

Low-fidelity Venezuelan equine encephalitis virus polymerase mutants to improve live-attenuated vaccine safety and efficacy

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Abstract

During RNA virus replication, there is the potential to incorporate mutations that affect virulence or pathogenesis. For live-attenuated vaccines, this has implications for stability, as replication may result in mutations that either restore the wild-type phenotype via reversion or compensate for the attenuating mutations by increasing virulence (pseudoreversion). Recent studies have demonstrated that altering the mutation rate of an RNA virus is an effective attenuation tool. To validate the safety of low-fidelity mutations to increase vaccine attenuation, several mutations in the RNA-dependent RNA-polymerase (RdRp) were tested in the live-attenuated Venezuelan equine encephalitis virus vaccine strain, TC-83. Next generation sequencing after passage in the presence of mutagens revealed a mutant containing three mutations in the RdRp, TC-83 3x, to have decreased replication fidelity, while a second mutant, TC-83 4x displayed no change in fidelity, but shared many phenotypic characteristics with TC-83 3x. Both mutants exhibited increased, albeit inconsistent attenuation in an infant mouse model, as well as increased immunogenicity and complete protection against lethal challenge of an adult murine model compared with the parent TC-83. During serial passaging in a highly permissive model, the mutants increased in virulence but remained less virulent than the parent TC-83. These results suggest that the incorporation of low-fidelity mutations into the RdRp of live-attenuated vaccines for RNA viruses can confer increased immunogenicity whilst showing some evidence of increased attenuation. However, while in theory such constructs may result in more effective vaccines, the instability of the vaccine phenotype decreases the likelihood of this being an effective vaccine strategy.

Key words: arbovirus; fidelity; vaccine; virus diversity; alphavirus.

1. Introduction

Due to the error-prone nature of the RNA-dependent RNA-polymerase (RdRp), RNA virus replication is characterized by a high mutation rate that results in increased genetic diversity of progeny viruses (Domingo et al. 2005). This diversity can be utilized by the virus to evade and escape the immune system, as well as to adapt to new hosts. Maintaining the correct amount of virus diversity of ~1 mutation per genome (Nebot et al. 2010) is of utmost importance to RNA virus survival within the host and during transmission. This is especially true for arboviruses (arthropod borne viruses), which utilize two distinct hosts to complete their transmission cycle. If the mutation rate of an RNA virus is too high, more unfit progeny will be produced due to the increased number of mutations, most of which are deleterious by chance. This results in a substantial decrease in viable genomes, and possible extinction of the viral population due to error catastrophe (Manrubia et al. 2010). Conversely, if the mutation rate is too low, little variation is produced, and the virus population becomes clonal and less able to adapt to diverse environments. Both scenarios reduce the overall fitness of the virus and can lead to extinction (Escarmís et al. 2008). This predicted lack of fitness with high- or low-fidelity replication has been demonstrated experimentally with a number of viruses, including poliovirus (PV) (Pfeiffer and Kirkegaard 2003; Vignuzzi et al. 2006), chikungunya virus (CHIKV) (Coffey et al. 2011; Rozen-Gagnon et al. 2014), West Nile virus (Van Slyke et al. 2015), St Louis encephalitis virus (Griesemer et al. 2017), human enterovirus 71 (Sadeghipour and McMinn 2013; Sadeghipour et al. 2013; Meng and Kwang 2014), foot and mouth disease virus (Arias et al. 2008; Zeng et al. 2013, 2014; Xie et al. 2014), SARS CoV (Eckerle et al. 2010; Graham et al. 2012; Graepel et al. 2017), influenza virus (Cheung et al. 2014; Pauly et al. 2017), Norovirus (Arias et al. 2016) and Coxsackie B virus (Gnädig et al. 2012; Levi et al. 2010; McDonald et al. 2016). These viruses were all subjected to treatment with nucleoside analogs such as Ribavirin or 5'Fluorouracil, which increases the mutation rate and can lead to virus extinction, or by the insertion of mutations found to change fidelity in other viruses. Virus populations circumvent extinction by developing resistance to the nucleoside analog, sometimes by developing mutations in the RdRp that either increase (high fidelity) or decrease (low fidelity/hypermutator) RdRp fidelity (i.e. the RdRp error-rate). When compared with unpassaged, wild-type (wt) viruses, fidelity mutants have similar growth kinetics *in vitro*, but are attenuated *in vivo* due to the alteration of diversity produced during replication, which hampers the ability of the virus to overcome bottlenecks in the host (Pfeiffer and Kirkegaard 2005; Vignuzzi et al. 2006).

The alphavirus genus, family *Togaviridae*, contains a number of important arboviruses that are distributed worldwide. Alphaviruses are classified as either Old World or New World viruses depending on their distribution. Venezuelan equine encephalitis virus (VEEV) is a New World encephalitic alphavirus that circulates continuously in an enzootic cycle in the forests and swamps of northern South America, Central America and Mexico, typically between rodents and mosquitoes. Spillover from this cycle is believed to cause tens of thousands of dead-end human infections annually (Aguilar et al. 2011). Periodically, the virus emerges to cause epizootic cycles in equids, which can also spill over into humans, sometimes resulting in hundreds of thousands of infections (Sudia et al. 1975; Wang et al. 2001; Brault et al. 2002; Weaver et al. 2004; Anishchenko et al. 2006). Prior to the 1960s, equine epizootics

involving high fatality rates were frequently recorded in VEEV-endemic areas. Since horses remain important components of the local agricultural economies within these regions, VEEV has had a major impact on the economic output of at-risk areas (Walton and Grayson 1988). Recent outbreaks in both Venezuela and Mexico demonstrate the ability of VEEV to re-emerge periodically from its enzootic niche (Rico-Hesse et al. 1995; Weaver et al. 1996; Oberste et al. 1998), and some earlier outbreaks spread as far north as Texas (Lord 1974; Sudia et al. 1975). Independent of these natural cycles of infection, there is great concern for the use of VEEV as a biological weapon due to its high infectivity via the aerosol route. To address this threat, multiple vaccines have been developed to protect against VEEV infection.

The first human vaccine described for VEE, TC-83, was produced following 83 serial passages of the Trinidad donkey VEEV strain (subtype IAB) in guinea pig heart cells (Berge et al. 1961). Sequencing of TC-83 revealed eleven nucleotide changes, but attenuation was attributed to only 2 of these mutations; one in the 5' UTR (untranslated region) and one in the E2 glycoprotein gene (Kinney et al. 1989, 1993). The reliance of TC-83 on only two point mutations for its attenuation increases the potential for reversion during vaccination, as well as instability during manufacturing. This concern has been supported by passaging in infant mice, where TC-83 reverted in a little as three intracranial passages (Mckinney et al. 1963). The retention of mosquito infectivity adds another safety concern for transmission from vaccinees to humans or equids, and the isolation of TC-83 from wild-caught mosquitoes in 1971 during an equine vaccination campaign underscores this risk (Pedersen et al. 1972). Due to these concerns, as well its incomplete immunogenicity (Mckinney et al. 1963; Alevizatos et al. 1967; Engler et al. 1992), TC-83 is only approved for equids, but it maintains investigational new drug status for at-risk military and laboratory personnel.

The presence of a high mutation rate is thought to be one reason for the persistence of RNA viruses in the environment. However, high mutation rates also have implications for live-attenuated vaccines (LAV's) such as TC-83, which typically generate long-lived immunity and are cost-effective. LAV replication has the potential to restore a virulent, wt phenotype via one or more reversions or pseudoreversions. Such events can lead to safety concerns and possible adverse events following immunization (Roukens and Visser 2008). However, further increasing the natural mutation rate is also hypothesized to decrease the ability of the virus to successfully revert or pseudorevert through Muller's ratchet (Muller 1964; Chao 1990), by the accumulation of deleterious mutations in asexual populations (e.g. viruses) during replication in the absence of efficient recombination. Additionally, deleterious mutations can also impair the ability of fitter viruses to replicate and increase in the population (Duarte et al. 1992; Escarmís et al. 1999; Escarmís et al. 2008). Thus, the RNA virus error rate is finely tuned to allow for optimal virus population health while also allowing for the production of mutations, which are key for adaptation to new hosts and environments.

The present study was designed to determine whether mutations in the TC-83 RdRp that alter virus fidelity, i.e. increasing the mutational spectrum, can attenuate the virus further, without sacrificing vaccine stability or immunogenicity. We discovered 3 RdRp mutations that conferred a hypermutator phenotype. Additionally, a variant with four mutations in the RdRp was found to have no evidence of a change in diversity after one cell culture passage, but shared many characteristics in common with the low-fidelity mutant. These mutants both

resulted in increased phenotypic stability compared with wt virus, as well as an increase in immunogenicity and protection in a mouse model.

2. Results

2.1 RdRp mutant identification

To identify mutations in the RdRp gene associated with changes in fidelity, TC-83 rescued from an infectious clone was used to initiate serial Vero cell passages in duplicate in the presence of Ribavirin, 5'Fluorouracil (5FU), or Azacytidine (Aza). Viruses were titered using plaque assays following each passage, and diluted to an MOI of 1 plaque forming unit (PFU)/cell for each subsequent passage. Following Passages 19 and 23, the RdRp gene (nsP4) was sequenced. Three mutations were identified in both 5FU passages, and all were located in the 5' end of the RdRp gene: a G5726C mutation resulting in the amino acid change G14R, an A5794G mutation resulting in the amino acid change E37G, and a G5970A mutation resulting in the amino acid change A96T (amino acid numbers refer to nsP4 residues).

2.2 Cloning of TC-83 mutants

The three mutations identified following the 5FU passages were cloned into the TC-83 backbone in conjunction with a high fidelity mutation that was previously identified in a related alphavirus, CHIKV (C483Y, which corresponds to position 488 in TC-83) (Coffey et al. 2011). Six mutants were created (Fig. 1A), four of which were individual mutants, and two with multiple mutations: a 3x mutant including G14R, E37G and A96T and a 4x mutant including G14R, E37G, A96T and C488Y. All clones were rescued by electroporation into baby hamster kidney (BHK) cells, and were titered using standard plaque assays on Vero cells (Beatty et al. 1995). All rescued mutants were viable, producing between 7 and 9 log₁₀ PFU/ml following electroporation (data not shown).

2.3 Replication kinetics

As the mutants included amino acid changes in the RdRp of VEEV TC-83, the viruses were subjected to standard replication curves on Vero cells (Fig. 1B). No mutants showed significant differences from TC-83 by repeated measures ANOVA. Since Vero cells are interferon-deficient, we used human embryonic kidney (HEK)-293 cells to examine the effects of interferon on the replication of fidelity-altered viruses. TC-83, the 3x mutant and the 4x mutant were subjected to one-step replication curves (Fig. 1C). As in Vero cells, no significant differences were observed between the viruses and their TC-83 parent.

2.4 Resistance to nucleoside analog treatment

To confirm the ability of the RdRp mutations to confer resistance to 5FU treatment (Fig. 1D), Vero cells were treated with 0, 10, 25, 50, 100, 200 or 300 µg/ml of 5FU and infected with parent TC-83 or an RdRp mutant using an MOI of 0.01. Repeated measures ANOVA found no significant differences between the RdRp mutants and parent TC-83 when Vero cells were treated with 0–25 µg/ml 5FU. However, treatment with higher 5FU concentrations caused titers for all single RdRp mutants to drop compared with parent TC-83, demonstrating increased sensitivity to the treatment. Of note, E37G was significantly different from parent TC-83 for all of the high 5FU concentrations ($p < 0.01$ for the 50 µg/ml treatment, $p < 0.0001$ for the

100–300µg/ml treatments). A96T was slightly more resistant, with titers significantly reduced compared with parent TC-83 when cells were treated with 100–300 µg/ml ($p < 0.0001$). G14R and C488Y were the most resistant to 5FU treatment of the single mutants. Titers were only significantly reduced compared with parent TC-83 for the 200–300 µg/ml treatments ($p < 0.5$ for G14R 300 µg/ml, $p < 0.0001$ for all others). Due to the increased sensitivity of these mutants to 5FU, it was unlikely that these single mutations caused the resistance observed during the initial fidelity mutant selection.

Unlike the single RdRp mutants, TC-83 3x and 4x generally had similar or slightly higher virus titers compared with parent TC-83, suggesting increased resistance to 5FU treatment. TC-83 4x titers were significantly higher during the 50 and 100 µg/mL treatments ($p < 0.01$ and 0.0001, respectively), and TC-83 3x titers were also significantly higher during the 100µg/mL treatment ($p < 0.05$).

To determine if the RdRp mutants reacted similarly upon exposure to another nucleoside analog, TC-83 and the RdRp mutants were exposed to various concentrations of Ribavirin (Fig. 1E). No significant difference was observed, except for the G14R mutant, which displayed increased sensitivity ($p < 0.01$ for 100 and 300 µM, $p < 0.001$ for 400 µM).

2.5 Virulence in infant mice

To assess virulence, individual TC-83 mutants were injected intracranially into 6-day-old CD-1 mice at a titer of ca. 5 log₁₀ PFU (backtiters ranged from 1.9×10^4 – 2.4×10^5 per dose). Mice were weighed daily and the time of death was recorded (Fig. 2A). The TC-83 3x mutant was significantly more virulent than the parent virus, with all animals succumbing by Day 2 compared with TC-83, where mice began succumbing at Day 3 ($p \leq 0.05$, Kaplan-Meier test). The mean time to death for parent TC-83 and all mutants except TC-83 3x was 5.36–5.54 days after infection, whereas TC-83 3x showed a mean time to death of 3.91 days.

Fidelity mutants have an impaired ability to overcome host bottlenecks, which results in reduced mortality and titer in certain organs (Pfeiffer and Kirkegaard 2005; Vignuzzi et al. 2006, 2008; Graham et al. 2012;). To test this, individual mutants were injected subcutaneously into 6-day-old CD-1 mice to measure their ability to cause mortality (backtiters ranged from 6.2×10^3 to 1.35×10^5 per dose). TC-83 3x and TC-83 4x exhibited significantly reduced mortality as reflected in survival (Kaplan-Meier test, $p \leq 0.05$) when injected subcutaneously, compared with the original TC-83, and compared with intracranial injection in 6-day-old CD-1 mice (Fig. 2B). The remaining mutants caused similar mortality to original TC-83, suggesting no change in their ability to traverse bottlenecks to cause fatal disease.

2.6 Illumina sequencing for virus population variants

To examine how different hosts in the arbovirus transmission cycle alter virus diversity, TC-83, as well as the 3x and 4x mutants, were passaged once using primate (Vero or HEK-293) or mosquito (C7/10 or U4.4) cells and compared with the parental virus (Fig. 3). TC-83 3x and 4x were the only RdRp mutants chosen for this analysis, because they were most likely to have altered fidelity as measured by a preliminary Illumina sequencing analysis measuring the diversity of one replicate of each RdRp mutant (data not shown), as well as the phenotypic tests examined in Figs 1 and 2. Illumina sequencing showed that the 3x

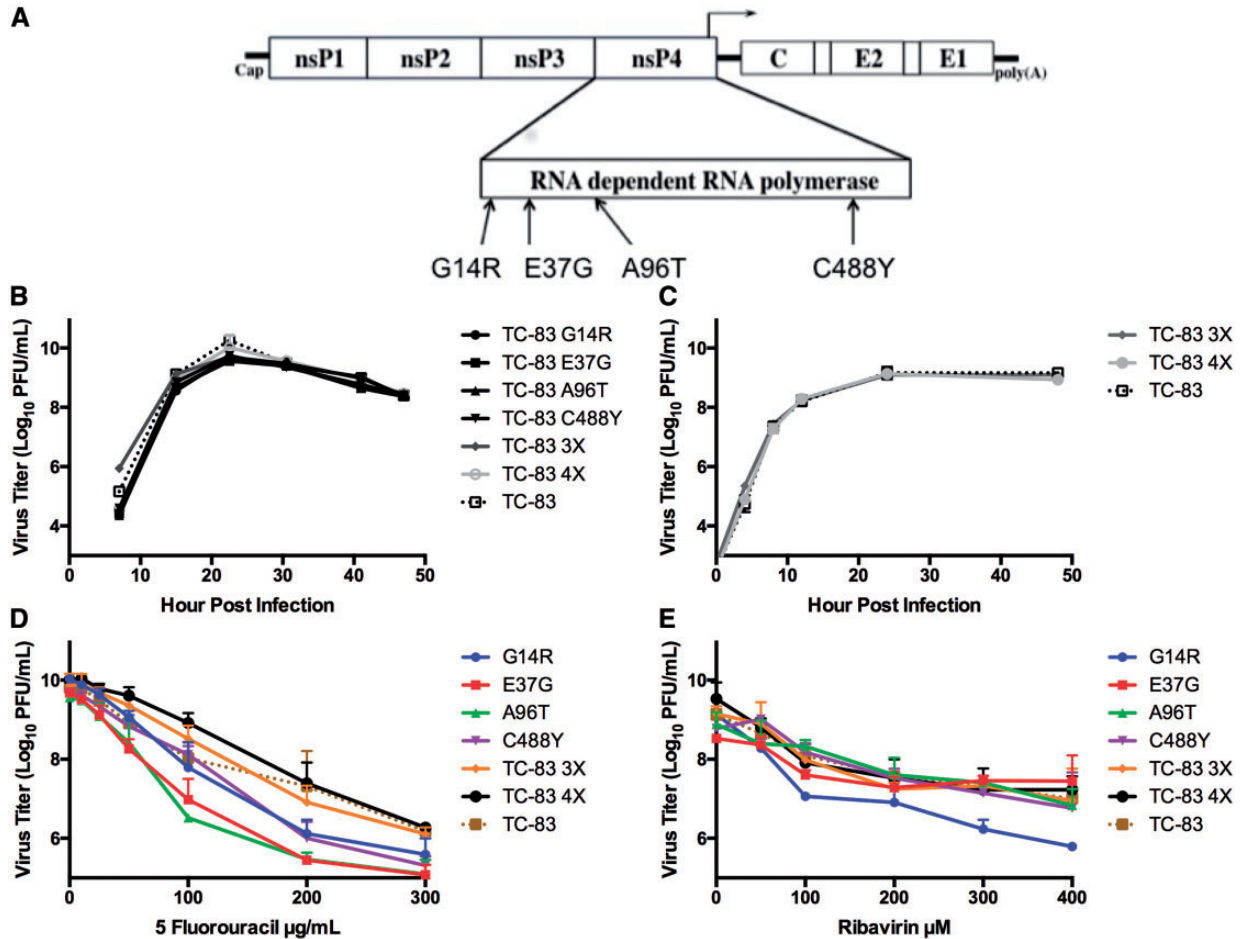


Figure 1. A schematic of the TC-83 genome showing the placement of the four mutations in the RdRp gene (A). Growth curves of TC-83 and the RdRp mutants in interferon negative Vero cells (B) or interferon competent HEK-293 cells (C). Experiments were performed in triplicate and titers were determined by standard plaque assay. Repeated measures ANOVA was used to determine statistical significance, and bars indicate standard deviation. Resistance of TC-83 mutants to treatment by 5FU (D). Cells were treated with 0, 10, 25, 50, 100, 200 or 300 $\mu\text{g/ml}$ of 5FU and infected using an MOI of 0.01; all experiments were performed in triplicate. Samples were titered by standard plaque assay. Statistical differences were determined by repeated measures ANOVA, and significance is detailed in the text. Resistance of TC-83 mutants to treatment by Ribavirin (E). Cells were treated with 0, 50, 100, 200, 300 or 400 μM Ribavirin and infected using an MOI of 0.01; all experiments were performed in triplicate. Samples were titered by standard plaque assay. Statistical differences were determined by repeated measures ANOVA, and significance is detailed in the text.

mutant produced a significantly higher amount of genetic diversity than parental TC-83, independent of the cell type used (Fig. 3A). This, along with the previously discussed results, indicated that the 3x mutant is a low-fidelity variant. Interestingly, the 4x mutant did not demonstrate a significant increase or decrease in diversity compared with TC-83. Cell type had no significant effect on virus diversity, at least not after one passage. When the number of nucleotide positions containing diversity was examined (Fig. 3B), the 3x mutant trended towards producing an increased number of positions with diversity, while the 4x mutant, again, looked much like the parent TC-83.

Lastly, because TC-83 3x and 4x only appear to be resistant to one nucleoside analog (Fig. 1D and E), it was important to determine if this resistance is due to an alteration in the types of mutations being made by these RdRp mutants (Fig. 3C). Indeed, both TC-83 3x and 4x produced much fewer U-C mutations. Because 5FU is a uracil analog and Ribavirin is not, this explains the difference in nucleoside analog susceptibility.

When examining peaks of diversity across the virus genome, some hotspots were conserved between the parent virus and RdRp mutants (Fig. 4, Supplementary Figs S1–S4). These included nsP2 C2627A (Asp-Glu) and A2634Del (Met-stop),

E2 T9561G (minus strand, Val-Gly), 6k C9978A (Ala-Asp) and E1 C11009A (minus strand, Arg-Ser). The parent TC-83 and, to a reduced extent, TC-83 4x both produced diversity hotspots in E1, but generally in different positions. In TC-83 4x, these consisted of C10032T (Ala-Val), T10356C (Val-Leu) and C11009A (Ala, minus strand, synonymous), while TC-83 had E1 hotspots at C10141T (Val, synonymous), C10371T (Ala-Gly), C10743G (Ala-Gly), C11009A (Ala, minus sense, synonymous) and T11269C (Asn, synonymous). Additionally, while there was no significant difference in diversity when different cell types were used (Fig. 3), TC-83 3x appeared to have an increased number of diversity hotspots in C7/10 cells than the other cell types.

2.7 Immunogenicity and protection against VEEV challenge

Immunogenicity of the mutants was tested in an adult murine model previously used to test the efficacy of TC-83 (Guerbois et al. 2013). Adult CD-1 mice, 7 weeks of age, were vaccinated with 5 log_{10} PFU in a 50 μl volume via the subcutaneous route. Animals were bled on days 1–3 after vaccination and weighed daily for 1 week and then again on days 11 and 14 (Fig. 5A).

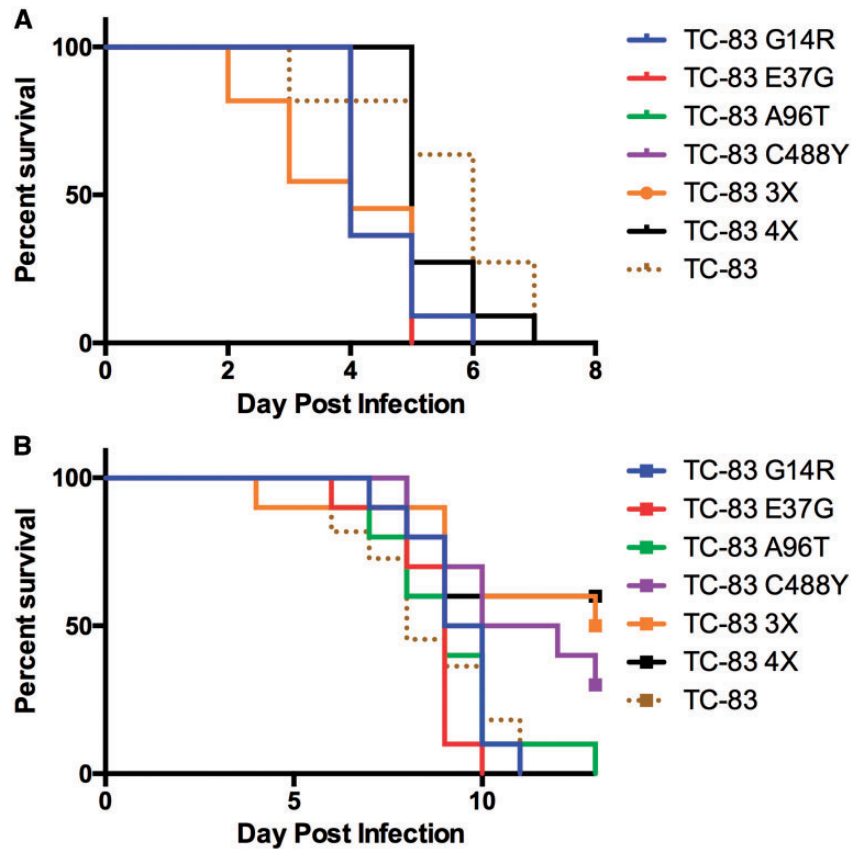


Figure 2. Survival curves for the TC-83 mutants in 6-day-old CD-1 mice following intracranial injection (A) and subcutaneous injection (B) of 10^5 PFU. Statistical differences were determined using Kaplan-Meier tests, and significance is detailed in the text.

Animals exhibited no weight loss and viremia was sporadic with no significant differences among any of the viruses (repeated measures ANOVA, Table 1). Four weeks post-vaccination, the animals were bled to assay neutralizing antibody titers (Fig. 5B). All vaccinated mice exhibited a strong immune response as determined by a plaque reduction neutralization test (PRNT₈₀). The 3x and 4x mutants produced significantly higher mean neutralizing antibody titers compared with TC-83 (repeated measures ANOVA, $p = 0.0049$ and 0.0012 , respectively). Following challenge at 6 weeks post-vaccination with VEEV subtype IC strain 3908, which was used previously for similar experiments (Guerbois et al. 2013; Rossi et al. 2013), animals were weighed daily and monitored for survival (Fig. 5C and D). All vaccinated animals showed complete protection from weight loss and death, whereas the sham-vaccinated animals all succumbed by Day 9 post-challenge.

2.8 Hypermutator variation in attenuation

With the identification of the 3x and 4x viruses as potential fidelity mutants, it was important to determine if these viruses were consistently less virulent in infant mice when subjected to population bottlenecks. This was especially important to confirm for the 3x mutant, because of its identification as a low-fidelity mutant, which might make this strain less phenotypically stable. To test this, TC-83, TC-83 3x and TC-83 4x clones were transcribed independently in duplicate. These constructs were rescued using BHK cells, and each produced between 7 and $9 \log_{10}$ PFU/ml following electroporation. To measure

virulence, 6-day-old CD-1 mice were injected intracranially or subcutaneously with 10^5 PFU using these rescued viruses (Fig. 6).

Intracranial survival curves (Fig. 6A) were similar to those in Fig. 2A, with most mice succumbing 5–7 days post-infection. Backtiters of the inocula ranged from 4.8 to 8.8×10^4 PFU per dose. Depending on the replicate group, TC-83 3x or TC-83 4x were slightly more or less virulent than the parental TC-83. This suggests that the RdRp mutations do not alter virulence when administered intracranially, and that the significant differences in lethality observed in Fig. 2A for TC-83 3x are likely dependent on the mutant spectrum produced post-electroporation or due to host variation.

Subcutaneous survival curves (Fig. 6B) were significantly different compared with Fig. 2B. Instead of half of the 3x and 4x mutants surviving subcutaneous inoculation, all individuals succumbed by 12 days post-infection. Inoculum back titers ranged from 3.5×10^4 to 1.1×10^5 PFU per dose. Although all mice succumbed, there was variation between the replicates. For example, while one of the TC-83 3x replicates survived an average of 7.9 days, the other replicate survived an average of 9.6 days. This and the intracranial survival curve results suggest that differences in the post-electroporation minority populations can result in variation in the degree of attenuation of low-fidelity viruses.

2.9 Genetic stability in vitro and in vivo

To confirm that the fidelity-altering mutations did not revert upon repeated replication, TC-83, TC-83 3x and TC-83 4x were subjected to five passages in Vero cells. Sanger sequencing of the RdRp revealed no mutations (data not shown).

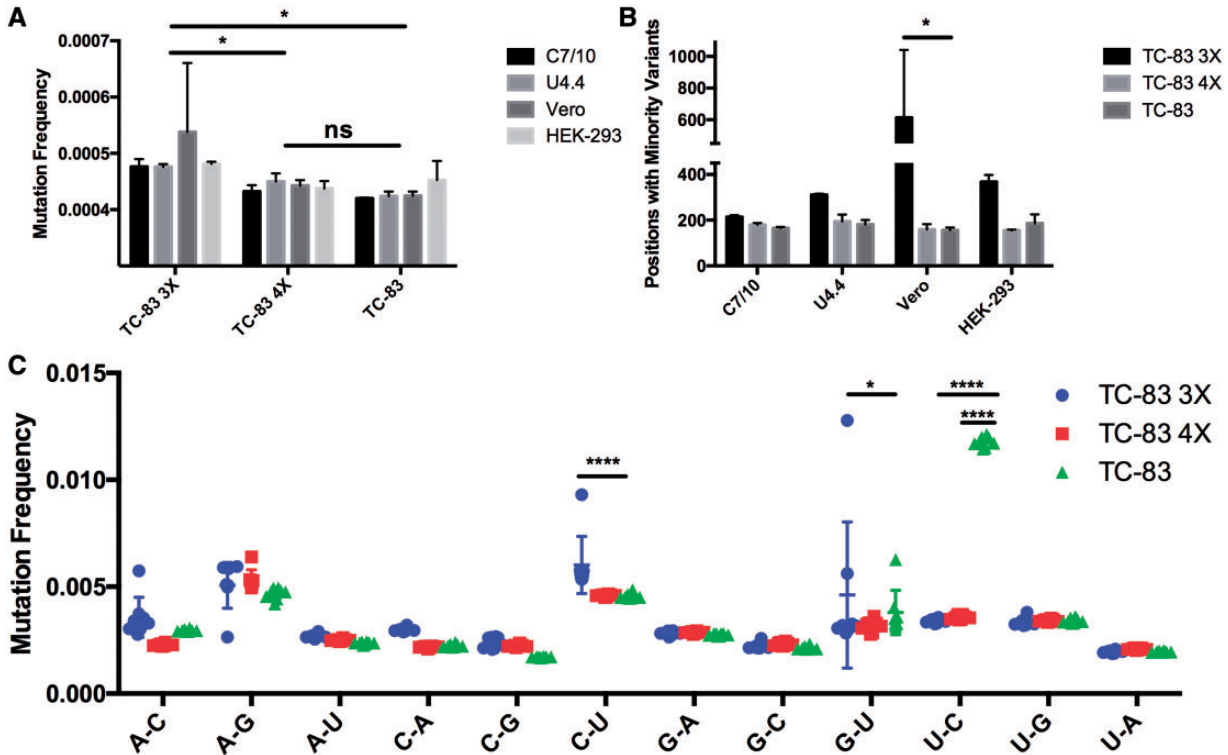


Figure 3. Illumina sequencing virus mutation frequency analysis of TC-83, 3x and 4x after 1 passage on Vero, HEK-293, C7/10 or U4.4 cells. Overall mutation frequency for each cell type (A). Number of genomic positions with minority variants present above a frequency of 0.001 (B). Mutation frequency of different mutations (C). TC-83 3x: blue; TC-83 4x: green; TC-83: red. Repeated measures ANOVA was used to determine statistical significance, $P < 0.05$ is represented by *, $P < 0.01$ is represented by **, $P < 0.001$ is represented by *** and $P < 0.0001$ is represented by ****.

The 3x and 4x RdRp mutants and TC-83 were also subjected to 10 serial intracranial passages in infant mice to determine if compensatory mutations accumulated *in vivo*. Passage 5 and 10 virus was injected subcutaneously into 6-day-old mice to assess changes in virulence that occurred during passaging. TC-83, TC-83 3x and TC-83 4x all gained virulence by passage 5 compared with their unpassaged counterparts as shown in Fig. 2B (Kaplan-Meier test, $p \leq 0.0001$) (Fig. 7A). At this point, the 3x and 4x mutants reached virulence equivalent to that of the original, unpassaged TC-83 with no significant difference in time to death. Interestingly, no significant changes in virulence occurred between passage 5 and 10 for any of the viruses. This indicates that TC-83 3x and 4x can only reach virulence equivalent to unpassaged TC-83, and are unable to increase in virulence past that point. Additionally, while not statistically significant, mice inoculated with TC-83 4x passage 10 survived up to 1 week longer than mice inoculated with TC-83 4x Passage 5. This suggests that additional passaging may result in decreased fitness for this construct.

2.10 Illumina sequencing for virus variants following passaging

Illumina sequencing following mouse brain passage 5 showed an increase in diversity for both TC-83 3x and TC-83 4x (Fig. 7B–F). This suggests that TC-83 4x may actually be low fidelity, and that multiple growth cycles may be required to truly assess fidelity changes. Curiously, while diversity hotspots increased for both TC-83 3x and 4x, the 6k hotspot observed after one cell culture passage was greatly reduced for all of the passage 5 viruses (Fig. 7D–F). Even though a different electroporation stock was used for infant mice passaging, TC-83 3x and 4x still exhibited

fewer U-C mutations (Fig. 3C, Supplementary Fig. S5), suggesting that this is a stable phenotype for these two RdRp mutants.

Although TC-83 3x and 4x were producing more diversity than the parent TC-83, fewer revertants occurred at the E2 mutation required for TC-83 attenuation (Fig. 7C) (Kinney et al. 1993). Due to the position of the 5'UTR attenuation mutation, coverage was too low to assess true differences in the potential for this site to also revert, but no reads contained the revertant mutation for any virus. Following passage 10 in mouse brains, Sanger sequencing was used to identify several SNPs (Table 2). TC-83 and TC-83 3x each produced two SNPs following 10 passages, while TC-83 4x had no consensus sequence changes. None of these SNPs reverted the TC-83 mutations that cause attenuation or the inserted RdRp mutations. Additionally, there were no convergent mutations found in all passage series.

3. Discussion

Infectious diseases transmitted by arthropod vectors are difficult to control, and protecting the population via vaccination tends to be more effective than trying to control or eradicate the vector population. Although LAVs tend to be cost-effective and immunogenic, LAVs have a significant Achilles' heel: the potential for reversion to wt virulence. This risk is particularly acute for RNA viruses, which replicate without proofreading, resulting in an increased ability to revert to the wt sequence or restore virulence via compensatory mutations (pseudoreversion). However, altering virus fidelity can theoretically overcome this major drawback. This approach has only been examined with fidelity mutants of PV and a low-fidelity ExoN mutant of SARS-CoV (Graham et al. 2012; Vignuzzi et al. 2008). While the high fidelity

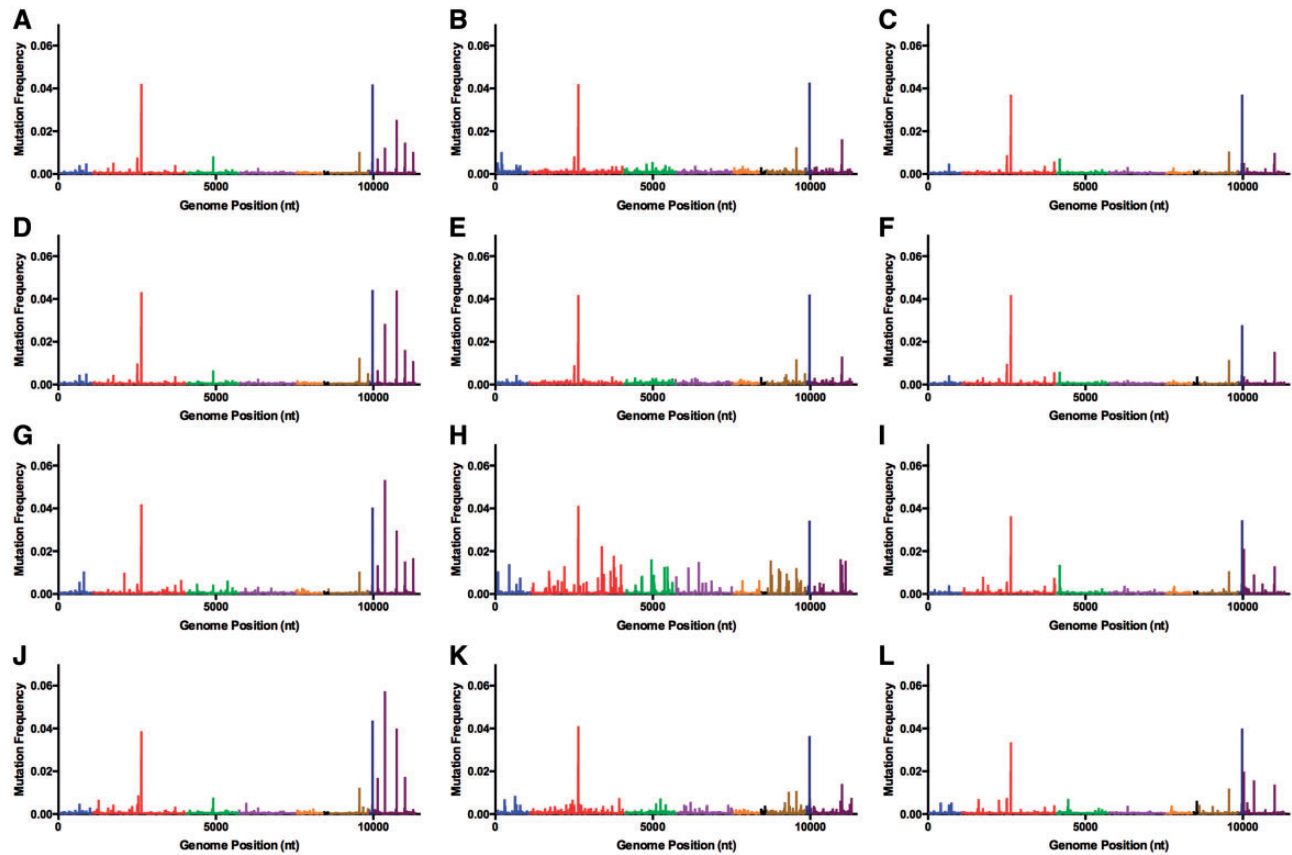


Figure 4. Illumina sequencing virus diversity hotspots for the coding regions of TC-83, 3x and 4x genomes after 1 passage on Vero, HEK-293, C7/10 or U4.4 cells. A representative replicate is pictured for each virus isolate from each cell type. For full results, see [Supplementary Figs S1–S4](#). TC-83 (A, D, G, J), TC-83 3x (B, E, H, K) and TC-83 4x (C, F, I, L). Vero (A, B, C), HEK-293 (D, E, F), C7/10 (G, H, I) and U4.4 (J, K, L). Genome organization is color-coded using the following: nsP1, blue; nsP2, red; nsP3, green; nsP4, purple; capsid, orange; E3, black; E2, gold; 6k, navy blue; and E1, maroon.

PV (Vignuzzi et al. 2008) and SARS-CoV ExoN (Graham et al. 2012) mutant both demonstrated increased immunogenicity and an improved safety profile compared to the parent viruses, this was not observed for the low fidelity poliovirus (Korboukh et al. 2014). However, fidelity mutants have only been generated using wt virus strains, which are unlikely to be sufficiently attenuated for vaccine use. We hypothesized that increasing the attenuation of a live-attenuated RNA virus vaccine by altering the RdRp fidelity would further improve its safety and efficacy. We addressed this by validating a series of low-fidelity mutations in a model system using the VEEV vaccine strain TC-83 to determine how this affects the stability and attenuation. Serial passaging of TC-83 in 5-FU identified three mutations in the RdRp, and validation showed that all three mutations were required to decrease virus replication fidelity. Additionally, we identified a 4x mutant that acted much like TC-83 3x, but showed no difference in diversity after one cell culture passage.

At present, there is no atomic resolution structure for an alphavirus RdRp, which limits our understanding of their mechanisms of fidelity alteration. However, all the mutations in the 3x mutant (G14R, E37G and A96T) were at the 5' end of the nsP4 gene, similar to the placement of the PV high fidelity G64S and low fidelity H273R substitution, which are both on the periphery of the RdRp protein rather than near the active site (Pfeiffer and Kirkegaard 2003; Campagnola et al. 2015; Korboukh et al. 2014). For PV, the presence of the G64S mutation does not significantly alter the structure of the polymerase, but it does decrease the incorporation rate of new nucleotides (Arnold et al. 2005;

Marcotte et al. 2007). In fact, the current theory is that this substitution simply stabilizes the polymerase rather than altering the active site. Alternatively, low fidelity H273R PV decreases fidelity by favoring an open state of the RdRp, which decreases the duration of this fidelity checkpoint, thus increasing the likelihood of NTP misincorporation (Moustafa et al. 2014). The presence of the low-fidelity mutations outside of the predicted active domain of the TC-83 nsP4 gene suggests a similar mechanism, but confirmation will require an atomic resolution structure.

There are two manners in which low-fidelity mutants have been isolated, either by mutating residues found to alter fidelity in other viruses or by passaging virus in the presence of a nucleoside analog. Of the latter (Arias et al. 2008; Pauly et al. 2017; Van Slyke et al. 2015), three low-fidelity mutants have been identified that are resistant to the nucleoside analog used for passaging, but are sensitive to other nucleoside analogs. In concordance with this, these low-fidelity mutants exhibit higher fidelity for the specific mutations selected by the nucleoside analog, but lower fidelity for other mutations.

Our 3x and 4x fidelity mutants appear to act similarly to these low-fidelity viruses, with resistance to 5'fluorouracil, but not ribavirin. When examining the mutation frequency of different mutations, the fidelity mutants produced a much lower amount of U to C transition mutations, which would be one of the mutations expected to be selected against during 5'FU passaging. This also may explain why the mutants are most resistant to 5'FU when using moderate amounts of the nucleoside analog, but then increase in susceptibility again at higher

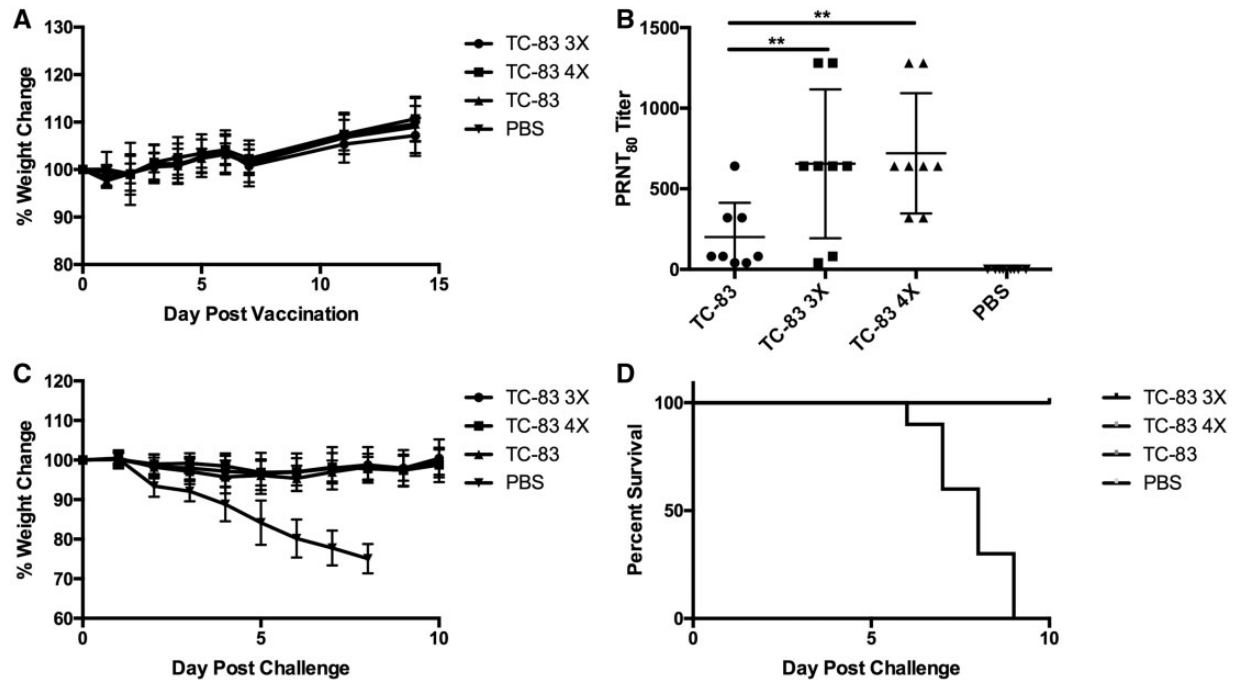


Figure 5. Weight change, immunogenicity and survival following vaccination and challenge. Five-week-old mice were injected with 10^5 PFU of TC-83, 3x, 4x or mock in a 50 μ l volume ($n = 8$ per vaccine treatment). Mice were monitored daily for percent weight change from the original weight at D0 (A). The effectiveness of the vaccine was determined by neutralizing antibody titer using serum from 6-week post-vaccination (B), and statistical differences were determined by repeated measures ANOVA, where $P < 0.01$ is represented by **. Mice were challenged with 10^5 PFU of VEEV strain 3908 and were monitored for percent weight change from the original weight at D0 (C) and survival (D).

Table 1. Viremia titers following vaccination with TC-83, TC-83 3x, TC-83 4x or mock.

	Day 1		Day 2		Day 3	
	No. positive	Average titer (\pm SD)	No. positive	Average titer (\pm SD)	No. positive	Average titer (\pm SD)
TC-83 3x	5/5	2.68 (\pm 0.52)	2/5	2.28 (\pm 0.21)	3/5	2.12 (\pm 0)
TC-83 4x	3/5	2.59 (\pm 0.49)	0/5	0	3/5	2.61 (\pm 0.59)
TC-83	4/5	2.94 (\pm 0.57)	1/5	2.73 (\pm 0)	2/5	2.47 (\pm 0.49)
PBS	0/5	0	0/5	0	0/5	0

concentrations, as there is no difference in the other uracil mutation frequencies.

TC-83 3x was clearly a low-fidelity mutant, as evidenced by the phenotypic tests, as well as the increased genetic diversity observed using Illumina sequencing. The 4x mutant, while exhibiting phenotypic similarities with other altered fidelity mutants, had no significant difference in virus diversity compared with the TC-83 parent after one cell culture passage. However, TC-83 4x may actually be a low-fidelity variant, as suggested by the increase in diversity that occurred during *in vivo* passaging, which exhibited gains in diversity nearly identical to TC-83 3x. Perhaps the C488Y mutation (Coffey et al. 2011), a high fidelity CHIKV mutation, offers an additive effect to initially increase virus diversity to that of wt virus, while exerting additional attenuation via an unknown mechanism. However, it appears that the C488Y mutation exerts less of an effect over multiple virus replication cycles, resulting in increased virus diversity compared with parent TC-83 after passaging *in vivo*. Alternatively, it could be that the cumulative effect of these mutations alters the ability of the virus to explore the most beneficial sequence space as the virus replicates, leading to Muller's ratchet and decreased virus health. Indeed, this may explain why the 4x mutant initially exhibited gains in virulence during

in vivo passaging, but later became more attenuated. It would be interesting to continue passaging this mutant to determine if the mutation threshold is eventually crossed, leading to error catastrophe and virus extinction. If this hypothesis is validated, the 4x construct is likely to be a safer LAV candidate than the 3x mutant. Indeed, a mixture of these two hypotheses might explain the observed TC-83 4x results.

Also of interest is the C488Y substitution that increased replication fidelity in CHIKV, but did not affect TC-83 fidelity, although it did attenuate TC-83 in the infant mouse model. This finding is the opposite of the outcome for Sindbis virus, which exhibited low-fidelity features when a low-fidelity CHIKV mutation was inserted (Rozen-Gagnon et al. 2014). However, Sindbis virus is more closely related to CHIKV than VEEV, so this may explain why the effects did not transfer to TC-83 even though the residue is highly conserved among alphaviruses (Coffey et al. 2011).

Illumina sequencing uncovered many noteworthy effects during RdRp mutant growth *in vitro*. In particular, the E1 diversity peaks identified in TC-83 were greatly decreased for the RdRp mutants. The E1 glycoprotein is responsible for the uncoating of the virus particle and escape of the genome into the cytosol. Some substitutions in the CHIKV E1 glycoprotein

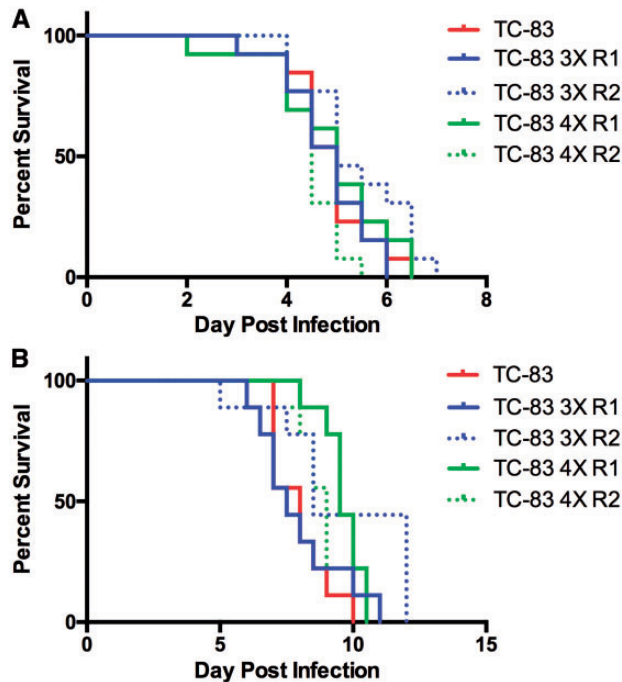


Figure 6. Survival curves for the TC-83 mutants in 6-day old CD-1 mice following intracranial injection (A) and subcutaneous injection (B) of 10^5 PFU from novel RdRp mutant virus rescues. R1 denotes replicate 1. R2 denotes replicate 2. Statistical differences were determined using Kaplan-Meier tests and are detailed in the text.

have been found to increase vector infectivity and transmission (Tsetsarkin et al. 2007, 2011; Vazeille et al. 2007). Diversity in this region may be important for increased vector fitness and/or host jumps.

When total genome diversity was analyzed for each construct, there was no significant difference in diversity when comparing cell types. This is in contradiction to the low-fidelity CHIKV mutants, which only showed increased diversity in vertebrate cells and not in mosquito cells (Rozen-Gagnon et al. 2014). However, while no significant difference was observed in overall diversity between the cell types, TC-83 3x produced much higher peaks in diversity in C7/10 cells than was observed in other cell types or viruses. This is intriguing, because it is hypothesized that mosquitoes are drivers of diversity, likely due to the sequence specificity of the mosquito antiviral RNAi response (i.e. siRNAs), although this has only been demonstrated for West Nile virus (Brackney et al. 2009, 2015; Grubaugh et al. 2015, 2017). This hypothesis does not appear to explain the increased diversity observed during the TC-83 3x C7/10 passages, because these cells lack a functional siRNA response. Additionally, no increased hotspots were observed for U4.4 cells, which do produce antiviral siRNAs. Further work is needed to determine how these diversity hotspots correspond to small RNA targeting in mosquito cells and examine the effects of the host immune response on virus populations.

As previous studies have shown that high fidelity PV mutations cause attenuation in mouse models by reducing dissemination to the central nervous system (Pfeiffer and Kirkegaard 2005; Vignuzzi et al. 2006, 2008), we tested our mutants in a lethal mouse model for TC-83. The 3x and 4x RdRp mutants initially displayed equivalent virulence to parent TC-83 when injected into a single tissue that can cause lethal damage (brain) and reduced virulence when injected via a multi-tissue

model (subcutaneous). However, when this experiment was repeated with virus rescued from different electroporation pools, the 3x and 4x mutants displayed greatly increased virulence compared with the initial subcutaneous outcome. This suggests that minority variants randomly generated during the transcription preceding electroporation can have large effects on the overall virus population health, and thus virulence. Importantly, TC-83 was always equal to or more virulent when injected subcutaneously compared with the RdRp mutants. While TC-83 is not lethal in an adult mouse model, it would be interesting to determine if the 3x and 4x mutants have consistently delayed and/or decreased dissemination compared with wt virus. This would allow for a greater understanding of how a low-fidelity vaccine would operate in a fully immunocompetent host.

When it was established that different pools of virus may exhibit different degrees of virulence, it became important to test whether the mutants would remain attenuated when allowed to experience multiple growth cycles. Perhaps counterintuitively, the 3x hyper mutator variant, while undergoing an initial increase in virulence during *in vivo* passaging, only reverted to the same level of virulence as unpassaged TC-83. This is likely due to an increased amount of unfit virions (e.g. defective interfering [DI] particles), which hamper wt virus virulence, as well as the inability of the 3x mutant to revert the inserted RdRp mutations. The passage 10 results for each virus were very similar to the passage 5 data, except for the 4x virus, which decreased in virulence so much that some infant mice were able to survive 7 extra days. This passaging data demonstrates that both of our mutants exhibit decreased risk of reversion to wt virulence compared with the parent TC-83.

As our study was designed to evaluate the effectiveness of RdRp mutations that decrease fidelity in vaccines, the mutants were put through a standard challenge model previously used for different VEEV vaccines (Guerbois et al. 2013; Rossi et al. 2013). Mice showed no adverse effects from vaccination, viremia was consistent with that expected for TC-83, and all mice were protected against lethal challenge. Additionally, the mutants induced higher antibody titers than TC-83. Our current hypothesis for this observation is that the low-fidelity RdRp creates an increased number of DI virus particles, which are well known to be strong immune stimulators (Dimmock and Kennedy 1978; Mercado-López et al. 2013; Tapia et al. 2013; Sun et al. 2015). As VEEV first amplifies in the draining lymph node (MacDonald and Johnston 2000), the presence of these DI particles may restrict dissemination from the draining lymph node, allowing for a greater opportunity for antigen presentation and therefore an increased immune response. This needs to be explored in future work.

Here, we demonstrated that the presence of low-fidelity mutations in a LAV resulted in reduced virulence in an infant mouse model and increased immunogenicity in an adult mouse model, both of which are desirable properties for LAVs. However, we also observed different outcomes in low-fidelity mutant attenuation *in vivo* when different pools of virus stock were used, which suggests that the low-fidelity virus population might not be initially stable. This demonstrates that there is great need to understand the role that minority variants play in phenotypic changes in vaccine development, as changes in the mutant spectrum may significantly alter the phenotype of vaccines. However, the passaging results imply that the virus population stabilizes after multiple growth cycles, so low-fidelity RNA virus vaccines may require passaging to ensure a consistent vaccination phenotype. Overall, this study demonstrates that while low-fidelity mutations may be a potential tool to further develop, the initial phenotypic instability observed post

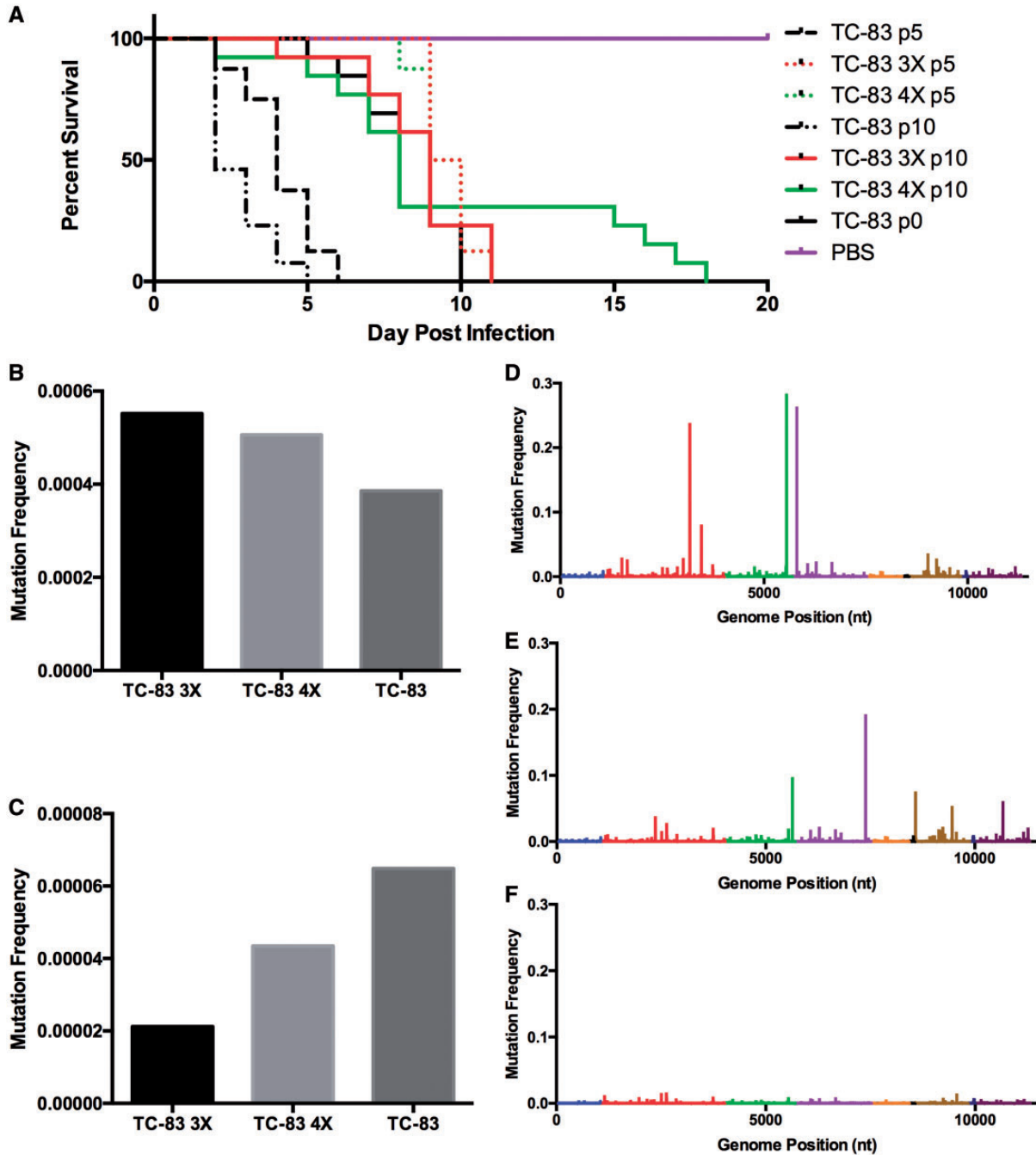


Figure 7. Survival of 6-day old CD-1 mice following subcutaneous injection with 10^5 PFU in a 50 μ l volume of Passages 0, 5 or 10 TC-83, 3x or 4x from infant mouse intracranial passages (A). Statistical differences were determined using Kaplan-Meier tests, and significance is detailed in the text. Illumina-sequencing diversity analysis was used to determine changes in virus diversity of passage 5 TC-83, 3x or 4x in infant mice (B). Mutation frequency of the E2 8922 mutation required for TC-83 attenuation (C). Illumina sequencing virus diversity hotspots for the coding regions of TC-83 (D), 3x (E) and 4x (F) genomes after five intracranial passages. Genome organization is color-coded using the following: nsP1, blue; nsP2, red; nsP3, green; nsP4, purple; capsid, orange; E3, black; E2, gold; 6k, navy blue; and E1, maroon.

electroporation suggests that this may not currently be a viable strategy for LAV development.

4. Materials and methods

4.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use

of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

4.2 Cell cultures and viruses

Vero (African green monkey kidney), HEK-293 and BHK cells were obtained from the American type cell collection (Bethesda,

Table 2. Changes in nucleotide sequence following p10 in mouse brain as verified by Sanger sequencing.

	Amino acid change	TC-83 3X	TC-83 4X	TC-83
nsP2	C3179T	X		
nsP4	T7208C			X
E2	A8805T D-L	X		
3'UTR	C11386T			X

MD) and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and Penicillin and Streptomycin (Pen/Strep) (100 U/ml) in a 37°C, 5% CO₂ incubator. C7/10 cells were maintained in DMEM supplemented with 10% FBS, 1% minimal essential medium Non-essential amino acids, 1% tryptose phosphate broth and Pen/Strep (100 U/ml) in a 30°C CO₂ incubator. U4.4 cells were maintained in Mitsushashi and Maramorosch media supplemented with 20% FBS, 2% Sodium Bicarbonate (7.5%) and Pen/Strep (100 U/ml) in a 30°C CO₂ incubator. Viruses were rescued from a TC-83 infectious clone as described previously and without further passage (Smith et al. 2008). Viruses were passaged on Vero cells unless otherwise stated. Titers of virus were determined by standard plaque assay using Vero cells (Beatty et al. 1995).

4.3 Mutagenesis of TC-83

Parental TC-83 was passaged in the presence of mutagens, Ribavirin (Sigma Aldrich, St Louis, MO), 5'Fluorouracil (5FU) (Sigma Aldrich) or Azacytidine (Sigma Aldrich) at concentrations of 40 mM, 80 µg/ml and 0.4 µM, respectively. Cells were pretreated 2 h prior to infection with the mutagens in duplicate, then infected with TC-83 at a multiplicity of infection (MOI) of 1 PFU/cell. Following a 1-h incubation period the cells were fed with DMEM supplemented with 2% FBS and Pen/Strep (100 U/ml) as well as the mutagen from the original pretreatment. Supernatants were harvested 2 days post-infection and stored at -80°C after being supplemented with 10% FBS. Viral titers were determined by standard plaque assay (Beatty et al. 1995). Viruses were sequenced using Sanger sequencing after Passages 10, 18 and 23 to determine if any mutations had occurred in the RdRp.

4.4 Manipulation of the TC-83 clone

Six TC-83 subclones containing mutations identified in the TC-83 RdRp or high-fidelity CHIKV RdRp were created by joining-PCR cDNA fragments from the TC-83 genome using designed primers. The sequence of each construct was verified prior to cloning into the genomic infectious clone backbone. All viruses were rescued after electroporation into BHK cells as described previously in Greene et al. (2005).

4.5 Testing of viral clones

To ensure that the inserted mutations did not interfere with viral replication, standard replication curves were conducted. Cells were infected at an MOI of 1 (HEK-293) or 0.01 (Vero) in 12-well plates and incubated for 1 h before being washed twice with phosphate buffered saline (PBS) and overlaid with 2 ml of DMEM supplemented with 2% FBS and Pen/Strep (100 U/ml). Virus was harvested by removing the entirety of the medium and replacing it with 2 ml of fresh medium. Harvested virus was supplemented with FBS to a final concentration of 20%, and

clarified by centrifugation at 3000 rpm for 5 min. The supernatant was then removed and stored at -80°C. Viral titers were determined by standard plaque assays (Beatty et al. 1995).

Rescued viruses were passaged five times on Vero cells to ensure genetic stability. Viruses were harvested 2 days post-infection and diluted 1:100 before infection of Vero cells in the subsequent passage. Following Passage 5, viral suspensions were placed into Buffer AVL (Qiagen, Valencia, CA) and RNA extracted using the column method as per the manufacturer's protocol. cDNA was produced using the Superscript III First Round Synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA was amplified for sequencing using the Phusion enzyme (NEB, Madison, WI) in a 50 µl volume following the manufacturer's instructions using primer sets that covered the RdRp region of the genome (primers available upon request). Products were sequenced on the ABI 3700 sequencer (ABI, 3500).

Rescued viruses were tested for susceptibility to 5FU or Ribavirin in triplicate. Cells were pretreated with 0, 10, 25, 50, 100, 200 or 300 µg/ml of 5FU or 0, 50, 100, 200, 300 or 400 µM Ribavirin for 2 h. The cells were then infected with 0.01 MOI of the RdRp mutants as well as TC-83. Medium containing the pretreatment amount of 5FU or Ribavirin was added to the infected cells 1-h post-infection. Virus was harvested 24-h post-infection and titrated in duplicate using standard plaque assays.

4.6 Virulence, immunogenicity and protection studies

To study virulence and attenuation, 6-day-old CD-1 mice (Charles Rivers, Wilmington, MA) were inoculated intracranially (IC) with 10⁵ PFU of virus in a volume of 20 µl, or subcutaneously (SC) with 10⁵ PFU in a volume of 50 µl. Animals were weighed daily for 2 weeks and monitored for survival.

In another experiment, 7-week-old CD-1 mice were vaccinated SC with the original TC-83 virus or one of the RdRp mutants with 10⁵ PFU in a volume of 50 µl, or with PBS for unvaccinated controls (n = 8 per cohort). Six-week post-vaccination, animals were challenged SC with 10⁵ PFU of wt epidemic VEEV strain 3908 (Suárez et al. 2005), with daily monitoring for signs of illness, survival and weight loss. Blood samples were collected from alternating cages of vaccinated mice for 3 days post-vaccination and 4 days post-challenge for viremia detection, as well as 5 weeks post-vaccination for antibody measurement by PRNT.

To assess genetic and phenotypic *in vivo* stability of the TC-83 RdRp mutants, each was subjected to 10 serial, IC passages in six-day-old CD1 mice at a dose of ca. 10⁵ PFU in a 20 µl volume per animal. Animals were euthanized 48-h post-inoculation, and their brains harvested and homogenized to determine viral titer by standard plaque assay (Beatty et al. 1995). The homogenized brain was diluted to produce the same inoculum dose and used to initiate the subsequent passage. Virulence of the mouse passages 5 and 10 viruses was compared with the parental strain by inoculating 6-day-old CD1 mice SC with 10⁵ PFU, as described earlier. Stability of the genomic sequences was assessed by RT-PCR on RNA extracted from mouse passages 5 and 10 viruses and sequenced using Illumina or Sanger sequencing, respectively.

4.7 Next generation sequencing

4.7.1 Library preparation

Viral RNA from cell culture extracts were fragmented by incubation at 94°C for 8 min in 19.5 µl of fragmentation buffer

(Illumina, San Diego, CA). First and second strand synthesis, adapter ligation and amplification of the library were performed using the Illumina TruSeq RNA Sample Preparation kit as per the manufacturer's protocol. For virus isolates from animals, PCR was first done using sequence-specific primers tiled across the VEEV genome to provide amplified products of ~1.5 kb in size that were used for library generation. Products were fragmented using transposons, then adapter ligation and amplification of the library was performed using the Illumina TruSeq RNA Sample Preparation kit under conditions described by the manufacturer (Illumina, San Diego, CA). Samples were tracked using the 'index tags' incorporated into the adapters as defined by the manufacturer.

4.8 Sequence assembly and analysis

4.8.1 Quality and filtration

The quality for each sample/dataset was assessed using FASTQC (Hoffmann et al. 2009). The paired-end reads were merged for each sample and then filtered to exclude reads with unknown characters (anything other than A, T, C, G) and low quality (<10 quality score), so that only high quality reads were used during analysis. Additionally, the first 16 bases of each read were trimmed due to nucleotide bias.

4.8.2 Reference sequence

The analysis was performed using VEEV strain TC-83, complete genome (GenBank accession no.: L01443.1) (R. M. Kinney et al. 1989).

4.8.3 Virus diversity analysis

To analyze the variant hotspots, each sample was run through a novel rare variant pipeline (available upon request). The pipeline first maps each read to the reference VEEV genome with perfect match, then unmapped reads are re-mapped with 1 mismatch and added to the final map. The 34 base long reads used in the analyses were validated as viral sequences and not host sequences by analysis of the longest subsequences shared explicitly (no mismatches allowed) and longest similar (1 mismatch allowed) between viral and host genomes. Diversity per position in the viral genome was calculated by taking a ratio of the reads mapped with mismatches and all reads mapped at that position. Positions in which the number of reads mapped with mismatches was higher than perfectly mapped reads or coverage was below 100 were excluded from diversity calculations. Mutation frequency per sample was calculated by summing per position diversities and normalizing by the number of positions for which diversity was calculated. Positions with non-zero mutation frequency were considered to be variant positions.

4.9 Statistics

GraphPad Prism was used to perform all statistical tests, which are described in the text.

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Data availability

50 Data are not publically available, but will be provided upon request.

Supplementary data

Supplementary data are available at Virus Evolution online.

Conflict of interest: None declared.

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