Dengue Non-coding RNA: TRIMmed for Transmission

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Dengue virus RNA is trimmed by the 5′→3′ exoribonuclease XRN1 to produce an abundant, non-coding subgenomic flavivirus RNA (sfRNA) in infected cells. In a recent paper in *Science*, Manokaran et al. (2015) report that sfRNA binds TRIM25 to evade innate immune sensing of viral RNA by RIG-I.

Mosquito-borne flaviviruses such as Dengue (DENV), West Nile (WNV), and Yellow fever have a major impact on human health and are a serious threat in developing countries. A complex network of interactions between hosts, mosquito vectors, and environmental and viral factors determines the transmission of these viruses in the field (Guzman and Harris, 2015). As a result, flavivirus epidemics can be highly dynamic, with different viral strains co-circulating and/or competing in the same area. However, the underlying interactions and factors driving the replacement of one flaviviral strain by another have not been clearly defined. The importance of mosquitoes in flavivirus strain replacement was exemplified in the well-documented WNV epidemic in the US in the period 1999–2003. The now-dominant WN02 virus, carrying an amino acid change in the structural envelope glycoprotein E, was transmitted more efficiently by *Culex pipiens* mosquitoes, and this likely contributed to replacement of the original NY99 strain (Kilpatrick et al., 2008). Elucidating the reasons for strain replacement may help us to understand flavivirus epidemiology and eventually mitigate virus spread.

In recent work, Manokaran et al. (2015) set out to find an explanation for the replacement of epidemic DENV2 from Puerto Rico (PR-1 clade) by emerging isolates (PR-2B clade) in the period 1995–2007. Phylogenetic studies of complete genome sequences demonstrated no significant difference between the structural proteins of PR-1 and PR-2B. Instead, the authors discovered that PR-2B viruses had significant nucleotide changes in their 3′ UTRs. PR-2B concomitantly expressed increased levels of non-coding, subgenomic flavivirus RNA (sfRNA) relative to viral genomic RNA (vgRNA). Correspondingly, the emerging Nicaraguan DENV2 NI-2B strain, which replaced the endemic NI-1 strain in 2005, also produced higher sfRNA: vgRNA ratios, providing a novel link between sfRNA production and viral epidemiological fitness.

SfRNA is a ~0.5-kb degradation product of viral mRNA turnover and is essential for flavivirus pathogenesis in vertebrates (Pijlman et al., 2008). Full-length sfRNA is formed when the 5′→3′ exoribonuclease XRN1 digests vgRNA but stalls at the conserved stem loop-II (SL-II) RNA structure in the 3′ UTR with a compact 3D fold and an important pseudoknot (PK) interaction (Figure 1; Chapman et al., 2014). XRN1 stalling at the downstream SL-IV and dumbbell-1 (DB1) RNA structures produces the smaller sfRNA2 and sfRNA3, respectively, although biological functions have primarily been linked to full-length sfRNA.

The vertebrate innate immune response against flaviviruses is triggered primarily by the sensing of viral RNA by retinoic acid-inducible gene-I-like receptors in the cytoplasm (RIG-I, MDA5). Binding of viral RNA to these RIG-I-like receptors (RLRs) triggers the downstream transcription of interferon response factor (IRF)-3/7 and eventually leads to induction of type I interferon (IFN). How flaviviruses cope with RLR signaling and subsequent immune activation remains unclear, although there is compelling evidence that sfRNA can effectively antagonize innate immune pathways (Figure 1).

In weanling mice, WNV sfRNA was shown to interfere with IFN-α receptor (IFNAR)-dependent and IRF-3/7-mediated IFN induction (Schuessler et al., 2012). An interaction between DENV2 sfRNA and the stress granule components G3BP1/2 and Caprin was shown to decrease IFN-stimulated gene (ISG) expression; however, a direct mechanism between IFN signaling and sfRNA is not known (Bidet et al., 2014). The present study by Manokaran et al. (2015) sheds light on the interference of sfRNA with IFN induction and provides evidence for binding of DENV2 sfRNA to the ubiquitin ligase tripartite motif protein 25 (TRIM25). TRIM25 normally polyubiquitynates RIG-I, a prerequisite for efficient signaling in response to non-self (viral) RNA recognition/sensing by RIG-I (Manokaran et al., 2015). RNA-immunoprecipitation showed that sfRNA of the emergent PR-2B virus binds more strongly to TRIM25, thereby preventing the ubiquitination-dependent activation of RIG-I (Figure 1). A similar strategy is used by influenza A virus, which targets the viral NS1 protein to TRIM25 to prevent RIG-I activation and subsequent IFN induction (Gack et al., 2009). Together, these findings form an intriguing example of convergent evolution of protein- and RNA-based viral products in different virus families to inactivate TRIM25.

The finding by Manokaran et al. (2015) raises the question of which RNA structures and/or sequence motifs in the DENV2 3′ UTR are responsible for the difference in IFN induction between PR-1 and PR-2B viruses. Although sfRNA secondary structures are highly conserved (Pijlman et al., 2008), its primary sequence is more variable. Interestingly, in-depth comparison of the 3′ UTR sequences revealed that just three conserved mutations (Figure 1, red dots) between the DENV PR-1 and PR-2B clade were responsible for increased sfRNA abundance and TRIM25 binding. Two mutations (A10301G, U10389C) did not disturb predicted pseudoknot nor stem-loop formation (Chapman et al., 2014; Pijlman et al., 2008); however, a third mutation (G10331A) would theoretically result in formation of a weaker pseudoknot

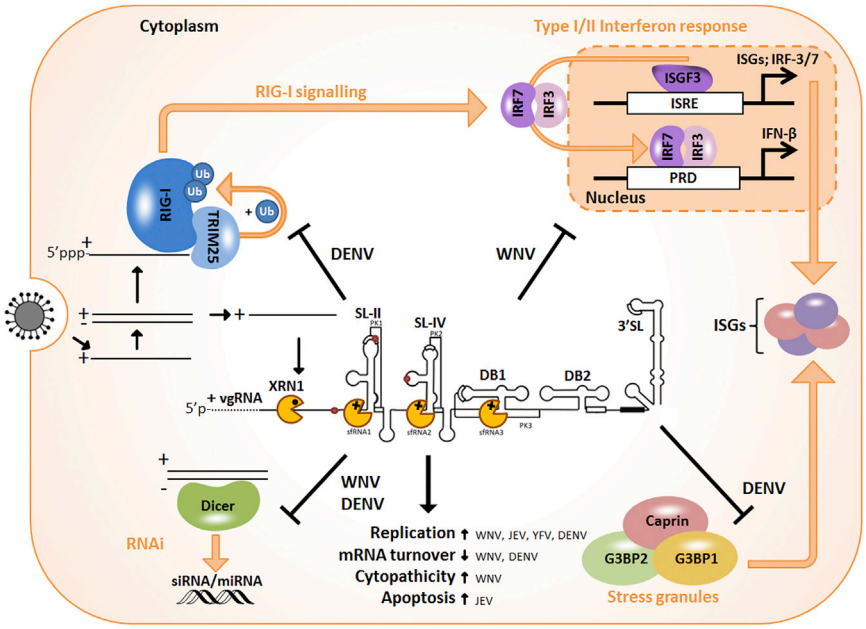

Figure 1. Inhibition of Innate Immune Pathways by Subgenomic Flavivirus RNA
Subgenomic flavivirus RNA (sfRNA) is formed after de novo (+) vgRNA synthesis by stalling of the host 5′→3′ exoribonuclease XRN1. sfRNA inhibits multiple innate immune pathways including the IFNAR-dependent IFN response, stress granule-initiated ISG expression, and RNAi pathways. Manokaran et al. (2015) now show in *Science* that sfRNA from DENV2 binds to the ubiquitin ligase TRIM25 and thereby inhibits vRNA recognition by the cytoplasmic non-self RNA sensor RIG-I. The sfRNA-TRIM25 binding is stronger for DENV2 isolates from the PR-2B clade and might explain the replacement of the endemic PR-1 clade by PR-2B.

in SL-II and perhaps decreased XRN1 resistance (Chapman et al., 2014). Indeed, in an earlier study with DENV2 strain 43, the very same 10331A/10389C mutations were shown to dramatically reduce full-length sfRNA expression with a concomitant increase in the 5′ truncated sfRNA species sfRNA2 and sfRNA3 (Liu et al., 2010). Curiously, the PR-2B strains in the present study by Manokaran et al. (2015) produce more full-length sfRNA, suggesting that XRN1 stalling is more complex than predictions from in silico RNA structure modeling would suggest. Further studies, e.g., northern blot analysis (Pijlman et al., 2008; Liu et al., 2010) or in vitro XRN1 stalling assays (Chapman et al., 2014) of the sfRNA species expressed by PR-1 and PR-2B strains, may help to clarify the molecular basis for enhanced full-length sfRNA expression by PR-2B.

Another question that arises from the discovery by Manokaran et al. (2015) is whether the sfRNA-TRIM25 interplay can fully explain the epidemiological strain replacements of DENV. Perhaps other factors in the natural transmission cycle are involved as well. SfRNA generation has been demonstrated for mosquito-borne, tick-borne, no-known-vector, and insect-specific flaviviruses (ISF). The conserved generation of sfRNA in ISF underscores the importance of sfRNA in insects. Indeed, WNV sfRNA was demonstrated to inhibit both miRNA and siRNA-mediated RNA interference, the primary invertebrate innate immune response, potentially by acting as a decoy-substrate for Dicer cleavage (Figure 1). Interestingly, insect host specialization leads to high mutation rates in the 3′ UTR of flaviviruses. For DENV2 it has been reported that the 3′ UTR undergoes extensive modification during replication in mosquito cells, especially in the SL-II/SL-IV region important for sfRNA formation (Villordo et al., 2015). It is possible that sequence differences between the 3′ UTRs of PR-1 and PR-2B viruses arose during DENV replication in mosquitoes and were maintained in the virus population due to superior innate immune suppression in the human host. Competition assays between PR-1 and PR-2B isolates in both vertebrate and mosquito models could simulate the DENV2 strain replacement that occurred between 1995 and 2007 and may provide further understanding of this phenomenon.

To conclude, the link between sfRNA and flaviviral fitness described by Manokaran et al. (2015) further emphasizes the pivotal role of non-coding sfRNA as regulator of antiviral innate immune pathways during flavivirus replication.

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