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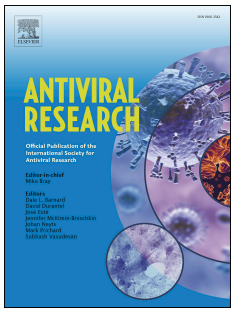

Subgenomic flaviviral RNAs: What do we know after the first decade of research

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Subgenomic flaviviral RNAs: what do we know after the first decade of research

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Abstract

The common feature of flaviviral infection is the accumulation of abundant virus-derived noncoding RNA, named flaviviral subgenomic RNA (sfRNA) in infected cells. This RNA represents a product of incomplete degradation of viral genomic RNA by the cellular 5'-3' exoribonuclease XRN1 that stalls at the conserved highly structured elements in the 3' untranslated region (UTR). This mechanism of sfRNA generation was discovered a decade ago and since then sfRNA has been a focus of intense research. The ability of flaviviruses to produce sfRNA was shown to be evolutionary conserved in all members of *Flavivirus* genus. Mutations in the 3'UTR that affect production of sfRNAs and their interactions with host factors showed that sfRNAs are responsible for viral pathogenicity, host adaptation, and emergence of new pathogenic strains. RNA structural elements required for XRN1 stalling have been elucidated and the role of sfRNAs in inhibiting host antiviral responses in arthropod and vertebrate hosts has been demonstrated. Some molecular mechanisms determining these properties of sfRNA have been recently characterized, while other aspects of sfRNA functions remain an open avenue for future research. In this review we summarize the current state of knowledge on the mechanisms of generation and functional roles of sfRNAs in the life cycle of flaviviruses and highlight the gaps in our knowledge to be addressed in the future.

1. Introduction

Flaviviruses have the unique ability to subvert host RNA degradation machinery for production of virus-derived noncoding RNA (subgenomic flaviviral RNA or sfRNA). This RNA was found to be produced by all flaviviruses tested to date (Pijlman et al., 2008; MacFadden et al., 2018). It is shown to inhibit host antiviral response and is required for viral pathogenicity (Pijlman et al., 2008; Esther Schnettler et al., 2012; Schuessler et al., 2012). In this review we summarise the available information on the structural determinants and molecular processes of sfRNA biogenesis in different ecological groups of flaviviruses, mechanisms behind the inhibitory effect of sfRNA on host antiviral response in arthropod and vertebrate hosts and discuss the role of sfRNA in evolution of flaviviruses. We also identify gaps in the current knowledge about sfRNA functions that are yet to be addressed to fully understand interactions between sfRNA, other viral processes, and host antiviral defence.

2. Diversity of genus Flavivirus

Flavivirus genus can be divided into several ecological groups: mosquito-borne flaviviruses (MBFs) that circulate between mosquito and vertebrates (avian, equine or human) hosts; tick-borne flaviviruses (TBFs) that are maintained in tick-vertebrate cycle; viruses that only infect vertebrates and are thought to be transmitted horizontally between vertebrates (no known vector flaviviruses, NKVFs), and insect-specific flaviviruses (ISFs) that infect mosquitoes and sand flies and are maintained in vertical transmission cycles (Blitvich and Firth, 2015). Arthropod-borne flaviviruses (ABFs, consisting of MBFs and TBFs) is the group of viruses that includes all human pathogens and until recently was the most studied group. However, other ecological groups of flaviviruses, and particularly ISFs, have recently attracted significant

attention due to their ability to inhibit replication of ABFs in co-infected mosquitoes and their potential use as agents of biocontrol (Bolling et al., 2012; Hall-Mendelin et al., 2016; Hobson-Peters et al., 2013). They are also considered to be a safe platform to generate recombinant vaccine candidates against pathogenic flaviviruses (Piyasena et al., 2017). Due to the medical importance of pathogenic flaviviruses, this group of viruses has been extensively studied and to date we have accumulated a wealth of knowledge on their ecology, molecular biology and processes involved in antiviral immunity and virus-host interactions.

3. Genome of flaviviruses and their 3'UTR

All flaviviruses have a relatively small genome of approximately 11kb in length, which has one large open reading frame (ORF) (Brinton, 2013). Organisation of flavivirus genome is schematically represented in Fig 1A. Genomic RNA of flaviviruses has type I cap at the 5'-end (Ray et al., 2006) and lacks ploy(A)-tail at the 3'-end (Brinton et al., 1986). ORF encodes for 3 structural (C, PrM and E) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Chambers et al., 1990). Structural proteins form viral particles, whereas non-structural proteins are involved in viral RNA replication and inhibition of host antiviral response (reviewed in (Roby et al., 2012)). Viral ORF is translated as a single polyprotein, which is cleaved into mature proteins by viral and host proteases. Cleavage at most sites occurs co-translationally except prM/E junction, which is cleaved post-translationally (Lobigs M., 1993). Viral ORF is flanked by 5' and 3'-untranslated regions (UTRs) that are required for replication of the viral genome (Khromykh et al., 2001; Ng et al., 2017) and translation of viral polyprotein (Holden & Harris, 2004; Chiu et al., 2005). 3'UTRs of all MBFs have conserved secondary structure (Fig 1B) and contain duplicated stem loop

elements (SLs) followed by one or two dumbbell structures (DBs) and terminal 3'-stem loop (3'SL) preceded by a short hairpin (sHP) (Clarke et al., 2015). Due to the complex secondary and tertiary structure, 3'UTRs of flaviviruses are resistant to digestion by the host 5'-3' exoribonuclease XRN-1 - the enzyme responsible for degradation of uncapped host and viral RNAs in the cytoplasm (Funk et al., 2010; Pijlman et al., 2008). This resistance prevents complete degradation of flaviviral genomes and results in accumulation of the abundant RNA fragments derived from the 3'UTR in infected cells (Fig. 1B,C) (Pijlman et al., 2008). These viral RNA species, referred as sfRNAs, were shown to be produced in arthropod and vertebrate hosts by all flaviviruses tested to date (Chapman et al., 2014; MacFadden et al., 2018; Pijlman et al., 2008) and to be required for viral pathogenesis and evasion of host antiviral response (reviewed in (Clarke et al., 2015; Roby et al., 2014)). The unique ability of flaviviruses to utilize host RNA degradation pathway for production of viral pathogenicity factor and the important functions of sfRNA in flavivirus life cycle attracted significant interest in recent years. This has led to rapid advance in our understanding of molecular mechanisms of sfRNA biogenesis and different functions of sfRNA as well as provided new insights into potential roles of sfRNA in flavivirus evolution and host adaptation.

4. Mechanism of sfRNA biogenesis.

4.1. sfRNA is produced as a product of incomplete degradation of viral genomic RNA by the host exoribonuclease XRN-1.

Similar to cellular RNAs, genomic RNA of flaviviruses can become a subject to degradation by the host mRNA decay machinery. Eukaryotic RNA degradation machinery consists of coordinated endo- and exoribonucleases and multiple auxiliary

117 factors (reviewed in (Garneau et al., 2007; Houseley and Tollervey, 2009)). It acts to
118 maintain the balance of cellular mRNAs, prevents translation of aberrant transcripts
119 and protects cells from exogenous infectious RNAs. As most cellular mRNA are
120 capped and polyadenylated, these RNA modifications serve as primary markers for
121 RNA quality surveillance and determine the fate of cellular transcripts (Bernstein et al.,
122 1989; Gao et al., 2000). Removal of either cap or poly(A)-tail is required to trigger
123 exonucleolytic mRNA degradation pathways. Deadenylation, which results from either
124 the enzyme activity of deadenylase or the edonucleolytic cleavage by nucleases such
125 as RNaseL and Ago, is usually the first step in eukaryotic mRNA decay (Schoenberg
126 and Maquat, 2012). RNAs lacking poly(A)-tail can be subjected to degradation by 3' ->
127 5' exoribonucleases in the multi-subunit RNA degradation complexes called exosomes
128 (Decker and Parker, 1993). In addition, deadenylation triggers decapping of mRNAs
129 and their degradation in 5' -> 3' direction by exoribonuclease 1 (XRN-1) (Tomecki and
130 Dziembowski, 2010), which is believed to be the major mRNA decay pathway in
131 eukaryotes (Garneau et al., 2007). Removal of the cap structure from cytosolic RNAs
132 is catalysed by decapping enzymes DCP1/DCP2 and involves a number of other
133 cofactors (Liu et al., 2002). XRN-1 recognises decapped RNA as they possess 5'-
134 monophosphate, which interacts with a positively charged pocket in XRNA-1 molecule.
135 XRN-1 then unwinds target RNA due to its ATP-dependent RNA-helicase activity and
136 rapidly digests bound RNAs by removing nucleotides from the 5'-end one by one
137 generating no intermediate products (Jinek et al., 2011). Enzymes required for 5' -> 3'
138 RNA decay such as XRN-1 and decapping proteins are localized in the cytoplasm and
139 can assemble into multiprotein granular formation called P-bodies or stress granules.
140 Assembly of P-bodies is often triggered by stress conditions associated with
141 accumulation of large amounts of RNA subjected to degradation, including RNA virus
142 infection (Lloyd, 2013).

At the 5'-end genomic RNA of flaviviruses contains a cap structure with a methyl groups in position N7 and at the 2'OH position of ribose of the first nucleotide (type I or m(7)GpppAmN cap) (Ray et al., 2006). The 3'-end of the viral genome does not have poly(A)-tail and terminates with a stem loop structure (3'SL), which is very conserved among all flaviviruses and has high thermodynamic stability (Dong et al., 2008). Interestingly, cellular mRNAs encoding for histones are also lacking poly(A) and contain 3' terminal stem loop (Zanier et al., 2002). This stem loop protects histone mRNAs from 3' -> 5' digestion via interaction with stem loop binding protein (SLBP) (Williams and Marzluff, 1995) and degradation of these mRNAs primarily occurs via 5' -> 3' mechanism dependent on 3'-oligouridylation. 3'SL of flaviviruses is also believed to protect viral RNA from 3' -> 5' exoribonucleases and determines RNA stability despite the lack of poly(A)-tail (Ford and Wilusz, 1999). However, it is currently unknown if 3'SL of flaviviruses has the same role as 3'SL of histone mRNA. Protection of 3'-end from 3' -> 5' degradations by 3'SL implies that endonucleolytic cleavage by RNase L (Samuel et al., 2006; Scherbik et al., 2006) and 5' -> 3' degradation by XRN-1 are likely to be the predominant pathways for flaviviral genomic RNA decay (Narayanan and Makino, 2013). However, flaviviruses evolved to block complete degradation of viral genomic RNA by XRN-1 at the beginning of 3'UTR in order to produce functional noncoding RNA.

Production of sfRNA by flaviviruses was first reported for MVEV (Urosevic et al., 1997) and later shown to be a common characteristics for flaviviruses in general. All ABFVs (Akiyama et al., 2016; Lin et al., 2004; Pijlman et al., 2008) and also recently ISFs and NKVFVs (MacFadden et al., 2018) have been shown to generate sfRNA. Biogenesis of sfRNA as XRN-1 dependent process (Fig 1B) was first described by our group for WNV in 2008 (Pijlman et al., 2008). Using recombinant constructs in which

genomic RNA of WNV with various deletions was transcribed from CMV promoter, we demonstrated that sfRNA is produced independently of virus replication and of viral proteins. We hypothesised that sfRNA is generated as the result of incomplete digestion of viral genomic RNA by XRN-1 and confirmed this hypothesis by demonstrating decreased production of WNV sfRNA in XRN-1-depleted cells, co-localization of XRN-1 with WNV sfRNA in infected cells, and the ability of XRN-1 to convert viral genomic RNA into sfRNA *in vitro* (Pijlman et al., 2008). Later the role of XRN-1 in generation of sfRNA was confirmed for other ABFs such as YFV (Silva et al., 2010), DENV (Chapman et al., 2014a) and ZIKV (Akiyama et al., 2016), as well as for ISFV Cell Fusion Agent Virus (CFAV) (MacFadden et al., 2018) and several NKVFs (MacFadden et al., 2018).

We determined that the 5'-end of WNV sfRNA aligns with the SL-II structure within WNV 3'UTR (Pijlman et al., 2008). Deletion or disruption of this structure by mutagenesis abolished generation of full-length sfRNA, indicating that SL-II was required for stalling XRN-1 and producing sfRNA. In addition, three smaller sfRNA species were detected in cells infected with WNV showing that downstream SL-IV and possibly dumb bell structures could also have the ability to stall XRN-1 (Funk et al., 2010; Pijlman et al., 2008). Later, using higher resolution gels, these smaller sfRNAs (named sfRNA-2, sfRNA-3, and in some cases sfRNA4) were shown to be also produced in cells infected with different flaviviruses (Fig 1C for DENV2) (Akiyama et al., 2016; Chapman et al., 2014a; Filomatori et al., 2017), indicating that in some instances XRN-1 can "slip" through the first resistant structure and stall at the downstream structural elements. Two sfRNA species has been also detected in YFV-infected mammalian cells, however sfRNA-2 of YFV had the same 5'end as sfRNA-1 and was truncated by ~100nts at the 3'end (Silva et al., 2010). The mechanism that

193 determines generation of sfRNA-2 by YFVs has not yet been characterized and
194 production of 3'-truncated sfRNA has not been reported for other flaviviruses.

195 **4.2. Structural determinants of sfRNA biogenesis are unique tertiary RNA**
196 **structures that differ between phylogenetic groups of flaviviruses.**

197 **4.2.1. Secondary and tertiary structures of XRN-1 resistant elements in**
198 **MBFVs**

199 Currently two classes of XRN-1 resistant elements (xrRNAs) have been
200 identified in 3'UTRs of MBFVs – xrRNAs formed by stem loops (SL) and those that
201 involve dumb bell (DB) structures (Fig. 2A) (Funk et al., 2010). All mosquito-borne
202 flaviviruses tested to date have been shown to contain at least one xrRNA (Clarke et
203 al., 2015). YFV seem to have the simplest set of xrRNAs with only one SL (SLA) and
204 one DB (SLB) (Silva et al., 2010), but majority of MBFs have duplicated XRN-1-
205 resistant elements (Villordo et al., 2016). ZIKV, for instance, has two SLs (SL1, SL2),
206 and one DB (Akiyama et al., 2016), Rocio virus is predicted to have one SL (SL1) and
207 two DB (DB1, DB2) structures (Setoh et al., 2018) and WNV and DENV have
208 duplication of both – SLs (SLII and SLIV for WNV, SLI and SLII for DENV) and DBs
209 (DBI, DBII) (Filomatori et al., 2017; Funk et al., 2010). These elements are usually
210 designated as xrRNAs 1 to 4 (in 5' to 3' direction) and are required for production of
211 sfRNAs 1 to 4, respectively, with sfRNA-1 being the longest (Fig 1B,C).

212 XRN-1 is a highly processive enzyme that has an RNA-helicase activity and is
213 generally capable of digesting structured RNAs. When the ability of SLII, SLIV and DBI
214 of WNV 3'UTR to stall XRN-1 was discovered, only the homopolymer G stretches of
215 9+ nucleotides were known to efficiently halt RNA digestion by XRN-1, whereas stem
216 loops were thought to have very little effect on processivity of 5' -> 3' exoribonucleases
217 (Poole and Stevens, 1997). It was therefore puzzling why stem loops within the

faviviral genomes have such a dramatic effect on XRN-1 processing and at the same time do not interfere with viral polymerase moving in 3' to 5' direction on the viral template (+) RNA strand while synthesising (-) RNA strand. It was hypothesised that stem loops of flaviviruses must be involved in formation of higher order tertiary structures to confer XRN-1 resistance (Funk et al., 2010). To address this matter, secondary and tertiary structures of MBFV 3'UTRs were assessed using computational folding prediction, enzymatic/chemical RNA structure probing and mutational analysis (Alvarez et al., 2005; Chapman et al., 2014a; Funk et al., 2010; Kieft et al., 2015a; Silva et al., 2010). In addition, crystal structures of MVEV and ZIKV xrRNAs have now been solved (Akiyama et al., 2016; Chapman et al., 2014).

First insights into the structural basis of XRN-1 resistance were provided by our study (Funk et al., 2010) and the study by Silva et al (Silva et al., 2010). These studies showed that XRN-1 resistant stem loops of WNV and YFV were involved in pseudoknot (PK) interactions (Figs 1B and 2A). PKs are RNA structures that contain two helical segments connected by single-stranded segments or loops. Most common type of PK is the H-type fold, in which nucleotides in the loop of a hairpin form intramolecular pairs with nucleotides outside of the stem, which leads to formation of a second stem and loop and results in a PK with two stems and two loops (Staple and Butcher, 2005). These type of PKs were shown to be formed between the loop regions of SLs/DBs of WNV and YFV and the downstream single stranded regions (Fig 2A) (Chapman et al., 2014a; Funk et al., 2010; Silva et al., 2010). Mutations in either loops of SLs or the interacting regions outside the SLs that prevented PK formation were shown to abolish production of corresponding sfRNA molecules, whereas mutations that didn't influence base pairing had no effect on the ability of the viruses to produce sfRNAs (Funk et al., 2010; Silva et al., 2010). Importantly, compensatory mutations

restoring PK interactions also restored XRN-1 resistance and generation of sfRNAs (Funk et al., 2010; Silva et al., 2010). Recently, similar PK-interactions were also demonstrated to confer XRN-1 resistance of xrRNAs in ZIKV(Akiyama et al., 2016).

However, somewhat different results were obtained when the ability of DENV SLs to form pseudoknots was tested by chemical probing (Chapman et al., 2014). Despite SLI of DENV (equivalent of WNV SLII) was predicted to be involved in PK-formation by computational analysis, chemical structure probing revealed that both RNA segments expected to interact were most likely unpaired (Chapman et al., 2014a). In addition, mutations that were predicted to prevent formation of PK were shown to reduce, but not completely abolish production of DENV sfRNA-1 (Filomatori et al., 2017). For DENV SLII (equivalent of WNV SLIV), PK-interactions were confirmed, however elimination of this interactions by mutations only reduced production of sfRNA-2 by less than 50% (Chapman et al., 2014a). These observations led to the conclusion that PK interactions could be rather transient and not always crucial for stalling XRN-1 (Chapman et al., 2014a; Kieft et al., 2015a).

Recently crystal structures of xrRNA-2 of MVEV (Chapman et al., 2014) and of xrRNA-1 of ZIKV (Akiyama et al., 2016) have been solved (Fig 2B), which has become a turning point in our understanding of the structural basis of XRN-1 resistance and sfRNA biogenesis. The structural analysis revealed that both xrRNA1 and xRNA2 form three-way junctions with coaxial stacking of helices P1 and P2, while helix P3 positioned at the acute angel to P1 (Fig 2B). Three-way junctions are structural elements of “branched” nucleic acids in which three double helical arms are connected at the junction point, with or without several unpaired bases in one or more of the three different strands (Lilley, 1998). Three-way junctions are common in highly structured nucleic acids such as rRNA and hammerhead ribozymes (Lilley, 1998). They can

acquire three major types of topology and usually involve formation of non-canonical base pairs such as base triplices and Hoogsteen hydrogen bonds (Lescoute, 2006). Surprisingly, three-way junctions that are formed in xrRNAs of MVEV and ZIKV have the unique topology that have not been previously observed in any other RNAs and cannot be classified into any of 3 known types of three-way junctions. They acquire the conformation in which 5'-end of the RNA passes through a ring-like structure (Fig 2B), which is somewhat similar to a knot (Akiyama et al., 2016; Chapman et al., 2014). The most likely model that explains XRN-1 resistance for this unusual fold suggests that the ring-like structure creates a mechanical block for XRN-1 (MacFadden et al., 2018). This model is based on the crystal structure of xrRNAs (Akiyama et al., 2016; Chapman et al., 2014) and the experiments in which the resistance of xrRNAs to a range of exonucleases unrelated to XRN-1 was tested (MacFadden et al., 2018). It assumes that the ring surrounding 5' end of RNA braces against the surface of the enzyme around the active site (Fig 2C) and prevents XRN-1 from assessing the next nucleotide, which blocks progression of XRN-1 in 5' to 3' direction (Fig 2C). The helicase activity of XRN-1 would not help to overcome this obstruction as the enzyme would need to pull 5'-end through the structure rather than simply unwind the helix (Chapman et al., 2014; Kieft et al., 2015a). The enzymes acting in 3' to 5' direction do not encounter this obstacle as they enter the structure from the outside, which explains why this structure for example does not halt viral RdRP. Formation of the ring-like topology was shown to require base triples and base pairs between the 5'-end of xrRNAs and three-way junction, and to be stabilised by a small PK and base pairing (Watson-Crick and non-canonical) within the junction (Fig 2B). All nucleotides required for the folding of ring-like structure were shown to be highly conserved amongst MBFVs (Akiyama et al., 2016).

PK between the apical loop of xrRNA-forming SL and the downstream complementary region of UTR was not evident in the crystal structure of MVEV xrRNA-2 although the RNA regions predicted to form PK were located in close proximity and ready to pair (Chapman et al., 2014). However, in more recent crystal structure of ZIKV xrRNA-1, PK was clearly defined (Akiyama et al., 2016). These results suggest that PKs can be transient and used to stabilise XRN-1 resistant fold rather than being XRN-1 resistant themselves (Akiyama et al., 2016; Kieft et al., 2015). Based on the cumulative data from structural and functional studies a model was proposed that suggests stabilising role of PKs and explains how a complex tertiary structure with RNA strand threading through the centre of the ring can be formed in the context of full-length genomic RNA. According to this model, the entire xrRNA structure is unfolded and ring is open until the 5'-terminal nucleotides of xrRNA pair with those in three-way junction. Once the pairing occurs, the junction forms around the 5'-end of xrRNA, causing the single stranded RNA segments of the loop and downstream region of 3'UTR to move into position where they can interact. As soon as they appear in the position that allows pairing, the PK is formed and the ring is "latched" in the stable XRN-1 resistant conformation (Kieft et al., 2015).

Acquisition of high-resolution crystal structures of SL-based MVEV and ZIKV xrRNAs significantly advanced our understanding of how flaviviruses achieve unique resistance to 5'->3' exonucleolytic digestion. However, the structural basis of XRN-1 resistance in DB structures, predicted to be responsible for generation of shorter sfRNA species, remains to be determined.

4.2.3. XRN-1 resistant elements in insect-specific flaviviruses (ISFs)

ISFs are phylogenetically heterogeneous group of flaviviruses that can only replicate in mosquitoes and are maintained via vertical transmission. Their ability to

318 produce sfRNA has not been tested until recently (MacFadden et al., 2018) and the
319 structural determinants of XRN-1 resistance have not been well characterized. Current
320 knowledge about sfRNA biogenesis in ISFs is based on the chemical probing of the
321 secondary structure in a single flavivirus Cell Fusion Agent Virus (CFAV) (MacFadden
322 et al., 2018), and predominantly using *in silico* analyses (MacFadden et al., 2018;
323 Roby et al., 2014; Villordo et al., 2016).

324 ISFs can be divided into two phylogeneticaly distinct groups (Fig 2A):
325 clade/lineage I or classic ISFs and clade/lineage II or dual-host associated ISFs (Hall
326 et al., 2016). Clade I ISFs are most phylogenetically distinct from ABFVs, are thought
327 to have evolved to replicate solely in insects, and are likely to represent the ancestors
328 of all flaviviruses (Hall et al., 2016). In contrast, dual-host associated ISFs display high
329 degree of sequence similarity to MBFVs and are thought to have diverged from
330 MBFVs by losing their ability to propagate in vertebrates and adapting to vertical
331 transmission in mosquitos (Hall et al., 2016).

332 As Clade II ISFs are very similar to MBFVs with a high degree of homology in
333 3'UTR, it is expected that they employ mechanisms of sfRNA biogenesis that
334 resemble those of MBFVs (Villordo et al., 2016). Computational sequence alignment
335 and secondary structure prediction for Clade II flavivirus Chaoyang Virus (CHAOV)
336 revealed that 3'UTR of this virus has high structural homology to the 3'UTR of MBFVs
337 and contain SL and DB structures capable of forming PKs. The SL element of CHAOV
338 3'UTR also shares with MBFVs the conserved nucleotides in the positions critical for
339 the formation of the ring-like three-dimensional xrRNA fold (Villordo et al., 2016). It is
340 therefore believed that Clade II ISFs would produce sfRNA similar to MBFVs utilising
341 this element as the structural determinant of XRN-1 resistance (Fig 2A) (MacFadden
342 et al., 2018; Villordo et al., 2016). However, production of sfRNA by Clade II

flaviviruses has not been experimentally demonstrated and no data exists to support the XRN1-resistant structures predicted by computer modelling (Villordo et al., 2016).

In contrast to Clade II ISFs, 3'UTRs of viruses from Clade I have very little sequence and structural similarity to those of MBFVs (Gritsun et al., 2014; Villordo et al., 2016). Computational prediction revealed that the only structural element shared between these two groups is the 3'-terminal stem loop (3'SL) (Gritsun et al., 2014). 3'UTRs of Clade I ISFs were also shown to be lacking sequences, that are strictly conserved in MBFVs xrRNAs. Moreover, Clade I ISFs is a very heterogeneous group that include three very distinct subgroups: viruses that infect *Aedes* mosquitos (AeISFs), *Culex*-associated viruses (CxISFs), and recently discovered viruses infecting only *Anopheles* mosquitoes (AnISFs) (Hall et al., 2016; Insect-specific, 2017). The first attempt to predict secondary structures within the 3'UTR of Clade I ISFs did not reveal elements capable of forming high-order structures, but instead demonstrated the presence of abundant short direct repeats and short hairpins (Gritsun et al., 2014). Similar results were also obtained in the later finding that involved secondary structure prediction and estimation of structural similarities based on tree alignment model (Villordo et al., 2016). This study, however, identified a putative duplicated structure with a potential to form PK interaction in *Aedes*-assosiated ISFs CFAV and AEFV (*Aedes* flavivirus), which could represent a potential xrRNA structure (Villordo et al., 2016).

Recently, experimental evidence for sfRNA production by CFAV has been obtained and the secondary structure of the 3'UTR element responsible for XRN-1 stalling has been resolved by chemical probing (MacFadden et al., 2018). Production of sfRNA in CFAV-infected C6/36 cells was detected by Northern blot. The 5'-end of CFAV sfRNA was determined by primer extension and found to align with an SL

structure (Fig 2A). The ability of this structure to resist XRN-1 digestion was demonstrated in *in vitro* assay thus confirming that this element is *bona fide* xrRNA (MacFadden et al., 2018). Secondary structure of CFAV sfRNA was then determined by SHAPE analysis and computational folding. It was shown to form three-way junction and PKs similar to those in MBFV xrRNAs (Fig 2A). The nucleotides at the 5'-end of CFAV xrRNA and in the critical positions of three-way junctions were different to those found in MBFVs xrRNAs (Fig 2A), but able to form similar base triples and base pairs. Thus, xrRNA of CFAV was suggested to also fold into a similar ring-like tertiary structure as xrRNAs of MBFVs despite lacking sequence similarity. In addition, sequence alignment identified second homologous structure in the CFAV 3'UTR, suggesting presence of a putative xrRNA-2. MacFadden and co-authors also demonstrated that nucleotides critical for formation of three-way junction, PK and ring-like structure identified in CFAV xrRNA were conserved between CFAV, *Aedes* flavivirus (AeFV) and Kamiti river virus (KRV) and suggested that all Clade I ISFs may employ similar to CFAV mechanism for sfRNA production (MacFadden et al., 2018). However, the sequence alignment of MacFadden et al. only included Aedes-associated ISFs - the only Clade I ISFs previously predicted to contain high-order structures in 3'UTRs (Villordo et al., 2016). The ability of Culex and Anopheles associated ISFs to produce sfRNA has not yet been tested and the secondary structures of their 3'UTRs remain either only predicted (CxISFs) or not assessed at all (AnISFs).

4.2.3. Structural determinants of sfRNA biogenesis in tick-borne and no known vector flaviviruses.

Tick-born flaviviruses (TBFVs) are another group of dual-host flaviviruses. They circulate between ticks and vertebrates and include several human pathogens

(reviewed in (LaSala and Holbrook, 2010)). Together with MBFVs they were among other flaviviruses demonstrated to produce sfRNA (Pijlman et al., 2008; Schnettler et al., 2014). TBFVs, however, have 3'UTRs that are rather dissimilar to 3'UTRs of MBFVs (MacFadden et al., 2018). XRN1-resistant structures in TBFVs were determined and analysed by chemical probing (Fig 2A) (MacFadden et al., 2018). They were shown to also contain three-way-junction and PK between apical loop and downstream 3'UTR sequence (MacFadden et al., 2018; Schnettler et al., 2014). However, the three-way junction was positioned on a longer stem compared to MBFVs and sfRNA start site was located in a bulging region within the stem, whereas in MBFVs it is preceding the stem region. The three-way-junctions in TBFVs could not be assigned to any known classes and thus their tertiary fold was impossible to predict without direct structural data (e.g. X-ray crystallography). Interestingly, the PK in TBFVs xrRNAs was shown to be critically important for XRN-1 resistance and sfRNA generation by mutational analysis (MacFadden et al., 2018).

No known vector flaviviruses (NKVFVs) are members of *Flavivirus* genus that exhibit restriction of replication to vertebrate (rodent or bat) host only (reviewed in (Blitvich and Firth, 2017)). This is a non-taxonomic group, which includes at least two phylogenetic subgroups of viruses – one related to MBFVs and one related to TBFVs (Blitvich and Firth, 2017). The ability of these viruses to produce sfRNA was tested only recently (Kieft et al., 2015; MacFadden et al., 2018). MBFV-like virus Yokose virus (YOKV) was found to contain conserved sequences responsible for xrRNA folding in MBFVs and predicted to have similar to MBFVs structure of 3'UTR (Fig 2A). It was suggested to be capable for sfRNA production via the same mechanism employed by MBFVs, but it was not experimentally tested (MacFadden et al., 2018).

NKVFVs that are similar to TBFVs were recently shown to produce sfRNA in infected cells (MacFadden et al., 2018). Montana myotis leukoencephalitis virus (MMLV), Apoi virus (APOIV), Modoc virus (MODV) and Rio Bravo virus (RIBV) were tested in this study. The 5'-ends of sfRNAs produced by these viruses were determined and shown to align with the structural elements similar to xrRNAs of TBFVs (Fig 2A). By the combination of *in vitro* XRN-1 resistance assay, SHAPE analysis and mutational study these NKVFVs were shown to contain the same structural determinants of XRN-1 resistance and sfRNA biogenesis as TBFVs (MacFadden et al., 2018).

The fact that TBFVs and related NKVFVs produce sfRNA but seem to have XRN-1 resistant elements different from those of MBFVs emphasizes the importance of sfRNA in the life cycle of flaviviruses replicating in variety of hosts and indicates that different groups of flaviviruses may have developed different ways to stall XRN-1. Crystallisation of xrRNAs for these viruses and generation of structural data is required to obtain more complete understanding on how they interact with and stall XRN-1 to generate sfRNAs.

4.3. Alterations between sfRNA isoforms and host adaptation

The majority of flaviviruses contain duplications of structural elements in the 3'UTR able to resist XRN-1 degradation. These can be SLs, DBs, or both. It has been known for a while that presence of these duplicated elements results in production of several sfRNA isoforms with different 5'-ends (sfRNA-1, sfRNA-2, sfRNA-3, etc), however the functional implications of producing different sfRNA species remained unclear (Villordo et al., 2016). However, recent studies provided the evidence that different sfRNA species can be beneficial for flavivirus replication in different hosts and

that DENV life cycle involves genetic alterations that switch between production of sfRNA-1 and sfRNA-2 depending on the host virus replicates in (Filomatori et al., 2017; Villordo et al., 2015). DENV contains four putative xrRNA resistant structures represented by two SLs (SLI and SLII) and two dumb bells (DBI and DBII) and produces three or four sfRNA species (Kieft et al., 2015). Villordo et al and Filomatori et al demonstrated that natural populations of DENV include viruses with mutations within SL-structures of the 3'UTR. Upon infection of mosquitos or passaging in mosquito cell line, the selective pressure favoured replication of viruses with mutations in SLII that disrupt xrRNA2. These viruses quickly overpopulated other genotypes and represented the vast majority of DENV population in mosquitos. In mammalian cells, however, the opposite effect was observed: viruses with mutations in xrRNA2 induced stronger type I IFN response and the selective pressure acted against the viruses with impaired xrRNA2 formation. As the result, viruses with intact SLII quickly became the majority of DENV population in mammalian host. In addition, they accumulated mutations that stabilize PK within xrRNA1. Disruption of xrRNA2 was shown to benefit DENV replication in mosquito cells, whereas presence of both intact xrRNAs was found to slightly improve viral fitness in mammalian cells (Villordo et al., 2015). These mutations were shown to alter the patterns of sfRNAs produced by DENV. Viruses with intact xrRNAs, adapted to mammalian host, were demonstrated to produce predominantly sfRNA-1, and, to some extent, sfRNA-3. However, mosquito-adapted viruses with mutations in xrRNA2, had reduced production of longer sfRNA-1 and sfRNA-2 and generated the abundance of shorter sfRNA-3 and sfRNA-4. Why mutations in xrRNA2 and not in xrRNA1 result in such profound changes in production of sfRNA-1 remains unclear. Allegedly, this involves not yet characterized interactions between SLI and SLII in which SLII stabilizes xrRNA1. Similar sfRNA patterns were also observed in DENV-infected mosquitos. Based on these observations and

correlation between sfRNA-1 accumulation and viral fitness in mammalian cells, the hypothesis was proposed that DENV replication in different hosts requires different sets of sfRNAs and that SLII/xrRNA2 structure acts as a genetic switch between their production during alternation between the hosts (Filomatori et al., 2017).

Therefore, it was suggested that duplication of structural elements in 3'UTRs of flaviviruses occurred in conjunction with transition to the dual-host life cycle to enable adaptation to switching between the hosts (Kieft et al., 2015; Villordo et al., 2016). However, further studies are required to validate this assumption as so far accumulation of adaptive mutations in xrRNA2 leading to switching between different sets of sfRNA species has only been demonstrated for DENV (Filomatori et al., 2017; Villordo et al., 2015). ZIKV, on the other hand, was shown to produce the same patterns of sfRNAs in both, mosquito and mammalian, cells (Filomatori et al., 2017). In addition, lack of sfRNA-1 was shown not to significantly affect replication of WNV in mosquitoes and mosquito cell lines, whereas replication of the mutant virus deficient in both, sfRNA-1 and sfRNA-2, was significantly reduced in mosquito but not in mammalian cells (Funk et al, 2010; Goertz, 2016). At the same time, production of sfRNA-1 was shown to be required for WNV pathogenicity in mice (Pijlman 2008, Funk 2010) and for WNV penetration of mosquito gut barrier and virus dissemination into salivary glands (Göertz et al., 2016). These observations emphasize the importance of different sfRNA species for replication of ABFVs in different hosts while also highlighting further need to investigate their role in determining tissue/host-specific replication, transmission and pathogenesis for each individual virus.

Duplicated SLs capable of folding into xrRNAs have recently been identified in clade I ISFs CFAV, KRV and AeFV by computational prediction with reference to a sequence that has experimentally validated structure (MacFadden et al., 2018; Villordo

et al., 2016). Generation of long sfRNA (sfRNA1) was also demonstrated for ISF CFAV (MacFadden et al., 2018). It is unclear in the context of the hypothesis suggesting structure duplications as a mechanism allowing adaptation to different hosts why viruses that don't alternate between different hosts also have duplicated 3'UTR structures and predominantly generate sfRNA-1. More detailed and extensive investigation of the role for different sfRNAs in the context of flavivirus evolution and virus-host interactions therefore represents important future research direction in this area.

Another important implication from the studies with host-adapted DENV variants is a possible role of xrRNA2 in biogenesis of sfRNA-1 (Filomatori et al., 2017). Previously sfRNA-1 was thought to be produced due to XRN-1 stalling at xrRNA-1 and this structure was believed to be self-sufficient barrier for XRN-1. However, DENV mutants with disrupted xrRNA-2 were shown to have impaired production of both sfRNA species – sfRNA-2 and sfRNA-1. Similar phenomenon was also observed previously when xrRNA-2 of WNV was mutated (Funk et al., 2010). This opens another avenue for future studies of the interplay between xrRNAs in sfRNA biogenesis.

5. Functions of sfRNA

Together with the discovery of mechanism for sfRNA generation, we reported the requirement of sfRNA for viral pathogenicity (Pijlman et al., 2008). WNV mutants lacking production of sfRNA1, or sfRNA1 and sfRNA2 were shown to exhibit reduced pathogenicity in mice (Pijlman et al., 2008). Later studies demonstrated the pivotal role of sfRNA in replication, dissemination and transmission of a wide range of flaviviruses (Chang et al., 2013; Donald et al., 2016; Filomatori et al., 2017; Junglen et al., 2017). In addition, host pathways targeted by sfRNA to facilitate virus replication,

dissemination and transmission were identified. The evidence were obtained that sfRNA impairs host mRNA turnover (Moon et al., 2012), inhibits RNAi and miRNA pathways (Moon et al., 2015b; Esther Schnettler et al., 2012; Schnettler et al., 2014), supresses type I IFN response in vertebrates (Chang et al., 2013; Schuessler et al., 2012) and Toll pathway in mosquitos (Pompon et al., 2017). In addition, sfRNA was shown to promote apoptosis of infected cells and virus-induced cytopathic effect (Liu et al., 2014; Pijlman et al., 2008). These known functions of sfRNA in flavivirus-host interactions are summarized in Fig 3. In this section we will analyse the current knowledge on the functional implications of sfRNA in the flavivirus life cycle and molecular targets of sfRNA.

5.1. sfRNA inhibits host exoribonuclease XRN-1 and dysregulates host mRNA turnover.

Considering the ability of the secondary structures in the 3'UTR of flaviviruses to stall XRN-1 (Funk et al., 2010), the effect of sfRNA on XRN-1 activity was among the first putative functions of sfRNA to be assessed. The competition experiments in which degradation of labelled reporter RNA by yeast, mammalian and mosquito XRN1 was assessed in the presence of sfRNA or unrelated competitor RNA, demonstrated that DENV and WNV sfRNA strongly inhibit activity of XRN-1 of any origin. XRN1 suppressor activity of sfRNA was shown to require monophospate at 5'-end and xrRNA secondary structures (Moon et al., 2012). Taking into account the important role of XRN1 in maintaining the balance of host RNA transcripts (Nagarajan, 2013), the effect of sfRNA on mammalian transcriptome was assessed. It revealed accumulation of uncapped mRNAs and increased stability of hundreds of host transcripts in cells infected with sfRNA-producing WNV in comparison to sfRNA-deficient mutant, suggesting that sfRNA strongly impairs mRNA turnover in flavivirus-

infected cells (Moon et al., 2012). It was speculated that inhibition of mRNA decay can misbalance production of antiviral proteins and pro-inflammatory cytokines that are predominantly encoded by short-lived mRNAs and thus prevent development of the functional innate immune response to the virus. Alternatively, excess mRNAs, including uncapped RNAs was suggested to potentially contribute to the cytopathic effect associated with sfRNA (Moon et al., 2012). Both these hypotheses can explain why flaviviruses evolved to inhibit host 5'->3' RNA decay but require further experimental validation. In addition, the effect of sfRNA on stability of viral genomic RNA can also be a potential target for future studies as inhibition of XRN1 by sfRNA may be required to maintain the balance between genomic RNA and sfRNA in infected cells.

5.2. sfRNA interferes with generation of siRNAs and miRNAs

Innate immune response to RNA virus infection in invertebrates, including mosquitoes, relies primarily on RNA interference (RNAi) pathway (Olson and Blair, 2015; Wu et al., 2010). RNAi response involves recognition of double stranded viral RNA by RNase III-like enzyme Dicer, which cleaves it into 21-nt double-stranded fragments (Bernstein et al., 2001; Fire et al., 1998). Another important class of Dicer-produced small RNAs is microRNAs (miRNAs) that are 18-24nt in length and, unlike siRNAs, are encoded by host genes and target endogenous mRNAs, establishing regulation of gene expression at posttranscriptional level (Lee, 1993). Both small RNA pathways have been extensively studied in the last two decades and several comprehensive reviews are available on this subject e.g. (Daugaard and Hansen, 2017; Ha and Kim, 2014; Wilson and Doudna, 2013).

Being generally similar, siRNAs and miRNAs are produced by different subtypes of Dicer (Lee et al., 2004) and act via different Ago proteins (Schott et al.,

2012). In invertebrates and plants Dicer 1 is responsible for the processing of miRNA precursors and Dicer 2 is required for siRNA generation (Lee et al., 2004). Vertebrates are believed to be lacking Dicer 2 and therefore incapable in processing viral RNA genomes into siRNAs (Cullen, 2014). Thus, siRNA pathway is limited to plants and invertebrates, whereas miRNAs are produced in all multicellular organisms (Chen and Rajewsky, 2007). Although RNA viruses have evolved to avoid direct miRNA targeting of their genomes (Cullen, 2013), flavivirus infection alters expression of numerous miRNAs in mosquito and vertebrate hosts that have a profound antiviral effect, acting indirectly via regulation of antiviral genes and host factors required for virus replication (Ashraf et al., 2016; Chen et al., 2014; Hussain et al., 2011; Kumari et al., 2016; Ouyang et al., 2016; Slonchak et al., 2015, 2014, Smith et al., 2017, 2012; Thounaojam et al., 2014; Zhou et al., 2014; Zhu et al., 2015).

Given the important role of siRNAs in antiviral defence in invertebrates, RNA viruses that infect insects have developed mechanisms to evade or inhibit RNAi response that rely on viral RNA silencing suppressor (RSS) proteins (Cirimotich et al., 2009; Lu et al., 2005; Nayak et al., 2010). The evidences for RSS activity of flavivirus proteins are currently conflicting as studies conducted with different flaviviruses assigned RSS to different viral factors. For instance, the RSS activity was demonstrated for NS4B and NS3 in DENV2 (Kakumani et al., 2013), whereas capsid protein was identified as RNAi inhibitor in YFV (Samuel et al., 2016). RSS activity was also shown for WNV, as cells carrying WNV replicon had reduced ability to develop shRNA-mediated gene silencing (Schnettler et al., 2012). In addition, decreased processing of pre-miRNAs into mature miRNAs was demonstrated in WNV-infected human cells using deep sequencing of small RNAs (Slonchak et al., 2015). However, RNA-binding activity required for RSS was not detected for any WNV non-structural

proteins or capsid protein while WNV sfRNA was shown to possess RSS activity (Schnettler et al., 2012).

The potential RSS activity of sfRNA was suggested based on highly structured nature of the falviviral 3'UTR containing multiple stem loops and double-stranded regions that could potentially interact with RNAi processing proteins (Schnettler et al., 2012). The effect of WNV sfRNA on siRNA and miRNA silencing was then assessed and the ability of sfRNA to inhibit RNAi-silencing of reporter gene was demonstrated in mosquito and mammalian cells (Moon et al., 2015b; E. Schnettler et al., 2012) and in mosquitoes (Moon et al., 2015b). The same effect was demonstrated for DENV2 sfRNA in mammalian cells (Moon et al., 2015b). Slight suppression of RNAi-mediated knockdown of reporter gene was also detected in *I. scapularis* (tick) cells expressing LGTV and TBEV sfRNAs (Schnettler et al., 2014). Furthermore, WNV sfRNA was also shown to interact with Dicer 2 in vitro (E. Schnettler et al., 2012) and to co-precipitate with Dicer and Ago2 in infected human cells (Moon et al., 2015b). In addition, the ability of Dicer to process sfRNA into small RNAs was demonstrated *in vitro* (Schnettler et al., 2012) and *in vivo* (Göertz et al., 2016). It was therefore concluded that sfRNA can act as a sink for the protein components of host RNAi machinery thus preventing their access to viral genomic RNA and RNA replication intermediates (Göertz et al., 2016).

Although the body of evidence suggesting inhibitory effect of sfRNA on RNAi pathway seems solid, the differences in RNAi silencing efficiency between the cells infected with WT and sfRNA-deficient flaviviruses were relatively mild, 2 to 3 fold or even less (Moon et al., 2015b; E. Schnettler et al., 2012; Schnettler et al., 2014). Moreover, generation of abundant virus-derived siRNA was detected in mosquito cells infected with sfRNA-producing wild type WNV (Göertz et al., 2016) and an up-

618 regulation of certain antiviral miRNAs was reported in WNV-infected human cells
619 (Slonchak et al., 2015; Smith et al., 2012) indicating that host cells can develop
620 functional RNAi and miRNA response regardless of the presence of sfRNA. In
621 addition, WNV deficient in production of sfRNA-2 showed no difference in replication
622 comparing to WT virus in either RNAi-competent or RNAi-deficient mosquito cell lines
623 (Göertz et al., 2016). Although replication of the WNV mutant with impaired production
624 of sfRNA-1 and sfRNA-2 was highly compromised comparing to WT in RNAi
625 competent cell line (Göertz et al., 2016), there was no evidence that this difference
626 was related to the effect of sfRNA on RNAi pathway as replication of the same viruses
627 in RNAi-deficient cells was not assessed. Therefore, it is currently unclear if RSS
628 activity of sfRNA is potent enough to determine the evasion of RNAi response by
629 flaviviruses. Further side by side comparison of the wild type and mutant flavivirus
630 deficient in production of all sfRNA species in RNAi- competent and RNAi-deficient cell
631 lines as well as quantification of virus-derived siRNAs produced in infection with both
632 viruses should clarify the biological relevance of RSS activity exhibited by sfRNAs.

633 **5.3. sfRNA inhibits RNAi-independent Toll antiviral pathway in mosquitos**

634 Intriguingly, recent study demonstrated that sfRNA-1 of WNV was critical for
635 replication of WNV in mosquito midgut and crossing the midgut barrier, while it did not
636 affect virus replication in either RNAi-competent or RNAi-deficient mosquito cell lines.
637 (Göertz et al., 2016). This indicates that sfRNA-1 could also inhibit RNAi-independent
638 antiviral pathways in mosquitoes. RNAi-independent antiviral defence in mosquitoes
639 relies on Toll, IMD and Jack-STAT pathways (reviewed in (Sim et al., 2014) and on
640 recently characterized Vago pathway (Paradkar et al., 2014, 2012).

641 Toll pathway is somewhat similar to NF-kB pathway of vertebrates and was
642 shown to be activated in DENV-infected *Aedes sp* mosquitoes (Xi et al., 2008). It

involves detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) PGRP-SA and PGRP-SD and subsequent signalling cascade which results in translocation of transcription factor Rel1 into the nucleus and activation of antiviral genes (Moon et al., 2015a). The IMD pathway was also shown to be involved in protection against DENV in mosquito cells (Sim and Dimopoulos, 2010) and functionally resembles cJun/JNK pathway of the vertebrates (Myllymaki et al., 2014). The Janus kinase/signal transducers and activators of transcription – signal transducer and activator of transcription (JAK-STAT) pathway in mosquitos is similar to interferon-induced JAK-STAT-signalling of vertebrates and has been shown to mediate the mosquito immune response to DENV but not ZIKV (Jupatanakul et al., 2017; Souza-Neto et al., 2009). Vago pathway acts in conjunction with JAK-STAT pathway and represents mosquito equivalent of RIG-I/MDA-5 signalling pathway. Vago pathway involves sensing of dsRNA by Dicer-2 and results in release of Vago peptide. Secreted Vago binds to the specific receptor and activates JAK-STAT pathway in bystander cells similar to IFN in vertebrates. This pathway has been shown to contribute to innate immune response against WNV in *Culex* mosquitos (Paradkar et al., 2012).

Recently, the first study adressing the effect of sfRNA on RNAi-independent antiviral pathways in mosquitoes was reported (Pompon et al., 2017). In this study mosquitos were infected with PR6452 and PR315022 strains of DENV2 that have low and high production of sfRNA per a copy of viral genome, respectively. The expression of innate immunity genes in salivary glands, bodies and carcases of infected mosquitos was then compared to those produced in uninfected mosquitoes. Infection with DENV2 strain, which produced high amount of sfRNA was shown to prevent activation of Toll-pathway component Rel1a and to inhibit expression of another Toll-pathway effector CecG in mosquito salivary glands, whereas increased expression of

both proteins was observed upon infection with the virus that generated small amount of sfRNA. The results were futher validated using chimeric viruses in which 5'UTR and coding sequence of PR6452 was combined with the 3'UTR of PR315022 and vice versa. The results showed that inhibition of Toll pathway-associated genes was caused by the 3'UTR sequence and not by the coding region. However, no significant correlaton between sfRNA production and expression of genes related to IMD, JAK-STAT and Vago pathways was observed in this study (Pompon et al., 2017).

These recent findings suggest that DENV sfRNA inhibits Toll pathway in infected *Aedes sp* mosquitos, however studies with other flaviviruses that replicate in different mosquitoe species need to be performed to determine if this function of sfRNA is unique to DENV or universall for all flaviviruses. In addition, it would be interesting to use flavivirus mutant completely deficient in sfRNA and compare the expression of wider range of genes related to IMD, JAK-STAT and Vago signalling. Modern methods of high-throughpout transcriptome and proteome profiling make this task relatively easy to achive and the reslts should give us furher insights into the effect of sfRNA on RNAi-independent innate immunity in mosquitos.

5.4. sfRNA inhibits type I IFN response in vertebrates

Within vertebrate cells flaviviruses encounter potent antiviral activity of the type 1 interferon (IFN) innate immune response, which has been a subject to a few comprehensive reviews e.g. (Cumberworth et al., 2017; Miorin et al., 2017). Briefly, IFN response begins with the detection of a viral PAMPs by a cellular pattern recognition receptor (PRR), that triggers a signalling cascade, which activates transcription factors known as IFN regulatory factors (IRFs) and NF-$_K$B (Quicke & Suthar, 2013). These transcription factors drive the expression of pro-inflammatory cytokines including type I IFN (Quicke & Suthar, 2013). Once produced and secreted,

type I IFNs bind to the IFN-α/β receptor (IFNAR) and activate JAK-STAT signalling cascade, which leads to expression of >300 IFN-stimulated antiviral genes (ISGs) (reviewed in (Randall and Goodbourn, 2008)).

To enable replication in vertebrate hosts flaviviruses have evolved multiple strategies to evade and inhibit type I IFN response, including inhibition of RNA sensing by PRRs, signal transduction to IRFs, and JAK-STAT cascade (reviewed in (Cumberworth et al., 2017; Diamond, 2009). Inhibitory activity against IFN response have been demonstrated for viral non-structural proteins NS5 (Best, 2017; Grant et al., 2016; Laurent-Rolle et al., 2010), NS4B (Muñoz-jordán et al., 2005; Wang et al., 2005), NS3 (Setoh et al., 2017, 2015; Wang et al., 2005), NS2B (Aguirre et al., 2012; Wang et al., 2005), NS2A (Liu et al., 2004; Setoh et al., 2015; Wang et al., 2005), NS1(Xia et al., 2018) and for sfRNA (Bidet et al., 2014; Chang et al., 2013; Donald et al., 2016; Manokaran et al., 2015; Schuessler et al., 2012).

The interferon antagonist activity of sfRNA was first suggested based on observation that although sfRNA-deficient WNV mutant replicated to lower titres and did not cause mortality in IFN-competent WT mice, both sfRNA-deficient and sfRNA-competent viruses, exhibited no difference in replication in IFN-deficient vertebrate cell lines e.g. Vero-76 and BHK-21(Pijlman et al., 2008). To address the possible role of sfRNA in evasion of type I IFN response, replication of WT and sfRNA-deficient WNV mutant was assessed in IFN-competent wild type mouse embryonic fibroblasts (MEF) and in MEFs deficient in transcription factors IRF3 and IRF7. In this experiment similar replication of WT and sfRNA-deficient mutant viruses was observed in IRF-3/7-deficient MEFs, whereas replication of the sfRNA-deficient mutant virus was significantly reduced in IRF-3/7-competent cells compared to that of WT virus (Schuessler et al., 2012). Moreover, replication of sfRNA-deficient WNV in IRF3/7-

719 deficeint MEFs was reduced drastically and in dose-dependent manner in response to
720 the addition of exogenous IFN-alpha, whereas the addition of IFN-alpha had lesser
721 effect on the replication of WT WNV. In addition, replication and neurovirulence of
722 sfRNA-deficient WNV was partially restored in mice lacking functional IRF-3/7 or
723 IFNAR (Schuessler et al., 2012). These experiments strongly indicated that sfRNA
724 inhibits IFN signalling and that this inhibition is happening downstream of IFN sensing
725 by IFNAR.

726 Moreover, it was shown that transfection of *in vitro* transcribed 5'-
727 monphosphate WNV 3'UTR RNA was able to rescue replication of Semliki forest virus
728 in IFN-treated cells, whereas the effect was not observed if mutated 3'UTR RNA
729 unable to be processed into sfRNA was transfected (Schuessler et al., 2012). This
730 indicates that sfRNA has a direct inhibitory effect on IFN signalling. Furthermore, other
731 studies showed that transfection of *in vitro* transcribed JEV sfRNA was shown to
732 reduce phosphorylation and nuclear translocation of IRF3 and IRF7 in JEV-infected
733 cells and led to ~2-fold decease in the expression of a reporter gene from IFN-β
734 promoter (Chang et al., 2013). Although JEV sfRNA findings suggests the additional
735 inhibitory effect of sfRNA on the IFN-pathway upstream of IFN secretion/sensing, the
736 performed experiments had some serious limitations. The *in vitro* transcribed JEV
737 sfRNA contained 5'-triphosphates, which is different from 5'-monophosphate-
738 containing sfRNA produced in infected cells. As 5'-triphosphates can be recognised by
739 PRRs as a PAMP, this can trigger the whole range of antiviral responses and lead to
740 rapid elimination of viral infection, thus producing the results that cannot be properly
741 interpreted. The effect of 5'-monophosphorylated sfRNA and of the infection with
742 sfRNA-competent and sfRNA-deficient flaviviruses on phosphorylation and nuclear

743 translocation of IRFs should be assessed to confirm the ability of sfRNA to suppress
744 signalling factors upstream of IFN production.

745 More recently the inhibitory effect of sfRNA on type I IFN response was also
746 demonstrated for DENV2 (Manokaran et al., 2015) and ZIKV infection (Donald et al.,
747 2016). In particularly, sfRNA production and expression of IFN-β was compared in
748 infection of human hepatocellular carcinoma cells with two groups (clades) of DENV2
749 strains from Puerto Rico - pre-epidemic clade PR-1 and epidemic clade PR-2B strains.
750 Epidemic strains were shown to contain mutations in the 3'UTR that resulted in
751 production of higher amounts of sfRNA per copy of genomic RNA than pre-epidemic
752 strains. Although both clades of DENV replicated at similar levels at later time points,
753 epidemic strains induced weaker production of IFN-β and replicated similarly in IRF3-
754 deficient cells while replication of pre-epidemic strains was increased in IRF3-deficent
755 cells. In addition, transfection of *in vitro* transcribed PR-2B sfRNA together with IFN-
756 response stimulator polyIC resulted in reduced expression of IFN-β compared to
757 transfection of polyIC with pre-epidemic strain sfRNA. Notably, transfection with any
758 DENV sfRNA reduced IFN-β expression compared to transfection with polyIC alone, or
759 with nonspecific RNA of the same size, thus further confirming IFN antagonist activity
760 of DENV sfRNA (Manokaran et al., 2015). Moreover, expression of DENV sfRNA from
761 plasmid DNA was shown to reduce by ~2-fold the expression on reporter gene
762 controlled by IFN-β promoter in polyIC-stimulated cells. Similar effect was also
763 observed if ZIKV sfRNA was expressed from plasmid DNA in the same system,
764 providing the evidence for IFN antagonist activity of ZIKV sfRNA (Donald et al., 2016).

765 Several attempts have been also made to identify the molecular targets of
766 sfRNA in RNA-sensing and IFN-signalling pathways. RNA binding proteins (RBPs)
767 G3BP1, G3BP2 and CAPRIN1 were identified as novel mediators of IFN response

against DENV2. These proteins were found to be required for translation of mRNAs encoding for ISGs such as PKR and IFITM2. Intriguingly, sfRNA produced in DENV2 infection was shown to co-localize with G3BP1, G3BP2 and CAPRIN1 by immunofluorescent analysis and RNA FISH, and to co-precipitate with these proteins in antibody pull downs. SLII was found to be required for interactions of DENV2 sfRNA with G3BP1, G3BP2 and CAPRIN1 and mutated *in vitro* transcribed sfRNA and DENV2 replicons lacking binding site were used to assess functional outcomes of sfRNA-RBPs interactions. Binding of DENV2 sfRNA to G3BP1, G3BP2 and CAPRIN1 was shown to inhibit translation of selected ISGs and to protect DENV2 replicons from antiviral activity of IFN-β (Bidet et al., 2014). This study was the first to link IFN antagonist effect of sfRNA with the specific molecular components of the IFN response pathway. Later, DENV2 sfRNA was also shown to interact with the ubiquitin ligase triptate motif protein 25 (TRIM25) and to inhibit deubiquitination of TRIM25 and subsequent ubiquitination of RIG-I, ultimately leading to inhibition of viral RNA sensing by RIG-I. More efficient binding of sfRNA to TRIM25 was linked to the weaker IFN response to epidemic PR-2B strains of DENV2 and was proposed to be responsible for the increased fitness of epidemic strains (Manokaran et al., 2015). Interestingly, the inhibitory effect of sfRNA on RIG-I pathway was also demonstrated by the experiment in which the expression of the reporter gene from IFN-β promoter was assessed in cells co-transfected with ZIKV or DENV sfRNA and the reporter plasmid upon treatment with RIG-I agonist. Cells expressing either of these sfRNAs showed ~2-fold lower IFN-β promoter activity than those expressing unrelated control RNA (Donald et al., 2016).

Thus, so far TRIM25 has been identified as molecular target of sfRNA upstream of IFN-β production while G3BP1/2 and CAPRIN1 have been identified as molecular

targets of sfRNA downstream of IFN-α/β signalling. However, it is likely that this is only the tip of the iceberg and we are still far from identifying complete map of molecular interactions that mediate IFN antagonist effect of sfRNA. First of all, binding of G3BP1/2 and CAPRIN1 was shown to occur only with sfRNA from clinical isolate of DENV2, and not for sfRNAs from DENV3, YF vaccine strain 17D or Kunjin strain of WNV (Bidet et al., 2014). Therefore, it is premature to extrapolate these findings to other flaviviruses. In addition, binding of TRIM25 to sfRNA was only assessed for DENV2 and was shown to be increased upon mutations in the 3'UTR specific to epidemic strain of DENV2 from Puerto Rico (Manokaran et al., 2015). The sequence of 3'UTRs is highly variable between flaviviruses although their structural organisation is rather conserved (Clarke et al., 2015; Göertz and Pijlman, 2015; Roby et al., 2014). Thus, considering high variability of the 3'UTR sequence and large effect of point mutations in the DENV2 3'UTR on TRIM25 binding it seems rather unlikely that this interaction will also occur with sfRNAs from other flaviviruses. In summary, a large body of evidence has been accumulated to date demonstrating the inhibitory effect of sfRNA on IFN response pathway, both upstream and downstream of IFN production, however, further studies are clearly required to identify molecular targets in IFN response pathway for sfRNAs of different flaviviruses.

5.5. sfRNA is required for viral cytopathicity and pathogenicity.

Infection with Flaviviruses has been shown to induce cytopathic effect in cultured cells and promote apoptosis via activation of several signalling cascades such as endoplasmic reticulum stress response and AKT/PI3K pathway (reviewed in (Okamoto et al., 2017)). It is generally believed that apoptosis of the infected cells is the part of host antiviral response aimed to clear the infection. However, the real role of apoptosis in flavivirus infection can be more complex than that as pro-apoptotic

818 activity has been reported for viral structural proteins C (Netsawang et al., 2010), M
819 (Catteau et al., 2003) and E (Prikhod'ko et al., 2001) and non-structural NS2A (Liu et
820 al., 2006; Melian et al., 2013), NS2B (Yang et al., 2009) and NS3 (Shafee and
821 AbuBakar, 2003)), suggesting that induction of apoptosis can be also required for viral
822 propagation.

823 Intriguingly, production of sfRNA appears to be paramount for flavivirus-induced
824 cytopathic effect. Mutants of WNV (Pijlman et al., 2008) and DENV (Liu et al., 2014)
825 deficient in generation of sfRNA-1 have been shown to have drastically reduced ability
826 to form plaques on Vero and BHK-21 cells, respectively. Crystal violate staining and
827 lactate dehydrogenase secretion assays further confirmed reduced cytopathicity of
828 sfRNA-deficient WNV by demonstrating that at 6 days post infection with 100%
829 infection rate it resulted in death of only 10% of cells versus 70% caused by sfRNA-
830 competent virus. Complementation with sfRNA produced *in trans* from the plasmid
831 partially rescued plaque-forming and cytopathic properties of sfRNA-deficient WNV
832 and DENV mutants, providing strong indication for the requirement of sfRNA for virus-
833 induced cytopathicity (Liu et al., 2014; Pijlman et al., 2008). In cells infected with
834 sfRNA-deficient DENV reduced cleavage of caspase 3 and of Annexin V translocation
835 of to the cell surface compared to the WT DENV infected cells were observed,
836 suggesting that sfRNA facilitates activation of caspase 3 - dependent apoptotic
837 pathways. This was accompanied by high phosphorylation of Akt and high expression
838 of anti-apoptotic protein Bcl-2 at the later time points post infection, whereas WT
839 DENV infection resulted in decreased expression of Bcl-2 and no detected
840 phosphorylated Akt after 48h post infection (Liu et al., 2014). These data indicate that
841 sfRNA may trigger apoptosis by suppressing Bcl-2, however the biological relevance
842 of this effect and its role in viral pathogenesis is yet to be determined.

Furthermore, sfRNA was shown to be required for WNV-induced neuropathogenicity in vertebrates as all mice infected with sfRNA-deficient virus failed to develop symptoms of encephalitis and survived the infection, which was 100% lethal in animals challenged with sfRNA-competent virus. Both groups of animals had similar viral loads in the brain, which indicates that lack of mortality in mice infected with sfRNA-deficient virus was not due to its inability to penetrate the blood-brain barrier and replicate in the brain (Pijlman et al., 2008). The likely explanation for the lack of neuropathogenicity associated with the loss of sfRNA is the inability of the virus to induce apoptosis and kill infected brain cells.

6. Conclusions and future directions

Generation of highly structured nuclease resistant noncoding RNA via halting digestion of viral genomic RNA by the host exoribonuclease XRN-1 is the evolutionary conserved process within flavivirus genus. This indicates for a crucial role of sfRNA in propagation of flaviviruses in all types of hosts. Despite conservation of sfRNA biogenesis by XRN-1, different taxonomical groups of flaviviruses employ somewhat dissimilar structural determinants for XRN-1 resistance (Fig 2A). Structural similarity of XRN-1-resistant elements generally correlates with evolutional relationships between flaviviruses. xrRNAs of MBFVs share structural but not sequence similarity with those of ISFs, while TBFVs have xrRNAs more similar to phylogenetically related NKVFs. This suggests that the ability to stall XRN-1 for generation of sfRNA appeared very early in evolution of flaviviruses before current taxonomic groups within the genus had diverged. Further evolution of XRN-1 resistance probably dictated accumulation of changes in the structure of xrRNAs related to adaptation for replication in different hosts. Why different structures of xrRNAs were selected in MBFVs/ISFs and

868 TBFVs/NKVFVs groups and whether these differences are in fact the result of
869 adaptation to different hosts remain to be elucidated.

870 sfRNA also appears to determine microevolution of flaviviruses as accumulation
871 of mutations in 3'UTR that shift the balance between genomic RNA and sfRNA have
872 been reported to contribute to the emergence of new epidemic strains of DENV. In the
873 future this property of flaviviruses can potentially be used for predicting flavivirus
874 outbreaks but it will definitely require more studies on the relationships between virus
875 fitness and sfRNA generation for different flaviviruses.

876 Highly conserved production of sfRNA is most likely determined by its ability to
877 inhibit major antiviral pathways in arthropods and vertebrates - RNAi and type I IFN
878 response, respectively. sfRNA was shown to be a dicer substrate and believed to
879 saturate the enzyme thus preventing its access to viral genomic RNA. The molecular
880 mechanisms that mediate IFN-antagonist activity of sfRNA, however, remain elusive.
881 DENV sfRNA interactions with RIG-I cofactor TRIM25 and translational activators of
882 ISGs have been reported but whether these interactions can be extrapolated to
883 sfRNAs of other flaviviruses remains unclear. Identification of other molecular targets
884 of sfRNA in IFN response pathway should be a priority direction in the field as it could
885 produce significant new knowledge required for full understanding of the critical role of
886 sfRNA in the flavivirus life cycle. Considering low sequence and high structural
887 conservation of flavivirus 3'UTRs, proteins that recognise structural (stem loops and
888 dumb bells) or biochemical (5'-monophosphate) motifs will be the most likely sfRNA-
889 interacting partners of functional importance. In addition, the effect of sfRNA on RNAi-
890 independent antiviral pathways in arthropods can be another attractive target to look at
891 in the future. Currently the inhibitory effect of DENV sfRNA on Toll-pathway has been
892 demonstrated in a single study and testing if sfRNA can modulate additional pathways

of mosquito antiviral response may advance our understanding of the mechanisms by which sfRNA facilitates replication of flaviviruses in invertebrate host.

Finally, the requirement of sfRNA for virus-induced apoptosis and neuropathogenicity may open a new avenue in the design of the attenuated vaccines. If sfRNA-deficient mutant flaviviruses prove unable to induce encephalitis in nonhuman primates while retaining their immunogenicity, similar to what is observed in mice (Funk et al, 2010), they can be considered as a novel promising flavivirus vaccine platform.

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Figures legends

Fig 1. Subgenomic flaviviral RNA is produced as a result of incomplete degradation of genomic RNA by host enzyme XRN-1. (A) Schematics of the flavivirus (DENV2) genome organization. (B) Schematic representation of sfRNA biogenesis via XRN-1 digestion. The schematics shows secondary structure of flavivirus (DENV2) 3'UTR with stem loops (SL) dumb bells (DB), short hairpin and 3'-terminal stem loop (3'SL); pseudoknots (PK) and sfRNA start sites. The model is based on SHAPE reactivity data from (Chapman et al., 2014a) (C) Northern blot demonstrating generation of several sfRNA species in DENV2-infected mosquito cells due to stalling of XRN-1 at different halt sites. (B) and (C) are reproduced with permission from (Filomatori et al., 2017).

Fig.2 Structural determinants of sfRNA biogenesis. (A) XRN-1 resistant structures formed by stem loops (SL) and dumb bells (DB) in the 3'UTRs of representative flaviviruses from different taxonomical groups of *Flavivirus* genus. Dendrogram shows phylogenetic relationships between flavivirus clades. Red lines indicate pseudoknots, orange lines show base pairs (internal small pseudoknots), blue lines show base triple and green lines show noncanonical base pairing. sfRNA start sites (where known) are indicated with a blue arrow. (B) Tertiary structure of xrRNA from 3'UTR of ZIKV. Different elements of RNA structures are color-coded. Bases holding the 5'-end inside the circle are shown in red. (C) Model of interactions between XRN-1 and xrRNA of ZIKV. (B) and (C) are reproduced with permission and minor modifications from (Akiyama et al., 2016).

Fig. 3. Functions of sfRNA in arthropod and vertebrate hosts. sfRNA inhibits XRN-1 and Dicer in both hosts, causing disruption of mRNA decay and siRNA/miRNA production, respectively. In vertebrates sfRNA inhibits IFN-α/β response and induces apoptosis. Inhibitory effect of sfRNA on IFN-α/β response is in part mediated by sfRNA binding to TRIM25 and to CAPRIN1/G3BP1/2 and inhibiting their functions in IFN induction and IFN signalling, respectively. In mosquitoes, sfRNA inhibits expression of Toll-pathway components CecG and Rel1a and supresses Toll-signalling in addition to inhibiting RNAi response.

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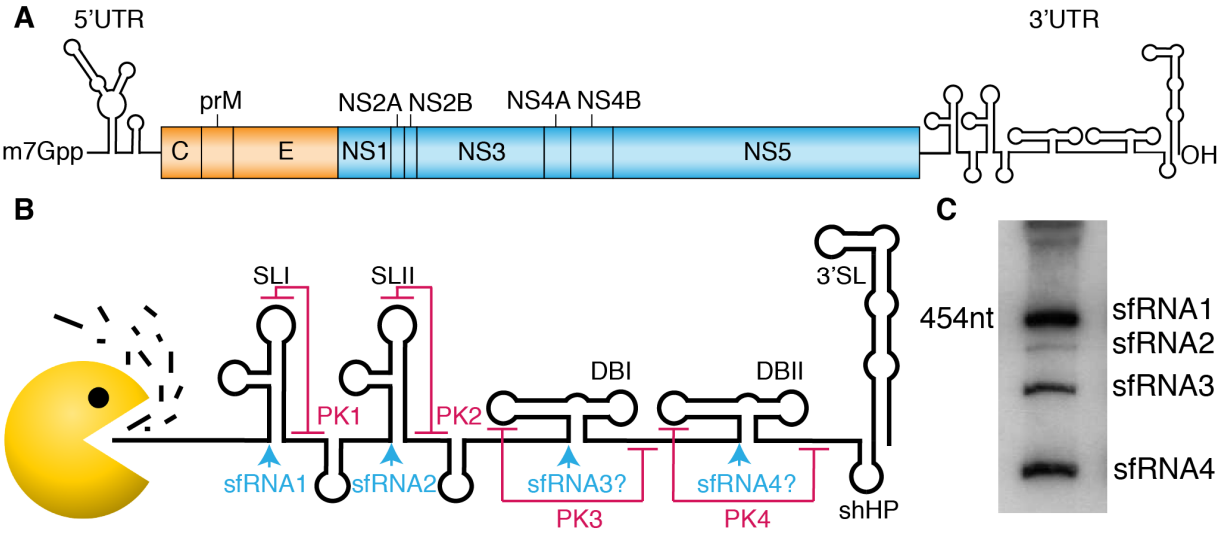

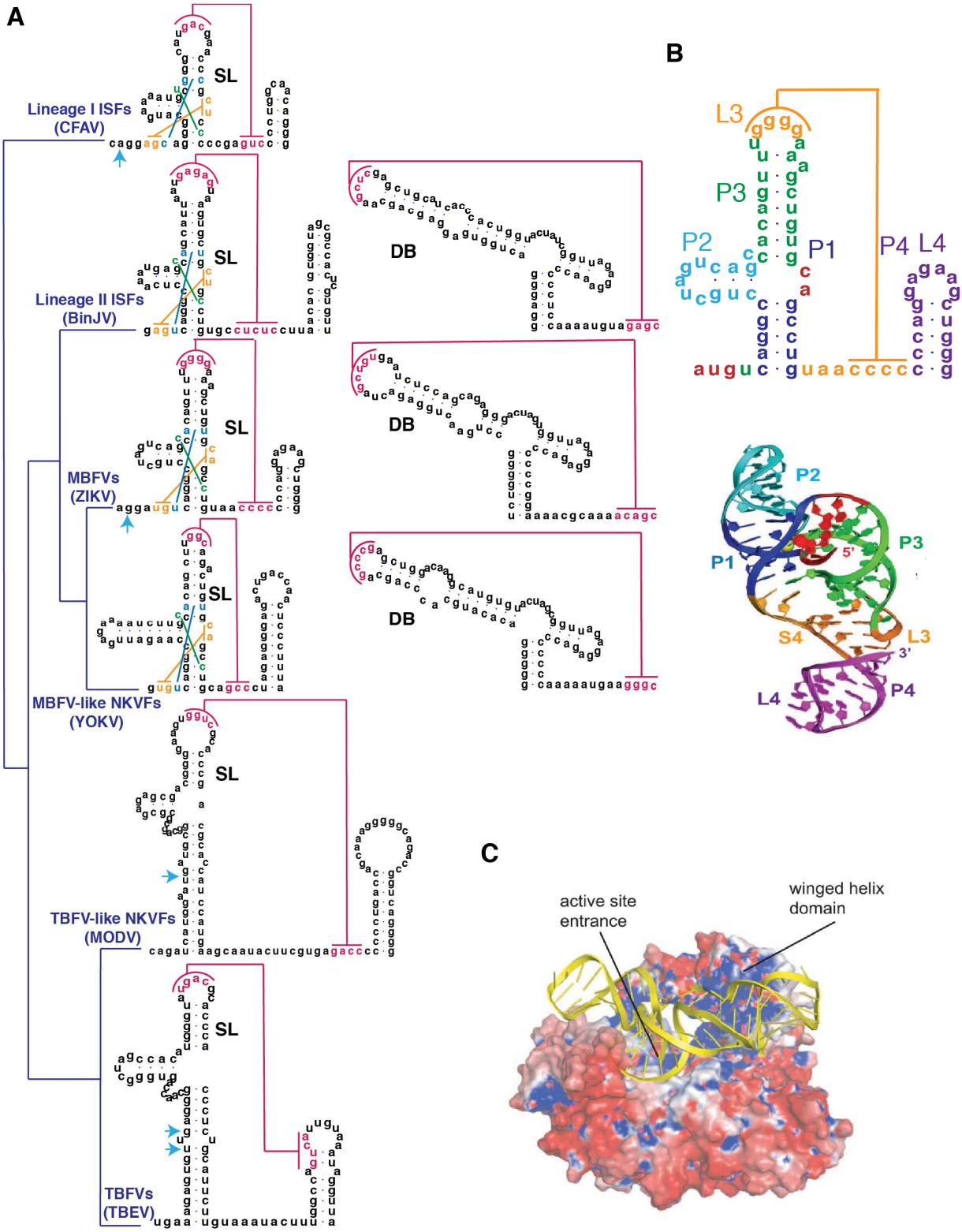
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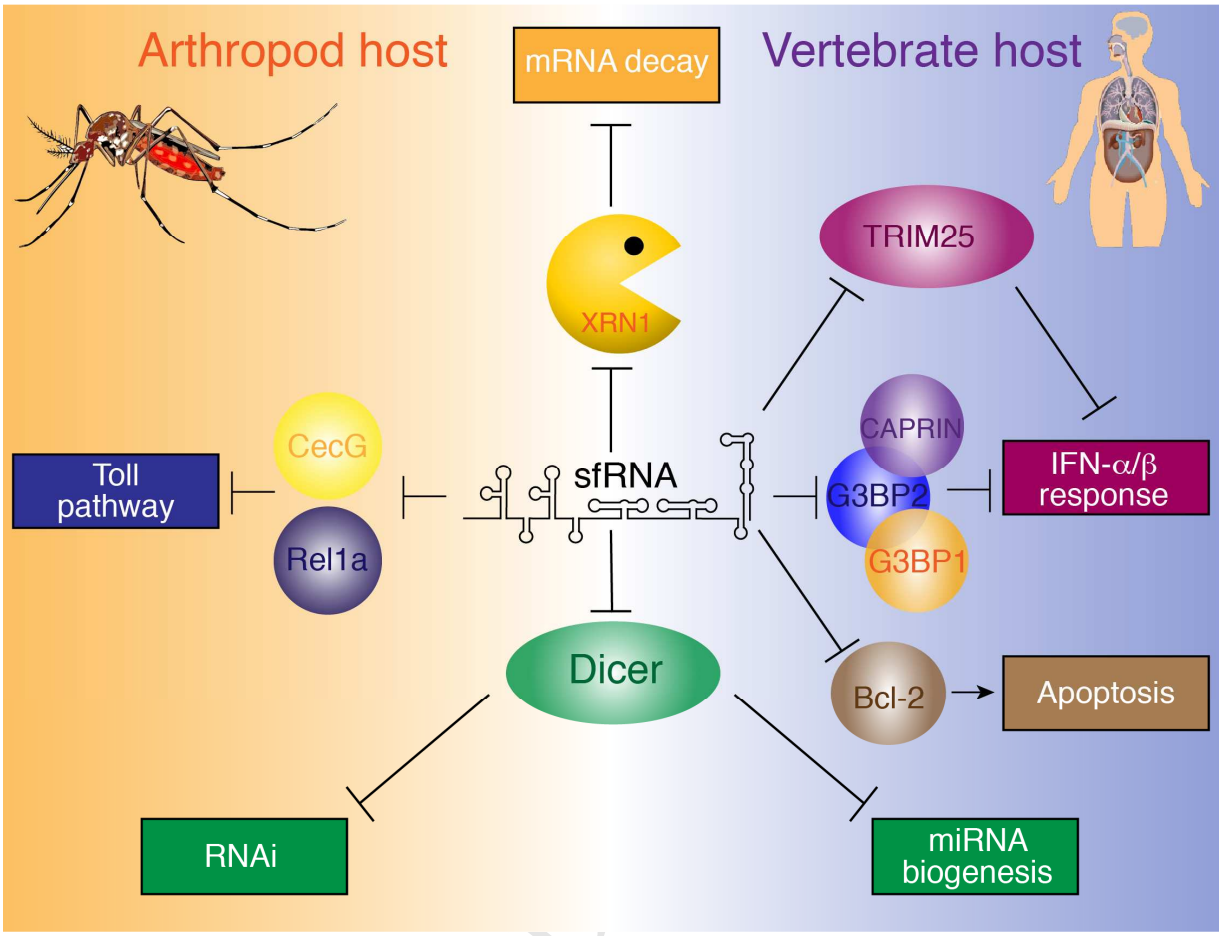
Arthropod host
mRNA decay
Vertebrate host
TRIM25
XRN1
CecG
Toll pathway
Rel1a
sfRNA
CAPRIN
G3BP2
G3BP1
IFN-α/β response
Dicer
Bcl-2
Apoptosis
RNAi
miRNA biogenesis

- sfRNAa are produced via incomplete digestion of flaviviral genomic RNA by XRN-1.
- RNA elements in 3'UTRs that form a 3-way junctions (xrRNAs) and pseudoknots are required to stall XRN-1 and produce sfRNAs.
- Generation of sfRNAs is highly conserved amongst all flaviviruses, whereas the structure of xrRNAs varies.
- xrRNA structures are duplicated in some flaviviruses resulting in the production of up to four sfRNA species of different sizes
- Different sfRNA species may be required for adaptation of some flaviviruses to replication in arthropod or vertebrate hosts
- sfRNA is required for pathogenesis of flaviviruses in vertebrates and transmission by arthropods
- sfRNA inhibits RNAi response and Toll pathway in arthropods and type I interferon IFN response in vertebrates.