mutagen resistance for other RNA viruses (4, 26, 27). A single amino acid change (C»Y) at nsP4 483 was identified in one of three ribavirin-treated replicates at p5 and in two of three 5-FU–treated replicates at p3 and p10 (Fig. 1*B*). Based on sequencing chromatograms, by p20, Y was the only amino acid present at nsP4 483 in two of the populations, whereas it existed in a mix with the WT C amino acid in the third. Full genome sequencing of selected p20 replicates revealed two other mutations: one in the E2 gene (E266K) arising in all passage conditions, and a nsP2 G641D mutation in the mutagen-treated populations. The E2 mutation is likely an adaptive mutation favoring growth in HeLa cells because it appeared in every passage condition regardless of whether mutagen was present. Although the nsP2 mutation may impart compound resistance by mechanisms not directly related to polymerase fidelity, here we focus only on nsP4 C483Y.

The appearance of nsP4 C483Y in both mutagen passage series coinciding with virus titers comparable with untreated CHIKV by p5 strongly suggests that this mutation is the principal mediator of mutagen resistance. To test this hypothesis, the infectious clone WT cDNA was modified to encode the C483Y polymerase. WT and C483Y stocks were generated in parallel and tested for their ability to resist ribavirin and 5-FU over a range of concentrations (Fig. 1*C*). C483Y resisted ribavirin and 5-FU treatment significantly better than WT at most concentrations tested. Maximum differences in resistance between C483Y and WT were 1 (40 μg/mL 5-FU) to 1.5 logs (200 μM ribavirin), within the range observed for higher fidelity variants of picornaviruses (1, 26). These results confirm that C483Y mediates ribavirin and 5-FU resistance.

C483Y Produces Fewer Mutations than WT CHIKV. To test the possibility that C483Y mutagen resistance results from increased polymerase fidelity, only previously observed for picornaviruses (1), the mutation frequencies were compared (Fig. 2*A*). In each of five independent replicates rescued from parallel BHK electroporations, where a mean of ≈55,550 nt were sequenced per replicate, C483Y consistently produced fewer mutations than WT (3.6 versus 5.1 mutations per 10,000 nt, $P < 0.001$). Thus, mutagen resistance is likely due to higher fidelity of C483Y, which produces a population with a lower baseline mutation frequency that lies farther from the error threshold and, therefore, better tolerates mutagen treatment. To determine whether the observed mutagen resistance indeed correlated with a lower mutational burden, we compared mutation frequencies of viruses treated with 200 μM ribavirin (Fig. 2*B*); the concentration at which the greatest difference in sensitivity between WT and C483Y was observed (Fig. 1*C*). In 200 μM ribavirin, mutation frequencies of both viruses were increased compared with those in the absence of ribavirin (Fig. 2*A* vs. Fig. 2*B*). Although the frequency of C483Y increased to 4.6 mutations per 10,000 nt, it remained within the range of the frequency for untreated WT that reached comparable titers (Fig. 1*C*). In contrast, the mutagenized WT frequency increased to 6.1 mutations per 10,000 nt and correlated with a more significant drop in virus infectivity (Fig. 1*C*).

To further confirm reduced heterogeneity of C483Y compared with WT CHIKV, one replicate of each virus (rescued as described above) was deep sequenced by preparing nine cDNA libraries corresponding to ≈1 kb RT-PCR amplicon fragments spanning the CHIKV genome that were independently generated from total viral RNA. The 12.7 million WT and 14.3 million C483Y quality-filtered sequences were aligned against the corresponding consensus sequence, resulting in a mean 54,000× genome coverage. The percentage of total reads that contained 0 (perfect match), 1, or 2 mutations versus consensus were compared (Fig. 2 *C*–*E*). For every library, C483Y had significantly more reads with 0 mutations compared with WT (Pearson correlation 0.82, two-tailed; $P = 0.003$). WT had significantly more reads with 1 (Pearson correlation 0.82; $P = 0.003$) and 2 (Pearson correlation 0.80; $P = 0.005$) mutations compared with C483Y. Taken together, these sequencing results strongly support that C483Y is a high fidelity variant of CHIKV.

To rule out that the lower mutation frequency of C483Y is the result of a replication defect, virus multiplication and RNA synthesis were assayed in one-step growth curves at 1 pfu/cell in mammal BHK and mosquito C6/36 cells. In both cell types, production of infectious particles by WT or C483Y did not differ significantly from 0 to 24 h (repeated measures ANOVA for curve; Fig. 2 *F* and *G*). Similarly, RNA production in BHK cells did not differ significantly between WT and C483Y (Fig. 2*H*). In C6/36 cells, C483Y produced a small but significantly higher amount of RNA than WT ($P < 0.02$) (Fig. 2*I*). Impaired C483Y virus or RNA production compared with WT CHIKV, therefore, does not explain its lower mutation frequency.

C483Y Mutation Does Not Significantly Impact in Vitro Fitness. To assess whether C483Y incurs a fitness cost in vitro, two types of

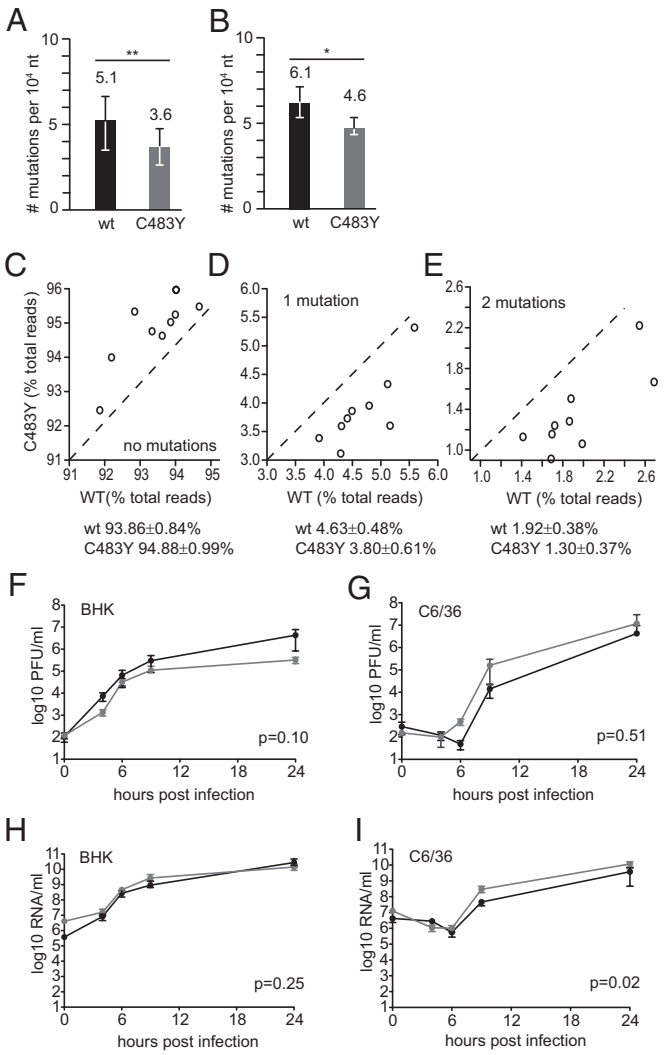

Fig. 2. (*A* and *B*) Mutation frequencies of CHIKV WT and C483Y. Five independent stocks per virus were generated in DMEM (*A*) and two independent replicates were generated in 200 μM ribavirin (*B*). A mean of 65 partial E1 sequences (≈55,550 nucleotides·replicate^{-1}) were obtained. The mean mutation frequencies (number of polymorphisms per 10,000 nt sequenced) ± SEM represent the sum of all replicates; the same pattern of lower mutation frequency for C483Y (gray bar) than for WT (black bar) was observed in each replicate. (X^2, $n = 5$ (*A*), $n = 2$ (*B*); $^{**}P < 0.001$, $^{*}P < 0.1$). (*C–E*) Genome-wide deep sequencing of CHIKV WT and C483Y. Nine cDNA libraries spanning the CHIKV genome were sequenced. For each cDNA library (open circle), the percentage of the total quality-filtered reads presenting 0 (*C*), 1 (*D*), or 2 (*E*) mutations compared with consensus were plotted as a function of C483Y (*x* axis) and WT (*y* axis). The dashed line delineates where equal numbers of mutations for WT and C483Y would fall. The percentage values ± SD for all libraries combined are noted below graphs. (*F–I*) One-step growth curves and RNA synthesis. BHK and C6/36 cells were inoculated in triplicate with CHIKV WT (black) or C483Y (gray) at 1 pfu/cell and supernatant titers (*F* and *G*) were determined by Vero plaque assay and genome copy measures (*H* and *I*) were determined from the same samples by quantiative RT-PCR. Mean values ± SD and *P* values (repeated measures ANOVA, $n = 3$) are shown.

competition assays were performed in mammal and mosquito cells. In direct assays (Fig. 3*A*), the relative fitness of WT was compared with that for C483Y via direct competition in the same well for three successive passages. The codon at nsP4 483 was used to differentiate the two competitors. After three passages in BHK cells, both nucleotides showed similar abundance in sequencing chromatograms, indicating equal fitness. In C6/36 cells, WT chromatogram peaks were slightly higher, suggesting that WT has a slight fitness advantage over C483Y in mosquito cells. In a more quantitative but indirect assay (Fig. 3*B*), fitness was determined by competing each virus against the same marked reference competitor under matching conditions. In BHK cells, both WT and C483Y showed neutral fitness with respect to the marked reference population and to each other, consistent with results from direct competition assays. In C6/36 cells, the fitness of C483Y was also neutral to the reference, whereas a slightly higher WT fitness compared with C483Y mirrored the direct competition assay results, but was not nearly as augmented as a CHIKV population that was adapted to mosquito cells (16). Together these results indicate that CHIKV C483Y is not less fit than WT in BHK cells and is slightly less fit in C6/36 cells, despite replicating as well as WT in mosquito cells (Fig. 2 *G* and *I*).

Chikungunya Virus C483Y Infection and Dissemination Are Impaired in a Mosquito Host. To determine whether the reduced genetic diversity of CHIKV C483Y affects vector infectivity, colonized *A. aegypti* from two sources were presented with matched-titer infectious bloodmeals containing WT or C483Y. Although a similar percentage of mosquitoes from both sources developed a disseminated infection, C483Y-infected *A. aegypti* from both colonies presented significantly lower mean body and leg titers by 0.5–1.0 log than WT-infected mosquitoes on day 7 (Fig. 4 *A* and *C*). However, there was no significant difference in mean transmitted titers of WT or C483Y on day 7 in mosquitoes from either source, and individuals salivated a similar range of doses of both variants, from ≈0.5 to 3 log pfu (Fig. 4 *B* and *D*).

Chikungunya Virus C483Y Viremia and Organ Titers Are Restricted in a Mammalian Model. To assess whether reduced genetic diversity of C483Y influences kinetics and magnitude of infection in vertebrates, 8-d-old C57BL/6 mice (28) were inoculated in parallel with a sublethal dose (160 pfu/mouse) of CHIKV WT or C483Y and sera, liver, muscle, and brain were harvested 5 d after inoculation (PI). Despite similar brain and muscle titers, no CHIKV was detectable in the liver of C483Y-infected animals, compared with a mean of 4.0 $\log_{10}$ pfu/mL in WT-infected mice ($P < 0.0001$) (Fig. 4*E*). Viremias in C483Y-infected mice were also significantly lower than WT-infected animals ($P = 0.005$); only one C483Y-infected neonate contained detectable viremia compared with all wt-infected mice whose titers ranged from 3.6 to 4.2 $\log_{10}$ pfu/mL. In a follow-up study to further examine viremia kinetics, each neonate was delivered a sublethal, yet higher, dose (560 pfu) of either CHIKV variant. No CHIKV was detected in serum from any C483Y-infected mice on day 7 PI, whereas all WT-infected mice were still viremic (mean 3.8 $\log_{10}$ pfu/mL) (Fig. 4*F*). Sequences flanking nsP4 483 in selected organs of C483Y-infected mice revealed that C483Y did not revert to WT.

Discussion

To date, in vivo infection dynamics of a higher fidelity variant have only been described for poliovirus (2, 3), raising the question of whether modulation of RdRp fidelity is unique to this family (*picornaviridae*) or universal for RNA viruses. Here, we identify a CHIKV RdRp mutation at C483Y that mediates resistance to two RNA mutagens. C483Y produced similar amounts of RNA and infectious virus compared with WT, indicating that replication was not impacted by this mutation. However, C483Y generated populations with reduced mutation frequency and restricted genetic diversity detected by classic Sanger and Illumina deep sequencing technologies. Although in vitro incorporation assays do not exist for alphaviruses, our results support that C483Y is a higher fidelity RdRp variant. In support of this claim, the resulting 30% reduction in mutation frequency of C483Y compared with WT CHIKV is within the

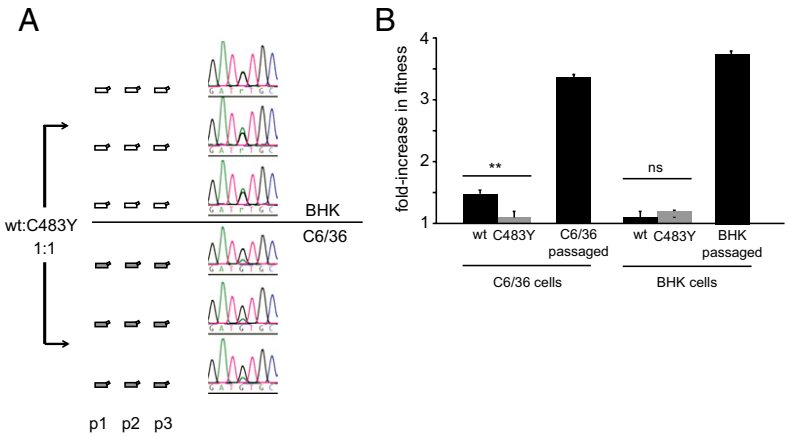

Fig. 3. Competition assays comparing relative fitness of CHIKV WT and C483Y. (*A*) Direct assays: Viruses were mixed at a 1:1 ratio and inoculated in triplicate into BHK or C6/36 at 0.1 pfu/cell for three passages, at which point nsP4 483 was sequenced. The abundance of each competitor was measured visually as the height of the nucleotide encoding either WT (G nt) or C483Y (A nt) in sequencing chromatograms. (*B*) Indirect assays: Each virus was mixed in a 1:1 ratio with the marked competitor presenting a silent SacII restriction site and inoculated in triplicate in BHK or C6/36 at 0.1 pfu/cell. The progeny RNA was RT-PCR amplified, and restriction fragment length polymorphism assays were performed to determine the abundance of each competitor. Fitness is represented as the output:input ratio of the CHIKV nsP4 483 variant:marked competitor. A fitness value >1 indicates that WT or C483Y is more fit than marked competitor. Mean values ± SD are shown (Student's *t* test, $n = 3$, $^{**}P < 0.001$). For comparison, viruses adapted to BHK and C6/36 cells via serial passage are included (16).

same range as reductions observed for higher fidelity variants of picornaviruses (4, 26). Because crystal structure data are not available for the RdRp of alphaviruses, we do not know how residue 483 modulates fidelity at the conserved GDD active site (nsP4 residues 465–467), although in linear sequence these amino acids are in close proximity. However, fidelity of the G64S poliovirus variant was modulated through an extensive network of H bonds distant from the active site (29, 30), indicating that proximity to the active site does not necessarily inform on possible effects on fidelity. Generation of other mutations at CHIKV nsP4 483 and adjacent sites may help better understand alphavirus RdRp activity in the absence of a structure.

Fidelity variants with WT-like replication kinetics are valuable tools to understand the roles of genetic diversity and mutation rates in virus fitness and may constitute an ideal experimental model to study virus evolution in general. The correlation between reduced genetic diversity and fitness in vivo was first demonstrated with higher fidelity poliovirus (2–4). However, the artificial nature of the poliovirus mouse model (transgenic expression of the virus receptor, necessity for high infection doses exceeding 10^6 particles for WT, and an inability to infect hosts naturally via the oral route) makes it difficult to validate the observed in vivo fitness costs. The current study presents two key advances: These experiments describe a fidelity variant in a natural infection, both in terms of host (invertebrate) and route (oral), and, although mice are imperfect models of human CHIK, infection of the vertebrate occurs through a naturally expressed virus receptor (unlike the poliovirus receptor transgenic murine model) at more biologically relevant, low infection doses. Despite exhibiting WT-like fitness in mosquito and mammal cells, the restricted genetic diversity of CHIKV C483Y imposes a significant fitness cost in invertebrates and vertebrates: Less virus infects and disseminates in mosquitoes, and neonatal mice exhibit truncated viremias and lower organ titers. These data suggest that genetic diversity of an RNA virus is necessary for maximal infection and dissemination, factors that strongly affect transmission. This last point is highlighted by truncated viremias in newborn mice. Shorter CHIKV viremia in a vertebrate would restrict the window of time during which a feeding mosquito can become infected to perpetuate transmission. Although the observed fitness reduction of C483Y may result from compromised adaptability in complex environments, we cannot exclude the possibility that it stems from costs of increased fidelity on other traits. For example, although increasing fidelity did not affect replication kinetics in cell culture, replication rates in different tissues and cell types in vivo, which were not measured here, may have been negatively impacted. Alternatively, rather than being more adaptable, the higher fitness observed for WT CHIKV might result from a greater incidence of positive epistasis among mutated genomes compared with C483Y, where interactions among mutations in the WT population may better mask effects of deleterious mutations (31).

For small rodent models of CHIKV infection, highly permissive newborn or adult IFN receptor-deficient mice show severe disease including fatality when infected with WT virus (28), a presentation that is more severe than human CHIK disease. In an immunocompetent human host, the difference between CHIKV C483Y and WT might be even more exaggerated, where fewer mutations in the C483Y population might result in a lower likelihood of variants capable of circumventing established innate and adaptive immune responses. Nevertheless, our results suggest that a CHIKV population with reduced genetic diversity via lower mutation frequency is less capable of outcompeting developing immune responses that control viremia in neonatal mice.

In *A. aegypti*, a reduced ability to infect and disseminate has implications for transmission and vector competence, although no reduction in the rate of transmission for C483Y compared with WT was observed here. In general, reduced infection of hosts by a less diverse arbovirus suggests that the viral mutation rate, despite being extremely high, is finely tuned to optimize virus survival. Too few mutations may lead to extinction when facing host environmental changes, immune responses, and anatomical or transmission bottlenecks, all obstacles to successful arbovirus perpetuation. Despite these observations, the fitness cost for C483Y in mosquitoes was not absolute and the reduction in vector infectivity and dissemination was moderate. Although mean titers were lower, variance within C483Y-infected mosquitoes was greater than for WT (Fig. 4 *A* and *C*, e.g.) and some C483Y-infected organs contained more CHIKV than WT (e.g., legs in Fig. 4*C* and saliva in Fig. 4*D*). Therefore, lower genetic diversity in the C483Y population probably only reduces, but does not exclude, the capacity to generate mutants required for high titer infections that favor transmission. This observation underscores the complex interplay between the ability to generate random

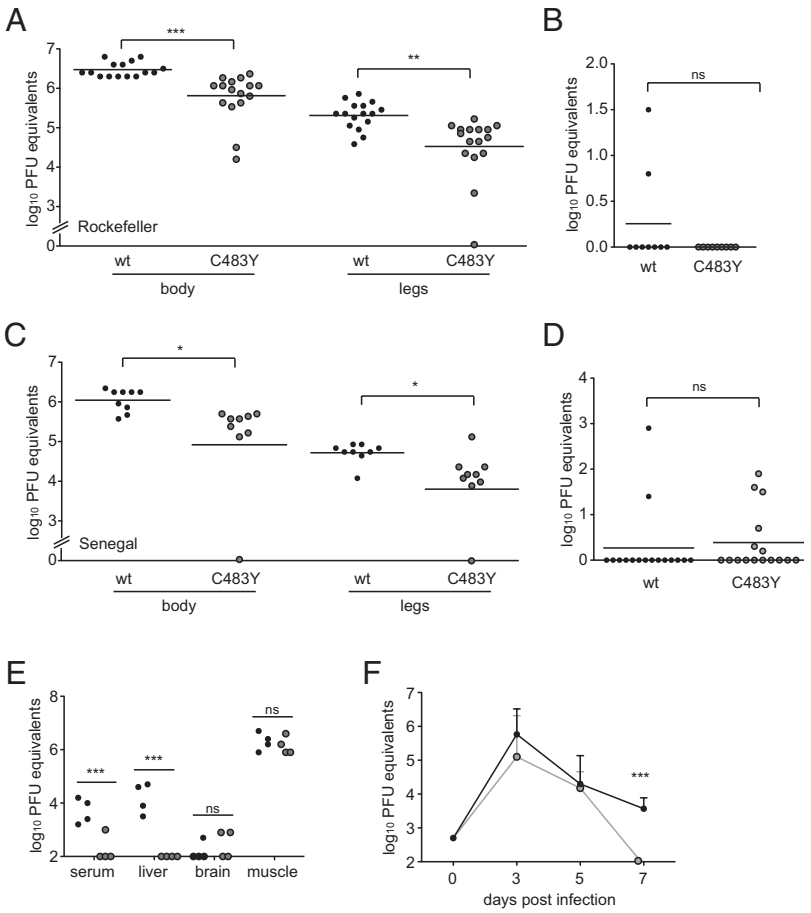

Fig. 4. Infection of WT CHIKV and C483Y in invertebrate and vertebrate hosts. (*A–D*) CHIKV titers in mosquitoes: *A. aegypti* from the Rockefeller colony (*A* and *B*) or a F_{23} colony from Kedougou, Senegal (*C* and *D*), were assayed 7 d after ingestion of infectious bloodmeals containing ≈5 $\log_{10}$ pfu/mL CHIKV WT or C483Y. Back titrations of bloodmeals and single mosquitoes harvested after feeding showed no significant differences in ingested titers. CHIKV pfu equivalents in homogenized bodies and legs/wings (*A* and *C*) and saliva samples (*B* and *D*) were measured by using quantitative RT-PCR. The limit of detection (0.3 $\log_{10}$ pfu equivalents) is shown as 0. Dots represent individual mosquito titers, lines show mean cohort titers (Student's *t* test, $n = 16$ for Rockefeller, $n = 9$ for Senegal; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*E*) CHIKV tissue titers in mice: 8-d-old C56BL/6 mice were inoculated s.c. with 160 pfu of CHIKV WT (black) or C483Y (gray) and organs were sampled on day 5. Dot show individual organs; lines indicate means (Student's *t* test, $n = 4$; $^{***}P < 0.0001$) The *y* intercept denotes the limit of detection (1.9 $\log_{10}$ pfu/mL). (*F*) Mean CHIKV viremias in mice inoculated with 560 pfu/mouse of WT or C483Y. Day 0 values show inocula measured by back titration. (Student's *t* test, $n = 5$; $^{***}P < 0.0001$).

mutations and the probability of generating specific mutations favored by selection.

In summary, we show that arbovirus RdRp fidelity can be modulated and results in fitness costs in vivo in invertebrate hosts and a vertebrate model. The CHIKV fidelity increase selected by our screen may be amplified by other amino acid changes. This study should encourage searches for fidelity modulating changes in the RdRp of other arboviruses. Fidelity variants are useful tools to study virus evolution, particularly for arboviruses that face two disparate sets of selective pressures where diversity could be especially advantageous for surviving host alternation.

Fidelity-changing mutations, coupled with other attenuating mutations obtained via conventional passage in cells, could prove useful for developing genetically stable live virus vaccines (4). For CHIKV, no licensed vaccine currently exists; developing one could prevent millions of human infections each year.

Experimental Procedures

Cells and Viruses. Mammalian cell lines BHK, HeLa and Vero were grown in DMEM with 10% inactivated calf serum (CS) and 1% penicillin/streptomycin (P/S) at 37 °C with 5% CO_2. C6/36 mosquito cells were maintained in L-15 medium with 10% CS, 1% P/S, 1% nonessential amino acids, and 1% tryptose phosphate broth at 28 °C with CO_2. WT CHIKV was rescued from an infectious clone made from the low-passage (two insect cell passages) strain 06-049 isolated in 2006 in Reunion Island, which possesses the E1 A226V mutation, as described (16). The nsP4 C483Y mutation was introduced into the WT infectious clone by site-directed mutagenesis (Stratagene XL-10 kit). Virus stocks were generated by electroporation of in vitro transcribed infectious RNA as described, using predetermined conditions that permit 95% transfection efficiency (16). In vitro transcribed RNA was measured qualitatively on gels and quantitatively by nanodrop (Thermo Scientific). Matched amounts of WT and C483Y RNA were electroporated in each parallel replicate. Passages and replication kinetics (for titer and RNA level measurements) were performed in parallel to ensure that disparities in mutation frequency could not be attributed to differences in experimental conditions. Sequencing of C483Y in stocks after electroporation and from selected tissue samples confirmed that the mutation was stable and that reversion to WT did not occur.

Mutagen Passages. Isolation of higher fidelity variants by mutagen passage was performed as described (25). Briefly, CHIKV was passaged 20 times in HeLa cells in triplicate in either 100 μM ribavirin or 10 μg/mL 5-FU. Cells were inoculated with WT CHIKV at 0.1 pfu/cell and virus harvested 72 h PI from supernatants was diluted to ≤0.1 pfu/cell and used in subsequent passages. Mean titers were determined by plaque assays on Vero cells.

Replication Kinetics and RNA Synthesis of CHIKV WT Versus C483Y. One-step growth curves were conducted in BHK and C6/36 cells as described (16). Titers were determined by Vero plaque assays. For CHIKV genome copy measures,

RNA was extracted from the same samples and quantitative RT-PCR was performed by using published protocols (16) adapted from Lanciotti et al. (32) that measure positive-strand RNA and amplify the nsP4 gene in a region distant from amino acid 483. A standard curve ($y = -3.12x + 42.55$; $R^2 = 0.96$) was generated by using two replicates of in vitro transcribed genomic RNA. For measurements in infected mosquitoes, a standard curve ($y = -3.47x + 34.49$; $R^2 = 0.97$) was generated from serially diluted CHIKV bloodmeals of known titer. Values represent averages of duplicate measures.

Direct and Indirect Competition Fitness Assays. ***Direct.*** CHIKV WT and C483Y were mixed in a 1:1 ratio and inoculated in triplicate wells containing BHK or C6/36 cells at 0.1 pfu/cell over three passages, after which RNA was extracted and a fragment flanking nsP4 483 was amplified by RT-PCR and sequenced. The abundance of each competitor was measured as height of the nucleotide encoding either WT (nucleotide G) or C483Y (A) in sequencing chromatograms. ***Indirect.*** Using established methodologies (16), the fitness of WT CHIKV was indirectly compared with that for C483Y by competing each variant in a 1:1 ratio against the same marked competitor, a WT CHIKV variant with a silent SacII restriction site at nsP2 amino acid 660. Forty-eight hours PI of BHK or C6/36 cells at 0.1 pfu/cell, RNA was extracted from cell culture supernatants and a restriction fragment length polymorphism assay was performed. Fitness is represented as the output:input ratio of the nsP4 483 variant:marked competitor CHIKV.

Sequencing. CHIKV RNA was extracted in TRIzol (Invitrogen), and amplicons were generated by using the Titan one-step RT-PCR kit (Roche). To determine mutation frequencies, a mean of 65 partial E1 sequences of ≈800 nt per replicate (primers flanking genome positions 9943–10746) were cloned into TOPO vectors (Invitrogen) and sequenced. The E1 gene was selected because of its relatively high tolerance for mutation. Mutation frequencies (mutations per 10,000 nt) were determined as described (25). For deep sequencing, cDNA libraries were prepared by RT (Superscript III; Invitrogen) and PCR (Phusion; NEB) amplifications of nine fragments spanning the CHIKV genome, fragmented (Fragmentase; NEB), clusterized, and sequenced with Illumina cBot and GAIIX technology by following manufacturer's instructions. Of the 30 million sequences obtained per virus, ≈15 million passed quality (maximum 2 mismatches per read, no gaps, no N's) and CHIKV-specific filtering and were aligned to reference sequences by using Razers (33). Total reads were used to determine the percentage of reads with 0, 1, or 2 mismatches with respect to consensus.

Mosquito Infections. Virus stocks diluted to ≈5 $\log_{10}$ pfu/mL were mixed 1:1 with artificial bloodmeals consisting of PBS-washed rabbit blood, warmed to 37 °C in water-jacketed membrane feeders, and presented to cohorts of 3- to 6-d-old female *A. aegypti* from a Rockefeller colony or a F_{23} colony from Kedougou, Senegal (subspecies *aegypti*). *A. aegypti* is a principal CHIKV vector (23) that exhibits high infection, dissemination, and transmission rates (34, 35). After 7 or 14 d incubation of engorged mosquitoes, the legs and wings were removed from each individual and its proboscis was inserted into ≈3 μL of C6/36 growth medium inside a 10-μL glass capillary for 30–45 min of salivation. The saliva, body, and legs/wings samples were homogenized in up to 300 μL of C6/36 medium in a MM300 homogenizer (Retsch) at 30 shakes per s for 2 min.

Mouse Infections. C57BL/6 mice (Janvier) were housed according to Institut Pasteur guidelines for animal husbandry in level-3 isolators. Eight-day-old neonates were inoculated s.c. in the back with either 160 ($n = 9$ per variant) or 560 ($n = 11$ per variant) pfu of CHIKV WT or C483Y. Three, 5, and 7 d PI, a subset of mice were killed. Blood was collected by decapitation and brains, livers, and right thigh muscles from the hip to the ankle were harvested at 5 and 7 d PI. Virus recovered from homogenized tissues (settings as for mosquitoes) in 300 μL of DMEM or in serum recovered from whole blood via centrifugation (825 × *g* for 5 min) was titrated by Vero plaque assay. Organ titers comprise values for the entire tissue.

Statistical Analyses. Statistical tests, noted where applied, were conducted by using GraphPad Prism. *P* values >0.05 were considered nonsignificant (ns).

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