Emerging roles of non-coding RNAs in vector-borne infections

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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Emerging roles of non-coding RNAs in vector-borne infections

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ABSTRACT

Non-coding RNAs (ncRNAs) are nucleotide sequences that are known to assume regulatory roles previously thought to be reserved for proteins. Their functions include the regulation of protein activity and localization and the organization of subcellular structures. Sequencing studies have now identified thousands of ncRNAs encoded within the prokaryotic and eukaryotic genomes, leading to advances in several fields including parasitology. ncRNAs play major roles in several aspects of vector–host–pathogen interactions. Arthropod vector ncRNAs are secreted through extracellular vesicles into vertebrate hosts to counteract host defense systems and ensure arthropod survival. Conversely, hosts can use specific ncRNAs as one of several strategies to overcome arthropod vector invasion. In addition, pathogens transmitted through vector saliva into vertebrate hosts also possess ncRNAs thought to contribute to their pathogenicity. Recent studies have addressed ncRNAs in vectors or vertebrate hosts, with relatively few studies investigating the role of ncRNAs derived from pathogens and their involvement in establishing infections, especially in the context of vector-borne diseases. This Review summarizes recent data focusing on pathogen-derived ncRNAs and their role in modulating the cellular responses that favor pathogen survival in the vertebrate host and the arthropod vector, as well as host ncRNAs that interact with vector-borne pathogens.

KEY WORDS: Non-coding RNAs, Vector-borne infection, Host–pathogen interactions

Introduction

Non-coding RNAs (ncRNAs) describe a class of transcripts without protein-coding capacity (Cech and Steitz, 2014). ncRNAs were historically referred to as 'junk' RNA and were not thought to perform any biological or cellular functions (Richard Boland, 2017). However, this assumption was challenged when the ENCODE project reported that ∼90% of all mammalian transcriptional output is ncRNA (Feingold et al., 2004; Hubé and Francastel, 2018). Functional studies of ncRNAs have now confirmed their importance in various biological processes (Cech and Steitz, 2014; Gil and Latorre, 2012). ncRNAs are divided into housekeeping and regulatory ncRNAs, and both have important epigenetic roles (Pang et al., 2018), being involved in the regulation of gene expression at the transcriptional and post-transcriptional levels, as well as in genome protection from foreign nucleic acids (Pang et al., 2018). Regulatory ncRNAs, the focus of this Review, are secreted in extracellular vesicles (EVs), namely exosomes (Silva and Melo, 2015), and they can be divided into two major classes based on transcript size (Box 1) – small ncRNAs [<200 nucleotides (nt); sncRNAs] and long ncRNAs (>200nt; lncRNAs) (Yang and Li, 2018). Even though most of the prokaryotic genome comprises coding sequences (Gil and Latorre, 2012), the non-coding portion may also serve a biological purpose. For example, bacteria express many potentially pathogenic sncRNAs, but these remain poorly understood due to a paucity of robust phenotypes in standard virulence assays (Westermann et al., 2016). So far, only a few ncRNAs from pathogens have been identified, including the subgenomic flaviviral RNA (sfRNA) from flaviviruses, Zika virus, Dengue viruses and West Nile fever virus, and small RNAs (sRNAs) from bacteria, such as riboswitches, *cis*-acting sRNAs and *trans*-acting sRNA from *Borrelia burgdorferi*, and the sRNAs Ysr141 and Ysr170 from *Yersinia pestis* (Bavia et al., 2016; Li et al., 2016; Villa et al., 2018). Other ncRNAs were also identified in parasites, such as tRNA- and rRNA-derived small RNAs from *Trypanosoma* and subtelomeric lncRNAs from *Plasmodium falciparum* (Fig. 1). The mechanism of action of these ncRNAs and their involvement in pathogen–host interactions will be discussed in the following sections.

Over the past two decades, rapid technological advances have facilitated the characterization of ncRNAs, which are now recognized as key players in several biological systems, including vector–host–pathogen interactions in parasitology. Vector-borne pathogens (including bacteria, nematodes, protozoa and viruses) have a primary vertebrate reservoir host and a primary arthropod vector that maintain their transmission cycle in nature (Pfeffer and Dobler, 2010). Arthropod vectors, such as mosquitoes, sandflies and ticks, infest a wide variety of vertebrate hosts and are responsible for several life-threatening human and animal diseases (Duvallet et al., 2017). When feeding on the host, blood-feeding arthropods secrete saliva containing a complex mixture of enzymes, protease inhibitors, histamine-binding molecules and prostaglandins, as well as ncRNAs, which modify and subvert host hemostasis and immune responses (Leitner et al., 2011). In addition, pathogens can be delivered into the vertebrate host through arthropod saliva (Schorderet-Weber et al., 2017); there, they interact with several vector salivary molecules, thereby facilitating the establishment of pathogen infection in the host (Rego et al., 2019). They are also able to avoid destruction by vector defenses using several strategies, including manipulating vector gene expression (Silmon De Monerri and Kim, 2014). In the context of vector-borne diseases, pathogen ncRNAs, their effects on host immune cells, and how immune-related cells maintain viability and competitiveness in various environmental niches have only just begun to be investigated. Infections with vector-borne pathogens are complex owing to the adaptive plasticity of pathogens, arthropods

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Box 1. Non-coding RNA biogenesis and functions

ncRNAs are broadly divided by length into long non-coding RNAs (lncRNAs) (>200 nt long) and small non-coding RNAs (sncRNAs) (<200 nt long). They are suggested to be secreted in host cells to manipulate gene expression and deregulate host defense pathways. LncRNAs are not translated into proteins, and include mRNA-like intergenic transcripts (lincRNAs), antisense transcripts of protein-coding genes and primary RNA polymerase II (Pol II) transcript-derived unconventional lncRNAs. sncRNAs represent a diverse set of molecules that control the expression of most vertebrate genes and include siRNAs, miRNAs and piwi-interacting RNAs (piRNAs). miRNAs have been extensively studied and their function in gene regulation is well understood.

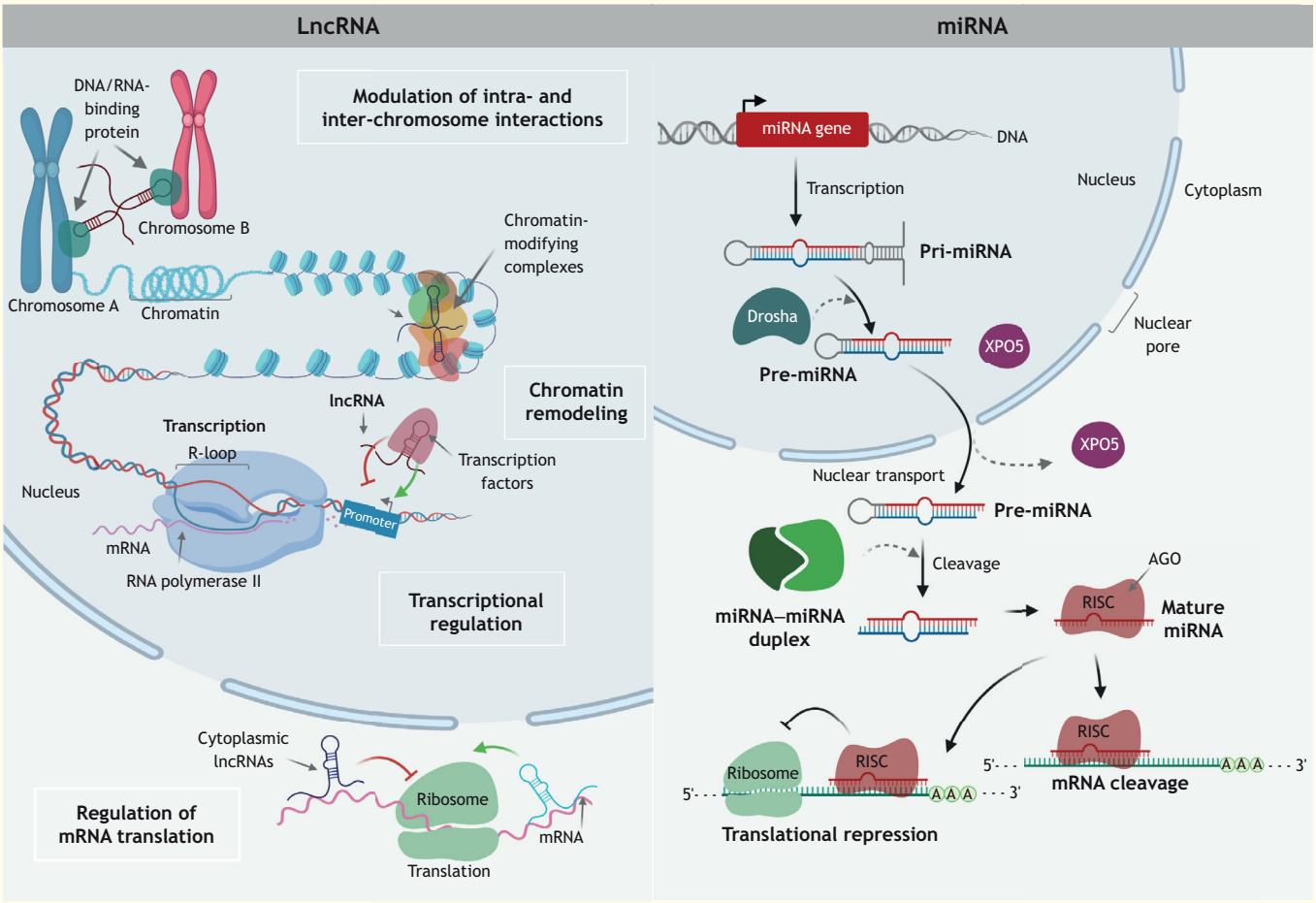
Long non-coding RNA

lncRNA synthesis and regulation mechanisms are still poorly understood. LncRNA biogenesis is controlled by cell type- and stage-specific stimuli through different mechanisms, such as cleavage by ribonuclease P (RNaseP) to generate mature ends, formation of small nucleolar RNA (snoRNA) and protein (snoRNP) complex caps at their ends, and the formation of circular structures (Dahariya et al., 2019) (see figure, left).

LncRNAs perform several cellular functions including the modulation of intra- and inter-chromosomal interactions through an association with DNA- and RNA-binding proteins, which in turn act on the spatial conformation of chromosomes and chromatin remodeling by the recruitment of chromatin-modifying complexes. LncRNAs can also regulate transcription by forming R-loops and interfering with the RNA polymerase II machineries or by regulating transcription factor activity. R-loops can tether lncRNAs in *cis* and recruit transcription cofactors to corresponding promoter regions (Yao et al., 2019) and appear to activate or inhibit the binding of transcription factors to the promoters of target genes (Dahariya et al., 2019). Furthermore, cytoplasmic lncRNAs can regulate mRNA expression by regulating mRNA stability or mRNA translation. They seem to interact with ribosomes by activating or inhibiting their activity (Dahariya et al., 2019) (see figure, left).

miRNA biogenesis

In the nucleus, miRNA biogenesis begins with the generation of the primary miRNAs (pri-miRNA) transcript from miRNA genes. Pri-miRNAs are subsequently cleaved by the endoribonuclease Drosha to generate precursor miRNAs (pre-miRNAs). Drosha cleaves the pri-miRNA duplex at the base of the characteristic hairpin structure of pri-miRNA. Once pre-miRNAs are generated, they are exported to the cytoplasm by exportin 5 (XPO5) through nuclear transport. In the cytoplasm, pre-miRNAs undergo further processing by the RNase III endoribonuclease Dicer and the TAR RNA-binding protein 2 (TARBP2). This processing involves the elimination of the terminal loop, resulting in a mature miRNA duplex (miRNA-miRNA duplex). Each strand of the mature miRNA duplex is loaded into the argonaute (AGO) family of proteins to form the RNA-induced silencing complex (RISC). In the RISC complex, mature miRNAs target specifically targeted mRNA due to their interaction with complementary sequences. Accordingly, they target mRNA cleavage via AGO endonuclease activity or function in translational repression by inhibition ribosome activity (O'Brien et al., 2018) (see figure, right).



and numerous actors in the vector–host–pathogen triad (Fig. 2). Coupled with the current antibiotic resistance crisis, this complexity not only makes vector-borne diseases very difficult to control but also challenging to eradicate (Rosenberg and Beard, 2011). Newly identified pathogen ncRNAs play an integral role in virulence expression and bacterial stress responses that are ultimately

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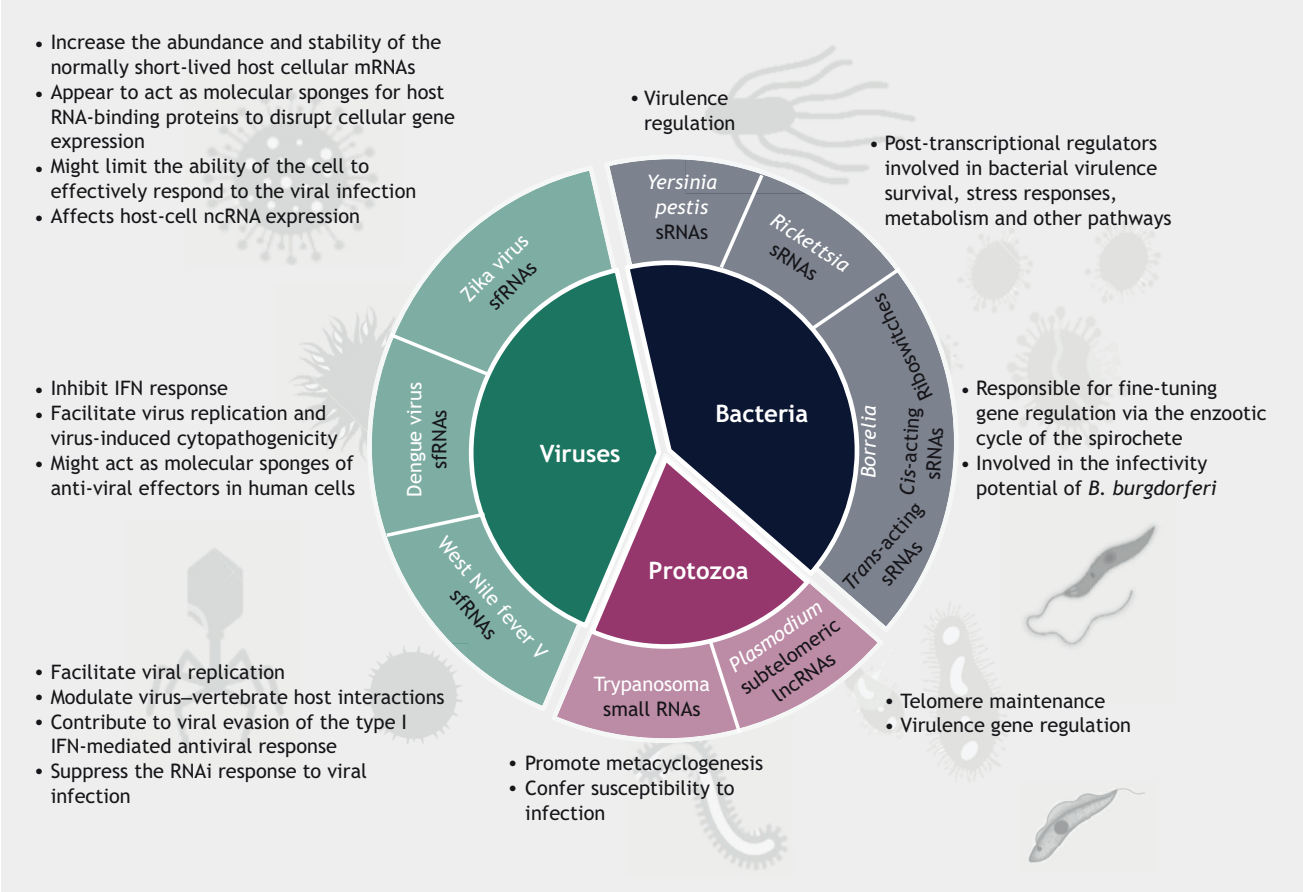

Fig. 1. Overview of pathogen non-coding RNAs and their potential roles in pathogen–host interactions. The different processes that ncRNAs from virus, bacteria and protozoa pathogens are involved in are listed in the scheme. A specific type of ncRNA, subgenomic flaviviral RNA (sfRNA) was identified from the Zika virus, Dengue virus and West Nile fever virus. These ncRNAs are suggested to play a role in facilitating virus replication and in affecting host ncRNA expression, among other functions. Bacteria including *Yersinia pestis*, *Rickettsia* and *Borrelia* appear to use sRNAs in bacterial virulence regulation. ncRNAs have also been identified in protozoa, such as subtelomeric lncRNAs from *Plasmodium*, and small RNAs derived from tRNAs or ribosomal RNAs from *Trypanosoma*, which might be involved in gene regulation during the infection. IFN, interferon.

advantageous for adapting and modifying host immune responses (Ahmed et al., 2016). As vector ncRNAs and their involvement in host interactions are reviewed elsewhere (Bensaoud et al., 2019), this Review focuses on recent data regarding pathogen–host interactions via ncRNAs. We will discuss the pathogens according to the vector by which they are transmitted and describe the possible functions of their ncRNAs in pathogen–host interactions, as well as future perspectives for this emerging field.

Non-coding RNAs in pathogens transmitted by mosquitoes

Mosquitoes are the leading arthropod vectors for human infectious agents (Higgs et al., 2017). Human urbanization has affected the natural environment of the mosquito, and continues to create habitats in which vectors for a wide variety of human and veterinary pathogens thrive, if not adequately controlled, causing an enormous hazard to public health (Neiderud, 2015). A specific type of ncRNA, sfRNA, is produced by positive-strand RNA viruses belonging to the genus *Flavivirus*, which are small, enveloped pathogens with non-segmented genomes consisting of single-stranded positive-sense RNA and include arthropod-borne human pathogens, such as dengue virus (DENV) and the West Nile virus (WNV) (Roby et al., 2014). sfRNAs are 300 to 500 nt long and are generated by incomplete degradation of the 3′-untranslated region (UTR) of the viral genome by the host cell 5′–3′ exoribonuclease Xrn1, which cleaves the viral RNA but stalls at defined locations in the highly folded 3′UTR (Pijlman et al., 2008). In this section, we discuss the most dangerous pathogens transmitted by mosquitoes highlighting the latest data regarding pathogen ncRNAs and their interactions with the vertebrate or invertebrate hosts.

Dengue virus

DENV is the most common cause of vector-borne viral disease in humans, with 50 to 100 million infections per year (Tuiskunen Bäck and Lundkvist, 2013). DENV is a single positive-stranded RNA virus of the family *Flaviviridae* (genus *Flavivirus*), transmitted by *Aedes* mosquito vectors (Guzman and Harris, 2015), and represented by four serotypes (DENV1–DENV4), which lead to a full spectrum of disease outcomes (Gubler, 1998). The mechanisms underpinning severe dengue are still poorly understood, partly owing to a lack of appropriate animal models to study dengue infection and disease (Qureshi and Saeed, 2019). Nevertheless, both viral and host factors play important roles during the course of the infection.

Like all arthropod-borne flavivirus infections, DENVs produce large quantities of sfRNA, whose specific function in viral replication is still elusive; however, it has been linked in related viruses to the inhibition of the interferon (IFN) response (Mazeaud et al., 2018). A recent large-scale *in silico* study suggested that

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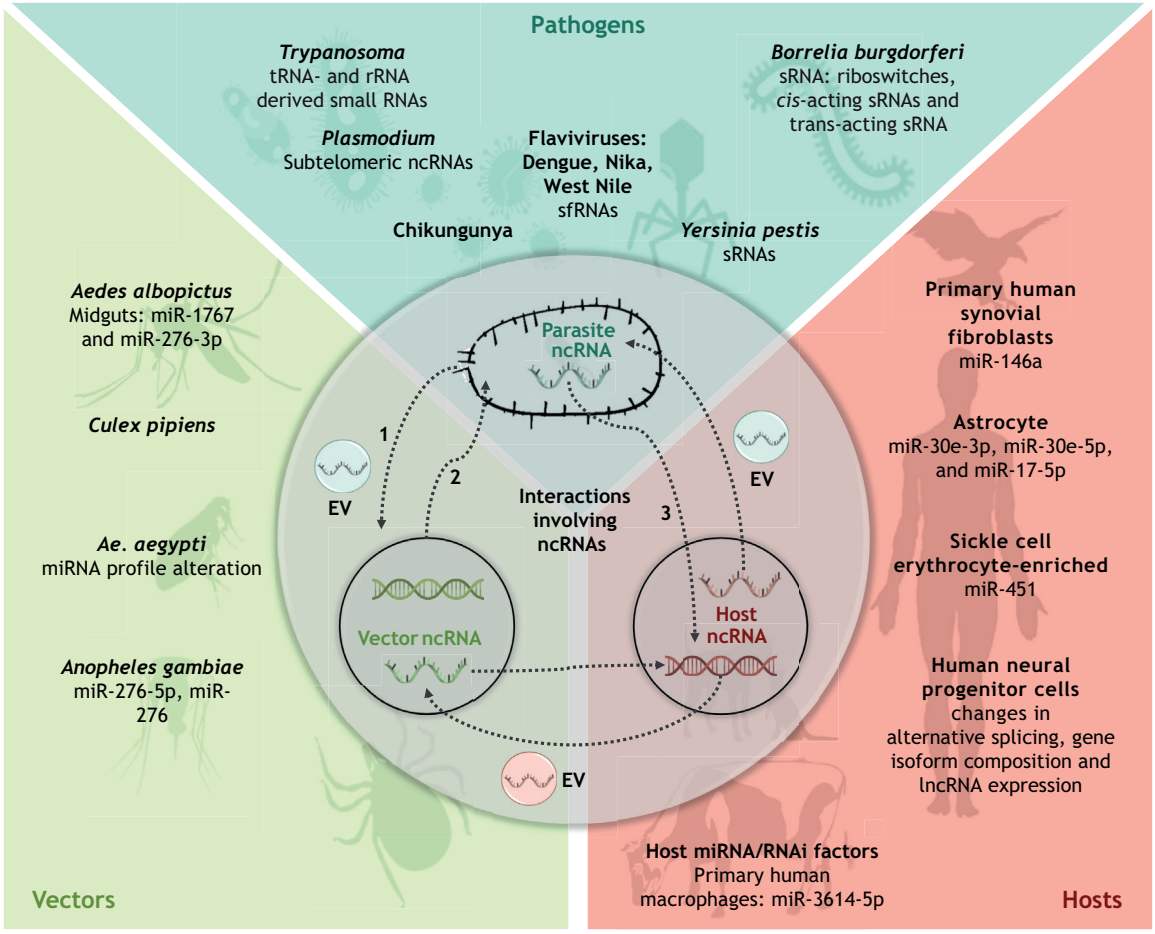

Fig. 2. Non-coding RNAs at the pathogen–vector–host interface. Interactions between pathogens and vertebrate hosts are complex, especially in the context of vector-borne diseases. When studying interactions of pathogens with the invertebrate vector and vertebrate host, several 'sub-interactions' need to be taken into account. (1) Pathogens and vectors. Pathogens avoid destruction by vector defenses using several strategies, including manipulating vector gene expression. (2) Vectors and pathogens. Blood-feeding vectors introduce pathogens directly into the blood of the vertebrate host through their saliva. Pathogens interact with several salivary molecules of the vector, which facilitate the establishment of the infection in the host. (3) Pathogens and hosts. When in the host, the infection is established, and pathogens tend to avoid the host immune system in order to survive and replicate. These sub-interactions involve several ncRNAs from pathogens (teal area) including sRNAs from flavivirus, *Yersinia pestis*, and *Borrelia burgdorferi,* subtelomeric ncRNAs from *Plasmodium*, and tRNA- and rRNA-derived small RNAs from *Trypanosoma*. Moreover, several miRNAs from vectors (green area) are implicated in these interactions, such as miR-1767 and miR-276-3p from *Aedes* albopictus and miR-276-5p, miR-276 from *Anopheles gambiae*. Finally, numerous miRNAs from the host (orange area) are expressed during the infection, such as miR-30e-3p, miR-30e-5p and miR-17-5p in astrocytes, miR-146a in primary human synovial fibroblasts, miR-3614-5p in primary human macrophages and miR-451 in sickle cell erythrocytes. Hosts also express lncRNA in human neural progenitor cells. EV, extracellular vesicle.

sfRNAs are important determinants of DENV adaptation, survival and epidemiological fitness (Finol and Ooi, 2019). This was highlighted by the sfRNA structures, which were under natural selection and were shown to be highly conserved despite their sequence divergence (Finol and Ooi, 2019). It has previously been shown that sfRNAs play a role in facilitating efficient virus replication and virus-induced cytopathogenicity in mice (Pijlman et al., 2008). The study showed that mice injected with altered flavivirus (FL) unable to produce sfRNA, such as FL-IRA (containing a 3-nt substitution abolishing sfRNA1 production) or FL-IRAΔCS3 (which is not capable of producing sfRNA1 or sfRNA2, which has minimal changes to RNA structure), showed no signs of encephalitis (one of the outcomes of DENV infection), demonstrating a crucial role for sfRNAs in determining viral pathogenicity (Pijlman et al., 2008). In another study, three conserved host RNA-binding proteins (RBPs), Ras GTPase-activating protein-binding protein 1 (G3BP1), G3BP2, and the cell cycle-associated protein 1 (CAPRIN1) were found to regulate the IFN response against DENV2 during infection (Bidet et al., 2014). Their antiviral activity was antagonized by the abundant DENV2 sfRNA, which bound to G3BP1, G3BP2 and CAPRIN1, inhibiting their activity and profoundly impairing interferon-stimulated gene (ISG) mRNA translation. Therefore, sfRNAs might act as molecular 'sponges' against anti-viral effectors in human cells (Bidet et al., 2014).

Interestingly, some studies have shown that host ncRNAs could also be involved in DENV infection. Viral effectors can interact with the host RNAi machinery by targeting host ncRNAs (Kakumani et al., 2013). For instance, DENV infection can lead to the downregulation of components of the host RNAi and microRNA (miRNA) machinery and interfere with the biogenesis of many known human miRNAs (Kakumani et al., 2013). Knockdown of host miRNA and RNAi factors, especially the endoribonuclease Dicer and the class 2 ribonuclease Drosha, significantly increases

the replication of the dengue virus genome, as the host RNAi–miRNA pathway is modulated by the viral nonstructural protein 4B (NS4B) (Kakumani et al., 2013). The mRNA levels of these genes were reduced in Huh7 liver cells infected with DENV2 (Kakumani et al., 2013). These results suggest that pathogen ncRNAs interact with the host RNAi machinery, which in turn can also limit dengue virus replication (Kakumani et al., 2013). Furthermore, another study analyzed the expression profiles of primary human macrophages challenged with DENV (Diosa-Toro et al., 2017), and this showed the differential expression of five miRNAs. Of these miRNAs, miR-3614-5p was upregulated in DENV-negative cells and its overexpression reduced DENV infectivity. Adenosine deaminase acting on RNA 1 (ADAR1) was identified as an miR-3614-5p target that promotes DENV infectivity at early time points 24–48 h) after infection (Diosa-Toro et al., 2017). Of note, some ncRNAs also appear to be involved in the arthropod vector–virus interaction. A recent study showed that miRNAs are differentially expressed in uninfected and infected midguts of *Aedes albopictus* at different time points, and the number of upregulated miRNAs was greater than the number of downregulated miRNAs (Su et al., 2019). When tested *in vitro*, upregulated miR-276-3p1767 and miR-276-3p from the vector enhanced DENV replication in C6/36 cells, while upregulation of miR-4448 led to a reduction in virus replication (Su et al., 2019).

Zika virus

Zika virus (ZIKV) is another mosquito-transmitted flavivirus that causes neurological complications and eventually severe congenital malformations, such as microcephaly (Musso and Gubler, 2016). Several studies have focused on virus–host interactions for vaccine development (He et al., 2017); however, ZIKV pathogenicity mechanisms, as well as those of other flaviviruses, are still poorly understood (Miner and Diamond, 2017). In a recent study, sfRNA was shown to accumulate in human cells during ZIKV infection, increasing both the abundance and stability of the normally short-lived host cellular mRNAs (Michalski et al., 2019). The same study also found that this sfRNA appears to act as a molecular sponge for host RNA-binding proteins to disrupt cellular gene expression and perhaps limit the ability of the cell to effectively respond to the viral infection (Michalski et al., 2019). Conversely, ZIKV was also shown to affect host-cell ncRNA expression. For instance, characterization of ZIKV replication in astrocytes and profiled temporal transcriptomic changes in host miRNAs during infection demonstrated that numerous genes involved in the unfolded protein response (UPR) pathway are dysregulated during infection (Kozak et al., 2017). However, other miRNAs, such as miR-30e-3p, miR-30e-5p and miR-17-5p, were upregulated, and have also been found to be upregulated in other flavivirus infection processes (Kozak et al., 2017). Moreover, transcriptomic analysis of ZIKV-infected human neural progenitor cells (hNPCs) revealed that there are changes in alternative splicing, gene isoform composition and lncRNA expression (He et al., 2017).

West Nile virus

WNV is an emerging viral pathogen that can cause fatal encephalitis and significant morbidity and mortality in birds, horses and humans (Rossi et al., 2010). WNV is the most widespread member of the arthropod-borne flaviviruses (Bollati et al., 2010; Weissenböck et al., 2010), which primarily cycle between *Culex* mosquitoes and birds (Diamond, 2009). Viral sfRNAs were also identified in mammalian cell lines (BHK-21) infected with WNV (Kunjin and NY99 strain) (Pijlman et al., 2008). Recent studies have shown that ncRNAs induced by viral infections might play a critical role in regulating the virus–host interaction during *Flaviviridae* infections. It was shown that the generation of sfRNAs in WNV not only facilitated viral replication but also induced cytopathic effects in cell culture and promoted viral pathogenicity in mice (Pijlman et al., 2008). By comparing the replication of wild-type (wt) WNVKUN virus to a mutant virus incapable of producing sfRNAs *in vitro* and in mice with various deficiencies in the innate antiviral response, it was possible to show that sfRNAs contribute to viral evasion of the type I IFN-mediated antiviral response (Schuessler et al., 2012). Conversely, WNV sfRNA suppresses cellular siRNA and miRNA production in infected cells by competing with Dicer substrates (Schnettler et al., 2012). SfRNA has also been shown to be a suppressor of the RNAi response to viral infection in insect cells (Schuessler et al., 2012). The infection of mosquitoes with the flavivirus WNV and an sfRNA-deficient mutant WNV demonstrated that sfRNAs determine the infection and transmission rates of WNV in *Culex pipiens*. A comparison of infection via the blood meal versus intrathoracic injection, which bypasses the midgut, revealed that sfRNA is important for overcoming the mosquito midgut barrier, and that the antiviral RNAi machinery processes sfRNAs into viral siRNAs (vsiRNAs) in mosquitoes. The study thus showed the pivotal biological function of sfRNA in arthropods (Göertz et al., 2016).

Chikungunya virus

Mosquito-borne viral infections, such as Chikungunya, cause severe febrile disease that can progress to long-term physical or cognitive impairment; it can also recur in epidemic waves with outbreaks becoming increasingly severe (LaBeaud et al., 2011; Rezza and Weaver, 2019). This infection is caused by the Chikungunya virus (CHIKV), a positive-sense single-stranded RNA virus transmitted to vertebrate hosts by *Aedes aegypti* and *Aedes albopictus* mosquitoes (Monteiro et al., 2019). Viral replication can hijack the vertebrate host machinery and use it to the advantage of the pathogen – the virus relies on the host machinery for translation of viral proteins by switching off cap-dependent translation (Girardi et al., 2018). Numerous ncRNAs have been shown to be involved in CHIKV–host-cell interactions, but little is known about CHIKV ncRNAs. Changes in host coding gene and miRNA expression were reported after CHIKV infection at various time points, with 152 miRNAs downregulated after CHIKV infection (Saxena et al., 2013). Another study showed that CHIKV exploits miR-146a, an NF-κB-dependent miRNA, to modulate the host antiviral immune response in primary human synovial fibroblasts (Selvamani et al., 2014). The induced expression of miR-146a downregulated signal transducers, such as the TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinases 1 and 2 (IRAK1 and IRAK2), and increased the replication of CHIKV. Interestingly, expression of TRAF6, IRAK1 and IRAK2 was restored when cellular miR-146a was inhibited (Selvamani et al., 2014).

Plasmodium

Malaria is a mosquito-borne infectious disease that affects humans and other animals, killing ∼400,000 people annually (World malaria report 2019; https://www.who.int/publications/i/item/9789241565721), with the parasite *P. falciparum* causing the most lethal form of human malaria (Urban et al., 2005). Several studies, mainly in *P. falciparum*, have been conducted to understand the contribution of ncRNAs in the expression of virulence genes. SncRNAs and lncRNAs are abundant during the blood stages of

P. falciparum infection (Vembar et al., 2014). Moreover, many *P. falciparum* ncRNAs, including subtelomeric ncRNAs, virulence gene-associated ncRNAs and natural antisense RNA transcripts have been shown to be important in host gene regulation (Vembar et al., 2014). High-resolution DNA tiling microarray analysis was used to characterize a family of 22 telomere-associated lncRNAs in *P. falciparum*, and homologous lncRNA-TARE loci were shown to be coordinately expressed after parasite DNA replication (Urban et al., 2006). These were suggested to play an important role in telomere maintenance, virulence gene regulation, and potentially other processes; however, further studies are needed in order to provide mechanistic insights into transcriptional regulation of *P. falciparum* (Broadbent et al., 2011). *P. falciparum* erythrocyte membrane protein 1, encoded by *var*, is an immunodominant antigen that mediates immune evasion in humans. At any given time, only a single *var* gene is expressed in each parasite (Jing et al., 2018). An antisense lncRNA (aslncRNA) derived from the *var* intron was identified as an activation factor for the corresponding *var* gene (Jing et al., 2018). These findings are highly relevant with regards to understanding gene expression control in *Plasmodium* and parasitic mechanisms of antigenic variation. Nevertheless, the mechanism underlying aslncRNA function needs further investigation to identify potential target binding domains.

Other studies have demonstrated the involvement of host ncRNAs in responses to *P. falciparum* infections. Erythrocyte miRNAs are translocated into *P. falciparum* in a sequence-specific manner, in particular the sickle cell erythrocyte-enriched miR-451 and let-7i, which inhibit parasite growth. Sickle cell erythrocytes exhibit cell-intrinsic resistance to malaria, in part through atypical miRNA activity, suggesting that these mechanisms may represent a unique host defense strategy against complex eukaryotic pathogens (Lamonte et al., 2012).

ncRNAs in pathogens transmitted by ticks

Ticks are vectors for a variety of pathogens that cause life-threatening zoonoses worldwide (Anderson and Magnarelli, 2008). Tick-borne diseases have a huge impact on human and animal health and consequently have socioeconomic implications, especially in countries in the southern hemisphere (Almazan et al., 2018). Several experimental studies have focused on tick-host-pathogen interactions to better understand pathogen transmission in order to improve the control and prevention of their associated diseases. Pathogen transmission from ticks to vertebrate hosts is assisted by salivary constituents that not only facilitate pathogen transfer but also promote infection by modulating host responses (Nuttall, 2019). Recently, ncRNAs have been shown to be key players in vector-host-pathogen interactions in tick-borne diseases, with some involved in the establishment of the infection in the host.

Borrelia burgdorferi

Lyme disease is an important and increasing global public health concern due to climate change and human activities (Dumic and Severnini, 2018; Van Hout, 2018). This disease is caused by spirochetes of the *Borrelia burgdorferi sensu lato* species complex, which are transmitted by infected *Ixodes* ticks (Rudenko et al., 2011). *B. burgdorferi* spirochetes are acquired by larval ticks when these are feeding on reservoir hosts; they persist in the tick midgut through the molt into nymphs, subsequently migrating to the salivary glands (Caimano et al., 2016). During nymph feeding, *B. burgdorferi* is transmitted to a vertebrate host, where the pathogen is capable of adapting to species-specific environments, including available nutrient resources and immune responses, by sensing its surroundings and altering its gene expression through several small ncRNA (sRNA)-dependent regulatory systems (Radolf et al., 2012; Samuels, 2011). These sRNAs are typically 50–500 bp ncRNAs of three different types: riboswitches, which are located upstream of mRNAs; *cis*-acting sRNAs, synthesized from the complementary strand of an open reading frame (ORF); and *trans*-acting sRNAs, transcribed from the intergenic regions with only partial complementarity to their target genes (Gottesman and Storz, 2011). A recent study adopted a direct approach to identify virulence-determining sRNAs by RNA sequencing *B. burgdorferi* in both the tick vector and vertebrate host, hypothesizing that sRNAs are responsible for fine-tuning gene regulation throughout the enzootic cycle of the spirochete (Lybecker and Samuels, 2017). The first intergenic sRNA involved in the infectivity potential of *B. burgdorferi* was later described; that study characterized an intergenic *trans*-acting sRNA from *B. burgdorferi*, called ittA, and showed that it is essential for optimal infection in a mouse model (Medina-Pérez et al., 2020). The effect of this sRNA on transcriptional and proteomic profiles was also tracked, with RNA-seq analysis determining that 19 transcripts were differentially expressed in a genetically inactivated ittA mutant relative to its parental counterpart (Medina-Pérez et al., 2020). At the host level, it was demonstrated that over 200 genes and 38 miRNAs were differentially expressed following 48 h of *B. burgdorferi* infection (Casselli et al., 2017). Pathway analysis of transcriptional changes revealed gene categories that included developmental pathways, chromatin assembly, cell-cell adhesion, and immune system processes. The expression of a subset of transcription factors and lncRNAs also changed, suggesting that regulatory networks might be altered following infection, resulting in long-term changes to the transcriptome (Casselli et al., 2017).

Rickettsia

Rickettsial diseases are caused by obligate intracellular Gram-negative bacteria of the order *Rickettsiales* (Portillo et al., 2015). They are transmitted by different arthropods, including mites, ticks, fleas and lice, and are associated with both human and plant diseases (Perlman et al., 2006). Rickettsial diseases are febrile illnesses that have varied clinical manifestations ranging from mild and self-limiting disease to life-threatening multi-organ failure (Stewart et al., 2019). Schroeder and colleagues predicted the existence of ∼1700 sRNAs in 13 different *Rickettsia* species across all four disease groups (ancestral, spotted fever, transitional and typhus) and confirmed the expression and biogenesis of six sRNAs in *R. prowazekii* (Schroeder et al., 2015). A follow-up study from the same group identified and cataloged *R. prowazekii* sRNAs expressed during host cell infection (Schroeder et al., 2016). These ncRNAs were hypothesized to be potentially critical post-transcriptional regulators involved in bacterial virulence, survival, stress responses, metabolism and other pathways (Schroeder et al., 2016). The same team analyzed the *R. prowazekii* transcriptome by strand-specific RNA sequencing and identified 67 *cis*-acting (antisense) and 26 *trans*-acting (intergenic) sRNAs expressed during infection of *Amblyomma americanum* (AAE2) tick cells (Schroeder et al., 2017). Comparative expression profiling of endothelial cells and AAE2 cells during *R. prowazekii* infection revealed differential upregulation of >150 rickettsial genes in both endothelial cells and AAE2 cells, and yielded evidence of host-cell-dependent utilization of alternative transcription start sites by 18 rickettsial genes. These results suggested differences in the expression of both sRNAs and the coding transcriptome during the infection of human endothelial cells and arthropod cells (Schroeder et al., 2017). Further investigations are now required

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to elucidate the importance of these novel sRNAs in regulating rickettsial pathogenesis.

Chowdhury and colleagues provided the very first experimental evidence suggesting altered expression of pulmonary host cell ncRNAs and their involvement in the host defense response (Chowdhury et al., 2019). The profiling of the expression of host lung cell lncRNAs during infection of mice with *R. conorii*, simulating the pathogenesis of human spotted fever rickettsioses (Fig. 3), distinguished two potentially active and highly upregulated enhancer lncRNAs (elncRNAs), *NONMMUT013718* and *NONMMUT024103* (Fig. 3). Additional in-depth analyses concluded that the genomic loci of *NONMMUT013718* and *NONMMUT024103* might regulate the expression of nearby protein-coding genes (PCGs), namely inhibitor of DNA binding 2 (*Id2*) and apolipoprotein 10b (*Apol10b*), respectively (Chowdhury et al., 2019). The regulatory role of these elncRNAs was confirmed during *R. conorii* infection *in vitro*, with induced expression of *NONMMUT013718* (*Id2*) in murine macrophages and *NONMMUT024103* (*Apol10b*) in endothelial cells clearly visible (Chowdhury et al., 2019). As expected, shRNA-mediated knockdown of both elncRNAs reduced the expression of their endogenous target PCGs.

In another study, Narra and colleagues performed a high-resolution transcriptomic profiling that revealed the presence of novel ncRNAs in *R. conorii* during host–pathogen interactions (Narra et al., 2016). Their study showed that two trans-acting sRNAs (Rc_sR35 and Rc_sR42) are differentially expressed during host–pathogen and vector–pathogen interactions, indicating that they have a role in survival and transovarial or transstadial transmission in arthropod vectors and regulation of virulence in the human host (Narra et al., 2016).

Taken together, these results have revealed ncRNA-based regulatory networks in the modulation of host gene expression. Further in-depth mechanistic inquiries of these versatile biological mediators in host–pathogen crosstalk and pathogenesis should reveal new strategies to counteract bacterial infections.

ncRNAs in other vector-borne diseases

Trypanosoma

Chagas disease is a tropical parasitic disease caused by the protist *Trypanosoma cruzi*, which is transmitted by Triatominae vectors. When an infected triatomine feeds, it pierces the skin and takes in a blood meal, defecating at the same time to make room for the new meal. The bite is typically painless but causes itching; consequently, scratching it introduces the *T. cruzi*-laden feces into the bite wound, initiating infection (Bern, 2015). Symptoms are usually mild and nonspecific during the acute phase; during the chronic phase, most patients remain asymptomatic but are infected for life. However, 30% of people suffer from cardiac disorders and up to 10% suffer

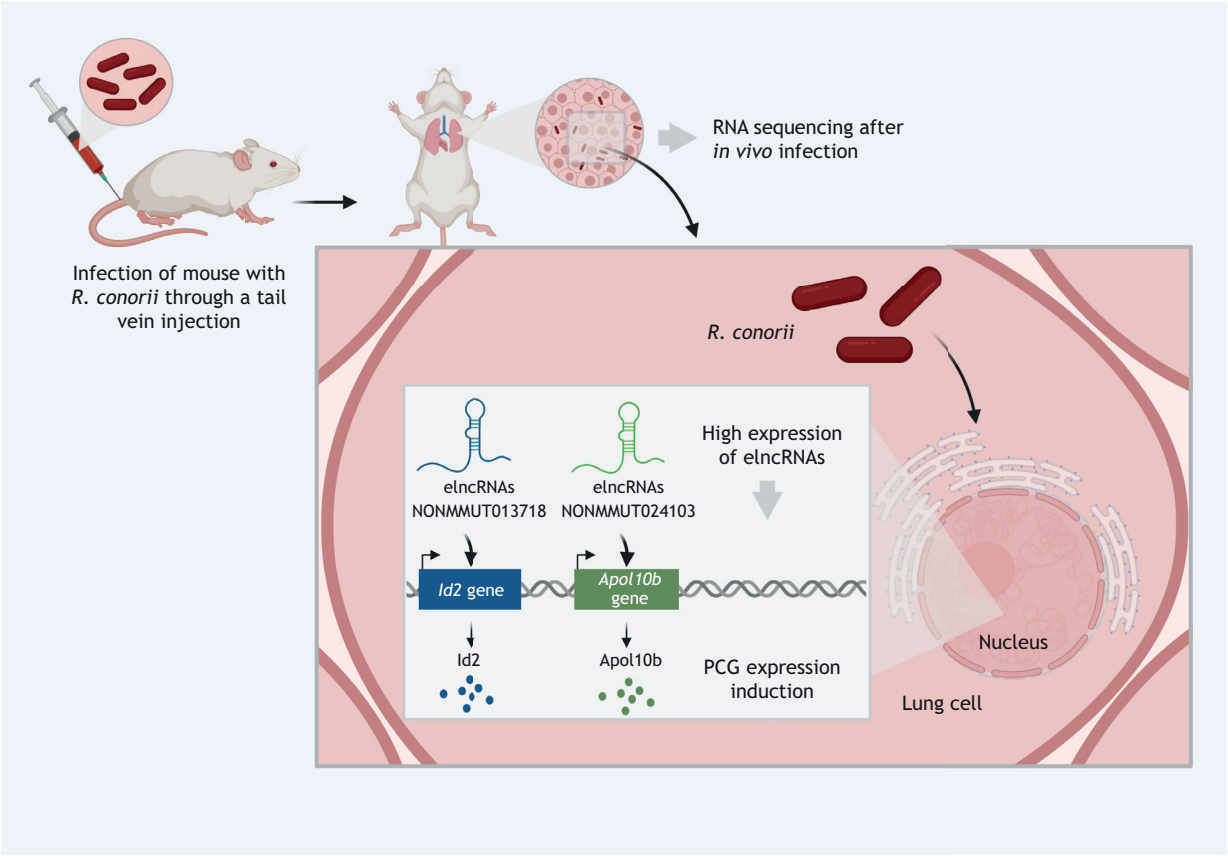

Fig. 3. Long non-coding RNA transcription and gene regulation in experimental models of rickettsial infection. The very first experimental evidence suggesting altered expression of pulmonary host cell ncRNAs and their involvement in the host defense response has only been recently obtained (Chowdhury et al., 2019). Here, mice were infected with high-dose *R. conorii* via tail vein injection. RNA sequencing of the lungs of infected hosts showed significant upregulation of two enhancer lncRNAs (elncRNAs), *NONMMUT013718* and *NONMMUT024103*. Additional experiments concluded that *NONMMUT013718* and *NONMMUT024103* induced expression of nearby protein-coding genes (PGCs), namely *Id2* and *Apol10b*, respectively. Thus, these results suggest that lncRNA-based regulatory networks contribute to the modulation of host gene expression and differentiation, as well as homing of T cells.

from digestive, neurological or mixed disorders (Teixeira et al., 2011). Through the analysis of the short transcriptomes of *T. cruzi*, several types of small RNAs have been identified, such as small RNAs derived from transfer RNAs (tRNAs) and small RNAs derived from ribosomal RNAs (Franzén et al., 2011). Subsequently, it has been shown that these RNAs can be delivered to other parasites and to mammalian-susceptible cells in EVs to promote metacyclogenesis and confer susceptibility to infection (Garcia-Silva et al., 2014). Changes in gene expression of host HeLa cells induced by extracellular vesicles from *T. cruzi* leads to the modification of the host cell cytoskeleton, extracellular matrix and immune responses pathways. Some genes are also modified by the most abundant tRNA-derived small RNAs in the EVs (Garcia-Silva et al., 2014). These data suggest that microvesicles secreted by *T. cruzi* could be relevant players in early events of the *T. cruzi*–host cell interplay. Sleeping sickness (African trypanosomiasis) is caused by *T. brucei*, mainly transmitted by the tsetse fly, which is found predominantly in sub-Saharan Africa. *T. brucei* develops as a chronic infection in mammalian hosts due to a sophisticated strategy of antigenic variation of its variant surface glycoprotein (VSG), which coats the parasite in order for it to escape antibody-mediated lysis (Mallick et al., 2008). mRNAs corresponding to VSG can be targeted by miRNAs secreted by *T. brucei* (Mallick et al., 2008). Furthermore, several miRNA hairpins have been found in clusters of multiple identical copies, targeting specific proteins (20S proteosome, GM6 and GRESAG 4.2), which demonstrates that these clustered miRNAs play an essential role in trypanosomiasis (Mallick et al., 2008). This mechanism can act as a genetic switch to modulate host–parasite interactions and may be exploitable as a therapy by providing useful clues towards the treatment of trypanosomiasis (Mallick et al., 2008).

Yersinia pestis

Yersinia pestis can infect humans via the Oriental rat flea *Xenopsylla cheopis* and cause the plague; it has been responsible for multiple epidemics throughout human history, and continues to adversely impact public health (Perry and Fetherston, 1997). Under pathogenic conditions, *Yersinia* induces the expression of multiple virulence genes that modulate the host immune response and promote pathogen survival (Cornelis, 2002). A total of 63 novel putative sRNAs were identified through deep sequencing of the *Y. pestis* sRNA-ome. Among them was the *Yersinia*-specific Ysr141 (Yersinia small RNA 141, also present in *Y. pseudotuberculosis* and *Y. enterocolitica*) (Schiano et al., 2014). Besides the expression of virulence genes, *Yersinia* expresses sRNAs that have been linked to virulence regulation. By using deep sequencing to compare sRNA expression in intracellular *Y. pestis* in the human macrophage cell line THP-1 to extracellular *Y. pestis* in the presence of THP-1 cells, several sRNAs were identified that are likely to function in virulence (Li et al., 2016). This study showed that knockdown of the sRNA Ysr170 attenuated infection efficiency in cell culture, reduced bacterial cell growth rate in response to stress and enhanced host immune responses during infection. Such results suggest a potential therapeutic use for molecules targeting functional sRNAs required for pathogen virulence (Li et al., 2016).

Overall, ncRNAs play vital roles in vector-borne infections. They participate in both pathogen developmental processes and host–parasite interactions. Advances in sequencing technologies and functional characterization have revealed many novel ncRNAs and implicated several of these RNAs in aspects of gene regulation in *Trypnosoma* or *Yersina*. However, most ncRNA candidates require further characterization in order to discern their function, especially in the infectious context.

Conclusion and perspectives

The diverse epidemiology and adaptive plasticity of the pathogen and the arthropod make vector-borne diseases difficult to control and even harder to eradicate. Therefore, there is an urgent need for a comprehensive understanding of the vector–host–pathogen triad at the molecular level. Understanding the complex events involving ncRNAs that occur during the transmission of arthropod-delivered pathogens at the host interface is expected to pave the way for the development of successful strategies to prevent or control vector-borne diseases. Pathogen ncRNAs are just starting to be discovered, as are their roles in pathogen–host interactions, but their main mechanism(s) of action are still poorly understood. For instance, sfRNA from flaviviruses appears to act as a sponge for host RNA-binding proteins, which affects host cell ncRNA expression and might limit the ability of the cell to effectively respond to the viral infection. Virulence gene regulation is also associated with subtelomeric lncRNAs from *Plasmodium*, which are suggested to play an important role in telomere maintenance and host gene regulation (Broadbent et al., 2011). Nevertheless, the mechanism involved in *P. falciparum*-mediated transcriptional regulation is still unknown. In contrast, *B. burgdorferi, Rickettsia*, *Yersinia pestis* and *Trypanosoma* appear to use several sRNAs for virulence regulation (Li et al., 2016; Villa et al., 2018). sRNAs, including riboswitches, *cis*-acting sRNAs and *trans*-acting sRNAs, from *B. burgdorferi* are hypothesized to be responsible for fine-tuning gene regulation throughout the enzootic cycle of the spirochete (Villa et al., 2018). Similarly, several sRNAs from *Rickettsia* and *Trypanosoma* have been linked to post-transcriptional regulators of virulence, growth, stress responses, metabolism and other pathways (Li et al., 2016; Villa et al., 2018).

Our understanding of the regulatory functions of ncRNAs in vector–host–pathogen interactions keeps improving as a result of the availability of high-throughput sequencing and tiling array technologies. In this Review, we discussed recently described ncRNAs from pathogens and their role in host–pathogen interactions in the context of vector-borne infections. However, there are several questions that remain unanswered. For example, ncRNA functions seem to be regulated in a spatiotemporal manner. However, the experiments performed thus far have not suggested a time-dependent effect or localization-dependent function for ncRNAs. Furthermore, ncRNAs have so far only been studied in vectors, pathogens or hosts separately. To date, no integrative study has been performed. Therefore, it might be possible that there are unknown ncRNAs whose expression levels do not change during infection but function by changing their localization. The techniques used so far can also generate bias in the results. Next-generation sequencing analyses using bulk (mixed population) samples disregard the heterogeneity of host cells or differences in the infection stages of the host cells, which increases the difficulty in identifying functional ncRNAs whose expression levels differ between each host cell. Even with new single-cell RNA-seq technology, sufficient read depths of rarely expressed RNAs are still missing, and vector, host or pathogen-derived RNA information could easily be lost. Thus, new integrative bioinformatics tools need to be developed to consider ncRNA specificity and, more importantly, their interactions with potential targets. At the experimental level, specific expression conditions of ncRNAs, whether from the pathogen, vector or host, can also produce misleading interpretations about the potential functional ncRNAs encoded in the genome. Overall, given the increasing data on the ability of regulatory RNAs produced by pathogens to regulate host gene expression, and also the possible regulatory function of

cellular RNAs on the pathogen, the role of RNAs in the vector or host–pathogen crosstalk might be even more sophisticated than previously anticipated.

Competing interests
The authors declare no competing or financial interests.

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