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Disruption of dengue virus transmission by mosquitoes

Alexander WE Franz[1], Velmurugan Balaraman[1] and Malcolm J Fraser Jr[2]

Current control efforts for mosquito-borne arboviruses focus on mosquito control involving insecticide applications, which are becoming increasingly ineffective and unsustainable in urban areas. Mosquito population replacement is an alternative arbovirus control concept aiming at replacing virus-competent vector populations with laboratory-engineered incompetent vectors. A prerequisite for this strategy is the design of robust anti-pathogen effectors that can ultimately be genetically driven through a wild-type population. Several anti-pathogen effector concepts have been developed that target the RNA genomes of arboviruses such as dengue virus in a highly sequence-specific manner. Design principles are based on long inverted-repeat RNA triggered RNA interference, catalytic hammerhead ribozymes, and trans-splicing Group I Introns that are able to induce apoptosis in virus-infected cells following splicing with target viral RNA.

Addresses
[1] Department of Veterinary Pathobiology, 303 Connaway Hall, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211, United States
[2] Department of Biological Sciences, 218 Galvin Life Science Bldg., University of Notre Dame, South Bend IN, 46556, United States of America

Corresponding author: Franz, Alexander WE (franza@missouri.edu)

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Introduction

Increasing global trade and human traffic cause arthropod-borne viruses (arboviruses) and their vectors to become ever more prevalent around the world [1–5]. Recent examples are mosquito-borne arboviruses such as dengue virus (*Flaviviridae*; *Flavivirus*; serotypes 1–4, DENV1–4) causing morbidity and mortality throughout the world's tropics, and the current emergence of chikungunya virus (*Togaviridae*; *Alphavirus*; CHIKV) in the Caribbean and the Americas [6,7•,8•]. No therapeutics are available for most of these arboviral diseases, and vaccines have been licensed against only a handful of arboviruses, with DENV or CHIKV being notable exceptions [9]. Current mosquito-borne arbovirus control efforts rely on intensive vector control measures such as removing potential mosquito breeding sites, use of insecticide treated bed nets/window curtains, and residual insecticide applications [6,10]. Using insecticides is becoming increasingly ineffective and unsustainable because many mosquito populations have developed elevated levels of insecticide resistance over time [11–13]. A promising, alternative strategy to suppress mosquito transmission of arboviruses such as DENV or CHIKV involves *Wolbachia*, an intracellular bacterial symbiont that lives inside insects and is transmitted vertically from mother to offspring [14]. *Wolbachia* has been successfully trans-infected into naïve *Aedes aegypti* populations [15•,16•]. Reproduction is inhibited when *Wolbachia*-infected males are mated with non-infected females, thus favoring spread of the symbiont within a population. Importantly, mosquitoes which are infected with certain *Wolbachia* strains have been shown to be resistant to arboviruses such as DENV and CHIKV [15•,16•]. *Wolbachia* can also negatively impact the longevity of infected mosquitoes. Currently, the effectiveness of *Wolbachia* as an arbovirus/mosquito control agent is being tested in field studies in Australia [16•,17].

Two novel genetic pest management concepts of arbovirus/mosquito control rely on the use of genetically modified mosquitoes. One of them is population reduction (elimination) based on Release of Insects with Dominant Lethality (RIDL) [18–20,21••,22], the other concept includes population replacement strategies, which will be the focus of this article. Population replacement implies that an arbovirus-competent mosquito population is replaced with laboratory generated, incompetent mosquitoes harboring specifically engineered antiviral effector genes [23–25,26••,27••]. Until now, the majority of research on disrupting mosquito-borne viral disease transmission has been concentrated on DENV and *Ae. aegypti* because of their role in transmitting the most clinically important arbovirus affecting humans [28]. Anti-DENV effector gene strategies include inverted-repeat (IR) RNA, catalytic hammerhead ribozymes (hRz), or trans-splicing Group I Intron (GrpI), which target and destroy exposed viral RNA genomes during their replication in the vector cell environment.

In the typical, urban disease cycle the four serotypes of DENV (DENV1-4) circulate between mosquitoes and humans [5,6,28]. Principal vectors are culicine mosquitoes, *Ae. aegypti* and *Ae. albopictus*. Following ingestion of a

viremic human bloodmeal, DENV enters the epithelial cells of the mosquito midgut and replicates in this tissue. Within 4–5 days, the virus starts disseminating from the midgut to secondary tissues such as hemocytes, fat body, nerve tissues, and the salivary glands [29–31]. Once the salivary glands are infected and virus is released into the salivary ducts, the mosquito is able to transmit the virus to a new human host. Besides overcoming several tissue barriers in the mosquito, DENV has to cope with several innate immune defenses, Toll, JAK–STAT, and RNA interference (RNAi), the last of which is the most potent antiviral immune pathway capable of eliminating the virus [32•,33•,34•,35,36•]. Because the midgut is the initial tissue of DENV infection in the mosquito, it also constitutes the ideal site for over-expressing/activating an antiviral effector [25,26••]. This way DENV can be attacked at an early, vulnerable step of its replication cycle before the virus has established any infection foci.

RNAi-based antiviral effectors

Dicer2 of the endogenous, antiviral exo-siRNA pathway senses the presence of long dsRNAs, as they arise during replication of positive sense RNA viruses like DENV (Figure 1) [32•]. Dicer2 initiates the RNAi pathway by cleaving the dsRNAs into 21 bp duplexes. With the help of the RNA binding protein R2D2, the 21 bp duplexes are unwound and one strand (guide strand) is incorporated into the RNA induced silencing complex (RISC), of which the endonuclease argonaute2 is the catalytic component. The guide strand then guides RISC to homologous RNA molecules, which are sliced with the help of argonaute2 resulting in the destruction of viral genomes.

An anti-DENV effector gene that efficiently triggers the RNAi pathway against the virus consists of an inverted-repeat (IR) DNA encoding virus-derived sequences of 300–600 bp length in sense and antisense orientation [25,26••,27••]. A small, functional intron placed between the sense and antisense sequences is spliced out during transcription, thereby supporting stable dsRNA formation and establishing the trigger for RNAi. This approach has been used to generate transgenic resistance to DENV2 (Figure 1). In this case the virus-derived cDNA sequences, 578 bp in length, corresponding to the prM-M encoding region of the viral genome are separated by an intron originating from the *sialokinin I* gene [37]. Regulatory elements of this IR effector gene include the transcription termination signal of Simian virus 40 and an endogenous, tissue-specific promoter such as the blood-meal-inducible *carboxypeptidase A* promoter of *Ae. aegypti* [38,39]. This promoter drives gene expression in midgut epithelial cells between 4 and 32 h following acquisition of a bloodmeal, an ideal time frame in which to tackle DENV2 before it is able to establish infection foci in this tissue. Germline transformation of the mosquito host is facilitated by insertion of the IR effector gene into a transgene insertion vector consisting of the non-autonomous class II DNA transposable element (TE), *mariner Mos1* [40–42]. This TE vector also contains an eye-specific selection marker such as enhanced green fluorescent protein (EGFP) to allow easy identification of transformants [43,44]. Unfortunately, TEs such as *mariner Mos1* follow quasi-random integration patterns due to their short recognition sequence motifs, which are abundantly present in the host genome. Consequently, TE-mediated transgene expression is often affected by position effect variegation, which could be overcome by using site-specific integration systems such as *PhiC31* or chromatin insulators such as the *scs scs'* elements of the Drosophila *gypsy* retrotransposon [45,46•,47,48•].

Using the Higgs White Eye (HWE) strain of *Ae. aegypti*, a transgenic line, Carb77, was previously generated according to the design principles described above [25]. Carb77 females, expressing the IR effector in midgut tissue of bloodfed females were highly resistant to orally acquired DENV2 but not to other serotypes of the virus or to other arboviruses, confirming the homology-dependence of this antiviral strategy. Carb77 females exhibited a midgut infection barrier to the virus, which could be circumvented by intrathoracic injection of the virus [25]. After 17 generations in laboratory culture, these Carb77 mosquitoes lost their resistance phenotype even though the transgene itself was not mutated [49], possibly due to hetero-chromatin rearrangement that silenced the IR effector gene. In a subsequent experiment a new transgenic line, Carb109, was generated, which harbored the identical transgene as Carb77 [26••]. Carb109 females proved to be refractory to various DENV2 strains for at least 33 generations. Importantly, the resistance phenotype was maintained after introgression of the Carb109 transgene into a genetically diverse laboratory strain (GDLS) derived from 10 different *Ae. aegypti* wild-type populations from DENV-endemic regions of Southern Mexico. Furthermore, fitness studies showed that introgression of the Carb109 transgene into GDLS via consecutive backcrosses resulted in transgenic hybrids that exhibited only minimal fitness loads. After five consecutive backcrosses without selection for transgenic individuals, the transgenic allele frequency of introgressed cage populations was in equilibrium. Thus, the strong fitness loads observed for the original Carb109 transgenic strain seem to have been associated with the genetic background of the highly inbred HWE strain, which had been the recipient for germline transformation.

The identical DENV2 targeting IR effector was also transgenically overexpressed in fat body tissue under control of the bloodmeal inducible *vitellogenin 1* promoter and in salivary glands from the *30K* promoter [27••,49]. Silencing DENV2 in fat body did not affect the mosquito's overall vector competence for the virus, indicating that this tissue can be circumvented by DENV2 during its route to the salivary glands. Constitutive silencing of DENV2 in the distal-lateral lobes of the salivary glands