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A molecular determinant of West Nile virus secretion and morphology as a target for viral attenuation

Justine Basset¹², Julien Burlaud-Gaillard³⁴, Maxence Feher⁵, Philippe Roingeard³⁴, Félix A. Rey⁶, Nathalie Pardigon¹#

¹ Institut Pasteur, Arbovirus Group, Environment and Infectious Risks Unit, Paris, France
² Université Paris Diderot, Sorbonne Paris Cité (Cellule Pasteur), Paris, France
³ Plateforme IBISA de Microscopie Electronique, PST ASB, Université de Tours and CHRU de Tours, Tours, France
⁴ INSERM U1259, Université de Tours and CHRU de Tours, Tours, France
⁵ Laboratory for Urgent Response to Biological Threats, Institut Pasteur, Paris, France
⁶ Institut Pasteur, Structural Virology Unit, Paris, France and CNRS UMR 3569 Virologie, Paris, France

# Corresponding author: nathalie.pardigon@pasteur.fr

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Corresponding author: nathalie.pardigon@pasteur.fr
ABSTRACT
West Nile virus (WNV), a member of the Flavivirus genus and currently one of the most common arboviruses worldwide, is associated with severe neurological disease in humans. Its high potential to re-emerge and rapidly disseminate makes it a bona fide global public health problem. The surface membrane glycoprotein (M) has been associated with Flavivirus-induced pathogenesis. Here we identify a key amino acid residue at position 36 of the M protein whose mutation impacts WNV secretion and promotes viral attenuation. We also identified a compensatory site at position M-43 whose mutation stabilizes M-36 substitution both in vitro and in vivo. Moreover, we find that introduction of the two mutations together confers a full attenuation phenotype and protection against wild-type WNV lethal challenge, eliciting potent neutralizing antibody production in mice. Our study thus establishes the M protein as a new viral target for rational design of attenuated WNV strains.

IMPORTANCE
West Nile virus (WNV) is a worldwide (re)emerging mosquito-transmitted Flavivirus causing fatal neurological diseases in humans. However, no human vaccine has been yet approved. One of the most effective live-attenuated vaccines was empirically obtained by serial passaging of wild-type yellow fever Flavivirus. However, such an approach is not acceptable nowadays and the development of vaccine rationally designed is necessary. Generating molecular infectious clones and mutating specific residues known to be involved in Flavivirus virulence is a powerful tool to promote viral attenuation. WNV membrane glycoprotein is thought to carry such essential determinants. Here, we identified two residues of this protein whose substitutions are key to the full and stable attenuation of WNV in vivo, most likely through inhibition of secretion and possible alteration of morphology. Applied to other flaviviruses, this approach should help in designing new vaccines against these viruses that are an increasing threat to global human health.

INTRODUCTION
West Nile virus (WNV) is a Flavivirus genus member. Most closely related to another genus member, Japanese encephalitis virus (JEV), but also similar to yellow fever virus (YFV), Zika (ZIKV) and dengue (DV) viruses, WNV is one of the most largely widespread neurotropic arthropod-borne viruses, causing severe neurological symptoms and even death (1, 2). Since
its first isolation in Uganda in 1937, recurrent and unpredictable WNV outbreaks have been detected in humans throughout the world generating health problems. Despite its global reemergence, however, there is currently neither treatment nor human vaccine available to cure or prevent the disease (1). In the hope of aiding the development of innovative rationally designed vaccines we focused our research on host-WNV molecular interactions and particularly viral particle assembly in the endoplasmic reticulum (ER) of the infected cell. WNV possesses two structural glycoproteins, the membrane protein (M), processed from a precursor protein (prM), and the envelope protein (E) (3). While the E protein mediates interactions between host cellular factors and the virus for attachment and penetration, prM supports E during folding and shields it from causing premature fusion in the acidic secretory pathway (4). As with other flaviviruses, WNV assembly occurs in the endoplasmic reticulum (ER) and requires interactions between prM, E and the nucleocapsid (5, 6). Following assembly, nascent virions bud into the ER lumen and are then translocated to the Golgi apparatus via trafficking vesicles (7). Once in the trans-Golgi network (TGN), prM is cleaved by the cellular furin, leading to the release of the pr peptide in the neutral pH extracellular environment and the formation of mature and infectious M-containing virions (8). Although Flavivirus assembly mechanisms have been largely investigated (9, 10), it is still unclear how nascent virions engage the host cell secretory pathway in order to exit the ER, reach the TGN, and then be released at the cell surface.

The flavivirus prM/M protein was recently shown to carry virulence determinants (11, 12). In the ectodomain of the M protein, the residue 36 (M-36) was suggested to be essential for proper Flavivirus viral morphogenesis, although the underlying mechanisms had not been evaluated (11, 13, 14). It has been shown, however, that among the 32 amino acid differences identified between the yellow fever virus vaccine strain 17D (YFV-17D) and the wild-type YFV Asibi strain, there is only one mutation in M at position 36, L36F (15). Noticeably, the same L36F substitution is found in another YFV vaccine strain the French neurotropic virus FNV (16). YFV L36F was shown to be partially responsible for the inability of YFV-17D to infect and disseminate in mosquitoes (14). Interestingly, in other flaviviruses M-36 is always occupied by a hydrophobic residue (Figure 1A): either by isoleucine (WNV, JEV, DV2 and DV4), alanine (DV1 and DV3) or leucine (YFV Asibi), and any substitutions of this M-36 residue always lead to a decrease in the production of virus-like-particles (VLPs) in mammalian cells (11, 13, 17). Additionally, we recently demonstrated that in JEV, substitution of isoleucine at M-36 for phenylalanine abolished infectious virus production by altering late steps of the viral cycle (11).
As specific recognition signals between cellular and viral components are required for viral particle assembly and egress, we investigated the possible involvement of residue M-36 in WNV assembly and secretion from the ER to the Golgi apparatus. We substituted the isoleucine residue for a phenylalanine at position 36 in the M protein of WNV, generating an attenuated virus that displayed an impaired secretion but that was not stable. We then introduced a sterically compensatory substitution in the same protein at position 43, M-A43G and obtained the stabilized double mutant M- I36F/A43G. This double mutant retained the specificities of the single mutant, eliciting a fully protective immune response against a lethal WT challenge in mice. We thus identified the M-36 residue as a molecular determinant of virulence that is crucial for efficient secretion of newly synthetized virions, and the M-43 residue that accommodates and stabilizes a substitution in M-36. Our study strongly suggests that export of newly formed WNV particles from the ER may depend on their morphology and identifies the M protein as a new target for the rational design of attenuated WNV strains to prevent WNV disease.
RESULTS

Mutation of M-36 affects WNV infectious cycle by potentially altering the M protein 3-dimensional structure

Mirroring the M-L36F mutation in the YFV 17D vaccine, we replaced isoleucine 36 of the WNV M protein with a phenylalanine (M-I36F) (Figure 1B). The resulting mutant virus was successfully produced in C6/36 cells electroporated with genomic RNA synthesized in vitro (see Material and Methods) (Figure 1C) and contrary to wild-type WNV, M-I36F mutant displayed a smaller foci phenotype in Vero cells, which is a potential attenuation marker (Figure 1D, M-I36F). Conversely, substitution of the parental isoleucine 36 with an alanine (Figure 1B, M-I36A) did not affect foci size (Figure 1D, M-I36A). More importantly, we observed that only the M-I36F mutation impaired WNV infectious cycle in mammalian neuronal SK-N-SH cells (Figure 1E) suggesting that the nature of residue 36 is essential for efficient viral particle production in these cells. While isoleucine, alanine and phenylalanine possess close chemical properties, only the phenylalanine has an aromatic ring. To examine how M-I36F might physically impact interactions with neighboring amino acids, we mapped either WNV M-I36F or WNV M-I36A into the recently published JEV M protein 4.3Å cryo-EM structure (18), revealing that M-I36F (Figure 1F), but not M-I36A (Figure 1G), clashes with an alanine residue (A43) located in the first transmembrane segment of M (TM1). Thus, while interactions between the two structural proteins E and M are seemingly conserved, M-I36F potentially disrupts the M protein 3-dimensional structure such that steric hindrance is introduced between the phenylalanine aromatic ring and the side chain methyl group of A43.

Compensatory mutation partially rescues M-I36F mutant to wild-type phenotype

To compensate the potential clash between the aromatic ring of residue 36 and the side chain of residue 43, we substituted the original A43 by a residue that has no methyl group, namely a glycine (M-A43G) in order to create more space, thereby generating a double mutant virus M-I36F/A43G. We recovered and amplified WNV M-I36F/A43G, M-A43G and wild-type viruses from mosquito C6/36 cells electroporated with genomic RNA synthesized in vitro (see Material and Methods). All viruses were found to form large foci on mammalian Vero cells (data not shown), and replicated similarly as assayed for RNA production, in Vero (Figure 2A) and C6/36 cells (Figure 2B), indicating that the M-I36F and M-A43G mutations alone or together did not
affect genome decapsidation and replication in mammalian and mosquito cells. When comparing infectious particle production in Vero cell supernatants, however, the titers of M-I36F as well as M-I36F/A43G variants were largely lower than that of wild-type and M-A43G viruses (1.42 logs and 0.93 logs respectively, Figure 2C). Yet the M-I36F/A43G titers were significantly higher than that of M-I36F (Figure 2C). Interestingly, when viruses were grown in C6/36 cells, no difference in titers was observed (Figure 2D). Genetic stability of the mutant viruses was tested by 10 serial passages in Vero cells. Full-genome analysis of M-I36F/A43G passaged up to 10 times revealed the presence of both M-I36F and M-A43G and no other mutation along the genome, while M-I36F alone had already reverted to WT sequence at passage 2 without compensatory mutation elsewhere in the genome (data not shown). A decrease in the amount of genomic viral RNA was observed over time in Vero cell supernatants infected with M-I36F or M-I36F/A43G mutants as compared to wild-type or M-A43G viruses (Figure 2E), that mirrored the decrease in infectious titers in mammalian cells (Figure 2C) and corroborating a decrease in the number of secreted particles. No change in the amount of genomic viral RNA in mosquito cells infected either with wild-type or any mutant viruses was detected (Figure 2F), again reflecting what we observed in terms of titers in these cells (Figure 2D). Interestingly, neither M-I36F nor M-I36F/A43G mutant virus infection of mammalian cells induced any cell death (Figure 2G, Vero cells, and 2H, SK-N-Sh cells), contrary to WT and A43G viruses. This result agrees with previous reports showing that residue M-36 can modulate the death-promoting activity of the M protein ectodomain of Flaviviruses (19, 20). No cell death induction was observed for either WT or any mutant viruses in infected C6/36 mosquito cells (data not shown). Altogether, these results indicated that the M-I36F mutation leads to an impaired WNV infectious cycle in mammalian cells, most likely due to the alteration of mutant viral assembly and/or egress, that can be partially rescued and completely stabilized by introduction of a second mutation relieving steric hindrance (M-A43G).

**M-I36F mutation strongly inhibits WNV efficient secretion**

As only a few M-I36F/A43G and M-I36F mutant particles were found in the supernatant of mammalian cells, we wondered whether the M-I36F mutation could interfere with proper budding and/or secretion of the viral particles. We examined mammalian cells infected with the different viruses by electron microscopy (Figure 3). Specific sub-cellular ultrastructural changes associated with the presence of each virus were observed in ultrathin sections of Vero
cells infected with either wild-type or mutant viruses (Figure 3, panels A, B, C and D).

Relatively few wild-type and M-A43G viral particles were observed within the cells, with the occasional particle found in the ER, indicating that the virions are secreted normally (Figure 3A and 3B, arrows). On the other hand, in the same cell type, infection with the M-I36F or M-I36F/A43G virus induced large ER swelling and massive accumulation of newly formed viral particles within the ER and ER-derived vesicles (Figure 3C and 3D, arrows). No such impairment of particle secretion with either mutant was observed in infected mosquito cells (Figure 3E, 3F). Importantly, the M-I36F and M-I36F/A43G mutant particles were released into the ER lumen of the infected mammalian cells and not retained at the ER membrane indicating that assembly and budding steps still occurred in the presence of the M-I36F mutation alone or associated with M-A43G (Figure 3A, 3B, 3C and 3D, zooms). The overall aspect of WNV M-I36F and M-I36F/A43G mutant particles seemed irregular as compared to wild-type and M-A43G mutant viruses in mammalian cells (Figure 3A, 3B, 3C and 3D, zooms), suggesting that WNV morphology was potentially altered by the M-I36F mutation. Indeed, the few secreted M-I36F/A43G virions into the supernatant of mammalian cells at 24h pi directly observed by standard negative staining electron microscopy seemed to display an altered morphology although the nucleocapsid and the lipid envelope were still well delineated (Figure 4C). While we were unable to obtain any image for M-I36F mutant due to an insufficient number of secreted particles, that of wild-type and mutant M-A43G virions showed typical characteristics of flavivirus particles (Figure 4A and 4B). The specificity of the particles produced from infected mammalian cells was confirmed using immunogold labeling with mAb 4G2 and the presence of WNV E protein at the surface of wild-type, M-A43G or M-I36F/A43G virions was unambiguously observed (Figure 4D, 4E and 4F), although less labeling was found at the surface of the double mutant virions. On the other hand, wild-type and mutant M-I36F, M-A43G and M-I36F/A43G virions collected from supernatants of mosquito cells all displayed the morphological characteristics of classic flaviviruses (Figure 4G, 4H and 4I). Taken together these data suggest that the M-I36F mutation affects virion secretion possibly by altering WNV morphology only in mammalian cells.

Atypical particle morphology of the M-I36F/A43G variant impacts WNV antigenic profile

Thus potential modification(s) of M protein structure caused by the M-I36F might lead to altered viral particle morphology with irregularly shaped mutant virions. We reasoned that
such atypical morphology of the mutant particles may impact the virion antibody recognition. We therefore first evaluated the recognition profile of wild-type and mutant virions by direct ELISA (Figure 5). While viruses produced in C6/36 cell supernatants are all similarly recognized by the mAb 4G2 that binds specifically the fusion loop of the E protein (21, 22) (Figure 5A), recognition of M-I36F/A43G virus collected in the supernatant of Vero cell is significantly decreased by approximately 1.2-fold for any antibody dilution when compared to wild-type and the M-A43G (Figure 5B). A similar significant decrease (ranging from 1.2 to 2-fold, depending on the antibody dilution) in recognition of WNV M-I36F/A43G produced in Vero cells by mAb 4G2 was obtained using indirect non-competitive ELISA (Figure 5D), while viruses produced in C6/36 cell supernatants are all similarly recognized (Figure 5C). Importantly, no difference in recognition by polyclonal anti-WNV antibody of wild-type and mutant viruses produced either in insect (Figure 5E) or mammalian cells (Figure 5F) was observed, indicating that despite a slight significant decreased recognition of protein E fusion loop, the general antigenic properties of WNV M-I36F/A43G mutant virus are conserved.

WNV surface epitopes are essential for both efficient recognition and cell attachment, and the proper folding of the E protein chaperoned by the M protein in the prM-E complex plays a critical role in them. We therefore tested the infectious capacity of our mutant and wild-type viruses under conditions allowing viral binding, but not internalization, to SK-N-SH mammalian cells or C6/36 mosquito cells by evaluating viral genomic RNA associated with the cell surface (Figure 5G and 5H respectively). Comparing viruses produced in mammalian cells and assayed at the surface of SK-N-SH or C6/36 cells, levels of M-I36F/A43G RNA were reduced by around 1-log as compared to that of the wild-type and M-A43G viruses (Figure 5G and 5H), indicating that the WNV double mutant M-I36F/A43G has impaired binding to host cells. Conversely, wild-type, M-I36F, M-A43G and M-I36F/A43G produced in insect cells showed no difference in RNA levels (Figure 5I and 5J). To confirm that the decreased infectious capacity of M-I36F/A43G mutant virus was not simply due to a lack of maturation, we tested for the presence of immature (prM) and mature (M) forms of the membrane glycoprotein at the surface of wild-type or mutant virions collected from Vero cell supernatants by Western Blot (Figure 5K). The presence of similar levels of prM and M for wild-type and mutant viruses alike suggested that all viruses undergo a similar maturation process. Taken together, the above results support the notion that virions harboring the M-I36F mutation have a possible altered morphology, while the main WNV antigenic properties are conserved.
In vivo effects of WNV M-I36F and/or M-A43G mutations

The in vitro properties of WNV M-I36F and M-I36F/A43G mutants encouraged us to test their phenotype in vivo. We first assessed pathogenicity in a well-established mouse model of WNV-induced encephalitis (23). In contrast to the high mortality rate observed among mice infected with either wild-type or M-A43G WNV (in which all 15 animals died), only 4 of 15 WNV M-I36F-infected mice died after being infected while all mice infected with M-I36F/A43G survived (Figure 6A). As expected, the wild-type, M-A43G and M-I36F-infected mice that died presented rapid weight loss beginning at day 6 pi (Figure 6B, purple, pink and red curves). Conversely, rather than weight loss, we observed normal weight gain among mice that survived the infection (Figure 6B, yellow and green curves). To investigate whether WNV M-I36F and M-I36F/A43G mutants were attenuated due to a less effective viral dissemination, we collected blood samples every other day following the infection, for 10 days, and assayed for viral load. The results showed viral loads peaked at day 3 for both the mutants M-A43G and M-I36F/A43G, but slightly later, at day 5pi, for wild-type and the M-I36F mutant (Figure 6C). At day 3 or 5 pi, however, blood viral loads of M-I36F survivors and M-I36F/A43G were 3.4- or 14.7-fold and 4- or 7.6-fold lower, respectively, compared to that of wild-type-infected mice (Figure 6C). Taken together these data indicate that the M-I36F/A43G and M-I36F viruses are rapidly cleared following infection. Sequence analyses of the entire M-I36F/A43G mutant genome collected from blood samples revealed no reversion to wild-type and no compensatory mutation (data not shown). This contrasts dramatically with the results of sequencing M-I36F viral genomes harvested from mice that did not survive the infection, which showed a reversion to the parental genotype (M-I36) (data not shown).

Altogether, these results demonstrate that the M-I36F mutation strongly attenuates WNV in vivo and that the presence of the M-A43G mutation allows for stable retention of M-I36F attenuation without negative impact.

Next, we investigated the immunogenic profile of the M-I36F and M-I36F/A43G mutants in mice (Figure 6, panels D and E). A single intraperitoneal injection of either M-I36F or M-I36F/A43G into adult BALB/c mice induced high levels of both WNV-specific IgG and neutralizing antibodies at day 27 post-infection (geometric mean titer = 102.86 and 110 respectively, Figure 6D and 6E respectively). Induction of a remarkably robust neutralizing antibody response to WNV M-I36F and M-I36F/A43G in mice led us to explore the protection afforded against a lethal challenge with the wild-type strain. Mice that had survived infection with M-I36F or M-I36F/A43G virus, or control mice injected with PBS, were
infected with 1000 FFU of wild-type WNV. Not surprisingly, all but one control mouse developed symptoms upon intraperitoneal challenge and died from the infection around 8 days pi. Importantly, none of the mice that had been injected with a single dose of the M-I36F/A43G mutant virus
exhibited symptoms after being challenged with wild-type WNV, and all survived the infection (Figure 6F, green curve). Such protection was also conferred to mice that had survived M-I36F mutant infection when the mutant virus did not revert (Figure 6F, yellow curve). These results demonstrate that the M-I36F and M-A43G mutation combination confers both full attenuation of WNV and full protection against wild-type WNV challenge in mice.

**DISCUSSION**

WNV is one of the most widely distributed arboviruses in the world (24). Responsible for encephalitis in equids and humans, this virus recently re-emerged in Europe during the 2016 and 2018 summers, causing an unusual number of human cases, including almost two hundred deaths (25). Currently no treatment or human vaccine is available. We focused our study on one of the two surface glycoproteins, namely the M membrane protein. Previously the M protein has been described as being essential for viral maturation and fusion steps (8).

In the present study we now reveal a role for this protein in WNV secretion by demonstrating that a phenylalanine for an isoleucine substitution at position 36 of the M protein (M-I36F) drastically decreases virion secretion out of the mammalian ER to the Golgi apparatus. This substitution appears to perturb the morphology of newly formed virions, which leads to massive ER accumulation of WNV progeny, while the same mutation does not seem to affect the morphology or secretion of virions during mosquito cell infection. We propose that the M-I36F substitution may cause steric hindrance that could directly affects the structure of the M protein. Importantly, in a mouse model of WNV infection, we found that the M-I36F mutant virus is strongly attenuated, resulting in the survival of most of the mice upon infection and all survivors producing neutralizing antibodies. As this mutation is unstable, stochastically reverting to wild-type within the mice, we demonstrate that we could achieve full stabilization of M-I36F by introducing a sterically compensatory substitution in the M protein, M-A43G. We found that this stable double mutant (M-I36F/A43G) retained the specific impaired secretion, viral attenuation, and resultant production of neutralizing antibodies found in the mice infected with the single M-I36F virus.

Clash between M-36 and M-43 residues likely affects viral secretion and morphology
Our results indicate that the M-I36F mutation directly affects WNV infectious cycle most likely by causing a clash between the phenylalanine residue and the alanine at position M-43 located in TM1. Similarly, substitution of residue M-36 also impairs JEV late steps, DV production and even YFV dissemination (11, 13, 14), suggesting that the nature of the amino acid M-36 is crucial for Flavivirus particle production. Phenylalanine chemical properties are close to those of the residues mainly found at position M-36 of other flaviviruses (A, I or L). Therefore, its introduction may not change the nature of the M protein helical region. However, introduction of a larger residue at position M-36 could lead to a steric hindrance. Due to its volume, phenylalanine at position M-36 might repel the M-A43 residue, that could disturb TM1 alpha helix positioning. M protein TM1 domain is known to be involved in viral assembly and secretion of Flavivirus likely through its interaction with TM2 domain (26–28). Interestingly, disruption of JEV TM1/TM2 interactions has been shown to decrease the secretion of JEV virus-like-particles (26). Our observations contend that introduction of a large residue such a phenylalanine at position M-36 is disruptive, while that of a small residue as an alanine is not. By potentially repulsing residue M-43 in TM1, phenylalanine could disturb interactions critical to proper viral biogenesis. We found that a compensatory substitution of the M-A43 residue for a glycine in TM1, a residue that does not have a side chain, partially restores a wild-type phenotype with a significantly greater quantity of viral particles secreted in cell culture supernatants, seemingly by relieving the clash.

M-36 residue is crucial for correct virus secretion by potentially altering viral morphology

Membrane curvature is known to be essential for Flavivirus morphology (29). Because our data potentially associate the M protein with viral morphology, and due to its location at the surface of the particle and its interactions with the ER-derived lipid membrane, it is possible that the M protein ectodomain mutation could directly affect the membrane curvature, thus resulting in abnormal viral budding. It has been shown that Flavivirus heterodimers of prM and E assemble laterally and their association induces ER membrane curvature in an isometric network (30). Therefore, introducing an aromatic phenylalanine residue that directly faces the ER membrane could, due to modified hydrophobic interactions, cause partial insertion of the M ectodomain into the ER membrane. As secretion and morphology of the mutant M-I36F and M-I36F/A43G virions from infected mosquito cells are normal, interactions between the M ectodomain and the ER membrane are probably unaffected by the I36F mutation in these
cells. Since mosquito cells grow at a lower temperature than mammalian cells (28°C versus 37°C), one hypothesis could be that temperature by itself might be responsible for the M-I36F mutant phenotype.
affecting viral assembly. However, preliminary data we obtained with WNV M-I36F mutant from mammalian cells cultivated at 32°C show that secretion is still altered as compared to that of wild-type virus (data not shown).

We demonstrated that it is the M-I36F mutation alone that causes alteration of WNV infectious cycle, as the M-A43G mutation alone has no effect by itself. Intriguingly, it has been shown that the substitution in DV1 virus-like particles of the M-L36 residue by an alanine increases prM and E glycoproteins interactions, leading to particle condensation (17).

Such a condensation may particularly affect the "breathing" of the particles, a dynamic phenomenon dependent on temperature ensuring the metastability of E dimers and transient exposure of this protein's buried domains. Since the WNV M-I36A substitution does not affect the viral cycle, we cannot conclude that such mutation alters or not a dynamic phenomenon of WNV particle "breathing".

Our results also indicate a lack of virus-dependent cell death in mammalian cells infected with M-I36F and M-I36F/A43G mutants, contrary to WT and M-A43G viruses. Residue M-36 is located in the pro-apoptotic domain ("apoptoM") of the M protein that was shown to be essential for apoptosis induction by Flaviviruses (19, 20). The M-I36F substitution in the M protein of YFV vaccine strain 17D abolished apoptosis induction, pointing to a crucial role for the M-36 residue. Importantly, the pro-apoptotic activity of M protein ectodomain has been associated with its transport along the secretory pathway and its localization in a post-Golgi compartment (20). The massive accumulation of WNV M-I36F mutants within the ER and the ensuing inhibition of particle secretion that we observed probably hampered the export of the M ectodomain from the Golgi apparatus to the plasma membrane, abolishing apoptosis initiation. Noticeably, introduction of M-A43G mutation in the M-I36F mutant, while partially rescuing WNV life cycle, did not restore death promoting activity.

**M-I36F and M-A43G mutations together fully attenuate WNV in vivo**

We demonstrate that the altered virulence of our mutant virions drives a strong viral attenuation in a mouse model of WNV-induced encephalitis (23, 31, 32). Although the phenylalanine substitution at position M-36 was naturally selected for in the YFV vaccine strain 17D (15), reducing the ability of the virus to spread in the mosquito (14), the role of this mutation in the mammalian host has yet to be evaluated. The M-36 residue may represent a common virulence factor to Flavivirus, however, since it has been demonstrated that introduction of a M-I36F mutation in JEV leads to production of a live attenuated virus in a murine model (11). Although
the quick reversion of M-I36F to wild-type underlines the low stability of this mutation when it is alone, the simultaneous presence of M-I36F and M-A43G mutations brings a stability to the resulting virus, without apparent reversion or compensatory mutation during the infection. Thus, the combination of these mutations leads to a double mutant virus with all the characteristics of a fully attenuated virus *in vivo*: suppression of lethality, limited weight loss, weak viremia, no neurological symptoms and production of neutralizing and protective immune response against a lethal challenge.

Curiously, the M-I36F mutation slightly alters antibody recognition by 4G2-monoclonal antibody, while the general antigenic properties of WNV M-I36F/A43G mutant virus are conserved (see Figure 5). The mAb 4G2 has been characterized to bind specifically the fusion loop of the E protein (21, 22). As the fusion loop is buried in the E dimer at the particle surface, mAb 4G2 can bind this epitope only when the E dimer dissociates. The M-I36F mutation we introduced may impair the accessibility of the E fusion loop either by masking it or by blocking it in a "buried" state. However, mAb 4G2 can also bind to immature patches in partially mature particles. Interestingly, such partially mature particles are readily found in our cell culture supernatants, as evidenced by the presence of both prM and M proteins in all viruses (Figure 5K). Importantly, the M-I36F mutation, while making virions less infectious, does not alter general antigenic recognition as shown both *in vitro* and *in vivo* (Figure 5F and 6D and 6E). Protection against flaviviruses is correlated to a large production of neutralizing antibodies (33–35). Neutralizing antibodies are generally most efficient when directed against a specific amino acid sequence in domain III of the E glycoprotein (36). Our *in vitro* data indicate that M-I36F and M-I36F/A43G mutant viruses have kept highly immunogenic epitopes and these mutations have not altered recognition of this E domain *in vivo*. On the other hand, antibodies directed against the domain II fusion loop of flaviviruses are generally poorly neutralizing, and may lead to an increase in the antibody-dependent enhancement phenomenon (ADE) (37, 38). It would be interesting to evaluate whether the presence of M-I36F et M-A43G mutations, by decreasing recognition of protein E fusion loop, may potentially reduce ADE.

Our study provides a robust proof-of-concept that M-I36F/A43G mutations may be used as a platform for the development of rationally-designed attenuated WNV strains. Of course, a vaccine to prevent WNV infection must protect against all genotypes, especially in the view of the recent emergence of lineage 2 neuroinvasive strains in Europe (39). It is known that prM and E proteins of one lineage cross protect against another lineage of WNV (40, 41). Therefore,
it would be of interest to test the protective efficacy of our lineage 1 double mutant virus on a circulating strain of lineage 2. Live attenuated vaccines against various virus infections have been empirically obtained by successive passages of wild-type virus strains and therefore may present significant risks of vaccinal accidents. To cope with the spread of Flavivirus worldwide, the development of rational vaccine design approaches is inevitable. Our study opens new perspectives for the development of live-attenuated vaccines based on molecular alteration of virulence determinants in viral genomes produced from infectious clones.

MATERIAL AND METHODS

Cells
Green monkey epithelial cells (Vero-E6), and human neuroblastoma derived cells (SK-N-SH) were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% of heat-inactivated fetal bovine serum (FBS). Aedes albopictus cells C6/36 were maintained at 28°C in Leibovitz medium (L15, Life Technologies) supplemented with 10% of FBS.

Production of recombinant WNV
The "two-plasmids" cDNA clone of WNV Israel 1998 strain produced in our lab (31) was used. Mutations M-I36F and/or M-A43G were directly introduced in pUC57-5'UTR-NS1 through PCR mutagenesis using primers 5'-AAAACAGAATCATGGTTTCCTGAGGAACCTGGG-3' and 5'-CCAGGGTTCCTCAAAGACATGATTCTGTTTTT-3' (M-I36F) or 5'-ACCCTGGATATGGACCTGGTGAGCAGC-3' and 5'-GCTGCCACCCAGTCCCATATTCAACGGTT-3' (M-A43G). Mutations are underlined.

The production of a full-length infectious clone was performed as already described (31), purified and transcribed in vitro using the mMessage mMachine SP6 kit (ThermoFischer Scientific). The resulting RNA was electroporated in C6/36 cells (400 V, 25μF, 800Ω) in OPTI-MEM medium (ThermoFischer Scientific). Cell culture supernatants were collected 72h post-electroporation and used to infect 10^7 C6/36 cells. Three-days pi, viral supernatants were amplified by infecting 5 x 10^7 C6/36 cells during 3 days before collection and utilization as final viral stocks. Full-length viral genomes were sequenced from cDNA obtained by
reverse transcription using Superscript II Reverse Transcription kit (Invitrogen) according to manufacturer’s instructions. cDNAs were then amplified by PCR using Phusion High Fidelity.
kit (ThermoFischer Scientific) and primers presented in Table 1.

**Antibodies**

Monoclonal antibody (mAb) 4G2 anti-Flavivirus E protein and HRP-conjugated mAb 4G2 were purchased from RD Biotech (Besançon, France). Polyclonal anti-WNV was isolated from intraperitoneal liquid of mice infected with WNV. Secondary antibody Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from Bio-Rad Laboratories. Secondary gold-conjugated goat-anti-mouse antibody was purchased from Aurion (Wageningen, Netherlands).

**M protein 3-fold structure**

M protein 3D structure data were obtained from the PDB (PDB accession number: 5wsn) and edited using PyMOL program.

**Quantitative RT-PCR**

Total RNA were extracted from samples using NucleoSpin RNA (Macherey-Nagel) according to manufacturer’s instructions. The RNA standard used for quantitation of WNV copy number was produced as already described (31). The quantitation of a given target RNA was performed using 2μl of RNA and the Power SYBR™ Green RNA-to-CT™ 1-Step (ThermoFisher Scientific) according to manufacturer’s instructions. The QuantStudio™ 6 Flex Real-Time PCR Instrument 384-well (Thermo Fisher Scientific) was used to measure SYBR green fluorescence with the following program: reverse transcription step at 48°C (30min), followed by an initial PCR activation step at 95°C (10min), 40 cycles of denaturation at 95°C (15s) and annealing at 60°C (30s). Results were analyzed using the CFX Manager software (Bio-Rad). Primers 5′- GCGGCAATATTCATGACAGCC -3′ and 5′- CGGGATCTCAGTCTGTAAGTC -3′ were used for viral genome quantitation. Target gene expression was normalized to the expression of GAPDH mRNA, measured using the primers 5′- GGTCGGAGTCAACGGATTTG -3′ and 5′- ACTCCACGACGTACTCAGCG-3′ (42).

**Titration**

Vero-E6 cells were seeded at 8×10⁴ cells per well in 24-well plates and incubated at 37°C for 24h. Tenfold dilutions of virus in DMEM were added to the cells and incubated for 1h at 37°C. Unadsorbed virus was removed, then 1ml of DMEM supplemented with 1.6%
carboxymethyl cellulose (CMC), 10 mM HEPES buffer, 72 mM sodium bicarbonate, and 2% FBS were added to each well, followed by incubation at 37°C for 2 days. The CMC overlay was removed, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.2% Triton X-100 for 5 min. Cells were then washed with PBS and incubated for 1 h at room temperature (RT) with anti-E antibody (4G2), followed by incubation with HRP-conjugated anti-mouse IgG antibody. The foci were revealed using the Vector VIP peroxidase substrate kit (Vector Laboratories) according to manufacturer’s instructions.

Analysis of the secreted particles by negative staining electron microscopy and immunogold labeling

The clarified viral supernatant was purified by polyethylene glycol precipitation followed by ultracentrifugation at 50000G, 4°C for 2 h (Ultracentrifuge Optima L-100 XP, Beckman) on iodixanol gradient (OptiPrep, Sigma Aldrich). Fractions of interest were then fixed (v/v) with paraformaldehyde 2% (Sigma, St-Louis, MO), 0.1 M phosphate buffer pH 7.2 overnight. Formvar/carbon-coated nickel grids were deposited on a drop of fixed sample during 5 min and rinsed three times with phosphate-buffered saline (PBS). After a single wash with distilled water, the negative staining was then performed with three consecutive contrasting steps using 2% uranyl acetate (Agar Scientific, Stansted, UK), before analysis under the transmission electron microscope (JEOL 1011, Tokyo, Japan).

For immunogold labeling, grids coated with the sample were washed and further incubated for 45 min on a drop of PBS containing 1:10 mouse monoclonal antibody against Flavivirus E protein (4G2). After 6 washes with PBS, grids were incubated for 45 min on a drop of PBS containing 1:30 gold-conjugated (10 nm) goat-anti-mouse IgG (Aurion, Wageningen, Netherlands). Grids were then washed with 6 drops of PBS, post-fixed in 1% glutaraldehyde, rinsed with 2 drops of distilled water, before being negatively stained and observed under the microscope as described above.

Ultrastructural analysis of the infected cells by transmission electron microscopy

24 h-infected Vero or C6/36 cells were trypsinized, rinsed once in PBS, and gently resuspended in cold fixation buffer containing paraformaldehyde 4% (Sigma, St-Louis, MO), 1% glutaraldehyde (Sigma), 0.1 M phosphate buffer pH 7.3, for 24 h. Cells were then placed in a mixture of (1:1) propylene oxide/Epon resin (Sigma) and left overnight in pure resin for samples impregnation. Cells were then embedded in Epon resin (Sigma), and blocks were
allowed to polymerize for 48 hours at 60°C. Ultra-thin sections of blocks were obtained with a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Sections were deposited on formvar/carbon-coated nickel grids and stained with 5% uranyl acetate (Agar Scientific), 5% lead citrate (Sigma), and observations were made with a JEOL 1011 transmission electron microscope.

**Mouse experiments**

Three-weeks old female BALB/c mice (Janvier) were housed under pathogen-free conditions in level 3 animal facility and protocols were approved by the Ethic Committee for Control of Experiments in Animals (CETEA) at the Institut Pasteur and declared to the French Ministry under no. 00762.02. Mice were infected intraperitoneally with 50 FFU of either wild-type, M-I36F, M-A43G, or M-I36F/A43G mutated virus in 50µL of DPBS supplemented with 0.2% bovine serum albumin. Mice were followed daily post-injection, survival rate, weight loss and clinical symptoms were monitored. Every two days pi (day 1, 3, 5, 7 and 9) blood samples obtained by puncture at the caudal vein were collected and tested for the presence of viral RNA. Mice that survived the infection were challenged with 1000 FFU of wild-type virus diluted in 50µL of DPBS + 0.2% BSA at day 28 pi. Mice mortality was followed over time. Blood was obtained by puncture at the caudal vein at day 27 pi, collected in tube containing EDTA and serum separated after centrifugation at 4000G, 10 min in order to perform ELISA and seroneutralization assays.

**Direct ELISA**

Viruses were purified by polyethylene glycol precipitation followed by ultracentrifugation at 50000G, 4°C for 2h (Ultracentrifuge Optima L-100 XP, Beckman) on iodixanol gradient (OptiPrep, Sigma Aldrich). Fractions of interest were then UV-inactivated. High-binding 96-well plates (Nunc) were coated with 2µg/mL of purified and inactivated viruses in 100µL of PBS-3% milk and 0.5% Tween 20 (PBS-milk-Tween) and incubated overnight at 4°C. Plates were washed five times with PBS containing 0.05% Tween 20. mAb 4G2, polyclonal anti-WNV antibodies, or sera obtained from mice blood were serially diluted 10-fold (morphology analyses) or 2-fold (mice experiments) starting at 1:100 dilution in PBS-milk-Tween, added to plates and incubated 1h at 41°C. After washing, plates were incubated with 100µL of HRP-conjugated goat anti-mouse IgG diluted 1:10 000 in PBS-milk-Tween for 1h at 41°C. Plates were washed again and 200µL of SIGMAFAST™ OPD (Sigma) substrate was added per well for 30min following manufacturer’s instructions. Luminescence was read on plate reader.
EnVision™ 2100 Multilabel Reader (PerkinElmer, Santa Clara, CA, USA) at a wavelength of 450nm.

Indirect ELISA
High-binding 96-well plates (Nunc) were coated with 5µg/mL of polyclonal anti-WNV antibody in 100µL of PBS-milk-Tween and incubated overnight at 4°C. Plates were washed five times with PBS containing 0.05% Tween 20 and 2µg/mL of purified and inactivated viruses were added to plates and incubated 2h at 41°C. After washing, 100µL of HRP-conjugated mAb 4G2 serially diluted 10-fold in PBS-milk-Tween were added to plates and incubated 1h at 41°C. Plates were washed and 200µL of HRP substrate, SIGMAFAST™ OPD (Sigma), was added per well for 30min following manufacturer’s instructions. Luminescence was read on plate reader EnVision™ 2100 Multilabel Reader (PerkinElmer, Santa Clara, CA, USA) at a wavelength of 450nm.

Seroneutralization assay
Serum samples were two-fold serially diluted in DMEM, with a starting dilution of 1:20. Each dilution was incubated with 50 FFU of wild-type WNV for 1h, under agitation, at 37°C. The remaining viral infectivity was evaluated by FFA on Vero-E6 cells. Sera collected from DPBS-injected mice served as negative controls. Neutralization curves were obtained and analyzed using GraphPad Prism 6 software. Nonlinear regression fitting with sigmoidal dose response was used to determine the dilution of serum that reduced the quantity of FFU by 50%.

Statistical analysis
Statistical analyses were performed using GraphPad Prism software. Non-parametric Mann-Whitney test was used to compared quantitative data and log-rank (Mantel-Cox) analysis was used for survival data analysis.

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AUTHORS CONTRIBUTION

Each named author has substantially contributed to conducting the underlying research and drafting this manuscript. Additionally, to the best of our knowledge, none have any conflict of interest, financial or otherwise. J.B., N.P. and P.R. designed the experiments. J.B., M.F. and J.B.G. performed the experiments. F.R. and J.B. conducted the structural analysis. J.B. and N.P. wrote the paper.
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LEGENDS:

Figure 1: The nature of M-36 residue impacts WNV infectious cycle by potentially disrupting the M protein 3-dimensional structure.

(A): Sequence comparison of M protein ectodomain and TM1 from different Flavivirus. Residue 36 location is indicated in red and that of residue 43 in blue. DV4 accession number MK506266.1, DV2 accession number MK506264.1, DV1 accession number MK506262.1, DV3 accession number MK506265.1, YFV Asibi strain accession number AY640589, YFV 17D strain accession number MN708489.1, Zika virus accession number MG827392.1, WNV accession number AF481864.1 and JEV accession number KF907505.1. (B): WNV membrane protein precursor (prM) organization showing ectodomain (ectoM) and part of transmembrane domain 1 (TM1) sequences. Residue at position 36 is indicated in red for WT virus, black for M-I36F or grey M-I36A mutant viruses. (C): Viral stocks were collected from C6/36 cell supernatants at times indicated and titrated by foci-forming assay (FFA) in Vero cells. No statistical difference was observed. (D): Foci morphology of wild-type WNV, M-I36F and M-I36A mutated viral stocks collected from C6/36 supernatants, observed on Vero cells. Vero cells were infected with the indicated virus and foci were observed 48h pi. (E): Growth curves of wild-type, M-I36F and M-I36A mutant WNV. SK-N-SH cells were infected with the indicated virus at a MOI of 1, cell supernatants were collected at indicated times for quantitation of virus titers by FFA using Vero cells. (F): Structure of M-E mature heterodimers (PDB accession number 5wsn). The insert zooms into the A43-F36 contact, with F36 highlighted in pink and A43 in green. The F36 aromatic ring clashes (in red) with the side chain of the A43 located in the TMD-1. (G): Same as (H) with alanine at position M-36. The insert zooms into the A36-A43 contact, with A36 highlighted in pink and A43 in green. No clash between A36 and A43 was observed. The image was generated using PyMOL. The data are representative of 3 independent experiments and error bars indicate standard deviation (SD). * p-value < 0.05; ** p-value < 0.01, *** p-value < 0.001.

Figure 2: Phenotypical characterization of WNV M-I36F and/or M-A43G mutant viruses in vitro

(A, B): Viral stocks of WNV wild-type and mutants M-A43G, M-I36F and M-I36F/A43G were used at a MOI of 1 to infect (A): Vero cells or (B): C6/36 cells. At the indicated time points, cells were harvested and levels of WNV genomic RNA were quantified by RT-qPCR.
Growth curves and genome quantitation of wild-type, M-I36F, M-A43G and M-I36F/A43G mutated WNV produced in Vero cells. Vero (C, E) and C6/36 cells (D, F) were infected with the indicated viruses at a MOI of 1, cell supernatants were collected at indicated times for quantitation of virus titers by FFA using Vero cells (C, D) or genome quantitation by RT-qPCR (E, F). (G, H): Cell viability. Vero (G) or SK-N-SH (H) cells were infected with the indicated viruses at a MOI of 1, cells were harvested at indicated times, cell viability was evaluated using CellTiter Glo and represented as a percentage of non-infected control cells. The data are representative of 3 independent experiments and error bars indicate standard deviation (SD). * p-value < 0.05; ** p-value < 0.01, *** p-value < 0.001.

Figure 3: M-I36F and M-I36F/A43G mutant particles are retained within the ER lumen of infected mammalian cells but not in mosquito cells.

(A, B, C, D, E): Vero cells were infected with wild-type or mutated WNV in positions M-36 and/or M-43 at a MOI of 10 and examined by transmission electron microscopy at 24h pi. (A): Vero cells infected with WNV WT. (B): Vero cells infected with mutated virus M-A43G. (C): Vero cells infected with mutated virus M-I36F. (D): Vero cells infected with double mutant virus M-I36F/A43G. (E, F): Mosquito C6/36 cells were infected with wild-type or mutated WNV in positions M-36 and/or M-43 at a MOI of 10 and examined by transmission electron microscopy at 24h pi. (E): C6/36 cell infected with WNV WT. (F): Same with double mutant virus M-I36F/A43G. Examples of viral particles located in the ER lumen are indicated by arrows. Inset bars: 100 nm.

Figure 4: Secreted mutant virions M-I36F/A43G display an altered morphology only when produced in mammalian cells.

(A, B, C, D, E, F): Wild-type and mutated viral particles collected from supernatants of Vero cells infected at a MOI of 10 for 24h, were concentrated and purified. (A, B, C): Particles were stained negatively with uranyl and observed by transmission electron microscopy. (A): WNV WT particles. (B): WNV M-A43G particles. (C): WNV M-I36F/A43G particles. (D, E, F): Viral particles were labeled by immunogold with an anti-protein E pan-flavivirus antibody (mAb 4G2) and observed by transmission electron microscopy. (D): WNV WT particles. (E): WNV M-A43G particles. (F): WNV M-I36F/A43G particles. (G, H, I): Wild-type and mutated viral particles collected from supernatants of C6/36 cells infected at a MOI of 10 for 24h, were concentrated and purified. Particles were stained negatively with uranyl and...
observed by transmission electron microscopy. (G): WNV WT particles. (H): WNV M-A43G particles. (I): WNV M-I36F/A43G particles. Bars = 100nm

**Figure 5: M-I36F mutation effects on WNV antigenic profile.**

(A, B): Wild-type and mutated WNV surface epitope exhibition was analyzed by direct ELISA. 200ng of different UV-inactivated viruses collected from C6/36 cells (A) or Vero cells (B) were coated and tested with increasing concentrations of mAb 4G2. (C, D): Same as (A) and (B) using indirect non-competitive ELISA. (E, F): Same as (A) and (B) but with increasing concentrations of polyclonal anti-WNV antibodies. (G, H): Infectious capacity of mutant virus M-I36F/A43G is impaired when the virus is produced in mammalian cells. SK-N-SH and C6/36 cells were placed at 4°C for 1h, then infected at a MOI (amount of viral genomic RNA) of 10 for 1h at 4°C with the indicated viruses produced in mammalian cells. (G): SK-N-SH cells were collected and viral genomes attached to the cell-surface were quantified by RT-qPCR. (H): Same as (G) with C6/36 cells. (I, J): Infectious capacity of WNV mutated at position M-I36F alone or associated with M-A43G is not affected when the virus is produced in mosquito cells. SK-N-SH and C6/36 cells were placed at 4°C for 1h, then infected at a MOI (amount of viral genomic RNA) of 10 for 1h at 4°C with the indicated viruses produced in mosquito cells. (I): SK-N-SH cells were collected and viral genomes attached to the cell-surface were quantified by RT-qPCR. (J): Same as (I) with C6/36 cells. (K): Levels of E, immature prM and mature M glycoproteins were tested under denaturing conditions by Western Blot using a polyclonal anti-WNV antibody. The same amount of viral RNA was loaded in each well. The histograms indicate the median value and the interquartile range determined from triplicate of three independent experiments. *p-value <0.05; ** p-value <0.01; *** p-value <0.001.

**Figure 6: Combined M-I36F and M-A43G mutations highly attenuate WNV and elicit WNV-specific humoral response in a mouse model.**

(A): Survival curves of 3-weeks-old BALB/c mice inoculated with 50 FFU of the indicated viruses by i.p. route. (B): Mice growth curve. Mice weight was measured every day pi and is represented as a percentage of the starting body weight. (C): Viral load in mice blood. Viral RNA loads were quantified by RT-qPCR. Dotted line indicates detection limit. (D, E): WNV specific-IgG and neutralizing antibodies were measured by ELISA and PRNT50 respectively. (F): Survivor mice were challenged with 1000 FFU of wild-type WNV at day 28 pi. Mice were monitored for clinical symptoms and mortality for 25 days. The data are representative
of at least two independent experiments and error bars indicate the SD. (* p-value < 0.05; ** p-value < 0.01, *** p-value < 0.001).

**TABLE:**

| Oligo Forward | Forward Sequence          | Tm  | Oligo reverse | Reverse Sequence          | Tm  | Size  |
|---------------|---------------------------|-----|---------------|---------------------------|-----|-------|
| 11-32F        | cttggtgacgtgacaaacttag    | 55  |               | caagcccccttcttgctc        | 58  | 787   |
| 501-520F      | gacggttaatgctactgaagc    | 56  | 1282-1300R    | ttccttgccaaaatgctc        | 55  | 800   |
| 1000-1018F    | ttggaagggttgtggag         | 56  | 1781-1800R    | aatgtggtgtaaattcaa        | 56  | 801   |
| 1500-1520F    | aaagctggagaatagtgaga     | 56  | 2278-2295R    | atggaacagtcccccaac        | 58  | 796   |
| 2000-2017F    | cattgaacgaaccaacgc       | 55  | 2766-2783R    | tgtgagcggttggaggtgtc      | 56  | 784   |
| 2500-2519F    | caagagctgagatggtgaaga    | 56  | 3281-3298R    | aatgcaactctacccgga        | 55  | 799   |
| 3001-3022F    | gaattgtagctgaagatcattg   | 57  | 3777-3799R    | ccaacataaaacagtctgtagctc | 58  | 799   |
| 3501-3518F    | cctctgctgctcaagagc      | 56  | 4280-4297R    | caatgctacgctgctcaa        | 57  | 797   |
| 4000-4017F    | ctacacccgctaaaccc        | 56  | 4781-4798R    | cctctgctgctcaagaaagc     | 57  | 799   |
| 4500-4519F    | ttggaagatagtaattgcataaca | 57  | 5277-5294R    | acaacacctgcttggtc         | 59  | 795   |
| 5003-5020F    | ccacagggacatagggctc      | 57  | 5783-5800R    | tgggttaactctgctgctg       | 57  | 798   |
| 5500-5520F    | gcacagagtt catactccacaca | 58  | 6282-6299R    | caacctcggctgctggtgta      | 59  | 800   |
| 6001-6018F    | aacagctgctgctcagctt     | 56  | 6783-6800R    | gaggagccgaacatatcg         | 58  | 800   |
| 6503-6520F    | tcctgctgctgctcagctt     | 59  | 7283-7300R    | aaccaagccgaccatggaacagc   | 58  | 798   |
| 7031-7049F    | caacagctgctgctgctgctt   | 55  | 7775-7792R    | aacgctgctgctgctgctg       | 58  | 762   |
| 7500-7517F    | aacagagcgcggccatcatttt  | 57  | 8258-8275R    | gggagcagcgcagatgctgctg    | 59  | 776   |
| 8000-8017F    | tgcctgctgctgctgcttcct   | 59  | 8663-8681R    | cattgcctgctgctggtggtcctgctggtgta | 58  | 682   |
| 8482-8499F    | ggggggggggggggggggggggg | 58  | 9283-9300R    | cagcctggtgcttgctgctgctg  | 59  | 819   |
| 9017-9034F    | ggggggggggggggggggggggg | 58  | 9782-9800R    | cattccactcagctcttgtggtcctgctggtgta | 55  | 784   |
| 9500-9517F    | ttgctagctgccctaaacaa    | 55  | 10282-10300R  | gttgagagccacagctgttggaaacagtctgctggtgta | 57  | 801   |
| 10002-10019F  | aagacgctgctgctgctgctgctt | 58  | 10783-10800R  | ttcgctgtctgctgctgctgctgctggtgta | 57  | 799   |
| 10504-10521F  | aagacgctgctgctgctgctgctgctggtgta | 60  | 11011-11028R  | atctgctgctgctgctgctgctggtgta | 57  | 525   |

**Table 1:** Primers used for the amplification and sequencing of the complete wild-type and mutant viral genomes.
Figure 1:

A

DV4: SVALTHSOKLETRAETNMSSEGKRAQVQESDIHLPQFVVLACPMAYIQGQGQR
DV2: SVALVHSVQGLETRTENMSSEGKRAQVQESDIHLPQFVVLACPMAYIQGQGQR
DV1: SVALAFHVONGLETRTENMSSEGKRAQVQESDIHLPQFVVLACPMAYIQGQGQR
DV3: SVALAVHVONGLETRTENMSSEGKRAQVQESDIHLPQFVVLACPMAYIQGQGQR
YFVasibi: AIDLPNHLQRLWQXMTRGEMEQGQLKIERFPRHPFVADTIALVAGSNHTQR
YFF-17b: AIDLPNHLQRLWQXMTRGEMEQGQLKIERFPRHPFVADTIALVAGSNHTQR
ZIKV: AVTLPHSTRLKRTQTHLEESQETTYKLKVENVFFYNPGQDFYAV(A)ILMLLSFDSTQR
WNV: SLYTVQIKSLNLVGMLDSTKATRKLVTKEYLHLPQFGVAVACSLNLLSFDSTQR
JEV: SVVQTVIKSLNLVGMLDSTKATRKLVIDGFAVAVACSLNLLSFDSTQR

B

S'UTR  Precursor  ectoM  IMI  TM  3'UTR

WT: SLTVQTHGESTLANKGAWLDSTKATRYLVTESWILRNPGFA
I36F: F
I36A: A

C

E

D

F

G
Figure 2:
Figure 5:

A

B

C

D

E

F

G

H

I

J

K

Env

55kDa

prM

25kDa

M

10kDa
