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Étude du mécanisme de régulation des alphavirus par les microARN cellulaires

Rôle de miR-124 dans l'infection par les alphavirus

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List of abbreviations

Ago	Argonaute
CHIKV	chikungunya virus
CLIP	crosslinking and immunoprecipitation
CNS	central nervous system
CP	capsid protein
dsRNA	double stranded RNA
EEEV	eastern equine encephalitis virus
GFP	green fluorescent protein
gRNA	genomic RNA
hNPCs	human neural progenitor cells
IFN	interferon
IP	immunoprecipitation
miRNA	microRNA
MOI	multiplicity of infection
mRNA	messenger RNA
nsP	non-structural protein
ONNV	O'nyong-nyong virus
ORF	open reading frame
RNA	ribonucleic acid
RRV	Ross river virus
SAV	salmonid alphavirus
sgRNA	subgenomic RNA
SINV	Sindbis virus
SPDV	salmon pancreas disease virus
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
WEEV	western equine encephalitis virus
WT	wild-type

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INTRODUCTION

I. INTRODUCTION

CHAPTER I. Alphaviruses as emerging and re-emerging pathogens

A. Alphaviruses

Alphavirus is the only genus belonging to the *Togaviridae* family. It represents viruses belonging to group IV of the Baltimore classification, namely RNA viruses with a genome in the form of a single-stranded positive-sense RNA molecule of between 10 to 12 kb in length containing two open reading frames (ORFs). Historically, the genus *Rubivirus* was also included within the *Togaviridae* family. However, due to differences in the mode of transmission, particle structure and genomic sequences and organization, it was recently moved to the *Matonaviridae* family (Chen et al., 2018). The *Alphavirus* genus contains up to 31 recognized species and is divided into at least 8 different complexes according to the antigenicity (Knipe and Howley, 2013).

Alphaviruses are arthropod-borne viruses, transmitted by different invertebrate vectors to vertebrate hosts. Depending on the virus, alphavirus infection can be associated with arthritic disease, rash and fever as is the case for chikungunya virus (CHIKV), Ross river virus (RRV) or Sindbis virus (SINV) in humans. Otherwise, it can be associated with neurological disease such as encephalitis caused by western, eastern, and Venezuelan encephalitis virus (WEEV, EEEV and VEEV) (Knipe and Howley, 2013).

Vertebrates such as humans, non-human primates, equids, rodents, reptiles, salmonids and birds represent the natural host of alphaviruses. However, not all of them are supposed to participate in the amplification and transmission of the virus. In amplifying hosts, typically birds, the infection is mainly asymptomatic while the virus replicates to high levels thereby allowing efficient uptake and transmission by the invertebrate vector. Usually, vectors are mosquitos, including *Aedes spp., Culex spp*. or *Anopheles spp*., but some alphaviruses have also been isolated in other arthropods such as lice or mites (Reeves et al., 1947; La Linn et al., 2001), suggesting a possible involvement in transmission. Mammal infections by these viruses happen due to spillover from the mosquito vector during blood-feeding. In addition to these cases of circulating

viruses, there are a few examples of alphaviruses that are restricted to one host. The insect-specific alphaviruses include Eilat virus (EILV) (Nasar et al., 2012), Agua Salud alphavirus (ASALV) (Hermanns et al., 2020) and potentially the EILV-related alphaviruses Taï Forest virus (TALV) (Hermanns et al., 2017) and Mwinilunga alphavirus (MWAV) (Torii et al., 2018). Finally, a number of alphaviruses form the group of aquatic alphaviruses and these include the southern elephant seal virus (SESV) (La Linn et al., 2001) and the salmonid alphaviruses (SAV) causing pancreas disease and sleeping disease to Atlantic salmon and rainbow trout (Deperasińska et al., 2018). Interestingly, the SAV do not require an invertebrate vector for transmission, viruses are transmitted horizontally by the water (Taksdal et al., 2015).

1. Origins of alphaviruses

1. 1. History

The first alphaviruses to be linked to the development of severe disease were the encephalitic viruses. In the early 1930s, WEEV, EEEV and VEEV were isolated from the brain of horses displaying symptoms of encephalitis in California, Virginia and New Jersey and Venezuela respectively. By then, it was already known that these viruses were transmitted by mosquitos. The arthritogenic viruses were not described until 1953 when CHIKV was first isolated from the blood of patients with severe arthritis in Tanzania and thus identified as the causative agent of this type of disease, rendering it the first alphavirus linked to such pathology (Knipe and Howley, 2013).

According to the geographical location of the first appearances of their infections, these viruses were first organized into two groups, “Old world” alphaviruses, mainly responsible of causing arthritic disease and “New world” alphaviruses, responsible of infections of the central nervous system leading to encephalitis. However, the associated pathology does not necessarily correlate with the geographical distribution of certain alphaviruses.

1. 2. Classification

Phylogenetic analysis based on sequence similarities of a region of the structural protein ORF encoding the envelope protein allowed the organization of alphaviruses in different complexes (Figure 1). EEE and VEE complex include viruses clustering together from the same clade. WEEV is more divergent and is believed to have arisen from a common ancestor by recombination of nsPs and capsid from a EEEV-like virus with a Sindbis-like Old World virus and thus forming a new complex (Weaver et al., 1993). Most of the arthritogenic alphaviruses are part of the Semliki Forest complex. While Middelburg virus, Ndumu virus and Barmah forest virus are genetically related to these viruses, they are considered to be part of their own individual subclades since their antigenic properties do not correspond to the previously defined complexes (Chen et al., 2018).

In terms of origin of these viruses, several hypotheses have been proposed. It was first stated that the striking genetic diversity of salmon pancreas disease virus (SPDV) compared to the other viruses could link the alphaviruses to a possible ancestral marine origin followed by emergence in terrestrial hosts. Due to the great capacity of adaptation of alphaviruses to different kind of vectors and a wide range of vertebrate hosts, the introduction to terrestrial hosts allowed a wider distribution of these viruses (Forrester et al., 2012). On the other hand, the divergence of SPDV and related SAV could be also due to a different adaptation to the aquatic hosts compared to the other alphaviruses due to the loss of genomic traits necessary for survival with the arthropod vector transmission to the vertebrate host (Chen et al., 2018).

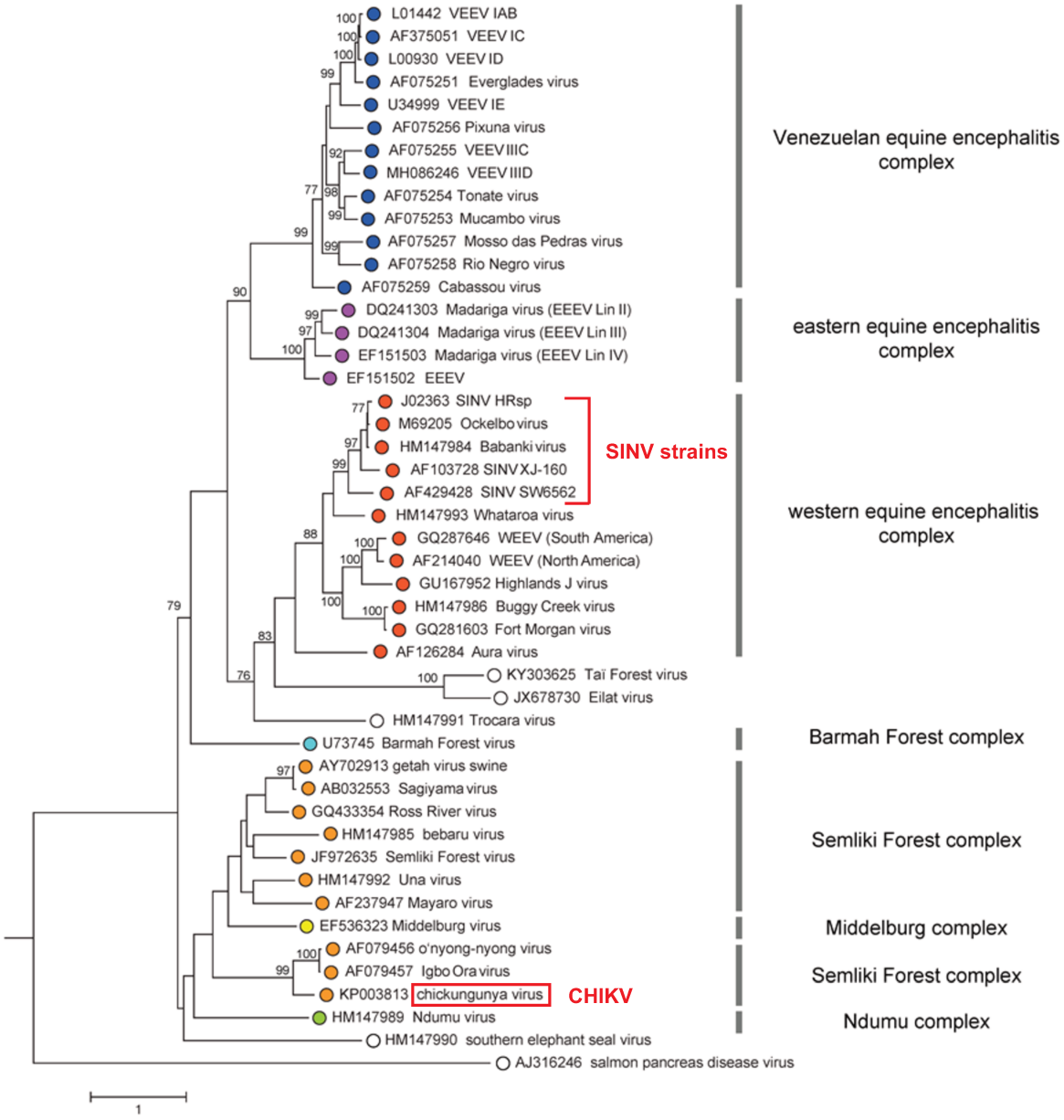

Figure I.1. Alphavirus phylogenetic tree and complexes.
Phylogenetic tree is based on nucleotide sequence alignment of conserved structural proteins region encoding the envelope protein. (Adapted from (Chen et al., 2018)).

2. Arthritogenic alphaviruses

Among the arthritogenic alphaviruses, the most studied and of medical interest is CHIKV. There have been two important epidemics associated with this virus extending worldwide and causing millions of cases. The first one began in 2004 in Kenya and spread within two years to several islands in the Indian Ocean, including the Seychelles and La Réunion, before arriving to the south-east Asia and southern Europe. In 2013, a second epidemic originated in the Caribbean Sea and spread towards the American continent. Besides CHIKV, other arthritogenic alphaviruses have caused sporadic outbreaks generally restricted in one geographical area. O'nyong-nyong virus (ONNV), Ross River virus (RRV) and Mayaro virus (MAYV) have remained endemic in Africa, Australia and South America respectively (Levi and Vignuzzi, 2019).

The infections are in most cases symptomatic with an acute disease associated generally with fever, polyarthralgia and rash. The word "chikungunya" comes from the Kimakonde and means "that which bends up", referring to the postures adopted by infected patients. Chronic manifestations have been reported following CHIKV infection, which leads to an overall debilitating disease most probably due to the inflammatory response resulting from the infection. In the most severe cases, CHIKV can also cause complications in the central nervous system (CNS) and encephalitis, especially in infants and elderly people (Gérardin et al., 2016).

Alphaviruses are usually transmitted by different vectors, however CHIKV has been shown to present adaptive mutations allowing it to be transmitted by other vectors, thereby increasing the efficiency of its spreading to other geographical areas (Weaver et al., 2020). Indeed, while *Aedes aegypti* is the main vector, a single amino acid mutation within E1 allowed adaptation to *Aedes albopictus*, present in rural areas and established in the American continent, Africa and Europe (Vazeille et al., 2007). This ability to evolve and improve transmission makes these viruses a public health issue and requires constant surveillance and preparation in case new outbreaks re-emerge.

3. Encephalitic alphaviruses

Encephalitic alphavirus infection is characterized by an onset of flu-like symptoms and a febrile illness that can later develop into the encephalitic disease. WEEV is typically transmitted from the natural host, birds, to horses and humans. Human infection is in the majority of cases asymptomatic but can cause important neurological damage, especially in children (Ronca et al., 2016). Similarly, VEEV infection rarely leads to severe neurologic disease in adults, while children are at risk of developing it. Interestingly, VEEV is also transmitted by mosquitos, however human to human transmission can eventually happen trough aerosols. Finally, EEEV is considered as the deadliest encephalitic alphavirus with the highest fatality rate estimated at 50-75% (Zacks and Paessler, 2010).

The strategy used by alphaviruses to reach the CNS is not completely understood. They are able to disseminate from the first site of infection after the mosquito bite towards the CNS by the bloodstream (Vogel et al., 2005). To establish infection in the brain, alphaviruses are able to cross the blood-brain barrier (BBB), which is formed of brain microvascular endothelial cells (BMEC), pericytes and neuronal cells. VEEV has been proposed to first use peripheral neurons as an entry route by propagating through olfactory neurons, in order to establish infection in the brain and induce opening of the BBB allowing direct entry of a second wave of infectious particles (Schafer et al., 2011). Recent studies supporting this hypothesis showed that the integrity of the BBB is in fact not compromised at early times of infection because IFN signalling inhibits replication in BMECs. Thus to cross the BBB, these viruses exploit caveolae, which are invaginations within BMECs and access the brain by caveolae-mediated transcytosis of free particles (Salimi et al., 2020).

4. Viral particle and genome structure

Alphaviruses are enveloped spherical viruses. The nucleocapsid is formed of the viral genome associated with a total of 240 capsid proteins monomers following a T=4 icosahedral symmetry. The envelope is made of a host-derived bilipid membrane

embedded with 80 glycoprotein spikes organized in trimers of E1 and E2 heterodimers. The virion has a size of 60 – 70 nm in diameter (Figure 2).

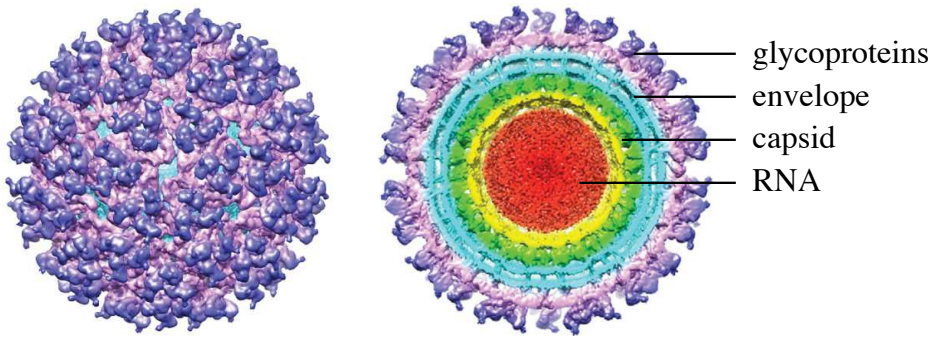

Figure I.2. Cryo-EM reconstruction of SINV viral particle at 7 Å resolution.
(A) SINV viral particle outer structure. (B) Cross-section of SINV viral particle. Glycoproteins (purple) are at the surface of the particle. The envelope (cyan) containing the nucleocapsid formed of the capsid protein (green) and the viral RNA (red). (Adapted from (Tang et al., 2011)).

The 49S genomic RNA is capped on the 5' end with a type 0 cap structure (7-methyl-GpppA) (Hefti et al., 1976) and polyadenylated on the 3' end. The genome is structured with two open reading frames (ORF) placed between a 5', an intergenic and a 3' untranslated regions (UTR). The UTRs are mainly regulatory regions that have a role during different viral cycle steps such as viral replication, translation of viral proteins as well as in evasion of host defense mechanisms or adaptation between the different vertebrate and invertebrate hosts (Hyde et al., 2015).

Both of the ORFs are translated into polyproteins that are processed by viral as well as cellular proteases in order to obtain the individual mature viral proteins. The first ORF (ORF1) represents about two thirds of the viral genome and codes for the non-structural proteins (nsP1-nsP4), involved in viral RNA replication and host defense evasion (Abu Bakar and Ng, 2018). The second ORF (ORF2) encodes the structural proteins allowing the encapsidation of newly synthesized genomic RNA and the formation of new viral particles: the capsid (CP), the envelope proteins (E3, E2 and E1)

and 6K (Figure 3). The structural proteins are expressed from a 26S subgenomic RNA (sgRNA), which corresponds to the 3' end of the genome containing only ORF2.

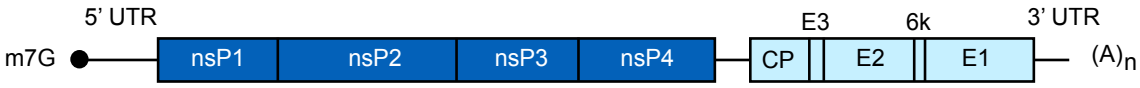

Figure I.3. Schematic representation of SINV genomic organization.
The two ORFs are flanked by 5' and 3' UTRs. Adapted from (López et al., 2020).

B. Genomic elements and viral proteins

1. 1. The 5' and 3' UTR

The sequence and length of 5' and 3' UTRs are quite diverse among the different alphaviruses. However, they share common functions and structural elements. Both UTRs contain promoter regions for negative- and positive-strand RNA synthesis during replication (Frolov et al., 2001; Hardy, 2006). Furthermore, an interaction between both ORFs is needed for negative-strand RNA synthesis. In addition to these regulatory functions, the 5' UTR can also interact with host factors such as the ISG Ifit1 to antagonize the antiviral response (Hyde et al., 2014). Translation factors such as eIF4E and eIF4F can bind the 5' UTR of the subgenomic RNAs to enhance viral protein translation during host translational shutoff.

The 3' UTR is important for viral RNA stability by repressing deadenylation (Garneau et al., 2008) or interacting with host factors such as the RNA-binding protein HuR (Sokoloski et al., 2010). Given the similarity of viral genomic RNA to cellular mRNAs, the 3' UTR can also be a potential target of cellular microRNAs, as is the case for the hematopoietic specific miR-142-3p, which binds to EEEV genomic RNA and modulates viral replication (Trobaugh et al., 2014). These aspects of regulation will be further discussed in Chapter III of the Introduction.

1. 2. Non-structural proteins

Non-structural proteins are the first viral proteins to be synthesized at the beginning of the cycle and have essential functions in the viral replication process (Rupp et al., 2015).

NsP1 is the viral capping enzyme. It has guanine-7-methyltransferase and guanylyltransferase activities responsible of the addition of a cap to viral RNAs allowing translation of viral proteins and protection of viral RNAs from degradation by cellular exonucleases. Furthermore, nsP1 is also involved in the anchoring of replication complexes to lipids in the cellular membranes during viral replication within viral replication factories thanks to the presence of a palmitoylation site and an amphipathic helix in nsP1 (Spuul et al., 2007).

The most essential function of nsP2 is the protease activity necessary for the processing of ns-polyprotein precursor. This is mediated by the papain-like cysteine protease domain present in the C-terminal domain of nsP2 (Vasiljeva et al., 2001). The N-terminal of nsP2 has a helicase domain, necessary for unwinding RNA secondary structures during replication. The helicase activity is promoted by the nucleoside triphosphatase (NTPase) activity of this domain. In addition, nsP2 initiates the viral RNA capping process by acting as an RNA triphosphatase before nsP1-mediated addition of the 5' cap. Finally, nsP2 is considered as a virulence factor for its involvement in mediating the cellular transcriptional and translational shut-off (Gorchakov et al., 2005). Furthermore, it is also involved in the evasion of innate immune response by suppressing type I interferon response (Frolova et al., 2002; Breakwell et al., 2007).

The specific function of nsP3 during alphavirus infection has not yet been fully elucidated. The protein is divided in three different domains: a macrodomain, an alphavirus unique domain (AUD) and a hypervariable region (HVR). The later has been implicated in the interaction with several host proteins such as the stress granule proteins G3BPs, important cofactors of viral replication. Furthermore, nsP3 is also a component of the viral replicase complex. It is necessary for negative-sense RNA and sgRNA synthesis although its precise function has still to be deciphered (Götte et al., 2018).

Finally, nsP4 is the RNA-dependent RNA polymerase (RdRp), the principal component of the replicase complex driving viral replication and the sole viral protein capable of RNA synthesis activity (Rubach et al., 2009).

1. 3. Structural proteins

The capsid protein directly interacts with the RNA genome to form the nucleocapsid core through an RNA binding domain localized in the N-terminal region (Mendes and Kuhn, 2018). The C-terminal region possesses a serine protease activity allowing the CP to autocatalytically cleave itself from the polyprotein precursor.

E3, E2 and E1 are the glycoproteins forming the spike. E3 drives the relocalization of the polyprotein precursor for processing and is involved in viral particle assembly (Parrott et al., 2009). With the exception of Semliki forest virus, E3 is not generally part of the spikes and is not incorporated into the mature virion (Vénien-Bryan and Fuller, 1994). E2 and E1 have very important roles in the early steps of infection. E2 N-linked glycosylation sites are involved in receptor and heparan sulfate binding. E1 mediates membrane fusion between the viral envelope and the cellular plasma membrane upon entry allowing release of the nucleocapsid into the cytoplasm (Kielian et al., 2010).

Initially, the 6K protein was thought to be incorporated into the virion. A slippery sequence present in the 6K coding sequence induces a -1 frameshift on protein translation. This rare event gives rise to a transframe form (TF) of 6K and avoids translation of E1 (Firth et al., 2008). Once the frameshift leading to TF was discovered, it turned out that the latter is actually the one present in the virion. The specific functions of these two individual proteins are still a topic of investigation (Snyder et al., 2013). TF has been shown to possess a palmitoylation site regulating its localization to the plasma membrane and is involved in the incorporation of this protein into the virion influencing budding (Ramsey et al., 2017). As for 6K, it is considered a viroporin, regulating ion levels to allow viral release (Gonza´lez and Carrasco, 2001). The role of these proteins has been associated with virulence, glycoprotein processing and viral particle assembly and budding but the exact mechanisms have not been understood yet (Jolene Ramsey and Suchetana Mukhopadhyay, 2017).

C. Viral cycle

1. 1. Attachment and entry

The broad tropism characterizing alphaviruses suggests that the different viruses adapt to different entry receptors. Alphaviruses mainly use ubiquitously expressed receptors to initiate the viral cycle (Figure 4). Different molecules such as class I major histocompatibility complex (MHC-I), the high-affinity laminin receptor, DC-SIGN or heparan sulphates have been described as important for binding but not used exclusively. NRAMP seems to be a SINV-specific receptor in mosquito as well as mammalian cells (Rose et al., 2011). Recently, Mxra8 was identified as a receptor for several arthritogenic alphaviruses such as CHIKV or RRV (Zhang et al., 2018).

After attachment of E2 to the receptor, the viral particle enters the cell by clathrin-mediated endocytosis. The acidic pH of the endosome triggers membrane fusion by inducing conformational changes and rearrangements of the glycoproteins. First, the spike's heterodimers dissociate and E1 homotrimerization induces fusion of the viral envelope and the cellular membrane, which allows release of the nucleocapsid into the cytoplasm (Kielian et al., 2010; Leung et al., 2011). After release of the nucleocapsid, the viral genomic RNA is uncoated by the action of ribosomes (Wengler et al., 1992).

1. 2. Viral protein synthesis and replication

The replication cycle of alphaviruses takes place entirely in the cytoplasm of the infected cell but in association with the cellular membranes (Figure 4). The infection induces important membrane rearrangements known as spherules (Frolova et al., 2010). In these spherules, viral replication takes place in a partially sequestered manner adding some protection to double-stranded RNA (dsRNA) intermediates from cellular sensing and activation of the innate immune response.

First, the non-structural proteins are synthesized directly from the 49S genomic RNA (gRNA). ORF1 is expressed in a cap-dependent manner to synthesize two polyprotein precursors: either polyprotein P123 or, P1234 produced after readthrough of

an opal stop codon after nsP3. However, the later form of the polyprotein is present in a lesser amount limiting nsP4 production. SFV and ONNV lack this opal stop codon and are the only cases of alphaviruses that produce exclusively P1234 (Strauss and Strauss, 1994). After proteolytic cleavage by nsP2 in *cis*, nsP4 is released and together with P123 forms the replication complex leading to the synthesis of a negative-sense viral RNA. This process leads to the production of a double-stranded RNA intermediate. Subsequent processing of nsP1 from the polyprotein and formation of a nsP1-P23-nsP4 complex allows the transition to positive-sense RNA synthesis. Subsequent processing of nsP2 and nsP3 leads to the replication complex formed of all individual nsPs giving place to the production of new copies of gRNA (Lemm et al., 1994).

An internal promoter present on the negative-sense RNA, within nsP4 coding region and the intergenic UTR, allows expression of the subgenomic RNA (sgRNA) (Ou et al., 1982; Levis et al., 1990). The sgRNA is capped at the 5' end, polyadenylated at the 3' end and ORF2 is flanked by a 5' and 3' UTR. The sgRNA is the most abundant viral RNA species during infection and allows translation of the structural proteins in the form of a polyprotein precursor.

Interestingly, some alphaviruses such as SINV and SFV possess a hairpin stem-loop structure in the sgRNA, within the CP coding region, that allows eIF2α-independent translation of structural proteins during translational shut-off after PKR activation. The structure known as the downstream loop (DLP), later renamed downstream stable hairpin (DSH), is placed 27 nucleotides downstream of the capsid initiation codon and makes translation possible by allowing direct entry of the ribosome and defining the correct AUG initiation codon for translation. Furthermore, this structure has been proposed to be one of the acquired genetic traits that allowed alphavirus adaptation from insects to vertebrate host. In agreement with this hypothesis, DSH loss-of-function studies showed that viruses evolve to reacquire a similar structure at the same position allowing eIF2α independence (Ventoso, 2012). However, this structure is not conserved among all alphaviruses.

The capsid is the first protein to be released from the polyprotein precursor by autocatalytic cleavage. The CP will then be able to interact with the genomic RNA to form the nucleocapsid. Genome packaging starts with the interaction of capsid protein with an encapsidation signal present in the genomic RNA, within nsP1 or nsP2 coding regions depending on the alphavirus, therefore allowing specific encapsidation of the 49S gRNA and not the 26S sgRNA (Weiss et al., 1989; Kim et al., 2011).

1. 3. Virion assembly, budding and egress

After release of the CP, the rest of the structural polyprotein precursor is relocated to the endoplasmic reticulum (ER) lumen due to the presence of a peptide signal in the N-terminal region of the E3 glycoprotein. The polyprotein is first cleaved by cellular signalases to give rise to 6K, E1 and E3-E2 precursor (pE2). E1 and E2 still in the form of pE2 dimerize and finally pE2 is the cleaved by furin protease in the *trans*-Golgi system before translocation of individual proteins to the plasma membrane.

Budding of nucleocapsids starts after interaction of CP with the cytoplasmic domain of E2 glycoprotein. Thus, newly formed viral particles obtain their cell-derived envelope from the cellular plasma membrane.

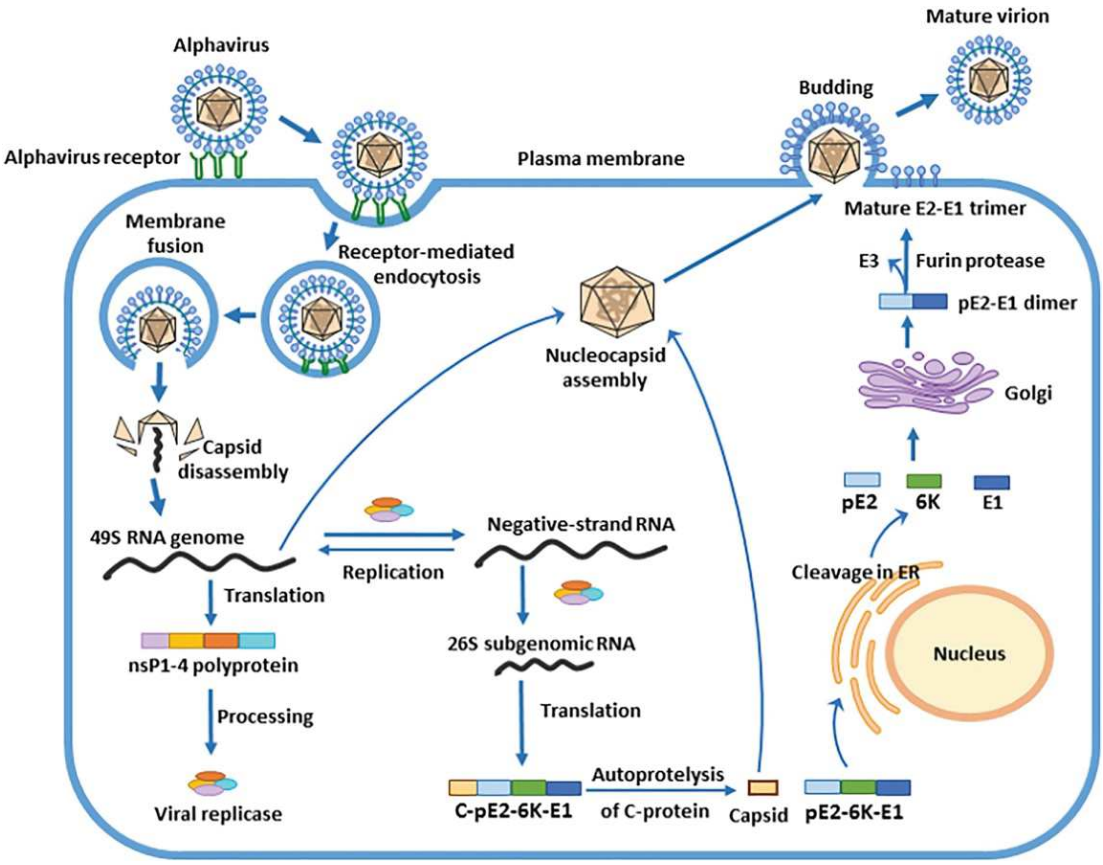

Figure I.4. Alphavirus life cycle.
After attachment to the cellular receptor, the infecting viral particle enters the cell by clathrin-mediated endocytosis. The viral envelope fuses with the endosome's plasma membrane releasing the viral nucleocapsid to the cytoplasm where the viral RNA is uncoated. Viral nsPs are translated directly from the viral genome forming the replication complex responsible of negative- and positive-strand RNA synthesis as well as sgRNA, encoding the viral structural proteins. The capsid protein assembles with newly synthetized genomic RNAs while the rest of the glycoprotein precursor undergoes several processing steps at the ER and later at the Golgi. Assembly of nucleocapsids and with mature glycoproteins at the plasma membrane allows budding for envelope acquisition and release of the new viral particles. From (Abdelnabi et al., 2020).

D. Sindbis virus – our model of study

1. Background

The name of the virus comes from the place where it was first collected in 1952, in the little town of Sindbis near Cairo, Egypt. It was isolated from *Culex spp*. mosquitos (Taylor et al., 1955), but it was not until many years later that SINV was related to human

infection and isolated from skin lesions (Malherbe et al., 1963). It is responsible of recurrent outbreaks in South Africa, Australia or northern Europe. It has been associated with Karelian fever, Ockelbo and Pogosta disease in Russia, Sweden and Finland respectively (Trent et al., 1988). SINV is one of the most widespread alphaviruses. The natural amplification host are birds particularly grouse in Finland and thrushes in Sweden (Lundström et al., 2001; Jalava et al., 2013), but SINV has also been isolated in crows in Germany (Eiden et al., 2014). Furthermore, migratory birds have also been proposed to contribute to the spread of the virus. The primary vector is *Culex spp*., nevertheless SINV has also been isolated in Culiseta and in Aedes mosquitos (Francy et al., 1989; Hesson et al., 2015; Lundström et al., 2019).

Very interestingly, pathogenesis in humans leads to the development of arthritic disease while infection in mice can lead to encephalitis, making SINV an excellent model for alphavirus studies.

2. Sindbis virus as a prototypical alphavirus

Since the discovery of alphaviruses, a great amount of efforts have been made to understand the molecular mechanisms of alphavirus infection. From early times, SINV has been the prototypical model of alphavirus used in many of these studies. This has been possible due to the ability of SINV to replicate efficiently in a wide variety of laboratory cell lines, of both insect and mammalian origin. These studies led to the discovery of most of the above mentioned molecular mechanisms of the alphavirus life cycle (Strauss and Strauss, 1994).

In addition, infection is also productive in animal models. Many studies have been conducted in mice with wildtype as well as neurovirulent SINV strains leading to important discoveries regarding the development of neurologic disease. Mice studies have been extremely useful for the understanding of neurovirulence determinants, innate immune responses to alphavirus infection as well as mechanisms leading to viral clearance (Atkins and Sheahan, 2016). Moreover, a model of SINV infection of zebrafish has also been successfully established for studies on the entry route of alphaviruses to the CNS (Passoni et al., 2017).

3. Pathogenesis in humans (arthritis) vs. mice (encephalitis)

The symptoms associated with SINV infection in humans are generally fever, rash, arthritis and myalgia. Following the mosquito bite, SINV is able to infect epithelial cells, leading to the skin lesions, fibroblasts and neighbouring tissues to the mosquito bite like muscle cells. Eventually, viremia is achieved and SINV can spread through the bloodstream to secondary sites of infection like joints, typically in the ankles (Adouchief et al., 2016). Symptoms associated with the infection are indeed due to cell damage caused by the virus but also as a consequence of the immune response triggered to clear the infection.

On the contrary, SINV infections in mice models are characterized by an age-dependent fatal neurological disease. While all new-born mice suffer from fatal encephalitis upon SINV infection, older mice develop encephalitis depending on the presence of virulence determinants in the SINV strain used (Tucker et al., 1993).

SINV neurovirulent strains have been developed in the laboratory in order to get more insight into the establishment of neuropathogenesis. In strains obtained by several passages in mice CNS, the mutations responsible for this adaptation were mapped to one residue (His-55) in E2 glycoprotein and two residues (Ala-72 and Asp-313) in E1 glycoprotein (Lustig et al., 1988). In fact, a His residue at position 55 in E2 increases binding to neuronal cells participating thus in the increased neurovirulence (Lee et al., 2002). Furthermore, recombinant neurovirulent SINV strains with His-55 have been shown to strongly enhance apoptosis induction in older mice's brains (Lewis et al., 1996).

In addition, the 5' UTR is also implicated in neuroinvasiveness (Dubuisson et al., 1997). Finally, studies comparing a natural neurovirulent strain, AR86 and the avirulent Girdwood strain identified also important virulence determinants in the nsP coding region such as a deletion of a region of nsP3 or the mutation of Ile-538 to Thr in nsP1 (Suthar et al., 2005).

CHAPTER II. Regulation of gene expression by microRNAs

A. Discovery of miRNAs, classification and overview of their role

The discovery of microRNAs (miRNAs) in animals arises from studies performed in *C. elegans* on the role of the lin-4 and let-7 genes in developmental larval stages transition. The products of these genes were identified as the source of small RNAs, of about 20 nucleotides in length, with complementary sequences on the 3' UTR of target mRNAs, thereby allowing their regulation at the translational level. The lin-4 small RNA was first identified as an essential regulator in early larval stages by downregulating LIN14 protein levels (Lee et al., 1993). The let-7 RNA was later shown to be expressed in late larval stages and to promote transition to adult cell fate by inhibiting LIN41 protein expression (Reinhart et al., 2000; Slack et al., 2000).

It was later discovered that let-7 was conserved in the animal kingdom (Pasquinelli et al., 2000), which prompted the search for other examples of such small regulatory RNAs. This led to the seminal discovery in *Drosophila melanogaster*, *Caenorhabditis elegans* and human cells of about a hundred small RNAs resembling let-7, which were miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). A common system for miRNA annotation was then defined as new miRNAs were identified. They are annotated sequentially with numbers in the order of their discovery. In addition, miRNA genes are grouped into miRNA families if they share sequence homology and identical seed regions, which are found in the 5' end of the miRNA (Ambros et al., 2003).

With the identification of new members, miRNA functions were not only limited to development but were broaden to many other cellular processes such as cell differentiation, proliferation and homeostasis. They act as post-transcriptional regulators of gene expression. Furthermore, dysregulation of their expression can participate to the development and progression of disease notably in cancer (Lin and Gregory, 2015; Rupaimoole and Slack, 2017). Indeed, oncomirs regroups sets of miRNAs enhancing tumor progression, while other miRNAs act as tumor suppressors (Esquela-Kerscher and

Slack, 2006). Finally, miRNAs can also participate the regulation of host-pathogen interactions, an aspect that will be developed in Chapter III of the Introduction.

B. Canonical miRNA biogenesis in animal

1. miRNA expression

One of the characteristics of miRNAs compared to other small regulatory RNAs is that they are encoded from the genome and thus produced from endogenous transcripts as opposed to siRNAs that derive from long double-stranded RNAs that are mostly of exogenous origin. They can be expressed under the control of their own promoter individually in monocistronic transcripts or in clusters if several miRNAs are processed from the same polycistronic transcript. Many miRNAs are present within intronic regions of protein-coding genes (Berezikov et al., 2007), and in these cases, their expression relies on the promoter of the coding gene. Furthermore, gene duplication events have led to the possibility that the same mature miRNA can be expressed from several genomic loci (Berezikov, 2011).

Genes encoding miRNAs are mostly transcribed by the RNA polymerase II and give rise to classical transcripts that are capped and polyadenylated (Lee et al., 2004). This transcript folds into a hairpin structure with unpaired flanking regions which is known as the primary miRNA (pri-miRNA).

2. miRNA processing events

In animals, the pri-miRNA is first processed in the nucleus by the Microprocessor, formed by the type III endoribonuclease Drosha (Lee et al., 2003) and its cofactor DGCR8 (diGeorge Syndrome Critical Region Gene 8) (Gregory et al., 2004). The complex is composed of one Drosha molecule together with a DGCR8 dimer and will recognise the substrate by structural as well as sequence-specific motifs (Nguyen et al., 2015). Drosha then cleaves the pri-miRNA at the base of the stem structure, forming the precursor miRNA or pre-miRNA. The pre-miRNA will be actively transported to the cytoplasm by

Exportin-5 in a Ran-GTP-dependent manner (Bohnsack et al., 2004; Lund et al., 2004). Once in the cytoplasm, the pre-miRNA will be processed again, this time at the level of the loop, by another type III RNase called Dicer (Hutvágner et al., 2001) and which forms a complex with its cofactor TRBP (Transactivation region (Tar) RNA binding protein) (Chendrimada et al., 2005). This cleavage gives rise to the mature miRNA duplex, characterized by having a 2-nucleotide overhang on the 3' ends due to the two processing cleavages (Lee et al., 2003; Zhang et al., 2004) (Figure 5).

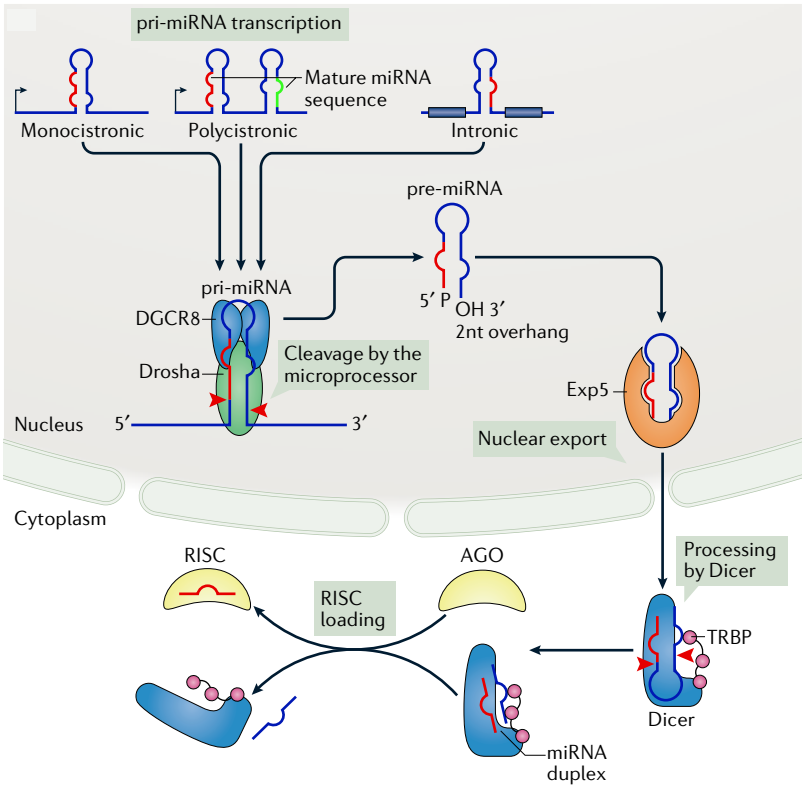

Figure I.5. Canonical miRNA biogenesis and formation of miRISC.
Transcribed pri-miRNAs are processed by Drosha with its cofactor DGCR8 in the nucleus into pre-miRNAs that will be transported to the cytoplasm by Exportin 5. The pre-miRNA will be processed in turn by Dicer with its cofactor TRBP to produce a mature miRNA duplex that will be loaded in Ago. One of the strands will remain loaded and act as the guide of the RNA-induced silencing complex (RISC). Adapted from (Treiber et al., 2019).

3. Non-canonical biogenesis of miRNAs

In some cases, miRNAs undergo a non-canonical biogenesis to be expressed. One example of non-canonical biogenesis are Drosha-independent miRNAs that can join the pathway after this processing step. For these miRNAs, the pri-miRNA consists of a small intron, which is excised by the splicing machinery to directly give rise to the pre-miRNA that is then exported to the cytoplasm and further processed by Dicer (Ruby et al., 2007; Babiarz et al., 2008). Other non-coding RNAs such as tRNAs, shRNAs or snoRNA can also lead to the production of miRNAs in a Drosha-independent manner (Babiarz et al., 2008; Ender et al., 2008). Finally, some examples of virus-derived non-coding RNAs resulting from Pol III transcripts are directly processed by Dicer. This is the case of the adenoviral VA-RNA (Andersson et al., 2005) and the herpesvirus MHV68 miRNAs derived from tRNAs (Pfeffer et al., 2005; Bogerd et al., 2010).

There are also Dicer-independent miRNAs, such as miR-451, whose primary transcript is processed by Drosha but yields a substrate too short for Dicer. This pre-miRNA is first loaded and cleaved by Ago2, and then further trimmed by the 3' to 5' exonuclease PARN (Poly(A)-specific ribonuclease) (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010; Yoda et al., 2013).

C. Formation of the effector complex miRISC

Once the mature miRNA duplex is formed, it will be loaded into one Argonaute protein (Ago) to form the effector miRNA-induced silencing complex (RISC). This process is done with the help of chaperon proteins such as Hsc70/Hsp90 chaperone machinery and in an active manner requiring energy from ATP hydrolysis (Iwasaki et al., 2010). At this point, one strand of the duplex (the passenger strand or miRNA*) is removed and only the guide strand or mature miRNA remains to exert its function. The choice of strand is a process based on the thermodynamic stability of the duplex, in which the Ago protein will select and retain the less stable 5' end pairing (Khvorova et al., 2003; Schwarz et al., 2003). It also depends on the suitability of the 5' end of the strand to be

accommodated into the Argonaute pocket binding the 5' end, generally preferring 5' pU or pA termini (Frank et al., 2010).

In mammals, there are 4 different Argonaute proteins (Ago1-4). Ago proteins are organized in four conserved domains: N-terminal, PAZ, MID and PIWI (Figure 6). All of the Ago proteins participate in miRNA-mediated silencing but only Ago2 has a slicer activity able to cleave target RNA (Liu et al., 2004; Meister et al., 2004). The presence of RNase H activity within the PIWI domain (Song et al., 2004) allows cleavage of the target at the residues opposite to the guide RNA nucleotides 10 and 11, when there is perfect complementarity with the target RNA. The miRNA is bound to Ago by interaction of the 5' phosphate with the MID domain and 3' OH end with the PAZ domain (Figure 6).

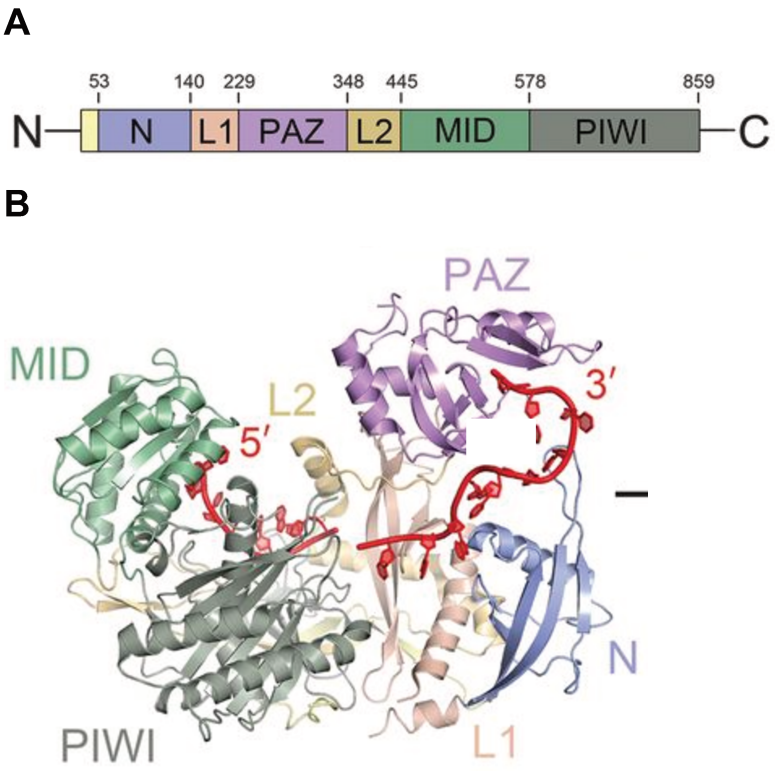

Figure I.6. Argonaute-miRNA complex crystal structure.
(A) Schematic representation of the domain organization of Argonaute proteins. (B) Front view of human Ago2 crystal structure loaded with a miRNA (in red). Adapted from (Schirle et al. 2014).

Most miRNAs are known to be very stable with long half-lives and they are stabilized by Ago proteins (van Rooij et al., 2007; Bail et al., 2010; Winter and Diederichs, 2011). Reversely miRNA levels also regulate the stability of Ago proteins (Smibert et al., 2013) thereby maintaining a homeostatic regulation of both.

D. Targeting of mRNA by miRISC

Once the miRISC is formed, gene silencing will take place by guiding the complex towards a target mRNA by partial complementarity to the miRNA and induce translational repression. Examples of miRNAs fully complementary to their target site are uncommon in animals but do exist. Such extensive complementarity will lead to cleavage of the target (Yekta et al., 2004). Most often however, recognition of mRNA targets by miRNAs is based on sequence complementarity of what is known as the miRNA seed, the nucleotides at position 2-7 pairing to a sequence on the target, typically in the 3' UTR of mRNAs (Lewis et al., 2003) (Figure 7).

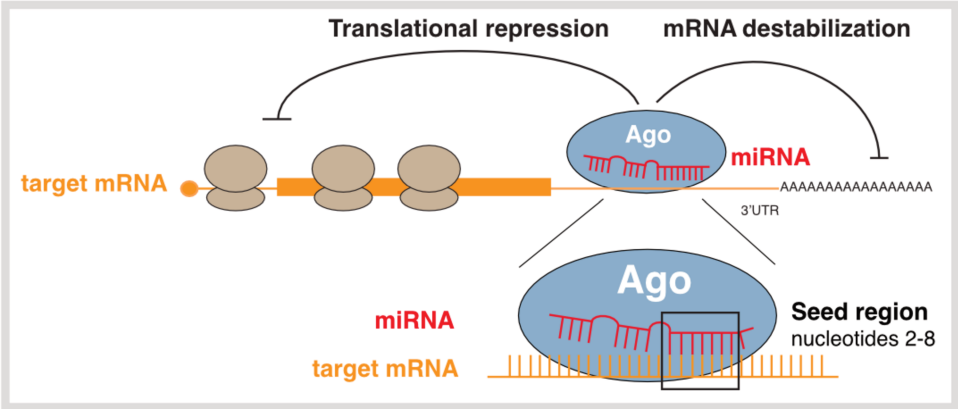

Figure I.7. Outcomes of miRNA targeting of mRNAs.
The miRNA guides Argonaute proteins towards the target mRNA and binds through the miRNA seed region. The principal functional outcomes in mammals are translational repression and mRNA destabilization of the target.

Single-molecule fluorescence studies allowed the establishment of a two-step model for mRNA targeting by the miRISC. First, Ago2 diffuses laterally along the target mRNA in search of a three-nucleotide motif complementary to nucleotides 2-4 of the miRNA seed region in a transient manner. This interaction eventually becomes stable once complementarity can be extended to the full seed region (Chandradoss et al., 2015).

1. Types of target sites

There are different types of targeting that can result in silencing of the target mRNA (Figure 8). The canonical sites are the ones involving pairing of the seed with an additional match, either until nucleotide at position 8 (7mer-m8 site), an adenosine at position 1 (7mer-A1 site) or both (8mer site). Canonical marginal sites include targeting mediated by only 6 nucleotides either involving the seed (6mer site) or slightly shifted involving nucleotides at position 3-8 (offset 6mer site) (Bartel, 2009).

Although these are the canonical targeting sequences, there are other possible sites. In all cases of seed region targeting, supplementary binding of the 3' end of the miRNA might be involved in the binding, these are called 3' supplementary sites. Finally, if the seed region is not continuous and includes bulges, it can be compensated by extensive pairing in the 3' end of the miRNA and still mediate repression, these are known as 3' compensatory sites, which are less common (Bartel, 2009). Other non-canonical targeting involves miRNAs in which there is no seed match or 3' compensatory sites but instead repression is mediated by centered pairing of 11-12 nucleotides of the miRNA (Shin et al., 2010).

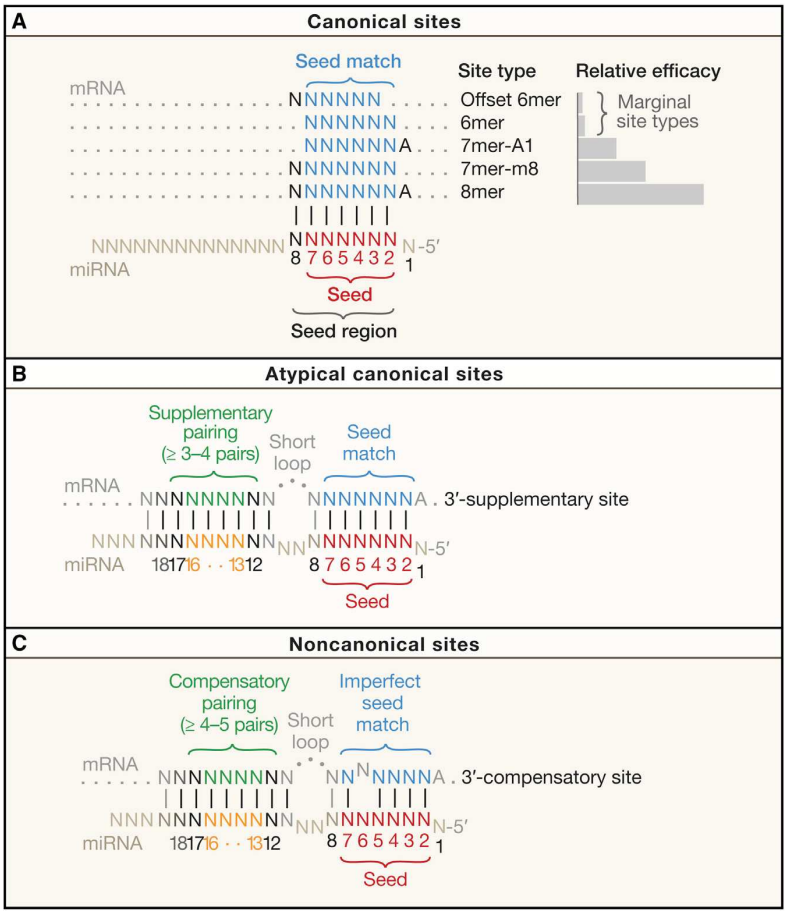

Figure I.8. Different types miRNA target sites.
(A) Canonical target sites involving the miRNA seed region (B) Atypical site with 3' supplementary binding in addition of the seed region (C) Non-canonical sites presenting bulges in the seed compensated by 3' binding. From (Bartel, 2018).

The different target sites show different efficacies, generally 8mer sites outrange 7mer sites while 6mer sites show the lowest efficacy. In a recent study, the laboratory of David Bartel developed a biochemical model for miRNA-target efficacy that confirmed the efficacy of seed binding. In addition, this study revealed the existence of miRNA-specific affinities for canonical sites as well as different affinities for non-canonical sites depending on the miRNA. Moreover, they showed the importance of dinucleotides of binding site flanking regions allowing accessibility for the targeting complex (McGeary et al., 2019). The final targeting outcome is thus influenced by target site context and surrounding elements (Grimson et al., 2007; Nielsen et al., 2007).

2. mRNA target site context

Targeting efficiency can be influenced by several features regarding the target site (reviewed in (Pasquinelli, 2012)). Sequence elements such as AU content can influence the efficacy of sites as AU-rich regions are preferentially found near conserved target sites (Grimson et al., 2007; Nielsen et al., 2007). The small size of miRNA target sequences involved in repression makes it possible for a mRNA to have several miRNA binding sites within its sequence. This allows for cooperative effects of miRNA regulation when sites are found in close vicinity (Grimson et al., 2007; Saetrom et al., 2007; Broderick et al., 2011). Furthermore, the position of the site within the 3' UTR is also important as effective sites are mostly found at the extremities of the UTR but with a minimum distance of 15 nucleotides downstream of the stop codon (Grimson et al., 2007). Although typically miRNAs bind to 3' UTRs, targeting is not restricted to this region and repression can also be achieved by miRNAs binding to coding regions (Hafner et al., 2010; Schnall-Levin et al., 2010) or 5' UTRs.

Finally, targeting efficiency can also be affected by the different RNA binding proteins (RBPs), which can bind the mRNA at or near the miRNA targeting site. The RNA binding protein HuR is a well-studied example and found in different CLIP studies bound generally to AU-rich regions near miRNA binding sites (Mukherjee et al., 2011). Binding of this RBP seems to have a dual role depending on the target and thus can positively or negatively influence miRNA targeting efficiency (Pasquinelli, 2012).

3. miRNA mode of action in animals

As mentioned earlier, binding of miRISC to its target leads to translational inhibition and mRNA destabilization. This action is mediated through the recruitment of the adaptor protein from the GW182 protein family. In vertebrates, there are three of these proteins termed TNRC6 (trinucleotide repeat containing 6) A, B or C. GW182 proteins are characterized by the presence of several tryptophan and glycine (GW) motifs. These motifs are responsible for the interaction with Ago through the W-binding pockets within the PIWI domain (Lian et al., 2009). GW182, as well as Ago can be found in mRNA

processing bodies, or P-bodies, which are cytoplasmic foci that include factors involved in mRNA degradation (Eulalio et al., 2007a; Standart and Weil, 2018). During miRNA silencing, P-body formation is triggered as some targeted mRNAs in silencing complexes can be relocalized to form these structures, which lack translational factors and where the targeted mRNA can be degraded (Eulalio et al., 2007b).

GW182-mediated repression happens by interaction of GW182 with the poly(A)-binding protein (PABPC) followed by recruitment of deadenylation complexes PAN2-PAN3 and CCR4-NOT to miRNA targets leading to mRNA destabilization by poly A tail degradation. GW motifs in GW182 proteins are responsible for binding to deadenylase complexes for their recruitment. Indeed, GW182 interacts directly with PAN3, the adaptor protein recruiting PAN2 to the target mRNA for deadenylation. It is proposed that PAN2-PAN3 complex acts on early steps. Thereafter, the CCR4-NOT complex takes over to ensure mRNA destabilization and decay by coupling deadenylation and decapping to mRNA degradation by the 5' – 3' exoribonuclease 1 (XRN1). In addition, the CCR4-NOT complex also recruits the DEAD-box helicase (DDX6), which binds the decapping complex participating to inhibition of translation (Figure 9) (reviewed extensively in (Jonas and Izaurralde, 2015)).

The mechanism by which translational repression takes place is not fully elucidated. However, it seems that it would be due to inhibition of translation initiation by interfering with eukaryotic initiation factor 4F complex (eIF4F). Recent studies showed that although translational repression happens rapidly (Béthune et al., 2012), the final contribution to silencing is relatively low. Indeed, the time needed for protein turnover allowing to see the repression effect is too long and by that moment repression is dominantly due to mRNA destabilization (Eichhorn et al., 2014).

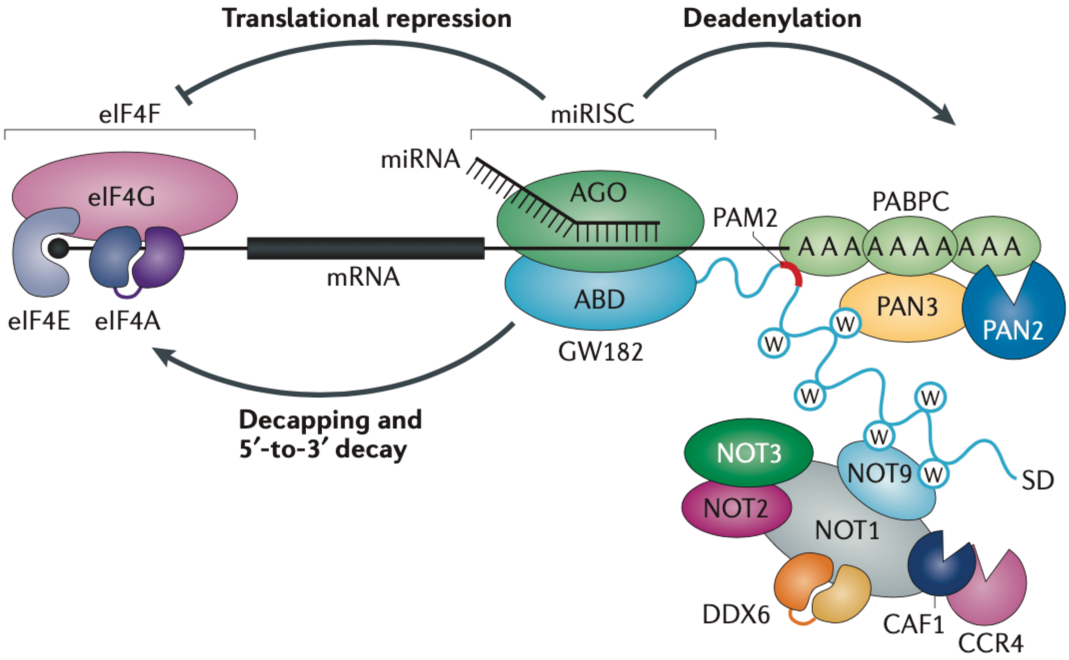

Figure I.9. Mode of action of miRNA-mediated repression of target mRNA. Interaction of Ago with GW182 protein induces recruitment of CCR4-NOT and PAN2-PAN3 complexes leading to deadenylation and decaping followed by RNA decay. Translational repression is obtained by interfering with the eukaryotic initiation factor 4F complex. From (Jonas and Izaurralde, 2015).

E. Regulation of miRNA expression and tissue-specific miRNAs

Because miRNAs are involved in the regulation of over 60% of cellular targets (Friedman et al., 2009), their expression is tightly controlled. Depending on their functions and the cellular pathways they regulate, some miRNAs might be highly expressed ubiquitously and others are only expressed at high levels in certain cell types (Landgraf et al., 2007). Some examples of these tissue-specific miRNAs are the muscle-specific miR-1 (Lee and Ambros, 2001), the liver-specific miR-122 or the neuronal-specific miR-124 (Lagos-Quintana et al., 2002). The latter is extensively studied during this project in relation with viral infection in different miR-124 expressing cells.

This tissue specificity is due to regulation at different levels of the biogenesis pathway. As products of RNA Pol II transcription, pri-miRNA expression is controlled

by elements classically implicated in transcriptional control of coding genes. This includes regulation by genomic elements and factors such as promoters, enhancers, repressors and interactions with different transcription factors. Epigenetic modifications, such as histone modifications or DNA methylation, can also participate in the regulation of miRNA expression. Some examples of this control of expression will be further discussed in regard to miR-124 expression in section 5 of this chapter.

In addition, processing of individual hairpins allowing accumulation of the mature miRNA form depends also on the accumulation and activity of Microprocessor and Dicer (extensively reviewed in (Ha and Kim, 2014; Treiber et al., 2019)).

F. The brain-enriched miR-124

miR-124 is a neuron-specific miRNA implicated in development of the CNS, specifically in the acquisition of neuronal cell identity during neurogenesis and its maintenance. Furthermore, miR-124 is included among the group of miRNAs termed "NeurimmiRs" because of their involvement in both neuronal and immune functions (Soreq and Wolf, 2011).

Since its discovery, it has been a deeply studied miRNA with research focusing on deciphering its neuronal functions but also for miRNA functional studies like effects of miRNA regulation at the level of the transcriptome (Lim et al., 2005a), spatial and temporal differential expression of miRNA and its targets (Stark et al., 2005) or targeting mechanisms (Grimson et al., 2007). Furthermore, it was also used in the earliest Ago-CLIP (Argonaute crosslinking and immunoprecipitation) studies for identification of miRNA targets and binding sites (Chi et al., 2009, 2012).

1. Discovery and tissue-specific expression

miR-124 was first identified in mice as a brain-specific miRNA. It is the most abundant miRNA in the brain, representing about 25% to 48% of all brain miRNAs (Lagos-Quintana et al., 2002). It is very conserved among different metazoan species with

conservation of the full mature miRNA sequence in different organisms including, nematodes, insects and vertebrates (Figure 10) (Conaco et al., 2006).

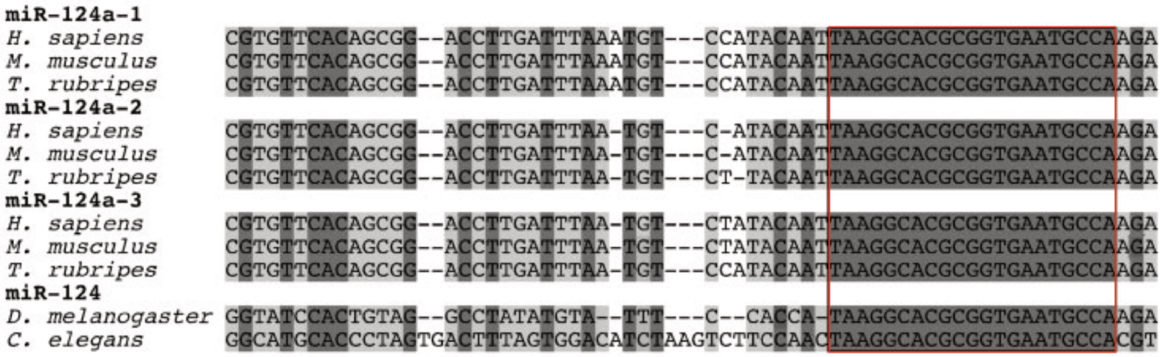

Figure I.10. miR-124 sequence conservation.
miR-124 precursor sequence alignment from different organisms. Conserved residues in all species are highlighted in dark grey while those conserved only in vertebrates are in light grey. The red square delimits the mature form. Adapted from (Conaco et al., 2006).

The extensive conservation of its full sequence highlights the importance of its functions as a regulator of neuronal gene expression and its implication in immune-related functions. In humans, miR-124 is expressed from three different genomic loci: miR-124a-1 (8p23.1), miR-124a-2 (8q12.3) and miR-124a-3 (20q13.33). In mice, the miR-124-1 gene is predominantly expressed giving rise to most of the accumulated mature form of miR-124 (Sanuki et al., 2011).

Together with other brain-specific miRNAs, miR-124 expression is repressed in non-neuronal cells by the action of the transcription factor RE1-Silencing (REST) that binds to genomic loci near miR-124 genes. Upon neuronal differentiation, REST-regulated genes are derepressed. miR-124 will in turn allow post-transcriptional regulation of non-neuronal transcripts participating in the acquisition and maintenance of neuronal identity (Conaco et al., 2006). In addition, epigenetic modifications also contribute to the regulation of miR-124 expression. The transcription factor EVI1 binds to miR-124 promoter regions and interacts with the histone deacetylase 1 (HDAC1) to

represses miR-124 expression, probably involving changes in histone acetylation in neuronal cells. This interaction is reduced in response to neuronal activity deprivation allowing miR-124 expression to participate in the induction of homeostatic synaptic plasticity (Hou et al., 2015). Methylation of miR-124 promoter regions has also been described as a result of exposure of neurons to the viral protein Tat1 (Periyasamy et al., 2018). Finally decreased miR-124 expression in several types of cancer in humans is associated with hypermethylation of miR-124 genes and thus inhibition of its tumor suppressor functions (Zhou et al., 2019).

Moreover, miR-124 expression is also controlled at a post-transcriptional level. Binding of polypyrimidine tract binding protein 1 (PTBP1), also known as hnRNP I, to a sequence upstream of the stem-loop inhibits further processing of pri-miR-124-1 by the Microprocessor in mouse embryonic stem cells, blocking transition to neuronal cells (Yeom et al., 2018).

2. Functions of miR-124 in the CNS

2. 1. Neuronal development

Neuronal progenitor cells derived from pluripotent stem cells can differentiate into three major cell types: neurons, astrocytes and oligodendrocytes. The latter belong to the glial cells, together with microglia, the resident macrophages in the CNS. However, microglia differ in origin as they are derived from hematopoietic progenitors instead of neuronal ones (Vieira et al., 2018). Glial cell functions include primarily maintenance of CNS homeostasis and immune defense.

The definition of cell identity can be modulated by tissue-specific miRNAs, such as miR-124, which participates in this process by down-regulating hundreds of targets. Ectopic expression of miR-124 in the non-neuronal HeLa cell line induces a change in the regulation of mRNA levels, shifting the non-neuronal transcriptional profile to a neuronal one (Lim et al., 2005a). Furthermore, miR-124 defines cell lineage differentiation from mouse embryonic stem cells. Its expression is increased as neuronal

precursor cells differentiate and it prioritizes neuronal lineage differentiation over glial cell type differentiation (Krichevsky et al., 2006). One of the mechanisms employed by miR-124 to drive neuronal differentiation is by alteration of alternative splicing in non-neuronal cells by repressing the PTBP1, which acts as a splicing repressor of neuronal-specific spliced forms of mRNA (Makeyev et al., 2007).

Another important level of regulation of neuron-specific transcriptome and promoting differentiation is to regulate transcription factors. A study revealed that miR-124 acts synergistically with miR-137 and miR-128, two other miRNAs highly expressed in the brain, to regulate murine neural stem cells (NSCs) differentiation. Transcriptomic analysis found that these miRNAs share common targets to regulate neurogenesis, the main component of this network being transcription factors. Particularly, Sp1 a transcription factor promoting proliferation was identified as a principal node of the network and its expression is strongly repressed by the cooperative action of the three miRNAs upon NSCs differentiation (Santos et al., 2016).

2. 2. Implications in neuronal disease

In addition to its role in CNS development, miR-124 is implicated in a wide variety of CNS disorders including, stroke, neurodegenerative diseases or brain tumors (Sun et al., 2015).

Down-regulation of miR-124 expression has been reported in some patients presenting Alzheimer's disease. In consequence, miR-124 targets are derepressed, PTBP1 and 2 among them, which lead to splicing anomalies potentially participating in the development of the disease (Smith et al., 2011). Similarly, deregulation of miR-124 targets has also been observed in Parkinson's disease, suggesting a possible implication in the pathogenesis (Sonntag, 2010).

In a wide variety of cancers, including breast cancer, prostate cancer, pancreatic cancer, or hepatocellular carcinoma, miR-124 expression is reduced, mostly by the action of epigenetic modifications. While this could be expected in cancers originating from non-neuronal cells, this deregulation of expression is also observed in brain tumors such as glioblastoma or neuroblastoma. The tumor suppressor activity of miR-124 can take

place in neuronal as well as non-neuronal tumoral cells and acts mainly by inhibiting cell growth and migration. This is due to regulation of the expression of important actors of the cell cycle. Cyclin-dependent kinases 6 and 4 (CDK6 and CDK4) are both targeted by miR-124 through binding to their 3' UTR. Decreased levels of these kinases block the cell cycle (Pierson et al., 2008; Deng et al., 2013).

Thus, miR-124 is currently seen as a miRNA with potential therapeutic properties for inhibiting tumoral cell migration and metastasis as well as a prognosis marker (reviewed in (Jia et al., 2019).

2. 3. Neuroimmune functions

Considering the numerous immune-related functions of miR-124, it is considered an anti-inflammatory miRNA (Gaudet et al., 2018) involved in maintaining homeostasis. In the CNS, it participates in the control of exacerbated immune response to different kinds of stress. The immune-related functions of miR-124 include modulation of innate and adaptive immune cells as well as regulation of the innate and adaptive immune responses (reviewed in (Qin et al., 2016)).

Like in neuronal cells, miR-124 is also expressed in microglia promoting microglia quiescence. In a mice model of experimental autoimmune encephalomyelitis (EAE), miR-124 acts by deactivating infiltrating monocyte and macrophages promoting differentiation to microglia. The molecular mechanism involves downregulation of the transcription factor C/EBP-α and consequently, its downstream target PU.1 leading to a decreased expression of macrophage activated markers (Ponomarev et al., 2011). In addition, miR-124 is differentially expressed in different subsets of dendritic cells suggesting a possible modulation in the development of dendritic cell lineages (Han et al., 2016).

Furthermore, miR-124 modulation of innate immune responses is possible through regulation of the JAK/STAT signaling pathway. The effector protein STAT3 is repressed by miR-124 and consequently, this regulation inhibits cytokine release, such as IL6 (Sun et al., 2013). In addition, by interfering with STAT3 expression, miR-124

participates in adaptive immune response to glioma cells and modulates T cell-mediated clearance of glioma (Wei et al., 2013).

3. Loss-of-function studies

In order to elucidate miR-124 essential and specific functions at the level of the whole organism, several loss-of-function studies have been pursued in various species. The first miR-124 mutant models obtained were in *Caenorhabditis elegans* and *Drosophila melanogaster* where there is one single copy of the mir-124 gene.

In *C. elegans*, although miR-124 mutants are viable and show no abnormalities in development (Miska et al., 2007), miR-124 contributes to the regulation of specific gene sets in sensory neurons defining the specificity of these cells (Clark et al., 2010). Similarly, miR-124 loss-of-function in *D. melanogaster* is viable and fertile, with no detrimental effects on neuronal differentiation. Nevertheless, these mutants showed neurophysiological defects like short lifespan, impaired larval locomotion and aberrant synaptic activity. In addition, derepression of miR-124 targets in the mutants reflected an incomplete transition in differentiation towards neuronal-specific gene expression (Sun et al., 2012).

In mammals, a partial loss-of-function mice model was established by disruption of only one copy of the three genes encoding miR-124, the major source of the mature miRNA form, gene *mir-124-1,* by deletion of the non-coding RNA 3 (Rncr3) containing pri-miR-124-1. Deletion mutants mice were viable at first but showed early postnatal death, reduced brain size, aberrant axonal development of hippocampal neurons and enhanced apoptosis (Sanuki et al., 2011).

In a human cellular model, deletion of miR-124 in induced pluripotent cells revealed that this miRNA is not essential for neurogenesis. Nevertheless, its deletion leads to morphological changes in neurons phenotype as well as increased apoptosis, similar to the mouse model. Very interestingly, in this study the authors show that in the absence of miR-124, other miRNAs can take over to ensure maintenance of the regulation and the networks (Kutsche et al., 2018).

Overall these models reflect the importance of miR-124 in neuronal-specific signature of gene expression modulating neurological functions not only by targeting specific mRNAs but also indirectly by modulating complex regulatory networks via repression of transcription factors.

4. Regulation of viral infection

Since miR-124 is involved in immune responses, it can be linked indirectly to viral infections and to certain immune responses to neurotropic viruses. This is the case upon Zika virus infection, which downregulates miR-124 expression in primary mouse neurons correlating with an increased expression of genes involved in antiviral and inflammatory response signalling pathways (Azouz et al., 2019). Japanese encephalitis virus (JEV) is attenuated by miR-124 through repression of the dynamin2 (DNM2), a cellular cofactor required for replication of JEV.

Finally, the methylation state of miR-124 promoters is influenced by retrovirus infection, leading to activation of microglia and development of neuropathogenesis. Exposure of microglial cells to HIV-1 Tat protein induces increased DNA methylation, including in miR-124 promoter leading to a downregulation of the miRNA expression and thus derepression of its target STAT3, leading to increased cytokine production, and the transcriptional repressor MECP2, maintaining a negative feedback loop (Periyasamy et al., 2018).

CHAPTER III. Implication of cellular miRNAs in the regulation of viral infection

In this chapter, I will summarize different effects of cellular miRNAs on the regulation of viral infection in the form of two reviews, written during my thesis, in which I am first author: "On the Importance of Host MicroRNAs During Viral Infection" published in *Frontiers in Genetics* and "Importance des microARN cellulaires dans la régulation des infections virales" published in *Médecine/Sciences*.

The first article focuses on the effects of miRNA on viral infection and the possible outcomes. Indeed, miRNAs can either have a proviral effect or an antiviral effect. In addition, viruses can be regulated by direct interaction of the miRNA with the viral RNAs, or indirectly, by repression of host target mRNAs coding for viral cofactors or implicated in different viral cycle steps. In a second part, we discuss the different techniques used nowadays to identify miRNA candidates. These include for instance analysis of miRNA differential expression during infection or the use of phenotypic screens with viral reporters. Finally, we also discuss the newest approaches used for target mRNA identification in order to understand the mechanistic of the regulation.

The second article was published in a French journal for popularisation of science for a wide audience of medical doctors, researchers or students. We thus described miRNA regulation of viral infection in an accessible manner for readers that are not necessarily specialized in the field. In addition, in this paper we further discuss the possible therapeutic applications that can derive from miRNA-based studies to develop vaccines and treatments.

A. Article 1: On the importance of host microRNAs during viral infection

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REVIEW
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On the Importance of Host MicroRNAs During Viral Infection

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Every living organism has to constantly face threats from the environment and deal with a large number of pathogens against which it has to defend itself to survive. Among those, viruses represent a large class of obligatory intracellular parasites, which rely on their host machinery to multiply and propagate. As a result, viruses and their hosts have engaged in an ever-evolving arms race to be able to maintain their existence. The role played by micro (mi)RNAs in this ongoing battle has been extensively studied in the past 15 years and will be the subject of this review article. We will mainly focus on cellular miRNAs and their implication during viral infection in mammals. Thus, we will describe current techniques that can be used to identify miRNAs involved in the modulation of viral infection and to characterize their targets and mode of action. We will also present different reported examples of miRNA-mediated regulation of viruses, which can have a positive outcome either for the host or for the virus. In addition, the mode of action is also of a dual nature, depending on the target of the miRNA. Indeed, the regulatory small RNA can either directly guide an Argonaute protein on a viral transcript, or target a cellular mRNA involved in the host antiviral response. We will then see whether and how viruses respond to miRNA-mediated targeting. Finally, we will discuss how our knowledge of viral targeting by miRNA can be exploited for developing new antiviral therapeutic approaches.

Keywords: microRNA, virus, post-transcriptional regulation, defense mechanism, host–pathogen interaction

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INTRODUCTION

Viral infections constitute a major threat for human health. As obligate intracellular parasites, viruses rely exclusively on the host cellular machinery to translate their genome, and therefore to replicate and propagate in their host and in the environment. *Per se*, they represent the ultimate example of selfish genes that are here solely to be amplified. Of course, this close dependency on a host organism also makes them vulnerable since they have to unveil their genome in the cells they infect. This results in an ongoing arms race in which the invading pathogen has to constantly evolve to find strategies to avoid detection and clearance by the host immune response. In parallel, the attacked organism has developed throughout evolution multiple ways to sense and fight back viral infections. As such, viruses can thus also be seen as crucial elements in the shaping of modern organisms.

In mammals, there are several layers of protection put in place to prevent the invading virus to establish a successful infection. One of the first line of defense is innate immunity, which is triggered by the recognition of foreign elements, brought in by the virus, and that include nucleic acids.

In vertebrates, upon sensing of specific pathogen-associated molecular patterns, such as double-stranded (ds) RNA or the presence of a 5′ triphosphate group, the cell responds by the activation of a signaling cascade that results in the induction of type I interferon (IFN) expression, which in turn triggers transcription of hundreds of interferon-stimulated-genes (ISGs) that include pro-inflammatory cytokines (Nakhaei et al., 2009). This chain of events creates an antiviral state that interferes with viral replication, blocks protein synthesis, induces cellular RNA degradation and ultimately leads to apoptosis of the infected cell (Barber, 2001).

In other organisms, including plants, arthropods and nematodes, the presence of exogenous long double stranded (ds)RNA activates another mechanism, known as RNA interference (RNAi), which represents their major antiviral defense system. In this case, long viral dsRNA molecules are recognized and processed into small interfering (si) RNAs by the type III ribonuclease Dicer. These siRNAs are then loaded into effector complexes that invariably contain a member of the Argonaute family. The activated complex, termed RNA Induced Silencing Complex (RISC), can be directed via sequence complementarity toward target RNAs (in this case viral messenger or genomic RNAs) to mediate their cleavage and degradation (Ding and Voinnet, 2007). Whether such an RNA-based defense mechanism is still functional and relevant in vertebrates remains a debated topic that will not be addressed here. Nonetheless, it is now widely accepted that another class of small non-coding RNAs do play important roles during viral infections in mammals. Indeed, the same machinery that was originally designed to clear the cell of unwanted nucleic acids, is also involved in the biogenesis of miRNAs, which have emerged in the last 15 years as one of the more relevant species of regulatory RNAs in higher eukaryotes.

In the canonical biogenesis pathway, miRNAs are transcribed by RNA Polymerase II into a primary precursor, called pri-miRNA, processed by Drosha and its co-factor DGCR8 into a precursor of about 70 nucleotides (nt). The pre-miRNA is then exported by the Exportin 5 protein from the nucleus into the cytoplasm, where it will be cleaved by Dicer into a ∼22 nt long miRNA duplex. One of the two strands is selected and loaded into an Argonaute protein (in human AGO1 to 4), to form the basic RISC. The mature miRNA functions as a sequence-specific guide to trigger the effector complex onto the 3′ untranslated region (UTR) of the target messenger RNAs (mRNAs). Upon binding to its target RNA, the Argonaute protein can then bind an adaptor protein, known as GW182 or TNRC6, which in turn interacts with factors that act on mRNA translation and stability [see **Figure 1** and Bartel, 2018 for review].

It is generally accepted that the main determinant of miRNA sequence specificity is its seed region. Initially discovered purely based on bioinformatic evidence (Lai et al., 2005; Lewis et al., 2005) before the determination of AGO2 structure brought more evidence (Schirle and MacRae, 2012), the minimal seed corresponds to a short region at the 5′-end of miRNAs (nucleotides 2–7), which displays perfect complementarity with its target site (referred to as the seed-match). The 8mer sites with an additional match to nucleotide 8 and an A in position 1 are the most effective canonical sites and those identified with increased confidence by target-prediction tools (Bartel, 2009; Agarwal et al., 2015). In some cases, additional base pairing toward the 3′ end of the mature miRNA (the 3′-compensatory site) may compensate for suboptimal pairing in the seed region (Grimson et al., 2007; Broughton et al., 2016). The definition of "seed rules" was extremely important to enable target predictions. Given the limited size of the interaction sequence, the regulatory potential of miRNAs is extremely flexible. Indeed, conservative estimates indicate that at least 60% of the human coding genome might be regulated by miRNAs (Friedman et al., 2009). This also means that miRNA-mediated regulation can in theory be expanded to every source of exogenous target RNA. However, one should be careful as to not extrapolate that all these potential targets are physiologically meaningful as recent reports indicate that only a handful of them could indeed have a true impact (Rausch et al., 2015; Denzler et al., 2016; Pinzón et al., 2017).

In this review, we focus on the involvement of cellular miRNAs in viral infection in mammals. We describe the current methods for identification of proviral and antiviral miRNAs and their targets. We review the principal mechanism of action of miRNAs which modulate viral infection and we examine the means employed by viruses to subvert or induce miRNA effects. Finally, we discuss about the miRNA-based therapeutic strategies as a promising emerging field in the context of infectious diseases and viral vector therapy.

FINDING THE NEEDLE IN A HAYSTACK: WHICH miRNAs ARE INVOLVED IN VIRAL INFECTION

According to the latest release of miRBase[1], the human genome contains almost 2000 miRNA precursors (Kozomara and Griffiths-Jones, 2014), each of which with the potential to regulate tens to hundreds of different targets, among them viral transcripts. Thus, finding the miRNAs playing important roles during a given virus infection can quickly become overwhelming. Conceptually, there are two main ways to identify these candidate miRNAs. One can first check if the infection has an impact on the miRNA profile of the cell or tissue studied. Indeed, if some miRNAs are strongly deregulated upon infection, it can be assumed that they might play a role during the viral cycle. The main limit with this approach though, is that it is impossible to know whether the observed miRNA regulation, which is a consequence of virus infection, is indeed meaningful for the virus, or if it is just an indirect effect without importance. The other possible approach is to go for a phenotypic screen in order to test, exhaustively if possible, the effect of overexpressing or blocking individually each miRNA on virus accumulation. This method has the advantage to be truly unbiased, but does have its limitation, especially concerning the blocking of miRNAs. Indeed, a specific cell line only expresses, at a functional level, a 100 different miRNAs at most, which means that it is easy to

[1]www.mirbase.org

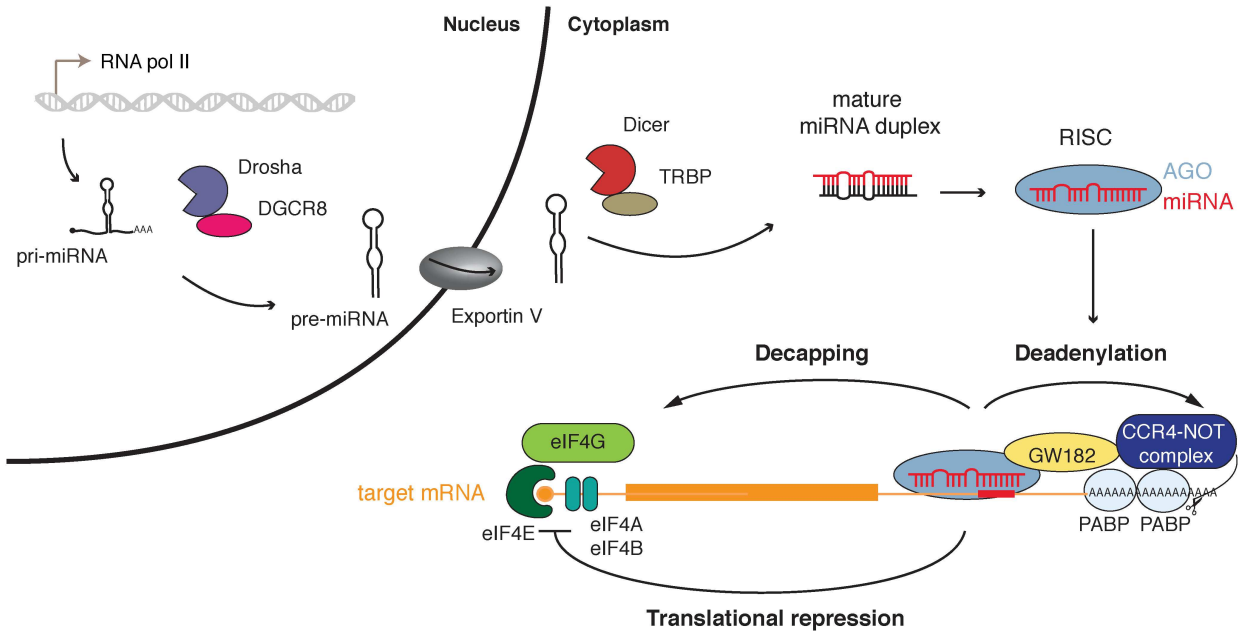

FIGURE 1 | miRNA canonical biogenesis and function. miRNA genes are transcribed by the RNA polymerase II into a primary precursor (pri-miRNA) which is processed in the nucleus by the Microprocessor (Drosha and its cofactor DGCR8) to produce a hairpin structure precursor (pre-miRNA) that will be exported to the cytoplasm by Exportin 5. The pre-miRNA is processed in turn by Dicer into the mature miRNA duplex that will be loaded in an Argonaute protein (AGO) within the RNA induced silencing complex (RISC). One of the strands remains bound to Ago and the complex can mediate post-transcriptional gene regulation by targeting mRNAs through binding of the miRNA seed region (nucleotides 2–8) to the target mRNA (binding site represented by a red rectangle). Adaptor protein GW182 is recruited by RISC and can interact with polyA-binding proteins (PABP) inducing recruitment of CCR4-NOT deadenylase complex. The target mRNA is destabilized by deadenylation and decapping leading to its degradation. Translation of targeted mRNAs is also repressed by inhibition of the preinitiation complex assembly.

overlook the effect of inhibiting a miRNA that is not naturally expressed in the cells used for the screen.

Impact of Viral Infection on miRNA Expression

Although a number of changes induced by viral infection on miRNA expression is most likely indirect, in some cases monitoring these variations can prove very informative. There are different techniques available to measure the impact of viral infection on miRNA expression (**Figure 2A**). The throughput of the classical ones (northern blot analysis, RT-qPCR and *in situ* hybridization) is generally not sufficient to determine the complete miRNA profile of a sample. Therefore, one should rely on the use of multiplexed RT-qPCR, microarrays or Next Generation Sequencing (NGS)-based genome-wide approaches to get a complete picture of the level of expression of every miRNA.

Li et al. (2017) performed miRNA profiling in cells infected with HCV using NanoString nCounter miRNA expression assays and microarray analysis. Among the modulated miRNAs, the top hits miR-25, miR-130a/b, and let-7a were downregulated by the virus, both in cultured cells and liver tissues of infected patients, suggesting that HCV counteracts their proven antiviral capacity by reducing their levels (Li et al., 2017). Profiling of 250 miRNAs in enterovirus (EV)71-infected cells by quantitative real-time PCR showed that miR-141 was induced upon EV71 infection. This miRNA turned out to be proviral (Ho et al., 2011). To identify the miRNAs involved in regulating antiviral signaling pathways, Ingle et al. (2015) performed microarray-based miRNA profiling in human cells infected with Newcastle disease virus (NDV). miR-485-5p was one of the most upregulated ones not only upon NDV infection but also in cells infected with Influenza A virus (IAV) H5N1 or transfected with a synthetic dsRNA, polyI:C (Ingle et al., 2015). Similarly, Rosenberger et al. (2017) used microarray to profile the expression of miRNAs in the lungs of mice infected with IAV and found miR-144 among the most significantly upregulated ones. Ectopic overexpression of miR-144 increased infectious virion production in cells infected not only with influenza virus but also with the negative-sense single-stranded (ss) RNA vesicular stomatitis virus (VSV) and the positive-sense ssRNA encephalomyocarditis virus (EMCV). In parallel, the transcriptome profile of influenza-infected wild-type and miR-144 over-expressing cells was compared and allowed the identification of the transcriptional network regulated by miR-144 (Rosenberger et al., 2017). In another study, both global cellular miRNA and mRNA expression was profiled in Japanese encephalitis virus (JEV)-infected human microglial cells using an Affymetrix microarray platform and identified key pathways associated with the differentially expressed miRNAs and inversely correlated mRNAs during JEV infection (Kumari et al., 2016).

The use of small RNA cloning and sequencing has been employed in several recent studies to identify up- or down-regulated miRNA upon infection with different viruses

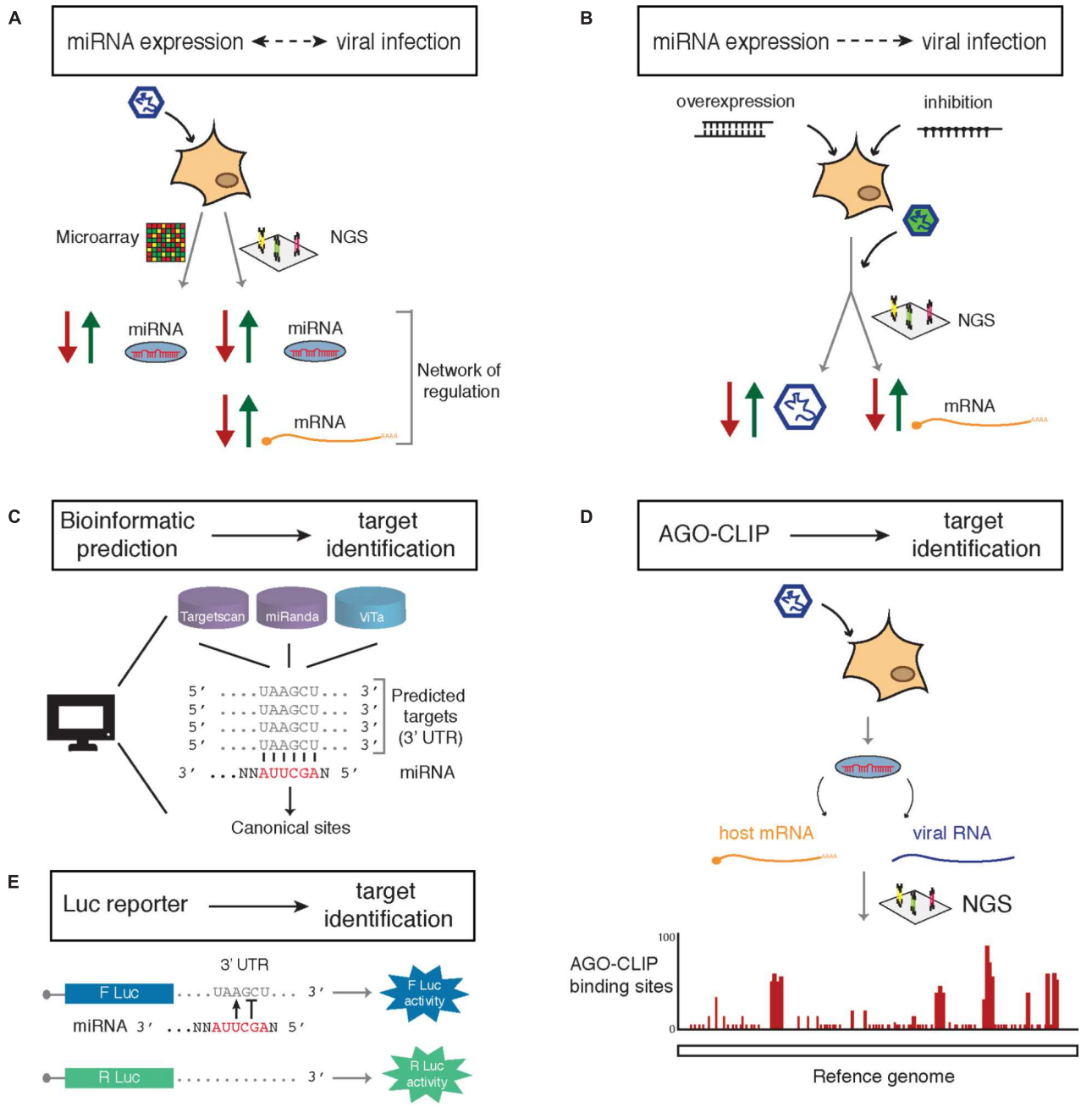

FIGURE 2 | Current approaches available for identification of miRNAs involved in the regulation of viral infection **(A,B)** and their targets **(C–E)**. **(A)** Reciprocal regulation of miRNA expression and viral infection allows identification of candidate miRNAs upregulated (green arrow) or downregulated (red arrow) by miRNA profiling through microarray. Sequencing (next generation sequencing, NGS) provides further information on regulated targets and reveals networks of gene regulation. **(B)** Virus-centered phenotypic approaches are based on miRNA regulation of the infection. Screens based on the overexpression or inhibition of candidate miRNA in the context of infection, generally using a reporter virus (indicated by the green color), allow a direct observation of the effect on the viral accumulation. This approach coupled to transcriptome profiling also identifies target genes of candidate miRNA. **(C)** Computational analysis for target identification of a given miRNA are based on the identification of seed-matches in the 3′ UTRs of cellular mRNAs. Bioinformatic predictions rely on the use of target prediction tools such as Targetscan or miRanda for cellular targets or ViTa for viral genomes and transcripts. **(D)** Biochemical isolation of AGO crosslinked to the miRNA and bound target followed by deep sequencing (AGO-CLIP) allows identification of miRNA specific targets, either cellular or viral, in a genome-wide manner and reveals the precise binding sites on the target. **(E)** Luciferase (Luc) reporter assays allow functional validation of a miRNA binding site based on the measure of the luciferase enzymatic activity when a potential binding site is present on the 3′UTR. Variants of luciferase (F, firefly; or R, Renilla) containing or not the binding site are used to estimate differential regulation.

(Motsch et al., 2012; Oussaief et al., 2015; Chen et al., 2016; Lodge et al., 2017). However, these studies did not necessarily confirm whether the regulated miRNAs were having pro- or anti-viral roles. When miRNA profiling is coupled with regular transcriptome analysis, this can provide useful information regarding the networks of regulated genes upon viral infection. Indeed, it is generally observed that the expression of a miRNA and of its predicted targets tends to be positively or negatively correlated, suggesting a frequent coordination between transcriptional or post-transcriptional regulation of a miRNA and its targets in gene networks (Tsang et al., 2007). As we will see below, many examples of positive and negative regulatory feedback loops between viral levels and miRNA expression have been identified, using NGS approaches or other techniques such as multiplexed RT-qPCR or microarray analyses.

Virus-Centered Phenotypic Screens

Viruses engineered to express a reporter protein (e.g., GFP) have been used as functional reporters to follow pro- and anti-viral miRNA activity in gain- and loss-of-function studies (**Figure 2B**). In general, the reporter activity can be measured upon overexpression or inhibition of miRNA expression, thereby providing a proxy to directly assess the impact on virus accumulation (see **Table 1** for currently available overexpression and inhibition tools).

Santhakumar et al. (2010) used either miRNA mimics or inhibitors and monitored viral growth by using viruses from all three herpesvirus families (α, β, γ) that encode green fluorescent protein (GFP) reporters. They identified host miRNAs with broad pro- and anti-viral properties, among which miR-199a-3p which leads to decreased viral growth in all three herpesviral subfamilies (Santhakumar et al., 2010). To test the extent of such miRNA antiviral activities in other viral infections, the same group conducted a screen on IAV and Respiratory Syncytial Virus (RSV) in human cells (McCaskill et al., 2017). Also, microscopy-based screen using miRNA mimics upon Dengue Virus (DENV), West Nile virus (WNV), and Zika Virus (ZIKV) identified several antiviral miRNAs against flaviviruses, including the miRNA miR-34, miR-15, and miR-517 families (Smith et al., 2017).

IDENTIFICATION OF miRNA TARGETS

Once a candidate miRNA has been found, it becomes essential to identify its targets to understand the molecular mechanisms underlying the effect on the virus. This can prove as difficult if not more as the initial identification of the miRNAs involved in regulating the virus of interest. Indeed, as mentioned above, miRNAs bind to their targets with limited complementarity, and the effect of their binding on the target RNA expression is usually mild. Several methods have been employed for miRNA target identification in vertebrates. The very same approaches are applicable to cellular targets involved in viral infection. However, they cannot all be directly transposed to the identification of virus-encoded targets, in case of a direct effect of the miRNA on a viral RNA.

Bioinformatic Predictions

A large set of miRNA target prediction tools based on sequence conservation and seed complementarity to 3′ UTR of host coding genes has been developed, such as TargetScan (Lewis et al., 2003, 2005), miRanda (John et al., 2004), PicTar (Krek et al., 2005), DIANA-microT (Kiriakidou et al., 2004) (**Figure 2C**). Although computational approaches are undoubtedly valuable in preliminary identification of miRNA target genes, they do not always identify the actual interaction between a miRNA and its target. Moreover, predictions do not include information about expression levels of miRNAs or their targets, leaving the question open about whether the miRNA-mRNA interaction is biologically relevant. Finally, the existence of non-canonical miRNA-target interactions extends the number of potential targets which are not necessarily considered by all bioinformatic tools.

In addition to the aforementioned limitations encountered to find cellular targets, bioinformatics predictions become especially difficult for the identification of miRNAs bound directly to virus. The main reason is that most of the prediction algorithms rely on the cross-species conservation of miRNA binding sites to select for the ones that were maintained during evolution. Therefore, the viral genomes are not included in the commonly used target repositories. In addition, the miRNA target sites are not necessarily contained in the 3′UTRs as for endogenous host targets. However, one group developed ViTa, a database for cellular miRNA targeting virus genomes and virus transcripts. This database contains information about known host miRNAs, known viral miRNAs, known and putative host miRNA target sites on viruses. It provides information such as human miRNA expression, virus tropism and virus comparisons (Hsu et al., 2007). Besides this report, initial efforts in the field identified first a biological effect by using gene reporter assays and then moved backward to the *in silico* search for identification of miRNA binding sites. Thus, in an early study, Dicer1-deficient mice were shown to be hypersusceptible to VSV infection, independently on RNAi or the interferon response. Fusing different portions of the VSV sequence in both positive (+) and negative (−) orientations to luciferase reporters, the authors identified the minimal region for regulation. Target prediction algorithms then allowed them to reveal potential target sites for miR-24 and miR-93 (Otsuka et al., 2007). Another example of prediction and identification of miRNA-viral target interaction concerns the antiviral miR-142 on Eastern equine encephalitis virus (EEEV). Target prediction algorithms identified binding sites for the haematopoietic-specific miR-142-3p in the 3′ Non-Translated Region (NTR) of the virus (Trobaugh et al., 2014). Indeed, EEEV is defective for replication in human and murine myeloid cells which express miR-142.

Experimental Approaches to miRNA Target Identification

To increase the low signal-to-noise ratio inherent to purely computational prediction approaches, different attempts have been made to measure the global effect of altering the level of one candidate miRNA. Methods like microarray or high-throughput sequencing indeed provide indirect relationships

TABLE 1 | Main tools to study miRNA gene function.

miRNA overexpression	miRNA mimic	Synthetic double-stranded RNA molecule mimicking the miRNA duplexes produced after Dicer processing. Designed to efficiently favor the loading of one miRNA strand (miR-5p or -3p) as a functional mature miRNA strand into RISC. Transient expression.
	Vector-based miRNA expression	miRNA precursor under the control of a strong RNA Pol II or Pol III promoter, processed by the biogenesis machinery. Expression of the miRNA often coupled with a fluorescent protein marker. Constructs can be cloned in lentiviral or adenoviral vectors to be packaged into viral particles to target hard-to-transfect cells or for *in vivo* purposes. Can be used for stable expression.
miRNA inhibition	AntimiR	Chemically modified, single-stranded antisense oligonucleotide that inhibits miRNA function by sequence complementarity. Modifications at the 2′ ribose position such as 2′-*O*-methyl and 2′ Fluoro (2′F)-RNA increase binding affinity and stabilize the molecule. A phosphorothioate backbone can be used to stabilize the molecule.
	Antagomir	2′-*O*-methyl modified antisense single−stranded RNA oligonucleotide, conjugated to cholesterol in 3′ end, that inhibits miRNA function by sequence complementarity. Developed as a pharmacological approach for silencing miRNAs *in vivo* (Krutzfeldt et al., 2005).
	Locked nucleic acid (LNA)	Chemically modified antisense RNA analog, in which the ribose sugar is locked by a methylene bridge joining the 2′-oxygen and 4′-carbon of the ribose to increase stability and specificity. The strong binding properties of LNAs make them particularly useful in anti-miRNA applications.
	miRNA sponges	Transcripts containing multiple tandem perfectly or imperfectly binding sites to a miRNA of interest. Act as competitive inhibitors of miRNA function. Can be engineered as fusions to a transgene in plasmid constructs via a strong promoter (Ebert and Sharp, 2010). Can be used either for transient or long-term loss-of-function studies both *in vitro* and *in vivo.*
	Decoy/tough decoy RNA	Antisense single-stranded RNA containing a microRNA binding domain (Decoy) or a stabilized stem-loop with two microRNA binding domains (TuD). Usually expressed from a strong Pol III promoter. Sequesters the miRNA into stable complexes through complementary base-pairing (Xie et al., 2012).
	Morpholino	Phosphorodiamidate morpholine oligomer (or morpholino) is an uncharged DNA analog in which morpholine rings replace the sugar moieties and non-ionic phosphorodiamidate linkages replace the phosphate linkages. Neutral charge of backbone reduces non-specific interactions with proteins.

between miRNAs and their targets. Experimental tinkering with endogenous miRNA expression should correspond to predictable changes in target expression, either at the RNA (Lim et al., 2005) or protein (Selbach et al., 2008) level. Thus, in order to identify cellular targets of miR-197 responsible for its antiviral effect on EV71, Tang et al. (2016) used a proteomics-based approach. McCaskill et al. (2017) and colleagues made use of a reverse-phase protein array (RPPA) to screen the expression levels of global signaling pathway markers to gain insights on the host pathways targeted by the miRNAs with antiviral properties against IAV. The advantage of conducting the RPPA is that it enables not only protein levels to be examined but also to distinguish the phosphorylation state relevant for the activation status of signaling pathways (McCaskill et al., 2017).

Other techniques provide a more solid evidence of what can be the target RNAs bound by miRNAs. These rely on the biochemical isolation of miRNA-target RNA complexes and can be either focused on one single miRNA (Easow et al., 2007), or more broadly on an Argonaute protein. The latter approach has been refined extensively in the past few years and is now based on the chemical cross-linking of one Argonaute protein to its target RNAs prior to its immunoprecipitation followed by deep sequencing (**Figure 2D**). There are several variations of this CLIP (Cross-Linking and ImmunoPrecipitation) technique, which allow the identification of miRNA target networks at a genome-wide level (Chi et al., 2009; Hafner et al., 2010). In addition to isolate physical interactions between the miRNA and its targets, these approaches provide deeper insights on the nature of binding site and in some cases can identify binding site locations with very high accuracy. CLIP data also revealed that a large portion of miRNA-target interactions *in vivo* are mediated not only through the canonical seed-match sites but also via non-canonical sites previously neglected by bioinformatic predictions. Interestingly, AGO-CLIP data are currently exploited to implement prediction tools. For instance, Agarwal et al. (2015) generated an improved quantitative model of canonical targeting based on available CLIP data. They showed that the vast majority of functional sites are canonical since non-canonical sites do not mediate detectable repression despite binding the miRNA. Of note, the appearance of databases such as miRTarbase which contains a curated collection of miRNA-target interactions with experimental support (Hsu et al., 2011; Chou et al., 2016) allows a step forward into the target validation compared to the mere prediction.

High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) experiments of human AGO during HCV infection showed robust AGO binding to the HCV 5′UTR at known miR-122 sites. Moreover, Luna et al. (2015) describe that HCV serves as a sponge for miR-122 from endogenous targets, suggesting that bi-targeting of either cellular

or viral RNAs is crucial for the final miRNA effect. A more recent paper characterized AGO binding landscapes for a number of medically important viruses (Scheel et al., 2016). Scheel et al. (2016) took advantage of an improved method of target identification based on miRNA-target chimera isolation in AGO, so called CLEAR-CLIP method (Moore et al., 2015) and showed a broad AGO coating for several RNA viruses.

Independently of the approach that is used to generate a list of putative targets, these have then to be experimentally validated. Reporter assays were among the first and more straightforward approaches to measure miRNA targeting (Doench and Sharp, 2004). The rationale behind the design of a miRNA reporter is very simple: the predicted miRNA target sequence is fused to the 3′UTR of a reporter gene and the reporter expression will be measured compared to a normalizer gene (**Figure 2E**). For a luciferase assay, the functionality of the target site can be monitored by measuring the luciferase enzymatic activity, while the expression of a normalizer gene will stay unaffected. Moreover, fluorescent reporters have also been chosen as functional read-outs of miRNA activity in living cells, working as biosensors for microscopy-based approaches and single-cell based analyses (Bassett et al., 2014). After the validity of the reporter regulation has been established, the effect of the miRNA on the endogenous target (either at the mRNA or at the protein level) should be measured, before ultimately assessing the effect of this target regulation on virus accumulation *in vitro* and/or *in vivo*.

EXAMPLES OF miRNA-MEDIATED REGULATION OF VIRAL INFECTION

We have now seen multiple ways by which the identity and implication of specific miRNAs in the replication cycle of viruses can be unveiled and we have briefly mentioned a few of them. In this part, we will describe in more details some selected cases of both positive and negative regulation of viruses mediated by miRNAs. Some of these cases are described in **Figure 3** and a more complete list (although not exhaustive) can be found in **Table 2**.

Direct Targeting of Viral RNA

The number of examples of viral regulation by direct binding of miRNAs targeting the viral genome remains limited. One possible explanation could be that if a miRNA target site with deleterious consequence for the virus would appear within a viral genome, the selection pressure would most likely remove this sequence quite rapidly in the virus progeny. This theory is backed up by the finding that a majority of direct host miRNA/viral RNA interactions results in a positive regulation of the viral cycle. In this case indeed, if the binding of the miRNA within the genome provides an evolutionary advantage and/or increases the viral fitness, then it will be maintained by the virus. However, there are some described cases where direct binding of a miRNA on a viral RNA does have a negative impact on the virus. As we will see, this can be explained by the mutual exclusion of the virus and the miRNA due to tightly controlled tissue-specificity. Actually, the idea that the tissue-tropism of some viruses can be partly due to miRNA expression is quite widespread.

The first discovered miRNA-virus interaction is the one involving miR-122 and HCV. HCV is a hepatotropic virus with a positive sense ssRNA genome. The liver-specific miR-122 is essential for the viral replication and positively regulates the virus by the direct interaction of the miRNA to the viral genome, which contains three different binding sites for miR-122 in the 3′ and 5′ UTRs. The regulatory function of miR-122 is exerted after binding to the 5′ UTR of the genome, upstream of the Internal Ribosomal Entry Site (IRES), and leads to increased viral RNA accumulation (Jopling et al., 2005) and enhanced viral protein translation after recruitment of the 48S ribosomal subunit (Henke et al., 2008). However, it seems that the dominant mechanisms leading to the positive effect of miR-122 binding is by protection of the genomic RNA 5′ extremity. Indeed, binding of miR-122-loaded AGO2 stabilizes HCV RNA by preventing its decay (Machlin et al., 2011; Shimakami et al., 2012) most likely caused by the XRN1 exonuclease (Li Y. et al., 2015). The positive effect of miR-122 on the virus certainly reflects the close co-evolution of HCV with its host and might be involved in defining the tropism of the virus, although it could also have appeared because of the liver tropism.

As mentioned previously, Scheel et al. (2016) performed a wide analysis of miRNA binding sites in several viruses using AGO2 CLIP. Among the tested viruses, they found that there were binding sites for the cellular miRNAs miR-17 and Let-7 in the 3′UTR of the bovine viral diarrhea virus (BVDV) genome (Scheel et al., 2016). Although the exact mechanism was not elucidated, the authors showed that this targeting of the BVDV genome had a positive impact on viral RNA and protein production. In addition, and similarly to HCV and miR-122, the sequestration of miR-17 by the viral RNA resulted in the de-repression of the miRNA cellular targets. These findings indicate that direct binding of viral RNAs by cellular miRNAs is more common than anticipated, although the physiological importance remains to be determined for a lot of these interactions.

Another example of a miRNA that partly plays a role in cell tropism is the haematopoietic lineage-specific miR-142, which restricts EEEV replication in myeloid cells. This miRNA binds directly to several conserved binding sites in the 3′UTR of the viral genome and thus restricts translation of non-structural proteins affecting subsequent viral replication in this lineage. Thus, the downregulation of virus accumulation in myeloid cells suppresses IFNα and β production, which allows the infection to occur and leads to the neuropathological features that characterize the viral disease (Trobaugh et al., 2014). Similarly, porcine reproductive and respiratory syndrome virus (PRRSV) can be directly targeted by both miR-181 and miR-130b cellular miRNAs. In this case as well these miRNAs can inhibit PRRSV replication. Studies on miR-181 family have shown that the target site is found on the region downstream of the ORF4 and inhibits the virus by targeting subgenomic RNAs. Expression of miR-181 family members is low in cells that are permissive for the virus. Over-expression of all four miRNAs belonging to this family inhibits PRRSV in a seed-dependent manner leading to 80% reduction in viral RNA accumulation and 30-fold decreased titers

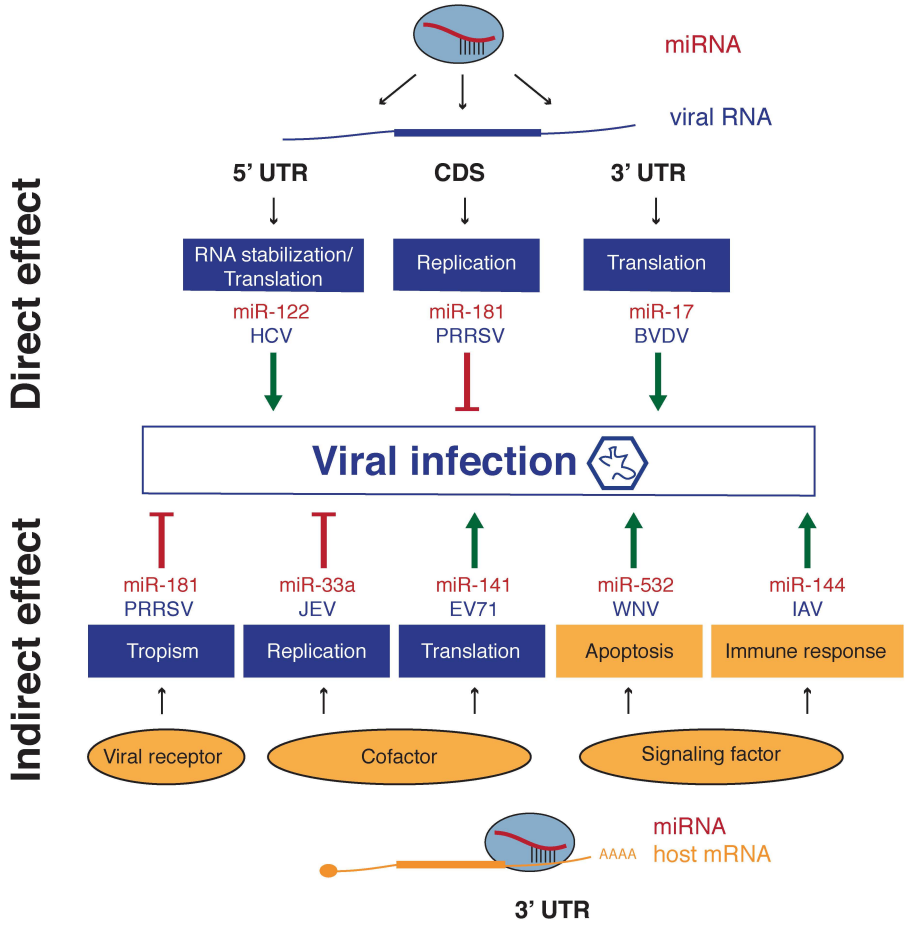

FIGURE 3 | Mechanism of action of miRNAs which modulate viral infection. **(A)** miRNA direct effect on virus regulation takes place by direct targeting of viral RNAs at different regions such as 3′UTR, 5′UTR or coding sequences. Binding leads to RNA stabilization, enhanced translation or impaired replication. **(B)** Indirect effect involves modulation of expression of a cellular transcript encoding a host factor required for one or several steps in the viral cycle. Modulation of receptor expression regulates entry of the virus affecting tropism and cofactors required for replication complexes or translation can impair or enhance viral replication and viral protein production respectively. miRNAs also participate to enhance or restrain cell responses to the infection for instance immune response or defense mechanisms such as apoptosis induction. Viral cycle steps are represented in blue, while host factors and associated pathways are labeled in orange.

in porcine alveolar macrophages (Guo et al., 2013). Despite the efficient antiviral effect of different miRNAs on PRSSV, further investigation is needed to have a better insight of the mechanisms of action of this type of regulation.

In addition, cellular miRNAs can also be involved in regulating the switch from lytic to latent infection in herpesvirus-infected cells by targeting viral mRNAs. Thus, the neuronal specific miR-138 binds to and regulate the immediate early transcript ICP0 in herpes simplex virus 1 infected cells, thereby helping in maintaining latency (Pan et al., 2014). In a similar manner, miRNAs from the miR-200 family regulate latency of the human cytomegalovirus by targeting the viral UL122 transcript (O'Connor et al., 2014).

Indirect Effect by Regulation of Cellular mRNAs

This form of viral regulation by host miRNAs is the most described in the literature. The indirect effect on the virus is in this case due to the targeting of mRNAs encoding host factors involved in one or several steps of the viral cycle or important elements in the establishment of the immune response and defense mechanisms.

Cellular Tropism and Viral Entry

The first step for a virion to start the infection is the permissiveness of a given cell by expression of viral receptors for entry. By regulating the expression of a receptor, miRNAs can therefore regulate the tropism of a virus for a cell type and influence the successful establishment of infection. The aforementioned virus, PRRSV, usually infects macrophages and dendritic cells and, to a lesser extent, monocytes. This tropism is in part due to the lower expression of the PRRSV receptor, CD163, at the surface of these latter cells, whereas it is expressed at higher levels once they differentiate into macrophages or dendritic cells. Gao and collaborators determined that miR-181 targets the 3′ UTR of CD163 mRNA and they showed an inverse

TABLE 2 | Examples of miRNA involved in viral infections.

microRNA	Virus	Effect	Target	Reference
miR-122	HCV	Proviral	Direct: viral 5′ UTR	Jopling et al., 2005; Lanford et al., 2010
miR-485	NDV and H5N1	Proviral/Antiviral	Indirect: RIG-I-mRNA Direct: H5N1 PB1 RNA	Ingle et al., 2015
miR-141	EV71	Proviral	Indirect: eIF4E mRNA	Ho et al., 2011
miR-142-3p	EEEV	Proviral/ Antiviral	Direct: viral 3′ NTR	Trobaugh et al., 2014
miR-17, let-7	Pestiviruses (BVDV)	Proviral	Direct: viral 3′ UTR	Scheel et al., 2016
miR-301a	JEV	Proviral	Indirect: IFN response	Hazra et al., 2017
miR-144	IAV, EMCV, VSV	Proviral	Indirect: TRAF6 mRNA	Rosenberger et al., 2017
miR-146a	HeV	Proviral	Indirect: RNF11 mRNA	Stewart et al., 2013
miR-24, miR-93	VSV	Antiviral	Direct: viral genes L and P	Otsuka et al., 2007
miR-221, miR-222	HIV-1	Antiviral	Indirect: CD4 mRNA	Lodge et al., 2017
miR-181	PRRSV	Antiviral	Indirect: CD163 mRNA	Gao et al., 2013
miR-181	PRRSV	Antiviral	Direct: viral ORF4	Guo et al., 2013
miR-130	PRRSV	Antiviral	Direct: viral 5′ UTR	Li L. et al., 2015
miR-542-5p, miR-24	IAV, RSV	Antiviral	Indirect: p38 MAPK pathway	McCaskill et al., 2017
miR-223	DENV-2	Antiviral	Indirect: STMN1 mRNA	Wu et al., 2014
miR-199, miR-214 and others	MCMV, HCMV, MHV-68, SFV	Antiviral	Indirect: ERK/MAPK, oxidative stress, and PI3K/AKT signaling	Santhakumar et al., 2010
miR-33a	JEV	Antiviral	Indirect: EEF1A1 mRNA	Chen et al., 2016
miR-34, miR-15 and miR-517	DENV, WNV, JEV	Antiviral	Indirect: Wnt pathway	Smith et al., 2017
miR-3614-5p	DENV	Antiviral	Indirect: ADAR1 mRNA	Diosa-Toro et al., 2017
miR-127-3p, miR-486-5p and others	IAV	Antiviral	Direct: viral genome	Peng et al., 2018
miR-25, Let-7, miR-130	HCV	Antiviral	Indirect: HCV co-factors	Li et al., 2017
miR-323, miR-491, and miR-654	IAV	Antiviral	Direct: PB1 RNA	Song et al., 2010
miR-532	WNV	Antiviral	Indirect: SESTD1 mRNA	Slonchak et al., 2016
Hs-154	WNV	Antiviral	Indirect: CTFC and ECOP mRNAs	Smith et al., 2012
miR-555	Poliovirus	Antiviral	Indirect: hnRNPC mRNA	Shim et al., 2016
miR-155	VSV, SeV	Antiviral	Indirect: SOCS mRNA	Wang et al., 2010
miR-197	EV71	Antiviral	Indirect: RAN mRNA	Tang et al., 2016

HCV, Hepatitis C virus; NDV, Newcastle disease virus; H5N1, Influenza A virus subtype H5N1; EV71, Enterovirus 71; EEEV, Eastern equine encephalitis virus; BVDV, Bovine viral diarrhea virus; JEV, Japanese encephalitis virus; IAV, Influenza A virus; EMCV, encephalomyocarditis virus; VSV, Vesicular stomatitis virus; HeV, Hendra virus; HIV-1, Human Immunodeficiency Virus 1; PRRSV, Porcine reproductive and respiratory syndrome virus; RSV, Respiratory Syncytial Virus; DENV, Dengue virus; MCMV, Mouse Cytomegalovirus; HCMV, Human Cytomegalovirus; MHV-68, murine gammaherpesvirus-68; SFV, Semliki forest virus; WNV, West Nile virus; SeV, Sendai virus.

correlation between the miRNA and the receptor expression levels. Through regulation of surface receptor expression, this miRNA hence affects the viral tropism and negatively regulates PRRSV (Gao et al., 2013). It is interesting to note that miR-181 is therefore exerting a dual action on this virus, both direct on the viral genome and indirect by regulation of CD163.

The Human Immunodeficiency Virus 1 (HIV-1) receptor, the CD4 surface protein, was also shown to be regulated by two miRNAs, miR-221 and miR-222. Interestingly, in this study, the authors found that the expression of these two miRNAs was low in productively infected macrophages, whereas it was higher in bystander macrophages. They also linked their upregulation to TNFα production in these cells, and confirmed that miR-221 and miR-222 could restrict entry of HIV-1 by downregulating CD4 expression (Lodge et al., 2017). Furthermore, the HIV-1 tropism is also determined by two different co-receptors, CCR5 or CXCR4. A signaling cascade involving miR-146a regulates CXCR4 expression in resting CD4+ T lymphocytes, making them less susceptible to HIV-1 infection. However, after T cell activation, the transcription factor PLZF downregulates the expression of miR-146a expression, leading to the expression of CXCR4 and hence rendering the cells susceptible to infection (Quaranta et al., 2015).

Cellular Cofactors Involved in Viral Replication

Another level where the viral cycle can be successfully impaired is at the replication step by regulation of cellular cofactors essential for the production of new copies of viral genomes. This is the case during infection by the flavivirus JEV. Chen et al. (2016) found that the expression of miR-33a was repressed in cells infected

with JEV, and that one of the targets of this miRNA was the elongation factor EEF1A1. Interestingly, the authors went on to show that EEF1A1 interacts with components of the replication machinery and contributes to the stabilization of the complex. Therefore, by preventing downregulation of EEF1A via miR-33a, the virus ensures that its replication can occur efficiently (Chen et al., 2016). In the case of poliovirus, replication relies on the recruitment of heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C) to the viral replication machinery. Shim et al. (2016) identified miR-555 as a miRNA with potent antiviral properties in a high-throughput overexpression screen, and showed that hnRNP C was regulated by this miRNA. However, in this case, it is not known whether the virus modulates the expression of this miRNA during infection.

As a last example, the enterovirus EV71 also partially relies on the downregulation of the cellular miRNA miR-197, which is involved in the regulation of the nuclear import factor RAN (Tang et al., 2016). RAN is important for the import of the viral protein 3D and 3DC, which plays an important role for the virus replication, although in an indirect manner.

Translation

Viruses also rely on the host machinery for translation of viral proteins. The *Picornaviridae* family gathers viruses with a positive sense RNA genome whose protein expression is cap-independent and rather depends on an IRES (Martínez-Salas et al., 2015). To hijack the cellular machinery and use it on their advantage, these viruses shut-off the cap-dependent translation. miRNAs can also be involved in this process. For instance, miR-141 has been the first miRNA described to participate in such a process and to have a positive effect on the viral infection (Ho et al., 2011). During EV71 infection, miR-141 expression is upregulated and the expression of the initiation factor eIF4E is repressed. Interestingly, unlike other translation initiation factors, eIF4E in necessary for cap-dependent translation but not for cap-independent translation. Ho and collaborators have shown by Chromatin ImmunoPrecipitation (ChIP) assays that the early growth response 1 (ERG1) transcription factor, which is induced upon infection, enhances miR-141 expression leading to the silencing of eIF4E, promotion of translational switch and increased viral production. This is a novel mechanism of translational switch during EV71 infection involving miR-141.

Immune Response

Innate immunity is the first line of defense against viral infection. After recognition of viral specific elements by different receptors an antiviral response sets in place triggering complex signaling pathways that lead to the activation of interferon response and production of cytokines. The involvement of miRNAs in the control of immune response has been studied extensively (see Taganov et al., 2007 for review), and it is not surprising that some miRNAs are specifically involved during viral infection to regulate the cellular response. This is the case of miR-144, which we mentioned earlier, and which interferes with the immune response allowing an increased replication of different RNA viruses. After validation of computational prediction, miR-144 was shown to act as a positive regulator of viral infection by targeting TRAF6 mRNA thereby regulating IRF7-mediated immune response. Furthermore, *in vivo* assays performed on mice where miR-144 expression is suppressed showed reduced viral infection suggesting a possible use of miRNAs to modulate the host immune response (Rosenberger et al., 2017). Another interesting example is miR-485, which is positively upregulated during infection by NDV or IAV and upon treatment with synthetic dsRNA. The upregulation of this miRNA results in a reduction of interferon and inflammatory cytokines such as IL6. The mechanism by which miR-485 is able to modulate antiviral response is through direct targeting of the RIG-I 3′ UTR thereby affecting the rest of the signaling cascade and the antiviral gene expression. Interestingly, upon influenza H5N1 infection at a high viral load, miR-485 action switches to control the virus and directly targets the viral gene PB1 coding for a RNA-dependent RNA polymerase protein required for replication (Ingle et al., 2015). The regulation of miRNA expression upon viral infection can also be due to the induction of the interferon pathway, which means that some interferon-regulated miRNAs can play a broad antiviral role (Pedersen et al., 2007). More recently, Robertson et al. (2016) identified miR-342 as an important regulator of multiple targets involved in the sterol pathway that is important in the macrophage interferon antiviral response. The authors also showed that this interferon-modulated miRNA was playing an antiviral role against several unrelated viruses (Robertson et al., 2016).

Apoptosis

The ultimate outcome of an efficient immune response is the induction of programmed cell death, which is set in place to avoid further viral production in a cell when the virus has not been successfully cleared. At the same time, apoptosis induction in late stages of infection can also help the virus to spread in the extracellular milieu or in neighboring cells. Following WNV infection, the expression of miR-6124 (referred to as Hs-154 in this paper) is induced. The authors found that this miRNA targets CTFC and ECOP, both anti-apoptotic factors, leading to apoptosis (Smith et al., 2012). Thus, in this case, cell death induction by WNV is partially mediated by the induction of a cellular miRNA. Another miRNA, miR-532, is involved in WNV replication in human cells as well as in mice brain and also interferes with apoptosis during infection. It was found to target TAB3, a protein involved in the NF-kB pathway, known to inhibit apoptosis and promote cell survival. Furthermore, it can also target SESTD1, a phospholipid binding protein localized in the plasma membrane and involved in the regulation of calcium transport by activating Ca^{2+} channels TRPC4 and TRPC5. During WNV infection, calcium can lead to cleavage of caspase 3, interfering with activation of FAK and ERK1/2 pathways, promoting cell survival and thus maintaining the optimal environment for the virus to complete its cycle (Slonchak et al., 2016). The last two examples perfectly illustrate the complexity of deciphering miRNA-mediated regulation during viral infection, since both pro- and anti-apoptotic functions occur simultaneously.

VIRUS-MEDIATED REGULATION OF miRNA ACTIVITY

As mentioned earlier in the introduction, co-evolution between viruses and their host results in an equilibrium to maintain the host alive despite viral infection. It is thus logical that in cases where cellular miRNAs have an antiviral effect, viral counter measures have been observed. In addition, in some cases, the host can also benefit from a modulation of miRNA activity in response to an infection. Several strategies have been reported that are either unspecific, in the sense that the miRNA biogenesis machinery or all cellular miRNAs are impacted upon viral infection; or targeted, since one or a few miRNAs are regulated specifically.

Non-specific Modulation of miRNA

One of the best ways to prevent detrimental targeting by small RNAs is to get rid of all of them. This seemingly radical approach is employed by the vaccinia virus (VACV), a large DNA genome virus, which expresses a poly-A polymerase known as VP55. This viral enzyme was known to be involved in the polyadenylation of the viral mRNAs until Backes et al. (2012) showed that it could also mediate poly A tailing of cellular miRNAs. The addition of these residues apparently results in the degradation of mature miRNAs and can be prevented when the RNA is 2′-*O*-methylated. The authors hypothesize that this strategy of inducing decay of small RNAs initially appeared in arthropod-infecting poxviruses, to remove siRNAs generated by Dicer to control infection. As a counter-counter-measure, insects would then have evolved to modify siRNAs by 2′-*O*-methylation in order to avoid their degradation. In mammals though, miRNAs are not 2′-*O*-methylated, and the benefit of degrading miRNAs for VACV remains elusive, although it could be indeed to prevent direct targeting of viral mRNAs by the host miRNAs. Adenovirus VA1 RNA was also reported some time ago as being able to perturb miRNA biogenesis by saturating Exportin 5 and Dicer (Lu and Cullen, 2004). This very abundant, multifunctional, viral non-coding RNA adopts a hairpin structure, which is also important for the inhibition of PKR or OAS1 (see Vachon and Conn, 2016 for review). The real importance of miRNA biogenesis alteration has not been readily addressed to date.

In other cases, a virus-induced response can lead to the alteration of key factors involved in miRNA activity, but with a net result that is negative for the virus. Thus, Argonaute proteins can be poly-ADP-ribosylated during stress conditions (Leung et al., 2011), including viral infection (Seo et al., 2013), which results in their inactivation. Although at first, it seems that limiting RISC activity might be beneficial for the virus if some miRNAs played antiviral roles, it is in fact the opposite. Indeed, Seo et al. (2013) showed that some ISGs are regulated by miRNAs of the miR-17 family, and therefore blocking Argonaute proteins by ADP-ribosylation results in the de-regulation of these antiviral factors to allow the cells to mount an effective response. Interestingly, antiviral signaling factors such as MAVS or RNase L are involved in the post-translational modification of AGO proteins, although the exact mechanism is not known. The identification of viral factors that could modulate the ADP-ribosylation machinery would be a nice validation of the importance of this pathway in the interplay between viruses and miRNAs.

Specific Regulation of miRNAs by Viruses

As we discussed above, there are cases where it is important for the virus to keep a miRNA target sequence, when it is beneficial during its replication cycle. This is the case with HCV, which has evolved to select a binding site for miR-122, thereby allowing the recruitment of AGO2 at the 5′ extremity of the viral genome to protect it from degradation. However, when a cellular miRNA directly targets a viral RNA, it does not make sense for the virus to maintain the target sequence if it limits its fitness. This is especially true for RNA viruses, which have a greater capacity to evolve quickly. Nevertheless, when the miRNA effect is indirect, it is more complicated to affect the targeting by the miRNA to get rid of its unwanted effect. There are cases where acting indiscriminately on the miRNA biogenesis machinery, as mentioned in the previous part, is not an option, either because the virus relies on this machinery to make its own miRNAs, or because it will have a too strong impact on the longer term. The mouse cytomegalovirus (MCMV) is a betaherpesvirus, which expresses a number of miRNAs (Dölken et al., 2007), and which was also shown to be negatively impacted by overexpression of the cellular miR-27 (Buck et al., 2010). However, in normal conditions, miR-27 does not have any effect on MCMV mainly because upon infection the level of the mature miRNA is dramatically reduced. This observation suggested that the virus somehow developed a strategy to actively degrade this miRNA since the level of the miRNA primary transcript or of the pre-miRNA was unaffected (Buck et al., 2010). It was later found that the virus expressed a transcript that contains a binding site for miR-27 that acts as a decoy to titer out the miRNA and induce its degradation (Libri et al., 2012; Marcinowski et al., 2012). This phenomenon, referred to as target RNA directed miRNA decay (TDMD), is known to occur when a target is almost perfectly complementary to the miRNA (Ameres et al., 2010; de la Mata et al., 2015; Haas et al., 2016). In the case of MCMV, it was indeed confirmed that being able to degrade miR-27 is important in the time course of *in vivo* infection, but it is possible that the virus does require regulation by miR-27 in the early stages of infection. Indeed, the level of expression of the viral transcript involved in the miRNA decay is key in this process, and it is only in late stages of infection that this RNA reaches sufficient levels in order to be able to fulfill its miRNA degradation role. Other viruses, such as Herpesvirus Saimiri (HVS) and human cytomegalovirus also make use of this strategy to remove specific miRNAs (Cazalla et al., 2010; Lee et al., 2013). The case of HVS is interesting since it was later shown that the virus also makes use of one of its own non-coding RNA, known as HSUR (Herpesvirus Saimiri U RNA), not only to repress some but also to recruit other miRNAs to specific targets through dual binding to cellular mRNAs and miRNAs (Gorbea et al., 2017).

CLINICAL APPLICATION OF miRNAs IN VIRAL INFECTION AND THERAPY

Using miRNAs as a tool to modify gene expression still holds many promises for the treatment of different human pathologies (Chakraborty et al., 2017) and, recently, it has become especially attractive in the field of infectious diseases. Indeed, miRNAs are very interesting molecules in the context of antiviral therapy as they show low immunogenicity and, for the ones that show cross-species conservation, they can be tested in various animal models in preclinical studies. Despite the availability of many different methods to inhibit (Li and Rana, 2014) or overexpress a given miRNA (Yang, 2015) *in vivo*, delivery remains a challenge for miRNA-based therapy in future clinical applications.

In addition, the silencing ability of the endogenous miRNA machinery is currently harnessed for the development of live attenuated vaccines. For instance, insertion of a tissue specific miRNA binding site in a given viral genome can disable viral replication in a specific cell type. Similarly, miRNA direct binding to its target sequence can be engineered to assure selectivity in viral cell tropism and to diminish toxicity in the case of oncolytic viruses.

miRNA Targeting as an Antiviral Therapy

An exciting example of miRNA-based treatment for antiviral therapy is represented by the use of inhibitors of miR-122 in HCV infection. An initial study showed that systemic delivery of a 15-nucleotide locked nucleic acid (LNA) oligonucleotide with phosphorothioate modifications (later on named SPC3649 or Miravirsen), complementary to the 5′ end of miR-122, results in sequestration of the endogenous miRNA in non-human primates without any associated toxicity (Elmen et al., 2008). Soon after, silencing of miR-122 by the antisense oligonucleotide Miravirsen was also achieved in chimpanzees with chronic HCV infection and provided long-lasting viral suppression (Lanford et al., 2010). Janssen et al. (2013) conducted a phase 2a study in chronic HCV infected patients who received 5-weekly injections of Miravirsen. The treatment resulted in a prolonged and dose-dependent reduction in HCV RNA levels (Janssen et al., 2013; van der Ree et al., 2014). More recently, assessment of miR-122 plasma levels in chronic HCV infected patients upon Miravirsen treatment demonstrated a significant, specific and prolonged decrease in miR-122 expression, close to detection limits in some cases. However, this was not always accompanied by a substantial reduction in the viral load (van der Ree et al., 2016). Finally, a *N*-acetylgalactosamine conjugated antisense oligonucleotide for miR-122, named RG-101, was developed to increase miR-122 sequestration by improving delivery in hepatocytes. In a phase 1B trial, RG-101 treatment resulted in substantial viral load reduction in all treated patients within 4 weeks (van der Ree et al., 2017).

An efficient delivery of miRNA mimic and/or inhibitor is crucial for *in vivo* therapy. Although not yet at the stage of clinical trials, *in vivo* studies in animals on the antiviral activity of miR-181 and miR-130 against PRRSV support the potential of miRNA intranasal inhalation for future therapies against respiratory viruses. Intranasal delivery of chemically modified miR-181 mimics in pigs caused a slower progression of PRRSV infection thus conferring some temporary protection against the virus (Guo et al., 2013). Moreover, piglets subjected to miR-130 intranasal delivery were able to control the infection and survived longer than controls infected with a lethal dose of the virus (Li L. et al., 2015).

In a very recent study, Peng et al. (2018) demonstrated that the intranasal administration of a combination of five chemically modified miRNA mimics corresponding to highly expressed miRNAs in respiratory epithelial cells was able to target the viral RNA, synergistically suppressed H1N1 replication and protected mice from viral infection. Another study showed that the neurotropic virus JEV induces miR-301 expression in neuronal infected cells which in turns impairs the antiviral host response. *In vivo* inhibition of miR-301 by intracranial injection of modified miR-301a morpholino (see **Table 1**) restores the IFN response improving survival of JEV infected mice by enabling IFNβ production, thereby restricting viral propagation (Hazra et al., 2017). Although very promising for the treatment of neurotropic viral infection, crossing the blood-brain barrier represents an additional difficulty for small RNA-based approaches in the future that will have to be addressed.

Attenuated Vaccines via miRNA-Directed Targeting

Live attenuated vaccines against human viral pathogens are amongst the most successful interventions currently available (Minor, 2015). The natural capacity of cellular miRNAs to inhibit viruses through direct targeting of viral RNAs can be exploited to generate new attenuated vaccines in a tissue specific manner by incorporating cell-specific miRNA target sequences into their genomes. Such a strategy can be very useful to design safe and effective live vaccines. Thus, as a proof-of-principle, insertion of complementary sequences for the neuronal-specific miR-124 into the poliovirus genome restricts its tissue tropism in mice and prevent pathogenicity of the attenuated viral strain (Barnes et al., 2008).

On the same line, an alternative approach based on miRNA-mediated gene silencing was applied to increase attenuation and improve vaccine safety for influenza A virus (Perez et al., 2009). The current live attenuated IAV vaccine is grown in eggs by conferring temperature sensitivity to the virus. Perez and colleagues engineered the virus by inserting non-avian miRNA responsive elements that mediated attenuation in mice, but not in eggs.

Finally, a recent study demonstrated that production of an IAV engineered to be targeted by miR-21, a ubiquitously expressed miRNA, is very efficient in a cell line knocked-out (KO) for this miRNA, while it is broadly attenuated in cells from a range of species across susceptible hosts including humans. The miR-21 KO cell has the potential to be used as a vaccine platform to build and grow viruses targeted by miR-21 and replace the common egg-based approaches for vaccine production (Waring et al., 2017).

Restriction of Viral Tropism in Cancer Gene Therapy

Modification of viral tropism is not only interesting for the development of live miRNA-attenuated vaccines, but also to develop safer replication-competent oncolytic viruses (Kaufman et al., 2015). Oncolytic viruses preferentially replicate in cancer cells and in turn trigger the activation of immune response against the tumor. However, as a side effect, they can induce toxicity in normal tissues. To overcome this issue, target sequences complementary to a specific miRNA can be integrated into the viral genome to reduce replication in normal cells, while maintaining the oncolytic potential in tumor cells. This was reported for the oncolytic picornavirus coxsackie A21 that causes lethal myositis in tumor-bearing mice. Addition of binding sites for the muscle-specific miR-206 and miR-133a reduced myotoxicity while maintaining oncolytic properties (Kelly et al., 2008).

Another example is given by the miRNA-mediated attenuation of Semliki Forest Virus (SFV) neurovirulence in mice. Since SFV is able to infect cells of the central nervous system (CNS), it has been of particular interest in viral therapy of brain tumors but on the other hand additional measures are needed to restrict viral replication in neurons. Neuropathogenicity of Semliki Forest virus can be selectively attenuated by inserting in its genome binding sites for the neuron-specific miR-124, which makes it a promising tool for cancer therapy in the brain (Ylosmaki et al., 2013).

Finally, the altered expression of specific miRNAs representing a hallmark of tumor cells has been used as a way to achieve tumor-specific replication of engineered oncolytic viruses. By taking advantage of the global decreased expression of Let-7 in tumor cells, Edge and colleagues demonstrated that incorporation of Let-7 miRNA complementary sequences within VSV genome eliminates replication and associated toxicity in normal cells but allows growth in cancer cells both *in vitro* and *in vivo* (Edge et al., 2008).

DISCUSSION

In this review, we have extensively covered the various aspects involving miRNAs during viral infection. We deliberately chose to focus on cellular miRNAs in order to avoid to over-complicate our message. However, it is important to keep in mind that some viruses encode their own miRNAs (see Kincaid and Sullivan, 2012 for a recent review), which adds another layer of regulation mediated by the virus. These viral small RNAs are clearly important during infection since they have been selected by the virus to modify the cell environment in a non-immunogenic manner. But, how important are host-encoded miRNAs during viral infection? As we discussed above, there are some cases where there is no doubt that one cellular miRNA has been selected to create an evolutionary advantage for the virus, and the best illustration of this is the role played by miR-122 during HCV infection. However, the contribution of host miRNAs as negative regulators of viruses remains a debated topic. Bogerd et al. (2014) postulated that the replication of many human viruses was unaffected by endogenous miRNAs. The authors generated a Dicer knock-out cell line that was then infected with a variety of viruses such as DENV, WNV, yellow fever virus, Sindbis virus, measles virus, influenza A virus, VSV or HIV-1 and compared the level of virus production with the parental cells infected with the same viruses (Bogerd et al., 2014). Since no significant difference between cells depleted of or expressing Dicer was observed, they concluded that host miRNAs had no impact on virus replication. One limitation of the study though was the choice of one single cell type (HEK293T cells), which would not take into account tissue-specific expression of certain miRNAs. However, for future studies, it will be important to consider these observations when assessing the effect on a given virus of miRNAs known to be expressed in HEK293T cells.

Another group also used a global approach to show that depletion of miRNAs did not have strong effects in term of antiviral response. Aguado et al. (2015) expressed the vaccinia virus VP55 protein that we mentioned previously as able to induce degradation of all cellular miRNAs and they measured the effect on the cellular response to viral infection. One of their main conclusions was that in cells expressing VP55, and thus devoid of mature miRNAs, the acute response to a challenge with dsRNA or IFN-β was overall unaffected. However, one clear difference in cells without miRNAs was an increased cytokine production when challenged, which would indicate that miRNAs do play important roles during chronic infection and activation of the immune response. In this study, the authors did not assess the impact of VP55 expression on a *bona fide* viral infection and only used synthetic challenges, but the expression of VP55 might have other miRNA-independent effects on viral replication that would complicate the interpretation of the results.

Although the opposite cannot be strictly ruled out, it only seems logical that cellular miRNAs would not play critical roles in acute infections, since typically a miRNA-mediated effect is more that of a fine-tuning than of an on/off switch. In addition, even if a cellular target playing an important role in antiviral response was strongly regulated at the mRNA level, the half-life of the protein would have to be very short in order to result in a meaningful effect in the early phase of infection. Therefore, when assessing the role of cellular miRNAs, we can safely say that they are crucial when they are proviral, or when a longer, persistent infection is established. We have also seen that viruses do have an impact on miRNA expression pattern, which is in favor of a real importance of at least these specific miRNAs during infection. Finally, with the advent of techniques that allow to validate the physical interactions between miRNAs and viral genomes, a number of examples where the miRNA acts by binding directly to the pathogen RNA has been recently reported (Scheel et al., 2016). Again, these examples only make sense biologically when the effect is beneficial for the virus, otherwise the virus will find a way to prevent inhibition (Cullen, 2013).

Can we harness the results obtained on the study of miRNA/virus interactions to design novel therapeutic approaches? We described the use of antisense oligonucleotides to block proviral miRNAs, which is the most straightforward and easy to implement way toward new drugs. But, miRNAs can also

be used as an entry gate into regulatory networks that could be explored to find new unconventional therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Agarwal, V., Bell, G. W., Nam, J.-W., and Bartel, D. P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *eLife* 4:e05005. doi: 10.7554/eLife.05005

Aguado, L. C., Schmid, S., Sachs, D., Shim, J. V., Lim, J. K., and tenOever, B. R. (2015). microRNA function is limited to cytokine control in the acute response to virus infection. *Cell Host Microbe* 18, 714–722. doi: 10.1016/j.chom.2015.11.003

Ameres, S. L., Horwich, M. D., Hung, J. H., Xu, J., Ghildiyal, M., Weng, Z., et al. (2010). Target RNA-directed trimming and tailing of small silencing RNAs. *Science* 328, 1534–1539. doi: 10.1126/science.1187058

Backes, S., Shapiro, J. S., Sabin, L. R., Pham, A. M., Reyes, I., Moss, B., et al. (2012). Degradation of host microrna by poxvirus Poly(A) polymerase reveals terminal RNA methylation as a protective antiviral mechanism. *Cell Host Microbe* 12, 200–210. doi: 10.1016/j.chom.2012.05.019

Barber, G. N. (2001). Host defense, viruses and apoptosis. *Cell Death Differ.* 8, 113–126. doi: 10.1038/sj.cdd.4400823

Barnes, D., Kunitomi, M., Vignuzzi, M., Saksela, K., and Andino, R. (2008). Harnessing endogenous miRNAs to Control virus tissue tropism as a strategy for developing attenuated virus vaccines. *Cell Host Microbe* 4, 239–248. doi: 10.1016/j.chom.2008.08.003

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Bartel, D. P. (2018). Metazoan MicroRNAs. *Cell* 173, 20–51. doi: 10.1016/j.cell.2018.03.006

Bassett, A. R., Azzam, G., Wheatley, L., Tibbit, C., Rajakumar, T., McGowan, S., et al. (2014). Understanding functional miRNA–target interactions in vivo by site-specific genome engineering. *Nat. Commun.* 5:4640. doi: 10.1038/ncomms5640

Bogerd, H. P., Skalsky, R. L., Kennedy, E. M., Furuse, Y., Whisnant, A. W., Flores, O., et al. (2014). Replication of many human viruses is refractory to inhibition by endogenous cellular microRNAs. *J. Virol.* 88, 8065–8076. doi: 10.1128/JVI.00985-14

Broughton, J. P., Lovci, M. T., Huang, J. L., Yeo, G. W., and Pasquinelli, A. E. (2016). Pairing beyond the seed supports MicroRNA targeting specificity. *Mol. Cell* 64, 320–333. doi: 10.1016/j.molcel.2016.09.004

Buck, A. H., Perot, J., Chisholm, M. A., Kumar, D. S., Tuddenham, L., Cognat, V., et al. (2010). Post-transcriptional regulation of miR-27 in murine cytomegalovirus infection. *RNA* 16, 307–315. doi: 10.1261/rna.1819210

Cazalla, D., Yario, T., Steitz, J. A., and Steitz, J. (2010). Down-regulation of a host microRNA by a *Herpesvirus saimiri* noncoding RNA. *Science* 328, 1563–1566. doi: 10.1126/science.1187197

Chakraborty, C., Sharma, A. R., Sharma, G., Doss, C. G. P., and Lee, S.-S. (2017). Therapeutic miRNA and siRNA: moving from bench to clinic as next generation medicine. *Mol. Ther. Nucleic Acids* 8, 132–143. doi: 10.1016/j.omtn.2017.06.005

Chen, Z., Ye, J., Ashraf, U., Li, Y., Wei, S., Wan, S., et al. (2016). MicroRNA-33a-5p modulates Japanese encephalitis virus replication by targeting eukaryotic translation elongation factor 1A1. *J. Virol.* 90, 3722–3734. doi: 10.1128/JVI.03242-15

Chi, S. W., Zang, J. B., Mele, A., and Darnell, R. B. (2009). Argonaute HITS-CLIP decodes microRNA–mRNA interaction maps. *Nature* 460, 479–486. doi: 10.1038/nature08170

Chou, C.-H., Chang, N.-W., Shrestha, S., Hsu, S.-D., Lin, Y.-L., Lee, W.-H., et al. (2016). miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Res.* 44, D239–D247. doi: 10.1093/nar/gkv1258

Cullen, B. R. (2013). How do viruses avoid inhibition by endogenous cellular MicroRNAs? *PLoS Pathog.* 9:e1003694. doi: 10.1371/journal.ppat.1003694

de la Mata, M., Gaidatzis, D., Vitanescu, M., Stadler, M. B., Wentzel, C., Scheiffele, P., et al. (2015). Potent degradation of neuronal miRNAs induced by highly complementary targets. *EMBO Rep.* 16, 500–511. doi: 10.15252/embr.201540078

Denzler, R., McGeary, S. E., Title, A. C., Agarwal, V., Bartel, D. P., and Stoffel, M. (2016). Impact of MicroRNA levels, target-site complementarity, and cooperativity on competing endogenous RNA-Regulated gene expression. *Mol. Cell* 64, 565–579. doi: 10.1016/j.molcel.2016.09.027

Ding, S. W., and Voinnet, O. (2007). Antiviral immunity directed by small RNAs. *Cell* 130, 413–426. doi: 10.1016/j.cell.2007.07.039

Diosa-Toro, M., Echavarría-Consuegra, L., Flipse, J., Fernández, G. J., Kluiver, J., van den Berg, A., et al. (2017). MicroRNA profiling of human primary macrophages exposed to dengue virus identifies miRNA-3614-5p as antiviral and regulator of ADAR1 expression. *PLoS Neglect. Trop. Dis.* 11:e0005981. doi: 10.1371/journal.pntd.0005981

Doench, J. G., and Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. *Genes Dev.* 18, 504–511. doi: 10.1101/gad.1184404

Dölken, L., Perot, J., Cognat, V., Alioua, A., John, M., Soutschek, J., et al. (2007). Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation. *J. Virol.* 81, 13771–13782. doi: 10.1128/JVI.01313-07

Easow, G., Teleman, A. A., and Cohen, S. M. (2007). Isolation of microRNA targets by miRNP immunopurification. *RNA* 13, 1198–1204. doi: 10.1261/rna.563707

Ebert, M. S., and Sharp, P. A. (2010). Emerging roles for natural microRNA sponges. *Curr. Biol.* 20, R858–R861. doi: 10.1016/j.cub.2010.08.052

Edge, R. E., Falls, T. J., Brown, C. W., Lichty, B. D., Atkins, H., and Bell, J. C. (2008). A let-7 MicroRNA-sensitive vesicular stomatitis virus demonstrates tumor-specific replication. *Mol. Ther.* 16, 1437–1443. doi: 10.1038/mt.2008.130

Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., et al. (2008). LNA-mediated microRNA silencing in non-human primates. *Nature* 452, 896–899. doi: 10.1038/nature06783

Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 19, 92–105. doi: 10.1101/gr.082701.108

Gao, L., Guo, X.-K., Wang, L., Zhang, Q., Li, N., Chen, X.-X., et al. (2013). MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. *J. Virol.* 87, 8808–8812. doi: 10.1128/JVI.00718-13

Gorbea, C., Mosbruger, T., and Cazalla, D. (2017). A viral Sm-class RNA base-pairs with mRNAs and recruits microRNAs to inhibit apoptosis. *Nature* 550, 275–279. doi: 10.1038/nature24034

Grimson, A., Farh, K. K.-H., Johnston, W. K., Garrett-Engele, P., Lim, L. P., and Bartel, D. P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell* 27, 91–105. doi: 10.1016/j.molcel.2007.06.017

Guo, X.-K., Zhang, Q., Gao, L., Li, N., Chen, X.-X., and Feng, W.-H. (2013). increasing expression of MicroRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. *J. Virol.* 87, 1159–1171. doi: 10.1128/JVI.02386-12

Haas, G., Cetin, S., Messmer, M., Chane-Woon-Ming, B., Terenzi, O., Chicher, J., et al. (2016). Identification of factors involved in target RNA-directed microRNA degradation. *Nucleic Acids Res.* 44, 2873–2887. doi: 10.1093/nar/gkw040

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., et al. (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141. doi: 10.1016/j.cell.2010.03.009

Hazra, B., Kumawat, K. L., and Basu, A. (2017). The host microRNA miR-301a blocks the IRF1-mediated neuronal innate immune response to Japanese encephalitis virus infection. *Sci. Signal.* 10:eaaf5185. doi: 10.1126/scisignal.aaf5185

Henke, J. I., Goergen, D., Zheng, J., Song, Y., Schüttler, C. G., Fehr, C., et al. (2008). microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J.* 27, 3300–3310. doi: 10.1038/emboj.2008.244

Ho, B.-C., Yu, S.-L., Chen, J. J. W., Chang, S.-Y., Yan, B.-S., Hong, Q.-S., et al. (2011). Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. *Cell Host Microbe* 9, 58–69. doi: 10.1016/j.chom.2010.12.001

Hsu, P. W.-C., Lin, L.-Z., Hsu, S.-D., Hsu, J. B.-K., and Huang, H.-D. (2007). ViTa: prediction of host microRNAs targets on viruses. *Nucleic Acids Res.* 35, D381–D385. doi: 10.1093/nar/gkl1009

Hsu, S.-D., Lin, F.-M., Wu, W.-Y., Liang, C., Huang, W.-C., Chan, W.-L., et al. (2011). miRTarBase: a database curates experimentally validated microRNA–target interactions. *Nucleic Acids Res.* 39, D163–D169. doi: 10.1093/nar/gkq1107

Ingle, H., Kumar, S., Raut, A. A., Mishra, A., Kulkarni, D. D., Kameyama, T., et al. (2015). The microRNA miR-485 targets host and influenza virus transcripts to regulate antiviral immunity and restrict viral replication. *Sci. Signal.* 8:ra126. doi: 10.1126/scisignal.aab3183

Janssen, H. L. A., Reesink, H. W., Lawitz, E. J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., et al. (2013). Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* 368, 1685–1694. doi: 10.1056/NEJMoa1209026

John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004). Human microRNA targets. *PLoS Biol.* 2:e363. doi: 10.1371/journal.pbio.0020363

Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309, 1577–1581. doi: 10.1126/science.1113329

Kaufman, H. L., Kohlhapp, F. J., and Zloza, A. (2015). Oncolytic viruses: a new class of immunotherapy drugs. *Nat. Rev. Drug Discov.* 14, 642–662. doi: 10.1038/nrd4663

Kelly, E. J., Hadac, E. M., Greiner, S., and Russell, S. J. (2008). Engineering microRNA responsiveness to decrease virus pathogenicity. *Nat. Med.* 14, 1278–1283. doi: 10.1038/nm.1776

Kincaid, R. P., and Sullivan, C. S. (2012). Virus-encoded microRNAs: an overview and a look to the future. *PLoS Pathog.* 8:e1003018. doi: 10.1371/journal.ppat.1003018

Kiriakidou, M., Nelson, P. T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., et al. (2004). A combined computational-experimental approach predicts human microRNA targets. *Genes Dev.* 18, 1165–1178. doi: 10.1101/gad.1184704

Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42, D68–D73. doi: 10.1093/nar/gkt1181

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., et al. (2005). Combinatorial microRNA target predictions. *Nat. Genet.* 37, 495–500. doi: 10.1038/ng1536

Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., et al. (2005). Silencing of microRNAs in vivo with "antagomirs. *Nature* 438, 685–689. doi: 10.1038/nature04303

Kumari, B., Jain, P., Das, S., Ghosal, S., Hazra, B., Trivedi, A. C., et al. (2016). Dynamic changes in global microRNAome and transcriptome reveal complex miRNA-mRNA regulated host response to Japanese encephalitis virus in microglial cells. *Sci. Rep.* 6:20263. doi: 10.1038/srep20263

Lai, E. C., Tam, B., and Rubin, G. M. (2005). Pervasive regulation of *Drosophila* Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. *Genes Dev.* 19, 1067–1080. doi: 10.1101/gad.1291905

Lanford, R. E., Hildebrandt-Eriksen, E. S., Petri, A., Persson, R., Lindow, M., Munk, M. E., et al. (2010). Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327, 198–201. doi: 10.1126/science.1178178

Lee, S., Song, J., Kim, S., Kim, J., Hong, Y., Kim, Y., et al. (2013). Selective degradation of host MicroRNAs by an intergenic HCMV noncoding RNA accelerates virus production. *Cell Host Microbe* 13, 678–690. doi: 10.1016/j.chom.2013.05.007

Leung, A. K. L., Vyas, S., Rood, J. E., Bhutkar, A., Sharp, P. A., and Chang, P. (2011). Poly(ADP-Ribose) regulates stress responses and microrna activity in the cytoplasm. *Mol. Cell.* 42, 489–499. doi: 10.1016/j.molcel.2011.04.015

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are MicroRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035

Lewis, B. P., Shih, I., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell* 115, 787–798. doi: 10.1016/S0092-8674(03)01018-3

Li, L., Gao, F., Jiang, Y., Yu, L., Zhou, Y., Zheng, H., et al. (2015). Cellular miR-130b inhibits replication of porcine reproductive and respiratory syndrome virus in vitro and in vivo. *Sci. Rep.* 5:17010. doi: 10.1038/srep17010

Li, Q., Lowey, B., Sodroski, C., Krishnamurthy, S., Alao, H., Cha, H., et al. (2017). Cellular microRNA networks regulate host dependency of hepatitis C virus infection. *Nat. Commun.* 8:1789. doi: 10.1038/s41467-017-01954-x

Li, Y., Yamane, D., and Lemon, S. M. (2015). Dissecting the roles of the 5' exoribonucleases Xrn1 and Xrn2 in restricting hepatitis C virus replication. *J. Virol.* 89, 4857–4865. doi: 10.1128/JVI.03692-14

Li, Z., and Rana, T. M. (2014). Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discov.* 13, 622–638. doi: 10.1038/nrd4359

Libri, V., Helwak, A., Miesen, P., Santhakumar, D., Borger, J. G., Kudla, G., et al. (2012). Murine cytomegalovirus encodes a miR-27 inhibitor disguised as a target. *Proc. Natl. Acad. Sci. U.S.A.* 109, 279–284. doi: 10.1073/pnas.1114204109

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., et al. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. doi: 10.1038/nature03315

Lodge, R., Ferreira Barbosa, J. A., Lombard-Vadnais, F., Gilmore, J. C., Deshiere, A., Gosselin, A., et al. (2017). Host MicroRNAs-221 and -222 Inhibit HIV-1 entry in macrophages by targeting the CD4 viral receptor. *Cell Rep.* 21, 141–153. doi: 10.1016/j.celrep.2017.09.030

Lu, S., and Cullen, B. R. (2004). Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and MicroRNA biogenesis. *J. Virol.* 78, 12868–12876. doi: 10.1128/JVI.78.23.12868-12876.2004

Luna, J. M., Scheel, T. K. H., Danino, T., Shaw, K. S., Mele, A., Fak, J. J., et al. (2015). Hepatitis C Virus RNA functionally sequesters miR-122. *Cell* 160, 1099–1110. doi: 10.1016/j.cell.2015.02.025

Machlin, E. S., Sarnow, P., and Sagan, S. M. (2011). Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3193–3198. doi: 10.1073/pnas.1012464108

Marcinowski, L., Tanguy, M., Krmpotic, A., Rädle, B., Lisniæ, V. J., Tuddenham, L., et al. (2012). Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication in vivo. *PLoS Pathog.* 8:e1002510. doi: 10.1371/journal.ppat.1002510

Martínez-Salas, E., Francisco-Velilla, R., Fernandez-Chamorro, J., Lozano, G., and Diaz-Toledano, R. (2015). Picornavirus IRES elements: RNA structure and host protein interactions. *Virus Res.* 206, 62–73. doi: 10.1016/j.virusres.2015.01.012

McCaskill, J. L., Ressel, S., Alber, A., Redford, J., Power, U. F., Schwarze, J., et al. (2017). Broad-Spectrum inhibition of respiratory virus infection by MicroRNA

mimics targeting p38 MAPK signaling. *Mol. Ther. Nucleic Acids* 7, 256–266. doi: 10.1016/j.omtn.2017.03.008

Minor, P. D. (2015). Live attenuated vaccines: historical successes and current challenges. *Virology* 47, 379–392. doi: 10.1016/j.virol.2015.03.032

Moore, M. J., Scheel, T. K. H., Luna, J. M., Park, C. Y., Fak, J. J., Nishiuchi, E., et al. (2015). miRNA–target chimeras reveal miRNA 3'-end pairing as a major determinant of Argonaute target specificity. *Nat. Commun.* 6:8864. doi: 10.1038/ncomms9864

Motsch, N., Alles, J., Imig, J., Zhu, J., Barth, S., Reineke, T., et al. (2012). MicroRNA profiling of Epstein-Barr virus-associated NK/T-cell lymphomas by deep sequencing. *PLoS One* 7:e42193. doi: 10.1371/journal.pone.0042193

Nakhaei, P., Genin, P., Civas, A., and Hiscott, J. (2009). RIG-I-like receptors: sensing and responding to RNA virus infection. *Semin. Immunol.* 21, 215–222. doi: 10.1016/j.smim.2009.05.001

O'Connor, C. M., Vanicek, J., and Murphy, E. A. (2014). Host microRNA regulation of human cytomegalovirus immediate early protein translation promotes viral latency. *J. Virol.* 88, 5524–5532. doi: 10.1128/JVI.00481-14

Otsuka, M., Jing, Q., Georgel, P., New, L., Chen, J., Mols, J., et al. (2007). Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* 27, 123–134. doi: 10.1016/j.immuni.2007.05.014

Oussaief, L., Fendri, A., Chane-Woon-Ming, B., Poirey, R., Delecluse, H.-J., Joab, I., et al. (2015). Modulation of MicroRNA cluster miR-183-96-182 expression by Epstein-Barr Virus latent membrane protein 1. *J. Virol.* 89, 12178–12188. doi: 10.1128/JVI.01757-15

Pan, D., Flores, O., Umbach, J. L., Pesola, J. M., Bentley, P., Rosato, P. C., et al. (2014). A neuron-specific host microRNA targets herpes simplex virus-1 ICP0 expression and promotes latency. *Cell Host Microbe* 15, 446–456. doi: 10.1016/j.chom.2014.03.004

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V., et al. (2007). Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* 449, 919–922. doi: 10.1038/nature06205

Peng, S., Wang, J., Wei, S., Li, C., Zhou, K., Hu, J., et al. (2018). Endogenous cellular MicroRNAs mediate antiviral defense against influenza A Virus. *Mol. Ther. Nucleic Acids* 10, 361–375. doi: 10.1016/j.omtn.2017.12.016

Perez, J. T., Pham, A. M., Lorini, M. H., Chua, M. A., Steel, J., and tenOever, B. R. (2009). MicroRNA-mediated species-specific attenuation of influenza A virus. *Nat. Biotechnol.* 27, 572–576. doi: 10.1038/nbt.1542

Pinzón, N., Li, B., Martinez, L., Sergeeva, A., Presumey, J., Apparailly, F., et al. (2017). microRNA target prediction programs predict many false positives. *Genome Res.* 27, 234–245. doi: 10.1101/gr.205146.116

Quaranta, M. T., Olivetta, E., Sanchez, M., Spinello, I., Paolillo, R., Arenaccio, C., et al. (2015). miR-146a controls CXCR4 expression in a pathway that involves PLZF and can be used to inhibit HIV-1 infection of CD4 + T lymphocytes. *Virology* 478, 27–38. doi: 10.1016/j.virol.2015.01.016

Rausch, M., Ecsedi, M., Bartake, H., Müllner, A., and Grosshans, H. (2015). A genetic interactome of the let-7 microRNA in *C. elegans*. *Dev. Biol.* 401, 276–286. doi: 10.1016/j.ydbio.2015.02.013

Robertson, K. A., Hsieh, W. Y., Forster, T., Blanc, M., Lu, H., Crick, P. J., et al. (2016). An interferon regulated microrna provides broad cell-intrinsic antiviral immunity through multihit host-directed targeting of the sterol pathway. *PLoS Biol.* 14:e1002364. doi: 10.1371/journal.pbio.1002364

Rosenberger, C. M., Podyminogin, R. L., Diercks, A. H., Treuting, P. M., Peschon, J. J., Rodriguez, D., et al. (2017). miR-144 attenuates the host response to influenza virus by targeting the TRAF6-IRF7 signaling axis. *PLoS Pathog.* 13:e1006305. doi: 10.1371/journal.ppat.1006305

Santhakumar, D., Forster, T., Laqtom, N. N., Fragkoudis, R., Dickinson, P., Abreu-Goodger, C., et al. (2010). Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13830–13835. doi: 10.1073/pnas.1008861107

Scheel, T. K. H., Luna, J. M., Liniger, M., Nishiuchi, E., Rozen-Gagnon, K., Shlomai, A., et al. (2016). A broad RNA Virus survey reveals both miRNA dependence and functional sequestration. *Cell Host Microbe* 19, 409–423. doi: 10.1016/j.chom.2016.02.007

Schirle, N. T., and MacRae, I. J. (2012). The crystal structure of human Argonaute2. *Science* 336, 1037–1040. doi: 10.1126/science.1221551

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58–63. doi: 10.1038/nature07228

Seo, G. J., Kincaid, R. P., Phanaksri, T., Burke, J. M., Pare, J. M., Cox, J. E., et al. (2013). Reciprocal inhibition between intracellular antiviral signaling and the RNAi machinery in mammalian cells. *Cell Host Microbe* 14, 435–445. doi: 10.1016/j.chom.2013.09.002

Shim, B.-S., Wu, W., Kyriakis, C. S., Bakre, A., Jorquera, P. A., Perwitasari, O., et al. (2016). MicroRNA-555 has potent antiviral properties against poliovirus. *J. Gen. Virol.* 97, 659–668. doi: 10.1099/jgv.0.000372

Shimakami, T., Yamane, D., Jangra, R. K., Kempf, B. J., Spaniel, C., Barton, D. J., et al. (2012). Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. *Proc. Natl. Acad. Sci. U.S.A.* 109, 941–946. doi: 10.1073/pnas.1112263109

Slonchak, A., Shannon, R. P., Pali, G., and Khromykh, A. A. (2016). Human MicroRNA miR-532-5p exhibits antiviral activity against west Nile Virus via suppression of host Genes SESTD1 and TAB3 required for virus replication. *J. Virol.* 90, 2388–2402. doi: 10.1128/JVI.02608-15

Smith, J. L., Grey, F. E., Uhrlaub, J. L., Nikolich-Zugich, J., and Hirsch, A. J. (2012). Induction of the Cellular MicroRNA, Hs_154, by West Nile Virus contributes to Virus-Mediated Apoptosis through repression of antiapoptotic factors. *J. Virol.* 86, 5278–5287. doi: 10.1128/JVI.06883-11

Smith, J. L., Jeng, S., McWeeney, S. K., and Hirsch, A. J. (2017). A MicroRNA screen identifies the Wnt signaling pathway as a regulator of the interferon response during flavivirus infection. *J. Virol.* 91:e02388-16. doi: 10.1128/JVI.02388-16

Song, L., Liu, H., Gao, S., Jiang, W., and Huang, W. (2010). Cellular MicroRNAs inhibit replication of the H1N1 influenza A virus in infected cells. *J. Virol.* 84, 8849–8860. doi: 10.1128/JVI.00456-10

Stewart, C. R., Marsh, G. A., Jenkins, K. A., Gantier, M. P., Tizard, M. L., Middleton, D., et al. (2013). Promotion of Hendra virus replication by microRNA 146a. *J. Virol.* 87, 3782–3791. doi: 10.1128/JVI.01342-12

Taganov, K. D., Boldin, M. P., and Baltimore, D. (2007). MicroRNAs and immunity: tiny players in a big field. *Immunity* 26, 133–137. doi: 10.1016/j.immuni.2007.02.005

Tang, W.-F., Huang, R.-T., Chien, K.-Y., Huang, J.-Y., Lau, K.-S., Jheng, J.-R., et al. (2016). Host MicroRNA miR-197 Plays a negative regulatory role in the enterovirus 71 infectious cycle by targeting the RAN protein. *J. Virol.* 90, 1424–1438. doi: 10.1128/JVI.02143-15

Trobaugh, D. W., Gardner, C. L., Sun, C., Haddow, A. D., Wang, E., Chapnik, E., et al. (2014). RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. *Nature* 506, 245–248. doi: 10.1038/nature12869

Tsang, J., Zhu, J., and van Oudenaarden, A. (2007). MicroRNA-Mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol. Cell.* 26, 753–767. doi: 10.1016/j.molcel.2007.05.018

Vachon, V. K., and Conn, G. L. (2016). Adenovirus VA RNA: an essential pro-viral non-coding RNA. *Virus Res.* 212, 39–52. doi: 10.1016/j.virusres.2015.06.018

van der Ree, M. H., de Vree, J. M., Stelma, F., Willemse, S., van der Valk, M., Rietdijk, S., et al. (2017). Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. *Lancet* 389, 709–717. doi: 10.1016/S0140-6736(16)31715-9

van der Ree, M. H., van der Meer, A. J., de Bruijne, J., Maan, R., van Vliet, A., Welzel, T. M., et al. (2014). Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. *Antiviral Res.* 111, 53–59. doi: 10.1016/j.antiviral.2014.08.015

van der Ree, M. H., van der Meer, A. J., van Nuenen, A. C., de Bruijne, J., Ottosen, S., Janssen, H. L., et al. (2016). Miravirsen dosing in chronic hepatitis C patients results in decreased microRNA-122 levels without affecting other microRNAs in plasma. *Aliment. Pharmacol. Therap.* 43, 102–113. doi: 10.1111/apt.13432

Wang, P., Hou, J., Lin, L., Wang, C., Liu, X., Li, D., et al. (2010). Inducible microRNA-155 feedback promotes Type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J. Immunol.* 185, 6226–6233. doi: 10.4049/jimmunol.1000491

Waring, B. M., Sjaastad, L. E., Fiege, J. K., Fay, E. J., Reyes, I., Moriarity, B., et al. (2017). MicroRNA-Based attenuation of influenza virus across susceptible hosts. *J. Virol.* 92:e01741-17. doi: 10.1128/JVI.01741-17

Wu, N., Gao, N., Fan, D., Wei, J., Zhang, J., and An, J. (2014). miR-223 inhibits dengue virus replication by negatively regulating the microtubule-destabilizing protein STMN1 in EAhy926 cells. *Microbes Infect.* 16, 911–922. doi: 10.1016/j.micinf.2014.08.011

Xie, J., Ameres, S. L., Friedline, R., Hung, J.-H., Zhang, Y., Xie, Q., et al. (2012). Long-term, efficient inhibition of microRNA function in mice using rAAV vectors. *Nat. Methods* 9, 403–409. doi: 10.1038/nmeth.1903

Yang, N. (2015). An overview of viral and nonviral delivery systems for microRNA. *Int. J. Pharm. Investig.* 5, 179–181. doi: 10.4103/2230-973X.167646

Ylosmaki, E., Martikainen, M., Hinkkanen, A., and Saksela, K. (2013). Attenuation of semliki forest virus neurovirulence by MicroRNA-Mediated detargeting. *J. Virol.* 87, 335–344. doi: 10.1128/JVI.01940-12

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B. Article 2: Importance des microARN cellulaires dans la régulation des infections virales

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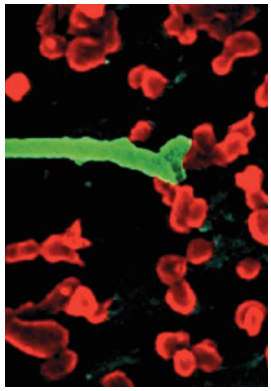
Importance des microARN cellulaires dans la régulation des infections virales

Paula López, Erika Girardi, Sébastien Pfeffer

› Parasites intracellulaires obligatoires, les virus dépendent d'un grand nombre de facteurs cellulaires pour accomplir leur cycle de multiplication. Parmi ceux-ci, les microARN (miARN) ont récemment émergé comme d'importants modulateurs des infections virales. Ces petites molécules régulatrices agissent comme des répresseurs de l'expression des gènes. Au cours de l'infection, ils peuvent agir sur des ARN cibles d'origine cellulaire mais aussi virale. Cette synthèse fait le point sur les différents mécanismes, directs et indirects, impliquant ces miARN dans la régulation des virus et aborde les possibles applications thérapeutiques qui peuvent en découler. ‹



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Vignette (Photo © Inserm- Thérèse Couderc).

Parasites intracellulaires obligatoires, les virus dépendent exclusivement de la machinerie cellulaire pour accomplir leur cycle de réplication et produire de nouvelles particules infectieuses. En réponse à cette invasion, la cellule met en place différentes stratégies de défense afin d'éliminer ce pathogène. De leur côté, les virus évoluent et s'adaptent aux différentes stratégies de défense de la cellule qu'ils infectent, ce qui conduit à une « course à l'armement » constante entre virus et cellule.

Une des voies cellulaires impliquée dans cette dynamique d'interaction entre les virus et leur hôte est la voie reposant sur les microARN (miARN). Ces petits ARN sont des ARN non-codants qui participent à la régulation, au niveau post-transcriptionnel, de l'expression de gènes jouant un rôle dans de nombreux processus cellulaires. Ils sont transcrits sous la forme d'un long précurseur dont la maturation s'effectue en deux étapes : dans le noyau, par le microprocesseur (Drosha et DGCR8 [*DiGeorge critical syndrome region 8*]), et, après export dans le cytoplasme, par Dicer et son cofacteur TRBP (*tat RNA-binding protein*). Le miARN mature est alors chargé sur une protéine Argonaute et dirigé vers son ARN messager (ARNm) cible grâce à la séquence « *seed* » correspondant aux nucléotides 2 à 8 qu'il contient. Cette séquence lui permet de guider la protéine Argonaute sur ses ARNm cibles par appariement de séquence, le plus souvent dans la région 3' non traduite (UTR) de ces ARNm. La fixation de la protéine Argonaute sur un ARNm induit l'inhibition de l'initiation de sa traduction et sa dégradation par le recrutement de protéines à l'origine de sa déadénylation *(Figure 1)* [1].

Dans cet article, nous passerons en revue les techniques couramment utilisées pour identifier les miARN impliqués dans les infections virales ainsi que leurs cibles. Nous décrirons ensuite quelques exemples de virus dont l'infection est modulée par des miARN et discuterons des possibles applications thérapeutiques résultant du ciblage de ces ARN régulateurs.

Identification des miARN impliqués dans le contrôle de l'infection virale

Le potentiel de régulation des miARN est très important : il existe en effet environ 2 000 gènes codant des miARN qui ont été annotés dans le génome humain. En raison du faible nombre de nucléotides requis pour leur interaction avec leur cible, chacun de ces miARN peut potentiellement participer à la régulation de nombreux ARNm, y compris ceux qui ont une origine virale. L'expression et l'abondance de ces molécules régulatrices diffèrent selon le type cellulaire étudié, ce qui a un impact sur le réseau de cibles potentielles. Une des premières approches à mettre en œuvre lorsqu'on veut identifier les miARN participant à la régulation de l'infection par un virus donné sera donc de déterminer le profil d'expression de ces miARN dans les cellules qu'il

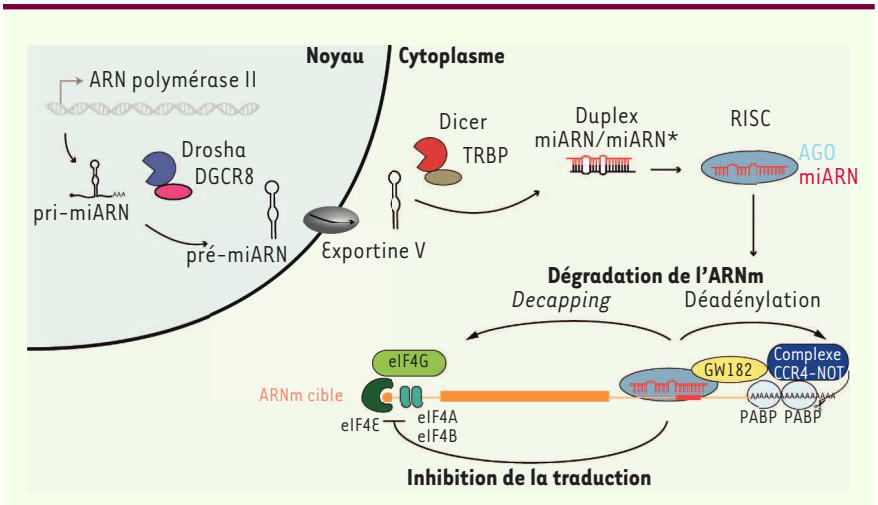

***Figure 1.* Biogenèse et fonction canoniques des miARN.** Les gènes de miARN sont transcrits par l'ARN polymérase II en précurseur primaire de microARN (pri-miARN) qui est clivé dans le noyau par le complexe Microprocesseur (Drosha et son cofacteur DGCR8) pour produire un miARN précurseur à la structure en épingle à cheveux (pré-miARN) qui est exporté vers le cytoplasme par l'exportine V. Le pré-miARN est ensuite clivé par Dicer en duplex de miARN, qui sera ensuite chargé sur une protéine Argonaute (AGO) dans le complexe de *silencing* induit par l'ARN (RISC). Un des brins reste lié à AGO (miARN mature) et joue un rôle clef dans la régulation des gènes au niveau post-transcriptionnel en ciblant les ARNm par liaison de la région « *seed* » (nucléotides en position 2-8) (le site de liaison représenté par un rectangle rouge). La protéine adaptatrice GW182 est recrutée par RISC et peut interagir avec les protéines de liaison à la queue polyA (PABP) induisant le recrutement du complexe de déadénylation CCR4-NOT. L'ARNm est déstabilisé par la déadénylation et le *decapping* conduisant à sa dégradation. La traduction des ARNm ciblés est également réprimée par l'inhibition de l'assemblage du complexe de préinitiation.

cible en l'absence d'infection et après l'entrée du virus en utilisant principalement le séquençage à haut débit [2]. D'autres approches, comme les puces à ARN, ont également été employées avec succès [3]. La comparaison des profils ainsi obtenus mettra en évidence des différences d'expression de certains miARN selon les conditions, indiquant leur possible implication dans l'infection. En parallèle, l'analyse globale de l'expression des ARNm pourra aider à identifier les différentes voies de régulation affectées par le virus.

Une autre approche consiste à évaluer par des études phénotypiques l'effet de la surexpression d'un miARN ou de son inhibition directement sur le virus. Les virus utilisés pour ce type d'approche sont souvent des virus modifiés génétiquement afin qu'ils expriment une protéine rapportrice, comme la GFP (*green fluorescent protein*). Cette approche a l'avantage de ne pas engendrer de biais, si on a la possibilité d'utiliser un criblage à haut débit ciblant tous les miARN connus [4]. Elle a cependant des limites, notamment pour identifier les miARN qui sont inhibés lors de l'infection. Un type cellulaire n'exprime qu'une centaine de miARN fonctionnels différents. Il est donc possible de ne pas pouvoir révéler l'intérêt du blocage d'un miARN particulier en raison de l'absence de son expression naturelle dans la cellule étudiée.

Identification des cibles du miARN

Le miARN candidat ayant été mis en évidence, l'étape suivante consiste à identifier ses cibles afin de comprendre son rôle dans la régulation de l'infection virale. Un grand nombre d'outils bioinformatiques permettent désormais de prédire les cibles des miARN identifiés. Ces outils, tels que TargetScan [5, 6], miRanda [7], PicTar [8], ou DIANA-microT [9] (cette liste n'est pas exhaustive) se fondent sur la complémentarité de séquences entre les 3'UTR des ARNm cellulaires et la séquence « *seed* » du miARN. Ils restent cependant limités aux cibles cellulaires. Afin de pouvoir prédire les cibles virales des miARN, PW Hsu *et al.* ont développé le logiciel ViTa qui permet l'analyse des cibles potentielles au sein des différentes séquences d'ARN viral [10]. Néanmoins, l'utilisation de ces logiciels de prédiction se heurte aux limites inhérentes à l'approche utilisée, ce qui conduit inévitablement à la prédiction de nombreux faux-positifs, des cibles potentielles mais qui ne sont en fait pas impliquées dans la régulation par les miARN [11]. Ils ne tiennent également pas toujours compte des interactions possibles qui ne sont pas canoniques.

D'autres possibilités, plus directes, existent afin d'identifier les cibles des miARN identifiés. Les complexes entre miARN et ARNm cibles peuvent en effet être isolés par des méthodes d'immunoprécipitation (associées à des pontages entre protéines et ARN par action des ultra-violets, par exemple) ciblant spécifiquement les protéines Argonautes liées aux miARN [12-14]. Ainsi isolés, les ARNm cibles présents dans les complexes isolés pourront être identifiés par séquençage à haut débit. Cette technique révèle l'ensemble des ARNm cibles d'un ou plusieurs miARN et permet d'entrevoir les voies de régulation impliquées au niveau génomique.

Quelle que soit l'approche utilisée, les cibles identifiées nécessitent d'être validées expérimentalement. Cela peut être réalisé dans un premier temps, à l'aide de systèmes rapporteurs reposant le plus souvent sur la luciférase, afin d'évaluer les changements d'expression de l'ARNm et/ou de la protéine liés au gène cible prédit, induits par la surexpression ou l'inhibition du miARN étudié.

Exemples de régulation de l'infection virale par des miARN

Il existe un grand nombre d'exemples de régulation de l'infection virale reposant sur les miARN. Ils peuvent agir de façon directe ou indirecte et avoir un effet positif ou négatif sur l'accumulation du virus *(Figure 2)*. Le *Tableau I* présente quelques exemples choisis dans la littérature qui rendent compte de tous les mécanismes de régulation possibles affectant une panoplie de virus taxonomiquement différents. Nous en décrivons ci-après quelques-uns plus en détails.

Effet direct : ARN viraux cibles des miARN

Ce type de régulation dans laquelle les ARN viraux sont directement ciblés par les miARN cellulaires est le moins couramment décrit et dans la plupart des cas, la fixation d'un miARN sur l'ARN du virus se traduit par un effet proviral. Ceci peut s'expliquer par le fait que, dans le cas d'une régulation négative, la pression de sélection conduit le virus à évoluer vers l'élimination de son génome du site de fixation du miARN. Ce site de liaison sera maintenu s'il confère un avantage au virus.

La première évidence d'une interaction directe entre un miARN et un ARN viral a été rapportée par Sarnow *et al.* qui ont décrit le rôle bénéfique du miARN hépatique miR-122 pour le virus de l'hépatite C (HCV). Le miR-122 guide en effet la fixation d'une protéine Argonaute au niveau de la région 5' non traduite du génome du virus, qui induit l'augmentation de l'accumulation des ARN viraux et la stimulation de la traduction des protéines virales [15-17] (➜).

(➜) Voir la Nouvelle de C. Mengardi et T. Ohlmann, *m/s* n° 6-7, juin-juillet 2015, page 612

La liaison de la protéine permet également la stabilisation du génome en le protégeant de l'activité de ribonucléases cellulaires [18]. Il s'agit donc d'un bel exemple de détournement d'un mécanisme cellulaire au bénéfice du virus. Il explique également, en partie, le tropisme tissulaire particulier du HCV pour les cellules hépatiques qui expriment spécifiquement le miR-122.

Effet indirect : régulation de cibles cellulaires

Ce type d'interaction permet la régulation de l'expression des facteurs cellulaires impliqués dans différentes étapes du cycle viral (facteurs antiviraux ou cofacteurs) ou d'éléments importants pour l'établissement de la réponse immunitaire en réaction à l'infection.

Tropisme cellulaire et entrée

L'entrée dans la cellule est la première étape du cycle viral. Elle définit également le tropisme du virus si un récepteur de surface spécifique lui est nécessaire. La régulation de l'expression de ces récepteurs cellulaires par les miARN peut ainsi permettre de moduler l'accès du virus à la cellule et le début de l'infection. C'est le cas du miR-181 qui réprime l'expression par les lymphocytes de la protéine de surface CD163 utilisée comme récepteur par le virus du syndrome respiratoire et reproducteur porcin (PRRSV). Cette modulation permet ainsi une régulation négative de l'infection virale [19].

Réplication

La réplication du génome viral, pour produire de nouvelles copies, représente une étape clé du cycle du virus qui peut également être altérée par la régulation de l'expression de cofacteurs cellulaires qui lui sont essentiels. Le virus de l'encéphalite japonaise (JEV) inhibe ainsi l'expression de miR-33a afin d'augmenter l'expression d'une de ses cibles, le facteur d'élongation EEF1A1 (*eukaryotic translation elongation factor 1 alpha 1*), nécessaire à la stabilisation du complexe de réplication viral [20].

Traduction

Afin de synthétiser les protéines virales nécessaires à la réplication et à la formation de nouvelles particules infectieuses, les virus dépendent exclusivement de la machinerie de traduction de la cellule. Les ARN viraux produits entrent cependant en compétition avec les ARNm de la cellule qu'ils infectent pour l'accès aux ribosomes. Les virus ont donc évolué afin de détourner les ribosomes cellulaires de leurs substrats habituels pour les rendre pleinement disponibles pour la synthèse de leurs propres protéines. Certains miARN participent à ce processus régulateur induit par le virus. C'est le cas lors de l'infection par l'entérovirus 71 (EV71) qui, comme de nombreux virus, utilise un site interne d'entrée du ribosome (IRES) pour initier la traduction de ses protéines et non une coiffe, comme l'immense majorité des ARNm cellulaires. Au cours de l'infection, le facteur ERG1 (*early growth response 1)* induit la surexpression de miR-141 qui réprime l'expression du facteur d'initiation eIF4E nécessaire à l'initiation de la traduction des ARN dépendant d'une coiffe. La synthèse des protéines cellulaires est alors arrêtée au profit de celle des protéines virales dont la traduction est indépendante d'une coiffe [21].

Réponse immunitaire

Lorsque la présence du virus est détectée par une cellule, une réponse antivirale est initiée par l'activation de voies de signalisation qui vont stimuler la réponse dépendante des interférons et la production de cytokines. Les miARN peuvent participer à la régulation de cette réponse [22]. Le miARN miR-144 agit, par exemple, comme un régulateur positif de l'infection de plusieurs virus dont le génome est constitué d'ARN : il cible l'ARNm codant TRAF6 (*TNF receptor-associated factor 6*), inhibant la réponse immunitaire reposant sur le facteur de transcription IRF7 (*interferon regulatory factor 7*) [23].

L'apoptose

La manifestation ultime de la réponse antivirale de la cellule est sa mort programmée afin d'éviter la pro-

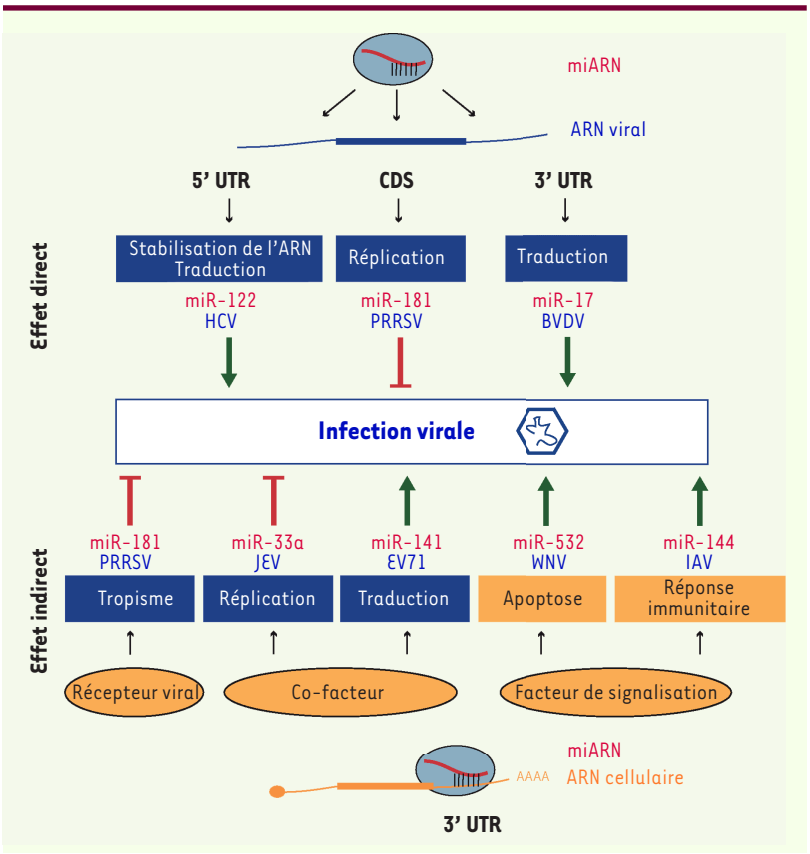

Figure 2. Exemples de mécanismes d'action des miARN modulant l'infection virale. A. L'effet direct du miARN sur la régulation du virus se produit par le ciblage direct des ARN viraux, soit du génome, soit des transcrits, dans différentes régions telles que 3'UTR, 5'UTR ou des séquences codantes (CDS). La liaison entraîne une stabilisation de l'ARN, une traduction améliorée ou une réplication altérée. ***B.*** L'effet indirect implique la modulation de l'expression d'un transcrit cellulaire codant un facteur hôte nécessaire pour une ou plusieurs étapes du cycle viral. La modulation de l'expression des récepteurs impacte l'entrée du virus, régulant ainsi son tropisme. Les cofacteurs nécessaires aux complexes de réplication ou à la traduction peuvent réduire ou améliorer la réplication virale et la production de protéines virales, respectivement. Les miARN participent également à l'amélioration ou à la répression des réponses cellulaires à l'infection, par exemple en inhibant ou stimulant la réponse immunitaire ou en stimulant des mécanismes de défense tels que l'induction de l'apoptose. Les étapes du cycle viral sont représentées en bleu tandis que les facteurs de l'hôte et les voies associées sont marqués en orange.

pagation du virus aux cellules environnantes. Certains virus utilisent cependant cette mort cellulaire qui, au contraire, facilite alors leur libération dans l'environnement extracellulaire. La régulation de facteurs contrôlant l'apoptose par des miARN peut ainsi être exploitée par ces virus. C'est le cas du virus du Nil occidental (WNV), dont l'infection induit l'expression du miARN Hs-154 qui cible deux facteurs anti-apoptotiques, CTFC (*CCCTC-binding factor*) et ECOP (*epidermal growth factor receptor-coamplified and overexpressed protein*), favorisant la mort de la cellule qu'il infecte [57].

Régulation de l'activité des miARN par les virus

Lorsqu'un miARN exerce un effet antiviral, qu'il soit direct ou indirect, le virus évolue afin de contourner cet effet inhibiteur. Cette évolution peut se traduire par l'apparition de mutations dans la séquence virale ciblée par le miARN mais elle peut aussi reposer sur des mécanismes moléculaires plus complexes. La réplication du virus de la vaccine n'est ainsi pas concernée par des régulations dépendant de miARN, dont il induit la dégradation globale et non spécifique. Sa protéine VP55 possède une activité polymérase qui ajoute une queue poly A aux miARN, ce qui déclenche leur dégradation par des ribonucléases [24]. Certains miARN sont également inhibés spécifiquement par des virus. C'est le cas de miR-27 dont la stabilité est fortement réduite par le cytomégalovirus murin (MCMV). Surexprimé expérimentalement dans les cellules, miR-27 présente un effet antiviral qui n'est pas observé dans les cellules qui ne sont pas transformées [25]. L'échappement à l'effet antiviral de miR-27 du MCMV repose sur l'expression d'un transcrit viral qu'il cible, qui induit la dégradation du miARN mature [26,27]. Ce phénomène peut apparaître dans des conditions particulières dans lesquelles l'ARN cible du miARN est fortement exprimé et présente un degré de complémentarité élevé avec le miARN [28].

Les miARN, cibles ou outils thérapeutiques

L'utilisation de miARN comme molécules thérapeutiques n'a pas conduit jusqu'à présent à l'élaboration d'un médicament. Les miARN restent néanmoins très prometteurs pour le traitement de plusieurs pathologies [29], y compris pour les maladies infectieuses. Leur utilisation comme traitement antiviral montre qu'ils présentent une très faible immunogénicité. Du fait de leur conservation au cours de l'évolution, ils peuvent être testés dans différents modèles animaux pour des essais précliniques. Des premiers essais ont ainsi été conduits afin de moduler l'infection par le virus de la grippe H1N1, qui peut être inhibé par plusieurs miARN spécifiques des cellules épithéliales respiratoires. L'administration intranasale de ces miARN a permis de contrôler l'infection et d'inhiber la réplication du virus chez la souris [30].

Bloquer un miARN jouant un rôle proviral est également envisageable. Plusieurs études portant sur l'utilisation

microARN	Virus	Effet	Cible	Références
miR-122	HCV	Proviral	Direct : 5'UTR viral	[15, 40-42]
miR-485	NDV et H5N1	Proviral/ Antiviral	Indirect : ARNm RIG-I Direct : ARN viral de H5N1 (gène PB1)	[3]
miR-141	EV71	Proviral	Indirect : ARNm eIF4E	[21]
miR-142-3p	EEEV	Proviral/ Antiviral	Direct : 3'UTR viral	[43]
miR-17, let-7	Pestiviruses (BVDV)	Proviral	Direct : 3' UTR viral	[38]
miR-301a	JEV	Proviral	Indirect : réponse IFN	[44]
miR-144	IAV, EMCV, VSV	Proviral	Indirect : ARNm TRAF6	[23]
miR-146a	HeV	Proviral	Indirect: ARNm RNF11	[45]
miR-24, miR-93	VSV	Antiviral	Direct : gènes viraux L and P	[46]
miR-221, miR-222	HIV-1	Antiviral	Indirect : ARNm CD4	[2]
miR-181	PRRSV	Antiviral	Indirect : ARNm CD163	[19]
miR-181	PRRSV	Antiviral	Direct : ORF4 viral	[47]
miR-130	PRRSV	Antiviral	Direct : 5'UTR viral	[48]
miR- 542-5p, miR-24 et autres	IAV, RSV	Antiviral	Indirect : voie p38 MAPK	[49]
miR-223	DENV-2	Antiviral	Indirect : ARNm STMN1	[50]
miR-199, miR-214 et autre	MCMV, HCMV, MHV-68, SFV	Antiviral	Indirect : voie ERK/MAPK, synthèse des prostaglandines, stress oxydatif, voie PI3K/AKT	[4]
miR-33a	JEV	Antiviral	Indirect : ARNm EEF1A1	[51]
miR-34, miR-15, and miR-517	DENV, WNV, JEV	Antiviral	Indirect : voie Wnt	[52]
miRNA-3614-5p	DENV	Antiviral	Indirect : ARNm ADAR1	[53]
miR-127-3p, miR-486-5p et autres	IAV	Antiviral	Direct : génome viral	[30]
miR-25, let-7, et miR-130	HCV	Antiviral	Indirect : cofacteurs de HCV	[54]
miR-323, miR-491, and miR-654	IAV	Antiviral	Direct : ARN viral (gène PB1)	[55]
miR-532	WNV	Antiviral	Indirect : ARNm SESTD1	[56]
Hs-154	WNV	Antiviral	Indirect : ARNm CTFC and ECOP	[57]
miR-555	Poliov	Antiviral	Indirect : ARNm hnRNPC	[58]
miR- 155	VSV, SeV	Antiviral	Indirect : ARNm SOCS	[59]
miR-197	EV71	Antiviral	Indirect : ARNm RAN	[60]

Tableau I. Exemples de régulation d'infections virales par des miARN. Virus de l'hépatite C : HCV ; virus de la maladie de Newcastle : NDV ; virus de la grippe A subtype H5N1, H5N1 ; Entérovirus 71 : EV71 ; virus de l'encéphalite équine orientale : EEEV ; virus de la diarrhée virale bovine : BVDV ; virus de l'encéphalite japonaise : JEV ; virus de la grippe A : IAV ; virus de l'encéphalomyocardite : EMCV ; virus de la stomatite vésiculeuse : VSV ; virus Hendra : HeV ; virus de l'immunodéficience humaine 1 : HIV-1 ; virus du syndrome respiratoire et reproducteur porcin : PRRSV ; virus respiratoire syncytial : RSV ; virus de la Dengue : DENV ; cytomégalovirus murin : MCMV ; cytomégalovirus humain : HCMV ; gammaherpèsvirus-68 murin : MHV-68 ; virus de la forêt Semliki : SFV ; virus du Nil occidental : WNV ; virus Sendai : SeV.

d'inhibiteurs du miR-122 comme traitement antiviral ont en effet été menées, d'abord avec le développement du Miravirsen (*Santaris Pharma*), qui bloque son activité [31, 32], et avec son optimisation, sous la forme du RG-101 (une forme conjuguée à une N-acétylgalactosamine favorisant sa pénétration dans les cellules hépatiques), qui ont permis de contrôler l'infection et de réduire la charge virale de patients infectés par le HCV [33].

Un point sur lequel des progrès restent néanmoins à faire concerne l'administration de ces molécules dans l'organisme. Celle-ci peut en effet poser problème selon le tissu qui est ciblé, particulièrement dans le cas des virus neurotropiques pour lesquels la barrière hématoencéphalique constitue un obstacle à la bonne pénétrance du médicament. Fondées sur le mode d'action des miARN, différentes applications peuvent également être envisagées non seulement pour le traitement des maladies virales, mais aussi pour leur prévention. Il est ainsi possible de détourner la fonction de miARN cellulaires afin de minimiser les risques possibles lors de l'utilisation de vaccins utilisant des virus atténués : l'insertion dans le génome du virus vaccinal d'un site de liaison pour un miARN exprimé de manière tissu-spécifique peut ainsi restreindre sa réplication et empêcher sa propagation dans certains types cellulaires. Cette approche a été testée avec le virus de la grippe dans le génome duquel l'insertion d'un site complémentaire au miARN ubiquitaire miR-21 a conduit à l'atténuation du virus dans plusieurs types cellulaires [34].

Enfin, l'ajout dans le génome viral de séquences cibles d'un miARN particulier peut également permettre de limiter la toxicité des virus oncolytiques [35]. Chez la souris, un inconvénient du traitement par le picornavirus oncolytique coxsackie A21 est le développement de myosites (ou myopathies inflammatoires, qui constituent un groupe de maladies rares auto-immunes du muscle). Ces effets secondaires touchant les myocytes peuvent être contrôlés en incluant des séquences cibles pour les miARN comme miR-206 et miR-133a, spécifiques des cellules musculaires [36].

Conclusion

Nous avons évoqué dans cette synthèse les différents moyens d'identification des miARN impliqués dans les infections virales, et les différents rôles qu'ils pouvaient jouer dans cette régulation. Les données de la littérature indiquent qu'il existe un certain nombre d'exemples de miARN cellulaires ayant des activités antivirales. Pourtant, le pouvoir inhibiteur des miARN dans le contexte d'infections virales reste un sujet controversé. Une étude menée par Bogerd *et al.* n'a en effet révélé aucune différence de production virale entre des cellules ne produisant pas de miARN (par délétion de Dicer) et la lignée parentale infectée avec différents virus [37].

D'autres virus utilisent les miARN pour leur réplication. Ils représentent donc une cible thérapeutique de choix puisqu'on peut envisager de développer de nouvelles thérapies qui les ciblent afin d'inhiber l'infection. Une étude récente indique que ce mode de régulation positive pourrait être plus utilisé qu'anticipé. Par des techniques d'immunoprécipitation de protéines Argonaute dans des lignées cellulaires infectées par différents virus, un autre mécanisme de régulation positive directe d'un virus par un miARN a en effet été révélé [38].

Un autre mode de détournement de la voie des miARN à l'avantage des virus, que nous n'avons pas abordé ici, a été montré. Il consiste en l'utilisation de la machinerie cellulaire par certains virus pour la synthèse de leurs propres miARN [39].

Il est encore trop tôt pour affirmer que les études sur l'importance des miARN dans le contexte d'infections virales permettront la mise au point de nouvelles approches thérapeutiques antivirales. Il est cependant clair que ces petits ARN régulateurs ne doivent pas être négligés. Peut-être faudra-t-il envisager de coupler l'inhibition d'un miARN proviral à une autre molécule, dans le but de proposer des outils plus puissants pour combattre les infections virales ? ◊

SUMMARY

Importance of cellular microRNAs in the regulation of viral infections

Viruses are obligatory intracellular parasites that rely on a wide range of cellular factors to successfully accomplish their infectious cycle. Among those, micro (mi) RNAs have recently emerged as important modulators of viral infections. These small regulatory molecules act as repressors of gene expression. During infection, miRNAs can function by targeting either cellular or viral RNAs. In this review, we will recapitulate what has been reported to date on this interplay between cellular miRNAs and viruses and the effect on the infection. Furthermore, we will briefly discuss the possibilities of interfering with the infection through the modulation of this pathway to develop novel antiviral therapies. ◊

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RÉFÉRENCES

1. Jonas S, Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet* 2015 ; 16 : 421-33.
2. Lodge R, Ferreira Barbosa JA, Lombard-Vadnais F, *et al.* Host microRNAs-221 and -222 inhibit hiv-1 entry in macrophages by targeting the CD4 viral receptor. *Cell Rep* 2017 ; 21 : 141-53.

RÉFÉRENCES

3. Ingle H, Kumar S, Raut AA, *et al.* The microRNA miR-485 targets host and influenza virus transcripts to regulate antiviral immunity and restrict viral replication. *Sci Signal* 2015 ; 8 : ra126.
4. Santhakumar D, Forster T, Laqtom NN, *et al.* Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. *Proc Natl Acad Sci USA* 2010 ; 107 : 13830-5.
5. Lewis BP, Shih I, Jones-Rhoades MW, *et al.* Prediction of mammalian microRNA targets. *Cell* 2003 ; 115 : 787-98.
6. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005 ; 120 : 15-20.
7. John B, Enright AJ, Aravin A, *et al.* Human microRNA targets. *PLoS Biol* 2004 ; 2 : e363.
8. Krek A, Grun D, Poy MN, *et al.* Combinatorial microRNA target predictions. *Nat Genet* 2005 ; 37 : 495-500.
9. Kiriakidou M, Nelson PT, Kouranov A, *et al.* A combined computational-experimental approach predicts human microRNA targets. *Genes Dev* 2004 ; 18 : 18 : 1165-78.
10. Hsu PW-C, Lin L-Z, Hsu S-D, *et al.* ViTa: prediction of host microRNAs targets on viruses. *Nucleic Acids Res* 2007 ; 35 : D381-5.
11. Pinzón N, Li B, Martinez L, *et al.* microRNA target prediction programs predict many false positives. *Genome Res* 2017 ; 27 : 234-45.
12. Dölken L, Malterer G, Erhard F, *et al.* Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay. *Cell Host Microbe* 2010 ; 7 : 324-34.
13. Chi SW, Zang JB, Mele A, *et al.* Argonaute HITS-CLIP decodes microRNA–mRNA interaction maps. *Nature* 2009 ; 460 : 479-86.
14. Hafner M, Landthaler M, Burger L, *et al.* Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 2010 ; 141 : 129-41.
15. Jopling CL, Yi M, Lancaster AM, *et al.* Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005 ; 309 : 1577-81.
16. Henke JI, Goergen D, Zheng J, *et al.* microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J* 2008 ; 27 : 3300-10.
17. Mengardi C, Ohlmann T. miR-122 continue de nous surprendre. *Med Sci (Paris)* 2015 ; 31 : 612-5.
18. Li Y, Yamane D, Lemon SM. Dissecting the roles of the 5'exoribonucleases Xrn1 and Xrn2 in restricting hepatitis C virus replication. *J Virol* 2015 ; 89 : 4857-65.
19. Gao L, Guo XK, Wang L, *et al.* MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. *J Virol* 2013 ; 87 : 8808-12.
20. Chen Z, Ye J, Ashraf U, *et al.* MicroRNA-33a-5p Modulates japanese encephalitis virus replication by targeting eukaryotic translation elongation factor 1A1. *J Virol* 2016 ; 90 : 3722-34.
21. Ho BC, Yu SL, Chen JJW, *et al.* Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. *Cell Host Microbe* 2011 ; 9 : 58-69.
22. Taganov KD, Boldin MP, Baltimore D. MicroRNAs and immunity: tiny players in a big field. *Immunity* 2007 ; 26 : 133-7.
23. Rosenberger CM, Podyminogin RL, Diercks AH, *et al.* miR-144 attenuates the host response to influenza virus by targeting the TRAF6-IRF7 signaling axis. *PLoS Pathog* 2017 ; 13 : e1006305.
24. Backes S, Shapiro JS, Sabin LR, *et al.* Degradation of host microRNAs by Poxvirus poly(A) polymerase reveals terminal RNA methylation as a protective antiviral mechanism. *Cell Host Microbe* 2012 ; 12 : 200-10.
25. Buck AH, Perot J, Chisholm MA, *et al.* Post-transcriptional regulation of miR-27 in murine cytomegalovirus infection. *RNA* 2010 ; 16 : 307-15.
26. Libri V, Helwak A, Miesen P, *et al.* Murine cytomegalovirus encodes a miR-27 inhibitor disguised as a target. *Proc Natl Acad Sci USA* 2012 ; 109 : 279-84.
27. Marcinowski L, Tanguy M, Krmpotic A, *et al.* Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication in vivo. *PLoS Pathog* 2012 ; 8 : e1002510.
28. Ameres SL, Horwich MD, Hung JH, *et al.* Target RNA-directed trimming and tailing of small silencing RNAs. *Science* 2010 ; 328 : 1534-9.
29. Chakraborty C, Sharma AR, Sharma G, *et al.* Therapeutic miRNA and siRNA: Moving from bench to clinic as next generation medicine. *Mol Ther Nucleic Acids* 2017 ; 8 : 132-43.
30. Peng S, Wang J, Wei S, *et al.* Endogenous cellular microRNAs mediate antiviral defense against influenza A virus. *Mol Ther Nucleic Acids* 2018 ; 10 : 361-75.
31. Janssen HLA, Reesink HW, Lawitz EJ, *et al.* Treatment of HCV infection by targeting microRNA. *N Engl J Med* 2013 ; 368 : 1685-94.
32. Ree MH van der, Meer AJ van der, Bruijne J de, *et al.* Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. *Antiviral Res* 2014 ; 111 : 53-9.
33. Ree MH van der, Vree JM de, Stelma F, *et al.* Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. *Lancet* 2017 ; 389 : 709-17.
34. Waring BM, Sjaastad LE, Fiege JK, *et al.* MicroRNA-based attenuation of influenza virus across susceptible hosts. *J Virol* 2017 ; 92 : e01741-17.
35. Kaufman HL, Kohlhapp FJ, Zloza A. Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev Drug Discov* 2015 ; 14 : 642-62.
36. Kelly EJ, Hadac EM, Greiner S, *et al.* Engineering microRNA responsiveness to decrease virus pathogenicity. *Nat Med* 2008 ; 14 : 1278-83.
37. Bogerd HP, Skalsky RL, Kennedy EM, *et al.* Replication of many human viruses is refractory to inhibition by endogenous cellular microRNAs. *J Virol.* 2014 ; 88 : 8065-76.
38. Scheel TKH, Luna JM, Liniger M, *et al.* A broad RNA virus survey reveals both miRNA dependence and functional sequestration. *Cell Host Microbe* 2016 ; 19 : 409-23.
39. Kincaid RP, Sullivan CS. Virus-encoded microRNAs: an overview and a look to the future. *PLoS Pathog* 2012 ; 8 : e1003018.
40. Sedano CD, Sarnow P. Hepatitis C virus subverts liver-specific miR-122 to protect the viral genome from exoribonuclease Xrn2. *Cell Host Microbe* 2014 ; 16 : 257-64.
41. Masaki T, Arend KC, Li Y, *et al.* miR-122 Stimulates hepatitis C virus RNA synthesis by altering the balance of viral RNAs engaged in replication versus translation. *Cell Host Microbe* 2015 ; 17 : 217-28.
42. Lanford RE, Hildebrandt-Eriksen ES, Petri A, *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010 ; 327 : 198-201.
43. Trobaugh DW, Gardner CL, Sun C, *et al.* RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. *Nature* 2014 ; 506 : 245-8.
44. Hazra B, Kumawat KL, Basu A. The host microRNA miR-301a blocks the IRF1-mediated neuronal innate immune response to Japanese encephalitis virus infection. *Sci Signal* 2017 ; 10 : eaaf5185.
45. Stewart CR, Marsh GA, Jenkins KA, *et al.* Promotion of Hendra virus replication by microRNA 146a. *J Virol* 2013 ; 87 : 3782-91.
46. Otsuka M, Jing Q, Georgel P, *et al.* Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* 2007 ; 27 : 123-34.
47. Guo X -k., Zhang Q, Gao L, *et al.* Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. *J Virol* 2013 ; 87 : 1159-71.
48. Li L, Gao F, Jiang Y, *et al.* Cellular miR-130b inhibits replication of porcine reproductive and respiratory syndrome virus in vitro and in vivo. *Sci Rep* 2015 ; 5.
49. McCaskill JL, Ressel S, Alber A, *et al.* Broad-spectrum inhibition of respiratory virus infection by microRNA mimics targeting p38 MAPK signaling. *Mol Ther Nucleic Acids* 2017 ; 7 : 256-66.
50. Wu N, Gao N, Fan D, *et al.* miR-223 inhibits dengue virus replication by negatively regulating the microtubule-destabilizing protein STMN1 in EAhy926 cells. *Microbes Infect* 2014 ; 16 : 911-22.
51. Chen Z, Ye J, Ashraf U, *et al.* MicroRNA-33a-5p Modulates japanese encephalitis virus replication by targeting eukaryotic translation elongation factor 1A1. *J Virol* 2016 ; 90 : 3722-34.
52. Smith JL, Jeng S, McWeeney SK, *et al.* A microRNA screen identifies the Wnt signaling pathway as a regulator of the interferon response during flavivirus infection. *J Virol* 2017 ; 91 : e02388-16.
53. Diosa-Toro M, Echavarría-Consuegra L, Flipse J, *et al.* MicroRNA profiling of human primary macrophages exposed to dengue virus identifies miRNA-3614-5p as antiviral and regulator of ADAR1 expression. *PLoS Neg Trop Dis* 2017 ; 11 : e0005981.
54. Li Q, Lowey B, Sodroski C, *et al.* Cellular microRNA networks regulate host dependency of hepatitis C virus infection. *Nat Comm* 2017 ; 8.
55. Song L, Liu H, Gao S, *et al.* Cellular MicroRNAs Inhibit replication of the H1N1 influenza a virus in infected cells. *J Virol* 2010 ; 84 : 8849-60.
56. Slonchak A, Shannon RP, Pali G, *et al.* Human microRNA miR-532-5p exhibits antiviral activity against West Nile virus via suppression of host genes SESTD1 and TAB3 required for virus replication. *J Virol* 2016 ; 90 : 2388-402.
57. Smith JL, Grey FE, Uhrlaub JL, *et al.* Induction of the cellular microRNA, Hs_154, by West Nile virus contributes to virus-mediated apoptosis through repression of antiapoptotic factors. *J Virol* 2012 ; 86 : 5278-87.
58. Shim BS, Wu W, Kyriakis CS, *et al.* MicroRNA-555 has potent antiviral properties against poliovirus. *J Gen Virol.* 2016 ; 97 : 659-68.
59. Wang P, Hou J, Lin L, *et al.* Inducible microRNA-155 Feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J Immunol* 2010 ; 185 : 6226–-33.
60. Tang WF, Huang RT, Chien KY, *et al.* Host microRNA miR-197 plays a negative regulatory role in the enterovirus 71 infectious cycle by targeting the RAN protein. *J Virol* 2016 ; 90 : 1424-38.

TIRÉS À PART

S. Pfeffer

THESIS OBJECTIVES

II. THESIS OBJECTIVES

Since the very beginning of their discovery, microRNAs (miRNAs) have been shown to function as important regulators of cellular physiology and development. Furthermore, these small non-coding RNAs can also present differential expression in specific tissues or conditions and their aberrant expression often correlates with different pathologies such as different types of cancer. Recently, miRNAs have also been related to host-pathogen interactions, by responding to or modulating bacterial and viral infections.

Emerging viral diseases are one of the major global public health concerns. More specifically, RNA viruses represent the main group of zoonotic pathogens responsible for many of the emerging human diseases. To date, there are no effective treatments to stop the spread of the majority of these viruses. The development of effective treatments or vaccines implies first a deep understanding of the different interactions between viruses and their host during infection.

These viruses not only possess an RNA genome, but they also produce other RNA molecules as intermediates during viral gene expression and replication, all susceptible of being targeted by cellular miRNAs.

Alphaviruses belong to the *Togaviridae* family, which gathers a large group of RNA viruses able of zoonotic spread and responsible of diseases such as arthritis or encephalitis. Examples of cellular microRNAs targeting these viruses exist affecting replication like miR-142-3p on EEEV (Trobaugh et al., 2014). In other cases, interactions have been identified with no further description of the physiological function like for CHIKV, VEEV or SINV (Scheel et al., 2016).

In relation with the increasing evidence linking miRNAs to viral infections, the objective of my PhD project is to contribute to the current knowledge of this particular area by investigating the involvement of cellular miRNAs in the regulation of

alphaviruses and by further characterising the molecular mechanisms set in place making this regulation possible.

The host laboratory previously performed a phenotypic fluorescence-based screen using a GFP reporter Sindbis virus to follow changes in the viral infection due to overexpression or inhibition of a library of known cellular miRNA. This screen allowed the identification of a list of significant miRNA candidates with a positive effect on SINV, and potentially other alphaviruses. Following these first results, the main objectives of this thesis project could be divided into three main axes:

(1) The validation of the miRNA candidates identified as positive regulators of viral infection when overexpressed. The first goal involved the validation of the phenotype observed from the screen by studying the viral production and viral RNA and protein synthesis in presence of these candidates.

(2) The characterization of the top candidate, the neuronal miR-124, in the regulation of alphavirus infection. This goal included first the development of a cellular system that would easily allow to study the effect of this miRNA on SINV infection and that could be used as a tool for further investigating the mechanism. Second, I aimed to validate the observations in a context where miR-124 was expressed endogenously such as neuronal cells.

(3) The analysis of the molecular mechanisms behind miR-124 regulation. This final objective gathered the identification and study of the possible RNA targets, either viral or cellular, mediating the regulation, by using biotinylated mimics, CLIP (crosslinking and immunoprecipitation) techniques of miRNA-target complexes and luciferase reporter assays.

MATERIAL & METHODS

III. MATERIAL & METHODS

A. Cell lines and primary cultures

Human miR-124-3p primary transcript (hsa-pri-miR-124) from miR-124a-1 gene in locus 8p23.1 and about 100 nucleotides of its flanking upstream and downstream sequences were cloned by Gateway technology (Invitrogen) in the lentiviral destination vector pCW57.1 (pCW57.1 was a gift from David Root (Addgene plasmid # 41393 ; http://n2t.net/addgene:41393 ; RRID:Addgene_41393)). HEK293T cells were transfected with this vector and 24 h post-transfection cells were subjected to selection pressure with puromycin at a concentration of 1 μg/ml for 72h. 1/10th of the culture was split and kept under selection for at least two weeks and 10 individual colonies were picked and cultured independently (HEK293T-miR-124, clones 1-10). The rest of the cells were collected and stored as a polyclonal cell line (HEK293T-miR-124).

Rat primary cerebellar granule cells (GCs) were obtained and prepared by Dr Sylvain Hugel (INCI, Strasbourg). Briefly, GCs were obtained from three different Wistar rat pups aged of 3 days. Cerebellum was dissociated first mechanically and then by trypsin digestion for 20 min at 37°C. Cells were resuspended in DMEM supplemented with 10% FBS, Penicillin and Streptomycin and 25 nM of potassium. Cellular debris was removed through a BSA density gradient and entire intact cells, as well as cellular clumps, were pelleted and washed in two centrifugation steps (6 min at 1000 revolutions per minute, rpm). Supernatant was removed and cells were resuspended in 10 ml of media and plated in collagen-coated plates, 200 μl of cells per well.

100 nM antimiR control (5' UCACAACCUCCUAGAAAGAGUAGA 3', IDT) or antimiR-124 (5' GGCAUUCACCGCGUGCCUUA 3', IDT) were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. 72 h post-transfection cells were infected with SINV-GFP at MOI 10^{-1}. 24 h post-infection, supernatants were stored at -80°C and total RNA was extracted using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

B. Luciferase reporter assay

Luciferase reporters were obtained by cloning one miR-124 binding site, imperfect match and seed mutant, in between a random sequence impairing folding of the site in psiCHECK-2 vectors by Gateway cloning. For miR-124 putative binding sites on the viral genome, a genomic region of about 230 nucleotides containing the site was cloned in psiCHECK-2 vectors. The mutant binding site was obtained by site-directed mutagenesis using forward (5' CGCATTTATCAGGACATCAGATGCACCACTGGTCTCA 3') and reverse (5' ATGTCCTGATAAATGCGGCGTTCGGGATGTCAATAGA 3') primers using the HiFi Clone Amp (TaKaRa) polymerase followed by incubation at 50°C for 15 min with the InFusion recombination enzyme (TaKaRa).

HEK293grip cells were cultured in Dulbecco's modified eagle medium (DMEM) with 4.5 g/liter glucose (Gibco, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS; TaKaRa), seeded in a 48-well plate (30000 cells/well) and transfected with 25 ng of vector, 250 ng of carrier pUC19 DNA and 20 nM of either control or miR-124 mimic. 24 h post-transfection, cells were lysed with 100 μl of 1x Passive Lysis Buffer (Promega) by rocking the plate at room temperature for 15 min followed by at least two freeze and thaw cycles. *Firefly* (Fluc) and *Renilla* luciferase (Rluc) activities were measured after incubation of 20 μl of lysate with the corresponding substrates from Dual-Luciferase Reporter Assay (Promega) in a white 96-well plate using a spectrophotometer (Safas Monaco).

C. Ago-APP for biotinylated mimics

HEK293T or Huh7.5.1 were transfected with 30 nM of biotinylated or classical mimic-CTL or 124 (IDT) using Lipofectamine 2000 (Invitrogen). In the biotinylated mimics, the biotin is coupled on the 3' end.

72 h post-transfection cells were infected with SINV-GFP MOI 10^{-3} (prepared as in (López et al., 2020)). 24 h post-infection cells were washed, scraped in PBS, pelleted for 5 min at 1000 rpm and lysed in 1x Lysis IP buffer (1x PXL: 1x PBS, 1% Igepal/NP40,

0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor (cOmplete Mini EDTA-free Protease Inhibitor Cocktail, Roche) or stored at -80°C until needed for IP.

50 μl of Dynabeads G (Invitrogen) per sample were washed 3 times with Bead Wash Buffer (BWB: 1x PBS, 0.02% Tween-20) and incubated with 10 μg of Flag antibody (Sigma) per sample in 1 ml of BWB at 4°C overnight rotating. Beads were washed three times in 1x PXL and resuspended in 1 ml of BWB for incubation with TNRC6 WT or AllAla peptide (Hauptmann et al., 2015) for 1 h at 4°C. Unbound peptide was removed by washing three times with 1x PXL.

Cell pellets were sonicated 5 times 30 sec on/off and then lysates were centrifuged 20 min at 10000 rpm at 4°C to remove cell debris. 500 μl of lysate were added to the beads and incubated for 3 h 30 min at 4°C rotating. Then, beads were washed four times with 1x PXL and complexes were eluted by competition with 100 μl of 100 μg/ml of Flag peptide incubated with the beads for 15 min on ice and 10 min at room temperature. IP product was transferred to a new tube and 500 μl of TRIzol was added to the beads. All samples were stored at -20°C.

For the PreScission protease elution, after the final wash, beads were resuspended in 100 μl of PBS and incubated with 2 μl (4 U) of PreScission protease (Sigma) for 16 h at 4°C in the shaker at 450 rpm. IP product was split in 1/3 for protein analysis and 2/3 for RNA analysis. Finally, 500 μl of TRIzol was added to the beads.

D. CLEAR-CLIP

The CLEAR-CLIP protocol used here was adapted from a previously published protocol (Moore et al., 2014).

1. Cell preparation and crosslinking

15 millions of HEK293T or 13 millions of 124+ cells were plated in 15 cm dishes and all were treated with 1 μg/ml of Doxycycline for 48 h and then mock or SINV-GFP infected (MOI 10^{-3}) for 24 h. Following infection, cells were washed with PBS and left in a minimal volume of PBS for UV crosslinking at 254 nm (Stratalinker, 400 mJ/cm^2, 30 sec

on ice and 200 mJ/cm^2). Cells were collected by scraping and each sample was obtained by pooling six dishes in the same tube. Cells were pelleted by centrifugation at 4°C for 5 min at 1000 rpm.

2. RNase digestion and Ago-IP

Ago proteins were immunoprecipitated using an adapted version of Ago-APP (described above). We used 200 μl of Dynabeads G per sample coupled with 40 μg of Flag antibody incubated in BWB for 1 h at room temperature. Loaded beads were washed three times with BWB, 2 ml per wash and resuspend in 1 ml of BWB to which 18 μg of T6B WT or AllAla peptide was added and incubated 1 h at 4°C rotating.

Crosslinked cells were resuspended in 4 ml of 1x PXL, sonicated 5 times 30 sec on/off and then treated with 120 μl of RQ1 DNase at 37 °C for 5 min. RNase digestion was performed with 40 μl of 1:5000 diluted RNase A at 37 °C for 5 min and then transferred to ice. RNase activity was quenched by adding 10 μl of RNasin Plus (Promega) to the lysates and centrifugation at 13000 rpm for 20 min at 4 °C. Supernatant was transferred to the antibody-bound beads and incubated for 90 min at 4 °C. The beads were washed with 5 ml of the following buffers (from (Moore et al., 2014):

- 2 washes with 1x PXL
- 1 wash with high-salt buffer
- 1 wash with high-stringency buffer
- 1 wash with low-salt wash buffer
- 2 washes with 1x PNK buffer 1 with 5 ml, the second with 1 ml, and beads were transferred to 1.5 ml microtubes.

3. 5’ end phosphorylation and chimera ligation

Phosphorylation of 5’ ends was performed with 1μl PNK (10U/μl, NEB), 1 μl of 100 mM ATP and 10 μl of 10x PNK buffer in a final volume of 100 μl for 20 min at 37 °C. Beads were washed three times in 1x PNK buffer and then chimera ligation was performed overnight at 16 °C shaking at 1000 rpm for 15 sec every 2 min with T4 RNA

Ligase I (10000 U/ml, NEB), 1 μl of 100 mM ATP, 1 μl of 10 mg/ml BSA and 10x T4 RNA ligase buffer in a final volume of 100 μl. Ligation was extended by adding 2.5 μl of fresh T4 RNA Ligase I and 1 μl of 100 mM ATP for another 4-6 h. The beads were then washed with 1 ml:

- Twice in 1x PXL
- Once in 1x PNK/EDTA/EGTA buffer-high stringency
- Twice in 1x PNK buffer.

4. 3' end dephosphorylation and 3' adaptor ligation

3' ends of RNA tags were dephosphorylated with 0.5 μl of CIP (10 U/ml, NEB), 8 μl of 10x CIP buffer and 2 μl of RiboLock RNAse inhibitor (Thermo Scientific) in a final volume of 80 μl for 20 min at 37 °C shaking at 1000 rpm for 15 sec every 2 min and then washed with 1 ml:

- Once with 1 ml of 1x PNK buffer
- Once with 1 ml of x PNK+EGTA buffer
- Twice with 1 ml of 1x PNK buffer.

5 μl of preadenylated 3' adaptor (20 pmol/μl, 5' 5Phos-NNNNTGGAATTCTCGGGTGCCAAGG (AmC7) 3') were incubated with 8 μl of T4 RNA ligase 2 truncated (NEB), 8 μl of DMSO, 2 μl of RiboLock RNAse inhibitor (Thermo Scientific) and 8 μl of 10x T4 homemade RNA ligase buffer in a final volume of 80 μl at 16°C overnight shaking at 1000 rpm for 15 sec every 2 min. Beads were then washed with 1 ml:

- Once with 1 ml of 1x PNK buffer
- Once with 1 ml of high-salt wash buffer
- Twice with 1 ml of 1x PNK buffer.

5. 5' radiolabeling of RNA tags, SDS-PAGE and nitrocellulose transfer

5' radiolabeling of RNA tags was performed with 4 μl of T4 PNK (NEB), 2 μl of P^{32}-ATP (6,000 Ci/mmol) and 10x PNK buffer in a final volume of 80 μl at 37 °C for 20 min shaking at 1000 rpm for 15 sec every 2 min. Then 1 μl of cold 10 mM ATP was added to each tube for an additional 5 min incubation. Then, the beads were washed three times with 1 ml of 1x PNK+EGTA buffer.

Samples were eluted from the beads by resuspending the beads on 20 μl of LDS loading sample buffer (7.5 μl of NuPAGE LDS sample buffer (Thermo Fisher), 22.5 μl of 1x PNK+EGTA buffer and 3 μl of sample reducing buffer (Invitrogen)). The mixture was incubated at 70 °C for 10 min shaking at 1000 rpm. Supernatants were loaded on an 8% Novex NuPAGE Bis-Tris gel (Invitrogen) and the gel was run at 190 V for about 2 h in the cold room. The gel was transferred to a nitrocellulose membrane for 1h15 at 100 V in 1x NuPAGE transfer buffer containing 20% ethanol. The membrane was exposed to an autoradiography film at -80°C for 5 days to obtain a signal.

6. Extraction of RNA tags

Ago:miRNA (~110 kDa) and Ago:miRNA:mRNA (~130 kDa) complexes were cut from the membrane at the expected size with the help of a scalpel and RNAs were extracted from the membrane by digestion with 200 μl of 4 mg/ml of Proteinase K (20 mg/ml, Roche) in 1x PK buffer at 37 °C for 20 min shaking at 1000 rpm. Then 200 μl of 1x PK/7 M urea solution were added to the mixture and incubated for another 20 min at 37 °C shaking at 1,000 rpm. RNA was purified by adding 400 μl of neutral PCI to the samples and incubated at 37 °C for 20 min, shaking at 1,000 rpm.

Samples were centrifuged (>12,000g for 10 min at room temperature) and the aqueous phase was collected to a new tube. RNA was precipitated with the help of 1 μl of GlycoBlue and 40 μl of 3 M sodium acetate (pH 5.2) in 1 ml of ethanol:isopropanol (1:1) for 2 h at -20 °C. The RNA pellet was washed twice in 70% ethanol by centrifugation (>12,000g for 10 min at 4 °C) and resuspended in 4.5 μl of RNAse-free water.

7. 5' adaptor ligation

1 μl of 5' RNA adaptor (20 μM, 5' GUUCAGAGUUCUACAGUCCGACGAUCNNNN 3') was added to the resuspended RNA together with 1 μl of T4 RNA ligase I (Biolabs), 1 μl of 10 mM ATP, 1 μl of 0.2 mg/ml of BSA and 1 μl of 1x T4 RNA ligase buffer at 16 °C overnight.

Ligated products were purified by phenol-chloroform extraction. Samples were centrifuged (>12,000g for 20 min at room temperature) and aqueous phase was retained. RNA was precipitated with the help of 1 μl of GlycoBlue, 1/10 of 3 M sodium acetate in 1 ml of ethanol:isopropanol (1:1) overnight at -20°C and pelleted by centrifugation (>12,000g at 4°C) for at least 20 min. The RNA pellet was washed twice with 1 ml of 70% ethanol (>12,000g for 10 min at 4°C) and resuspended in 13 μl of RNase-free water.

8. RT-PCR

Each sample was divided in 10 μl and 2.5 μl aliquots (for RT+ and RT- control respectively). Retrotranscription was performed using the SuperScript IV Reverse Transcriptase (Invitrogen). 2 μl of 5 μM RT specific primer (5' GCCTTGGCACCCGAGAATTCCA 3') and 1 μl of 10 mM dNTP were added to the 10 μl of RNA and was annealed at 65 °C for 5 min. Then RT mix containing 1x SSIV buffer, 0.1 M DTT, RNAsin Plus and the SuperScript IV enzyme or the water control were added to the reactions. Samples were incubated at 50 °C for 10 min and the reaction was inactivated by incubating all samples at 80 °C for 10 min.

PCR was performed using the Phusion High–Fidelity DNA Polymerase (Invitrogen) with 2.5 μl of cDNA, 2 μl of 10 μM forward (5' AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA 3') and barcoded reverse primers, 1 μl of 10 mM dNTPs, 10 μl of 5x Phusion buffer and 0.5 μl of Phusion polymerase (1U/μl) in a final volume of 50 μl. Every sample was amplified for different cycles, either 10, 15, 20 or 30 cycles. The PCR program was set up as follows:

- Initial denaturation 98°C for 30 sec.
- 10, 15, 20 or 25 cycles of: 98 °C for 10 sec, 50 °C for 20 sec, 72 °C for 20 sec.

9. Analysis and gel purification of PCR amplification products

Samples run for 15, 20 or 25 cycles were used to assess the length of PCR products and the optimal quantity of amplicon for sequencing. PCR products were loaded on a 10% acrylamide gel and run in 1x Tris-Borate-EDTA (TBE) buffer at 170 V for 2 h 30. The gel was incubated in 1x TBE containing ethidium bromide for 10 min and DNA was visualized with a UV transilluminator.

Samples run for 10 cycles were stored at -20°C and then were amplified for 8 more cycles to reach the optimal PCR cycle amplification for sequencing. PCR products were purified by PCI and precipitated with 1 μl of GlycoBlue, 1/10 of 3 M sodium acetate and 1 ml of ethanol:isopropanol (1:1) at -20 °C overnight. cDNA was pelleted by centrifugation (>12,000g at room temperature) for 30 min and washed twice with 70% ethanol for 10 min. Pellets were resuspended in 10 μl of water.

Samples were gel-purified in a 10% acrylamide gel run in 1x Tris-Borate-EDTA (TBE) buffer at 170 V for 2 h 30 and stained with ethidium bromide and 1x TBE solution for 10 min for DNA visualization on a UV transilluminator. Fragments around 200 bp were cut from the gel, crushed and extracted from the gel by incubation in 400 μl of 0.4 M NaCl at 4 °C, shaking at 1400 rpm overnight. Fragments were further purified in a spin X column and precipitated with 1 μl of GlycoBlue and 1 ml of 100% ethanol for 4 h at -20 °C. DNA was pelleted by centrifugation (>12,000g at room temperature) for 30 min and washed twice with 70% ethanol for 10 min. Pellets were resuspended in 16.2 μl of water and resuspended to send 15 μl of 5 ng/μl for sequencing.

10. High-throughput sequencing

Libraries were sequenced on the Illumina HiSeq 4000 platform with 50-nucleotide single-end reads.

RESULTS

IV. RESULTS

With the aim of studying the implication of host cellular miRNAs in the regulation of SINV infection, we performed a high-throughput gain and loss of miRNA function genome-wide screen. The screen identified a list of sixteen miRNA candidates that had a positive effect on the virus and we validated some of them as being pro-viral on SINV. Very interestingly, a large number of the identified miRNAs are specifically expressed in the central nervous system (CNS).

During my PhD project, I focused my efforts on understanding the role of the top candidate identified in the screen, namely the neuron-enriched miR-124, which is also widely conserved across evolution. Furthermore, it has important developmental and immune functions in the brain, which makes it a good candidate for the regulation of neurotropic viruses.

In order to get more insight into the regulation of SINV cycle by miR-124 and its molecular mechanism, I employed several experimental systems and tools to study this miRNA and its effect on alphavirus infection. In the upcoming chapters, I will present the experiments performed during my thesis regarding this research project.

The first chapter contains all the results that I obtained concerning the establishment of several cellular models (primary cells and cell lines) in which the functional study of miR-124 and SINV regulation was set up to eventually decide on the best model for the follow-up of the project.

The second chapter recapitulates all the results assessing miR-124 function on both SINV, the prototypical alphavirus, and CHIKV, a re-emerging alphavirus of medical importance. These results are presented in the form of an article that has been published in *Journal of Virology* in which I am first author.

In the third chapter, I present additional experiments performed to go deeper into the understanding of the molecular mechanism behind miR-124 regulation and notably the identification of miR-124 cellular and viral targets by a CLIP approach. Finally, I

briefly discuss the tools that I have put in place to further analyse the impact of miRNA on SINV viral replication, which can be useful for future work regarding miR-124 as well as other interesting candidates.

CHAPTER I. Setting up systems to study the impact of miR-124 on SINV

The strategy employed to select cellular miRNAs involved in the regulation of alphavirus infection was to perform a gain-of-function screen based on transient transfection of miRNA mimics to overexpress candidate miRNAs in human hepatic Huh7.5.1 cells before infecting them with SINV-GFP. In this screen, we could identify a phenotypic effect on SINV accumulation for a limited number of miRNAs, including miR-124, as assessed by an increase in fluorescence intensity. In addition, the measured effect of miR-124 ranked it as the top candidate in the list of miRNAs that had a pro-viral effect on SINV-GFP.

Given the fact that miR-124 is a neuron-specific miRNA, we had to rely on transient transfection of the miRNA mimics to overexpress it in non-neuronal cells for subsequent validations. While transfection of small molecules is very efficient in most cell lines used in the laboratory cell lines, I rapidly realized that there were some variations between experiments. For this reason and in order to bypass this limitation of transient transfection, we sought to establish the best cellular system stably expressing miR-124 to study its implication in viral infection. Thus, I tested primary neuronal cultures, endogenously expressing miR-124 cells, and I also generated several cell lines expressing miR-124 ectopically.

A. Endogenous expression

1. Rat cerebellar granule cells (GCs)

As previously mentioned, miR-124 is a conserved neuron-specific miRNA, thus I decided to conduct the study of its involvement in physiologically relevant cells, where this miRNA is endogenously expressed. Furthermore, it has been shown that SINV infection causes an age-dependent encephalitis in murine models. In suckling mice, SINV infection is lethal, while in adults the infection is mostly asymptomatic (Johnson et al., 1972). Thus, for our studies I investigated the involvement of miR-124 in the regulation

of SINV infection by inhibiting the miRNA in neuronal cells isolated from rats of different ages.

In collaboration with Dr Sylvain Hugel (Institut des Neurosciences Cellulaires et Intégratives, Strasbourg), I obtained primary cultures of cerebellar granule cells (GCs) isolated from adult or 3-day old rats. GCs are neurons present in the granular layer of the cerebellar cortex and they are the most abundant type of cells in this region, representing up to 90% of cells. These cells participate in cerebellar physiological functions involving mainly motion and coordination, but also in some cognitive processes such as learning (Gruol et al., 2016). In humans and rodents, GCs neurogenesis takes place postnatally. Their great abundance in the cerebellar cortex and their late development was put into advantage for the establishment of a neuron model that could be grown *in vitro*, resulting in a homogeneous neuronal population. Throughout the last decades, GCs have been widely used in neurobiology as a model to study a variety of neuronal functions, developmental processes as well as neuronal pathologies (Contestabile, 2002).

I used these primary cells to inhibit the endogenous miR-124 by transfecting cells with antimiR-124 or antimir-CTL and infecting them with SINV-GFP. Using northern blot analysis, I could show that the accumulation of miR-124 was efficiently lowered upon transfection of antimiR-124 oligonucleotides compared to the control antimiRNAs (Figure 1).

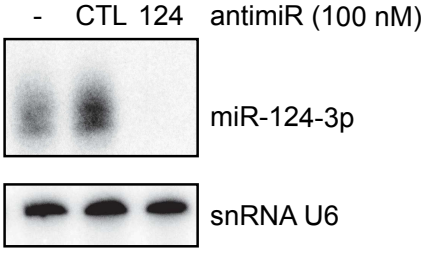

Figure IV.1. miR-124 expression in rat cerebellar granule cells (GCs) upon antimiR transfection.
Northern blot analysis of miR-124 expression in adult rat GCs transfected with antimiR control (CTL), directed against miR-124 (124) or non-transfected (-).

However, when I assessed the effect of miRNA inhibition on the viral infection, I could not observe any difference in terms of viral particle production in GC cultures derived either from adult or 3-day old rats (Figure 2A and C).

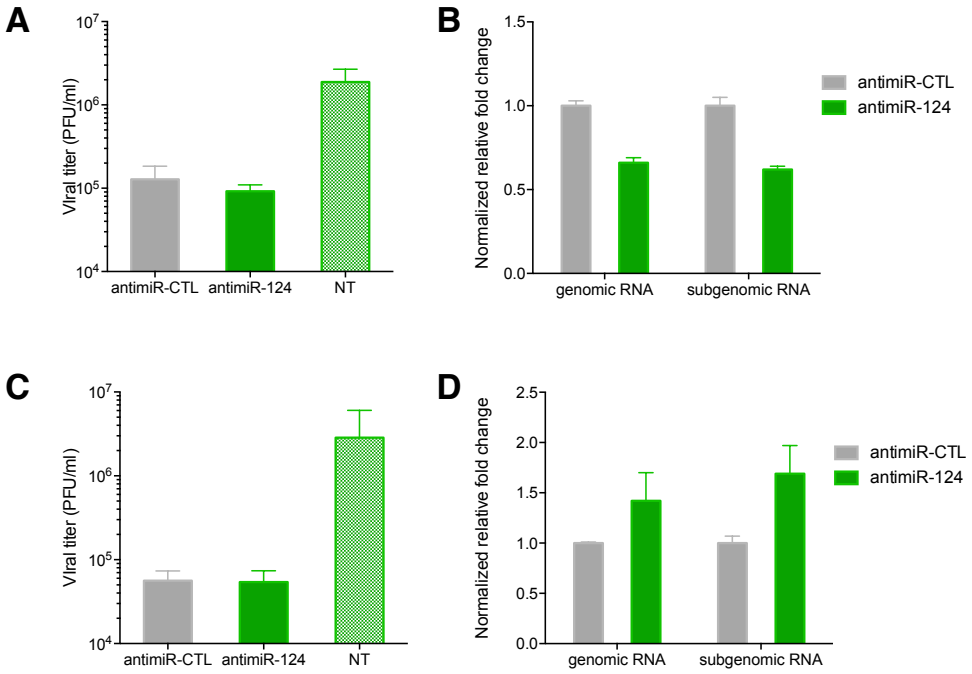

Figure IV.2. Effect of miR-124 inhibition on SINV infection in rat cerebellar granule cells (GCs).
Viral titers and viral RNA accumulation of SINV-GFP infection in adult rats (A and B) and 3-day old rats (C and D). Data are presented as mean + SEM (viral titer quantification) or SD (RNA accumulation) of technical replicates from one single experiment.

Surprisingly, we observed that transfection itself resulted in a drop of viral titers by ten-fold irrespective of the antimiRNA used. Concerning the viral RNA accumulation, I observed that transfection of the antimir-124 resulted in a reduction of about 40% in genomic and subgenomic viral RNA accumulation in cells from adult rats (Figure 2B) and a slight increase in cells from 3-day old animals (Figure 2D). Taken all together, these results show that miR-124 can be very efficiently blocked with antimiR-124 compared to the control in GCs. However, these results suggest that viral infection was not productive in these cells following transfection and that additional improvements will have to be made in order to optimize the infection.

2. Human Neural Progenitor cells (hNPCs)

We also attempted to study the involvement of the endogenous miR-124 in human cells. To this end, we employed human neural progenitor cells (hNPCs) in collaboration with Dr Muriel Coulpier (Ecole Nationale Vétérinaire d'Alfort, Paris). These neural stem cells are able to self-renew and can be cultured and differentiated to a mixed population of neuronal and glial cells (Brnic et al., 2012). Furthermore, they represent an excellent model for the study of human CNS infection by neurotropic viruses (Fares et al., 2020).

I first verified the impact of hNPCs differentiation on miR-124 expression level by northern blot analysis. I observed that upon 14 days of differentiation (D14) miR-124 expression was higher compared to proliferating cells (D0) but lower than the expression in different adult mouse brain regions such as cortex (cx) or cerebellum (cb) (Figure 3).

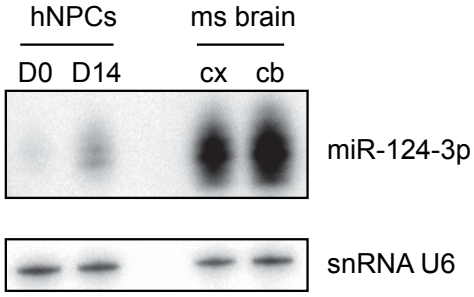

Figure IV.3. miR-124 expression in differentiated human neuronal progenitor cells (hNPCs).
miR-124 levels in proliferating (D0) or 14-day differentiated (D14) hNPCs. Expression from mouse brain cortex (cx) and cerebellum (cb) were used as a positive control. snRNA U6 is used as a loading control.

We also determined the best condition for SINV infection of these cells. Infections were performed by our collaborators with SINV-GFP at different multiplicities of infection (MOI) and the outcome of infection was assessed phenotypically by observation of GFP positive cells at for several time points post-infection (pi). As expected, at 24 hpi we observed the highest number of GFP positive cells at MOI of 1 compared to MOI of 0.1 and 0.01, with an increase in GFP signal at 48 hpi for all MOIs tested. Finally, the GFP level decreased at all MOIs at 72 hpi, probably due to the cell death caused by the viral infection (Figure 4).

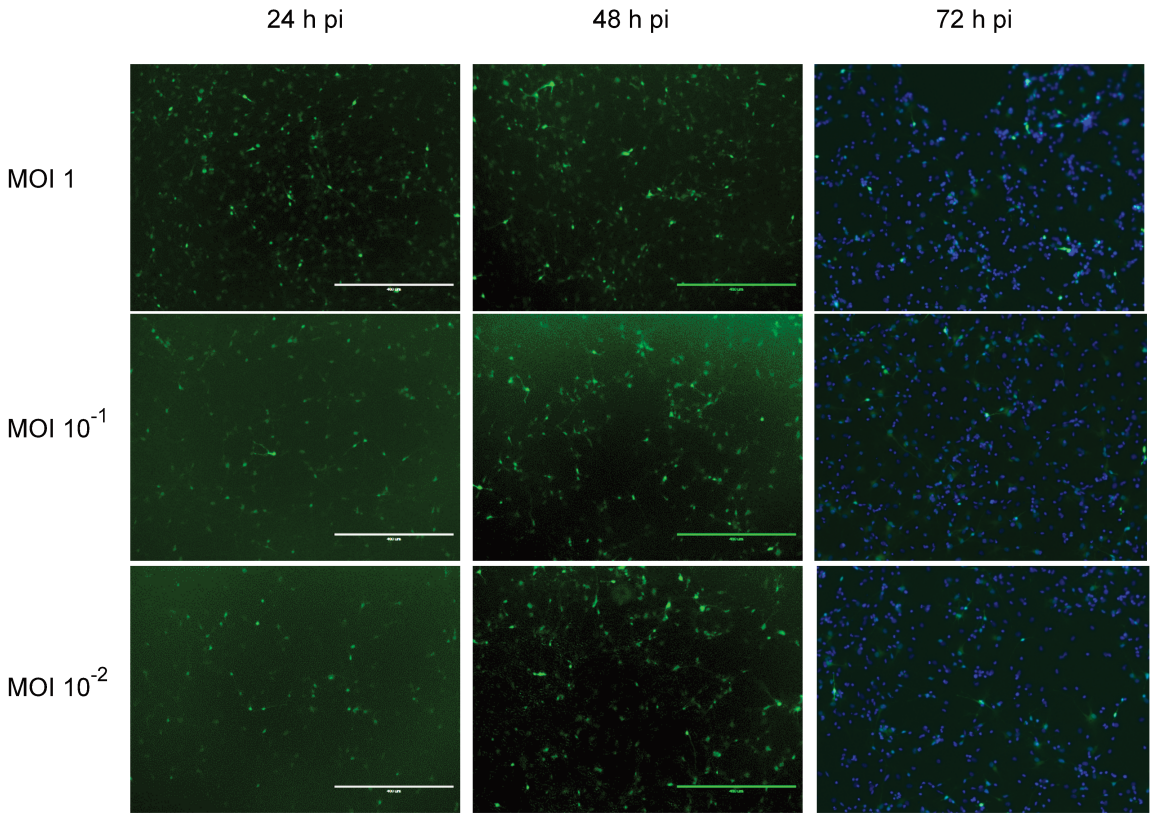

Figure IV.4. hNPCs are infectable by SINV-GFP.
Fluorescence microscopy images of SINV-GFP infected hNPCs with different MOIs at 24, 48, or 72 h post-infection (pi). The right panel at 72 h pi corresponds to the merge of Hoechst staining of cell nuclei and GFP signal from infected cells.

Altogether, these results show that miR-124 expression is naturally induced upon 14 days of differentiation of hNPCs, although not reaching levels as high as in mouse brain regions. In addition, we could show that D14 differentiated cells are infectable by SINV-GFP, making it a promising model for future studies. However, the use of these cells in research is strictly controlled requiring authorizations for their applications and they might be subject to ethical concerns regarding their origin.

3. Neuroblastoma cell line SK-N-BE(2)

Ultimately, we found the right neuronal cell type allowing me to progress in the study. Given the great efficiency of the antimiRNA approach in blocking miR-124 in all

previous experiments, I used this strategy to conduct SINV studies in this background. I chose the human neuroblastoma cell line, SK-N-BE(2), where miR-124 is expressed and its expression can be induced upon differentiation of these cells (Laneve et al., 2007). However, once differentiated, these cells are very difficult to transfect (Karra and Dahm, 2010). Thus, I set up the optimal experimental conditions allowing transfection of antimiRs for efficient miR-124 inhibition while inducing differentiation, in order to have neuronal cells to be as close as possible to cells present in the brain in conditions of infection in the CNS. These results are part of the experiments presented and discussed in Chapter II of Results.

B. Ectopic expression

1. Generation and characterization of a HEK293T doxycycline-inducible stable cell line expressing miR-124

In order to study the effect of miR-124 on SINV infection, we decided to establish a cell line easy to manipulate and to amplify that would express miR-124 under the control of a doxycycline(Dox)-inducible promoter. The aim was to be able to control and induce miR-124 expression to monitor its effect on SINV infection in one simple setup. I transfected HEK293T cells with a vector expressing miR-124 and put them under puromycin selection to select for stable integration events. One polyclonal and ten monoclonal HEK293T stable cell lines were screened by northern blot analysis to measure miR-124 expression and its induction by Dox treatment for 24 h. Among the isolated clones, we obtained one false positive (clone 1) and nine clones expressing miR-124 at different levels in Dox-treated and control conditions (Figure 5). Clone 7 was chosen to pursue our study, being the one having the highest difference in expression between mock- and Dox-treated conditions.

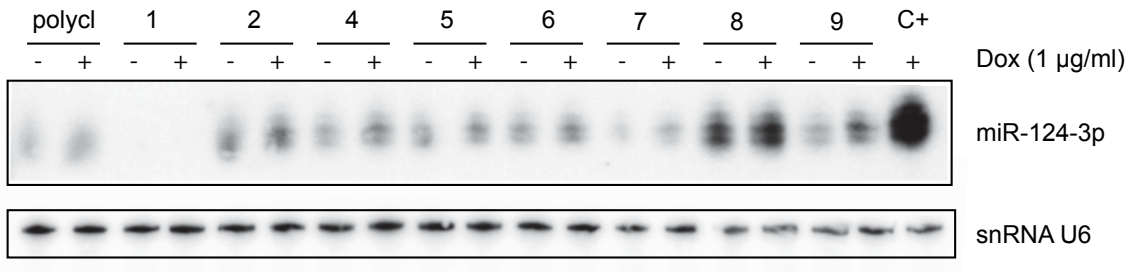

Figure IV.5. Screening of monoclonal HEK293T stable cell lines expressing miR-124 after Dox treatment.
Northern blot analysis of miR-124 expression in HEK293T-miR-124 upon mock (-) or Dox (+) treatment for 24 h. The positive control (C+) corresponds to the expression of miR-124 in HEK293T cells after transient transfection of the vector and induced with 1 μg/ml of Dox. snRNA U6 is used as a loading control.

To further characterize this clone, I induced the miRNA expression with different doses of Dox ranging from 0 to 5 μg/ml for 24 or 48 h (Figure 6A). I could show that miR-124 expression was induced already at 0.5 μg/ml at 24 h of Dox treatment and that higher doses of Dox did not induce stronger expression. At 48 h, miR-124 expression was strongly induced compared to control already at 0.5 μg/ml and again with no major differences in expression with higher doses of Dox. Furthermore, to study the miRNA stability, the cells were treated with 1 or 2 μg/ml of Dox for 24 or 48 h. Dox was then removed and cells were incubated for another 24 h before sample collection (Figure 6B). This allowed to verify that the expression of miR-124 was stable in time even after Dox removal. Of note, in all these experiments I also observed the expression of miR-124 in the mock-treated sample, which indicated that the Dox-inducible promoter was leaky.

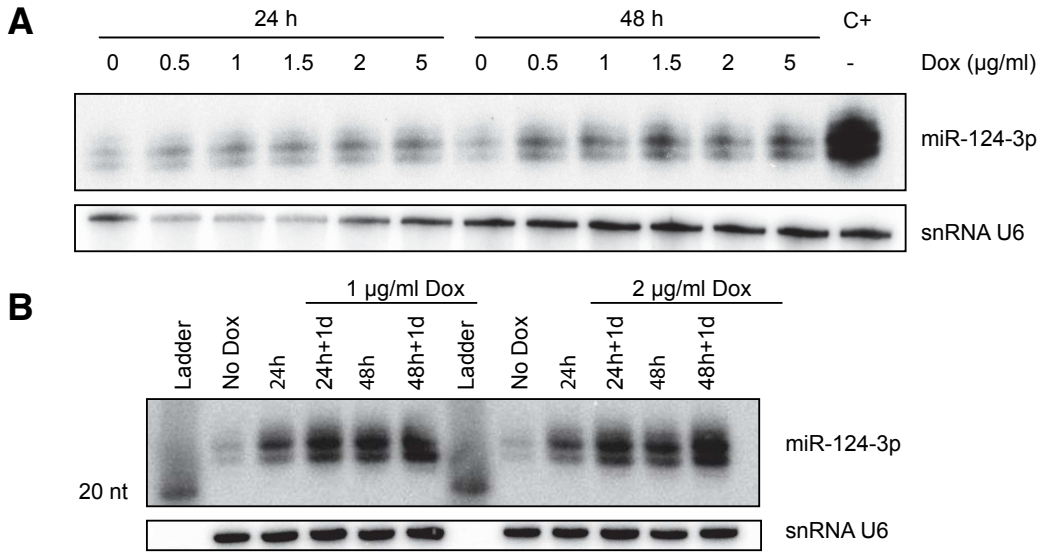

Figure IV.6. Characterization of miR-124 expression induction and stability of clone 7.
Northern blot analysis of miR-124 expression upon increasing doses of Dox for 24 or 48 h (A) or upon treatment for 24 or 48 h and then incubation in antibiotic-free media for another 24 h (B). The positive control (C+) corresponds to the miR-124 expression in the adult mouse cortex region. snRNA U6 is used as a loading control.

Considering that I did not detect a dose-dependent increase in expression of miR-124 with different doses of Dox, I decided to perform all subsequent experiments with this cell line by treating cells with 1 μg/ml of Dox for 48 h to limit undesired toxic effects of the antibiotic treatment on the cells.

1. 1. Effect of miR-124 overexpression on SINV infection

To assess miR-124 effect on SINV, I induced miR-124 expression in clone 7 as indicated above and I measured viral production by plaque assay as well as viral RNA accumulation (genomic, subgenomic or both) by RT-qPCR analysis (Figure 7). I could see that viral titers increased only slightly and non-significantly upon Dox treatment (Figure 7A). RT-qPCR analysis of viral RNA expression also showed a very mild

increase of subgenomic RNA accumulation in cells treated with Dox, and no effect on genomic RNA (Figure 7B).

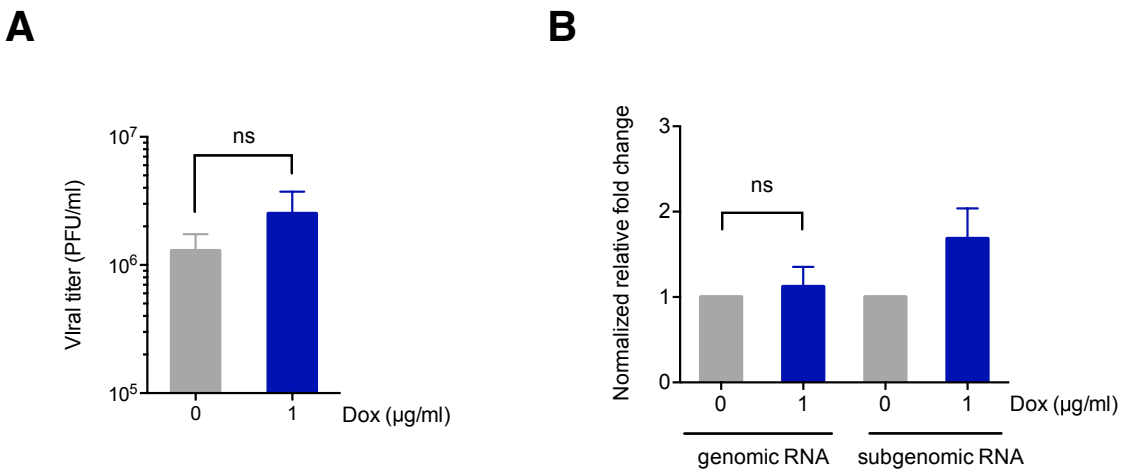

Figure IV.7. Effect of induced miR-124 expression on SINV-GFP infection in clone 7.
Viral titers (A) and viral RNA accumulation (B) of SINV infection produced upon mock or Dox treatment. Data are presented as mean + SEM of three independent experiments except for the subgenomic RNA accumulation which is two replicates, in this case no statistical analyses could be done. ns: non-significant, unpaired Student's *t* test.

1. 2. Effect of miR-124 inhibition on SINV infection

Another method used to assess the importance of a miRNA in virus accumulation is to block it with an antisense oligonucleotide and then measure the effect on the infection. I thus transfected specific miR-124 inhibitor (antimiR-124) or a control (antimiR-CTL) in "clone 7" cells, in which miR-124 expression had been induced or not with 1 μg/ml of Dox treatment. Following transfection, cells were infected with SINV-GFP and I measured the effect of miR-124 inhibition on viral particle production and viral genomic RNA accumulation.

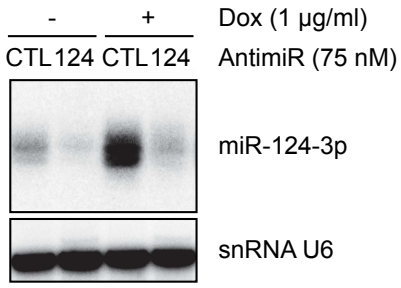

Figure IV.8. miR-124 accumulation upon antimiR transfection of clone 7 cells.
Northern blot analysis of miR-124 expression in mock- (-) or Dox- (+) treated cells transfected with an antimiR control (CTL) or antimiR-124 (124). snRNA U6 is used as a loading control.

Although I could observe a significant reduction of miR-124 expression following its inhibition in Dox-treated or mock cells by northern blot analysis (Figure 8), it seems that this did not translate in a very prominent effect on viral titer (Figure 9A) or genomic RNA accumulation (Figure 9B).

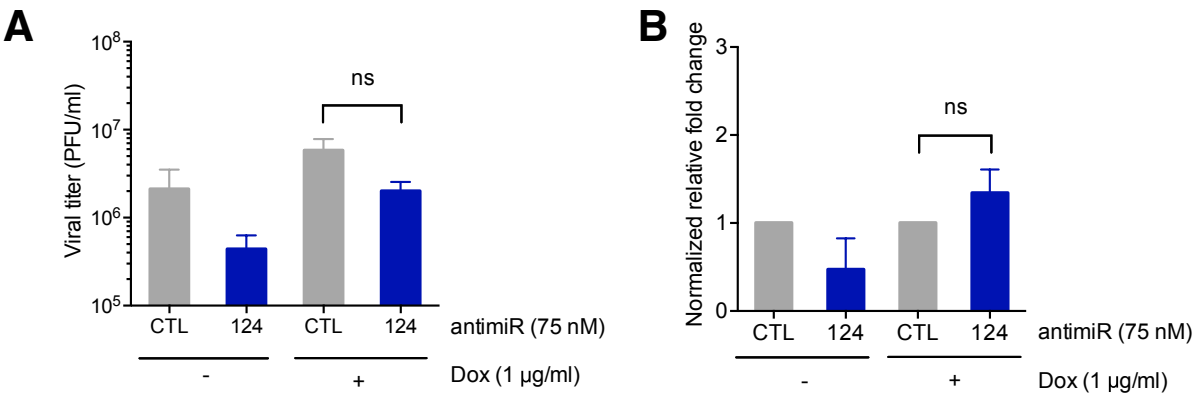

Figure IV.9. Effect of miR-124 inhibition on SINV infection.
Viral titers (A) and viral genomic RNA accumulation (B) of SINV-GFP infected clone 7 cells at an MOI 10^{-3} for 24 h upon miR-124 inhibition after expression induction with 1 μg/ml of Dox. Error bars represent mean + SEM of three independent experiments except for -Dox which is two replicates. ns: non-significant, unpaired Student's *t* test.

Overall, I concluded that even if the levels of miR-124 reached after induction in this cell line were high, we detected a basal expression already in the cell line without Dox treatment, probably due to leaky promoter activity. This could also explain why in our inhibition experiment we were not able to efficiently block the miRNA. Hence, I

decided not to pursue my research with this cellular model, which was not robust enough for the purpose of our experiments.

2. Generation and characterization of a Huh7.5.1 stable cell line expressing miR-124

Given the limitations of the doxycycline-inducible promoter in our experimental setup, I used the experience gathered until here to generate another miR-124 expressing vector under the control of a constitutive promoter which I employed to produce lentiviral particles and establish a new cell line by lentivirus transduction.

For this new stable cell line, I used Huh7.5.1 cells to be as close as possible to the genetic context in which the initial screen was performed. Integration of the lentivirus in the genomic DNA allowed a stable and constitutive expression of miR-124. In this experimental setup, I used the previously designed antimiRNA molecules to specifically inhibit miR-124 and monitor the effect on the virus. The characterization of this cell line and the results obtained in this system are described in the article published in *Journal of Virology*, and upon which Chapter II of Results is based.

CHAPTER II. High-throughput fluorescence-based screen identifies the neuronal miRNA miR-124 as a positive regulator of alphavirus infection (Published article)

Ultimately, I succeeded at setting up and characterizing the most appropriate models to conduct our study of miRNA-virus interaction. These results are recapitulated in this chapter and will be presented in the form of my first-author article published in *Journal of Virology* and included below.

In the article, we present first the miRNA genome-wide screen and its validations. Briefly, a library of synthetic RNA molecules of either mimics or antimiRs were transfected in a human hepatoma cell line (i.e. Huh7.5.1 cells) in order to overexpress or block all of the known cellular miRNAs. Following infection of these cells with a SINV strain engineered to express the GFP (SINV-GFP), we evaluated the effect on viral infection by automated image analysis of the percentage of GFP positive cells as well as GFP intensity. This led to the identification of our candidate list, made up of pro-viral miRNAs, many of them tissue-specific miRNAs and not expressed in the cell line used.

For the study of the top candidate, the brain-specific miR-124, we used Huh7.5.1 cells that we engineered to stably express the miRNA as well as the human neuroblastoma cell line SK-N-BE(2). Inhibition studies in these cell lines confirmed the effect of miR-124 on SINV and validated the identification of miR-124 as a novel pro-viral factor.

Concerning the mechanisms of regulation, we could narrow the pro-viral role to a mechanism involving increased viral protein synthesis and leading to a higher viral particle production but we did not observe a regulation at the level of viral replication.

While considering a possible direct regulation by interaction between miR-124 and SINV RNA, we conducted bioinformatic analyses on SINV genome sequence that showed the presence of three predicted binding sites. I introduced point mutations into the viral genomic RNA to disrupt one of the putative binding sites. Experiments with the

mutant virus showed that it is no longer affected by miR-124 and validated the involvement of this particular binding site on the viral genome in the regulation process.

In addition, we show that the regulatory functions of miR-124 can be extended to other alphaviruses. Indeed, experiments with CHIKV confirmed that it is also positively regulated by miR-124. Interestingly the lack of an optimal miR-124 binding site raises the possibility that these viruses can be regulated differently. CHIKV would therefore be indirectly regulated by modulation of one or several cellular targets controlling the infection.

Overall, this study provides further evidence of the implication of miRNAs in modulating viral infection and more particularly it uncovers a novel role for miR-124 in the regulation of alphaviruses, known to have the potential of infecting the CNS. It thus broadens the potential regulatory mechanisms set in place during viral infection and sheds light on new avenues for future studies regarding RNA regulation in host-pathogen interactions.

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VIRUS-CELL INTERACTIONS

High-Throughput Fluorescence-Based Screen Identifies the Neuronal MicroRNA miR-124 as a Positive Regulator of Alphavirus Infection

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Paula López and Erika Girardi contributed equally. Author order was determined on the basis of increasing seniority.

ABSTRACT MicroRNAs (miRNAs) are small regulatory RNAs which act by modulating the expression of target genes. In addition to their role in maintaining essential physiological functions in the cell, miRNAs can also regulate viral infections. They can do so directly by targeting RNAs of viral origin or indirectly by targeting host mRNAs, and this can result in a positive or negative outcome for the virus. Here, we performed a fluorescence-based miRNA genome-wide screen in order to identify cellular miRNAs involved in the regulation of arbovirus infection in human cells. We identified 16 miRNAs showing a positive effect on Sindbis virus (SINV) expressing green fluorescent protein (GFP), among which were a number of neuron-specific ones such as miR-124. We confirmed that overexpression of miR-124 increases both SINV structural protein translation and viral production and that this effect is mediated by its seed sequence. We further demonstrated that the SINV genome possesses a binding site for miR-124. Both inhibition of miR-124 and silent mutations to disrupt this binding site in the viral RNA abolished positive regulation. We also proved that miR-124 inhibition reduces SINV infection in human differentiated neuronal cells. Finally, we showed that the proviral effect of miR-124 is conserved in other alphaviruses, as its inhibition reduces chikungunya virus (CHIKV) production in human cells. Altogether, our work expands the panel of positive regulation of the viral cycle by direct binding of host miRNAs to the viral RNA and provides new insights into the role of cellular miRNAs as regulators of alphavirus infection.

IMPORTANCE Arthropod-borne (arbo) viruses are part of a class of pathogens that are transmitted to their final hosts by insects. Because of climate change, the habitat of some of these insects, such as mosquitoes, is shifting, thereby facilitating the emergence of viral epidemics. Among the pathologies associated with arbovirus infection, neurological diseases such as meningitis and encephalitis represent a significant health burden. Using a genome-wide miRNA screen, we identified neuronal miR-124 as a positive regulator of the Sindbis and chikungunya alphaviruses. We also showed that this effect was in part direct, thereby opening novel avenues to treat alphavirus infections.

KEYWORDS microRNA, miR-124, RNA virus, arbovirus, alphavirus, Sindbis virus, chikungunya virus, neuron, host-virus interaction

Infectious diseases, and among them viral diseases, remain a leading cause of morbidity and mortality worldwide. In addition to the direct consequences of viral infections, many health disorders are indirectly linked to viruses. Although vaccines are

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available for some viruses, this is not the case for a large number of them, and it is essential to find novel antiviral compounds to fight them. Alphaviruses, from the *Togaviridae* family, are arthropod-borne viruses (arboviruses) transmitted to vertebrates by a mosquito vector and form a group of widely distributed human and animal pathogens. They are small, enveloped, positive single-stranded RNA viruses. Their RNA genome of ~11 kb is capped and polyadenylated. It has two open reading frames (ORFs) encoding nonstructural and structural proteins. ORF2 is expressed through the production of a subgenomic RNA from an internal promoter in the minus-strand RNA replication intermediate. In addition to protein-coding sequences, alphavirus RNAs contain important regulatory structures, such as the 5′ and 3′ untranscribed regions (UTRs) (1).

Alphaviruses represent an emerging public health threat, as they can induce febrile and arthritogenic diseases, as well as other highly debilitating diseases such as encephalitis (2). Sindbis virus (SINV) is considered the prototypical alphavirus and is widely used as a laboratory model. Although the infection has been mainly associated with a rash, arthritis, and myalgia in humans (3), SINV displays a neuronal tropism in developing rodent brain cells and is associated with encephalomyelitis (4). Another virus from the same genus is chikungunya virus (CHIKV), which causes outbreaks of severe acute and chronic rheumatic diseases in humans (5). CHIKV has also been reported to affect the human nervous system, causing encephalopathy in newborns, infants, and adults (6). Due to its ability to quickly spread into new regions, CHIKV is classified as an emergent virus (7, 8) for which preventive or curative antiviral strategies are needed.

MicroRNAs (miRNAs) are small, 22-nucleotide-long noncoding RNAs which act as guides for effector proteins to posttranscriptionally regulate the expression of target cellular mRNAs (9) but also viral RNAs (10–17). These small RNAs have been identified in almost all eukaryotic species, and a number of them are conserved throughout evolution (18). They derive from longer precursors, which are transcribed by RNA polymerase II, and are sequentially processed by the RNase III enzymes Drosha and Dicer. The mature miRNA is then assembled in a protein of the Argonaute family to guide it to target RNAs. Once bound to its target, the Argonaute protein regulates its expression by recruiting proteins to inhibit translation initiation and induce its destabilization by deadenylation (19). The main determinant of miRNA sequence specificity is its seed sequence, which corresponds to a short region at the 5′ end of miRNAs (nucleotides 2 to 7) (20). Perfect pairing of the miRNA seed with the target RNA represents the minimal requirement for efficient Argonaute binding and function. In some cases, additional base pairing toward the 3′ end of the mature miRNA (so-called 3′ compensatory sites) may compensate for suboptimal pairing in the seed region (21, 22).

Identifying miRNAs that alter virus replication has illuminated roles for these molecules in virus replication and highlighted therapeutic opportunities. Target predictions based on the concept of "seed" initially identified binding sites for liver-specific miR-122 in the 5′ UTR of hepatitis C virus (HCV), which turned out to be positively regulated by this miRNA (23). Further work from different teams later showed that miR-122 can positively regulate the virus by increasing the stability and translation of the viral RNA (24, 25). Interestingly, the use of inhibitors of miR-122 in HCV infection is currently in a clinical trial as an miRNA-based antiviral therapy (26).

Here, we performed a genome-wide miRNA overexpression and inhibition screen to identify cellular miRNAs involved in the regulation of SINV infection. We found 16 miRNAs with a positive effect on virus accumulation. Among them, miR-124-3p was shown to positively regulate the virus by increasing viral structural protein translation and viral production, and we identified a binding site for this small RNA in the viral genome. miR-124 is the most expressed, conserved, and specific microRNA in the central nervous system (CNS), and it has been described as a master regulator of neuronal differentiation (27). It is upregulated as neuronal progenitors exit mitosis and begin to differentiate (28), and its expression has been shown to be sufficient to drive cells toward the neuronal pathway (29). Mutations in the miR-124 seed region or in the

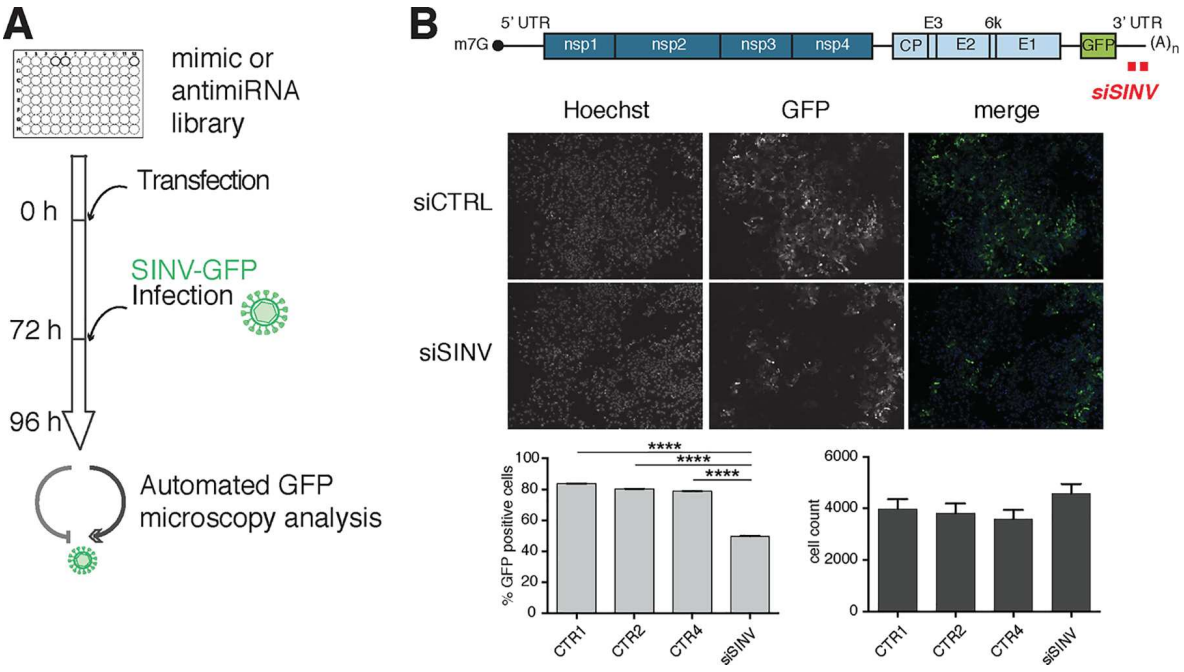


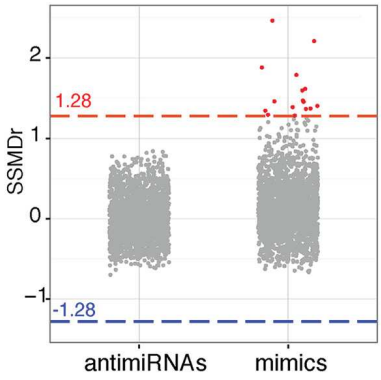
FIG 1 Design of the phenotypic fluorescence-based screen and validation with siRNA directed against SINV-GFP. (A) Schematic representation of the screening protocol. Huh7.5.1 cells were reverse transfected with a complete library of mimics or antimiRNAs corresponding to all identified human miRNAs, and 72 h posttransfection, they were infected with SINV-GFP at a low multiplicity of infection (MOI of 10^{-3}) for 24 h. The infection level was analyzed by automated microscopy analysis based on GFP fluorescence. (B, top) schematic representation of SINV-GFP genomic structure with the repeated region within the 3′ UTR of SINV (starting at positions 12345 and 12437) that is targeted by the siRNA SINV (siSINV) used as a functional control for the genome-wide screen. (Middle) Representative fluorescence microscopy images of SINV-GFP infected Huh7.5.1 cells after transfection with a control siRNA or with the siRNA directed against the virus (siSINV). (Bottom) Histograms represent the percentages of GFP-positive cells (left) and cell counts (right) to evaluate toxicity after negative-control siRNA (CTR1, CTR2, and CTR4) or siSINV transfection. The means ± standard errors of the means (SEMs) from three independent experiments are presented. ****, $P < 0.0001$, unpaired Student's t test.

binding site on the viral RNA abolished regulation. We also proved that the miR-124 inhibition strategy via antisense oligonucleotides reduces both SINV and CHIKV production in human cells expressing this miRNA. These studies highlight a novel role for miR-124 in arbovirus infection and suggest that targeting alphavirus infection via miRNA modulation could be used therapeutically.

RESULTS

Identification of miRNAs involved in the regulation of SINV infection by fluorescence-based screening. To identify miRNAs that are involved in the regulation of SINV infection in a systematic and comprehensive way, we performed a fluorescence microscopy-based high-throughput screening using two libraries of ~2,000 human miRNA mimics and ~2,000 antimiRNA oligonucleotides (Fig. 1A). Control miRNA mimics or antimiRNAs with no sequence homology to any known human miRNA were used as negative controls. A small interfering RNA (siRNA) against the 3′ UTR of SINV, able to decrease green fluorescent protein (GFP) expression without inducing cell death, was used as a functional positive control (Fig. 1B). Huh7.5.1 cells were transfected with either the library of miRNA mimics or antimiRNAs, and 72 h later, the cells were infected with a SINV strain encoding the green fluorescent protein (GFP) (Fig. 1A). The number of GFP-positive cells and the GFP intensity were measured 24 h postinfection (hpi) by automated image analysis, and the robust strictly standardized mean difference (SSMD* or SSMDr) value was calculated for each miRNA to identify significant hits. While the antimiRNA screen did not reveal any significant hit and the mimic screen did not identify any miRNAs with a negative effect on GFP accumulation, we identified 16 miRNA mimics that increased the GFP signal significantly (SSMDr > 1.28) without affecting cell viability (Fig. 2A). All of the top hit miRNAs were undetectable or expressed at a very low level in Huh7.5.1 cells as assessed by small RNA sequencing

A



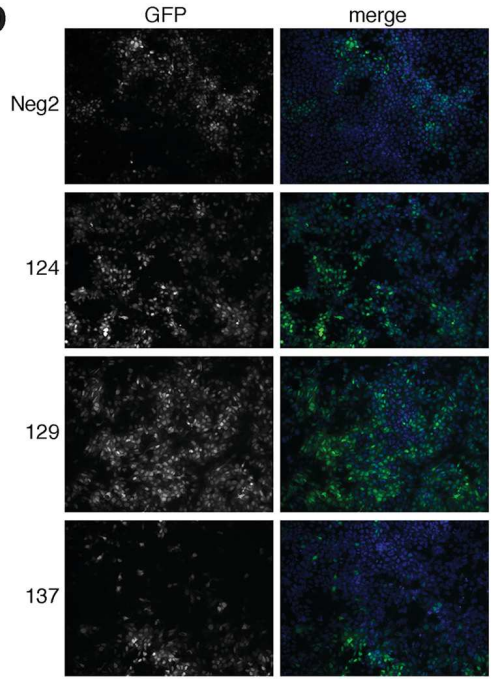
B

Mature microRNA	*SSMDr*	*Total toxicity*	*Expression (rpm)*
hsa-miR-124-3p	2.47	0.53	-
hsa-miR-129-5p	2.21	0.06	19.81
hsa-miR-205-5p	1.88	0.55	-
hsa-miR-3910	1.79	0.30	-
hsa-miR-1244	1.61	0.43	-
hsa-miR-485-3p	1.60	0.34	0.29
hsa-miR-3682-5p	1.47	0.48	-
hsa-miR-30b-3p	1.46	0.61	9.61
hsa-let-7a-2-3p	1.46	0.65	-
hsa-miR-3163	1.40	0.28	7.09
hsa-miR-665	1.39	0.32	-
hsa-miR-506-3p	1.37	0.38	-
hsa-miR-624-3p	1.37	0.66	11.26
hsa-miR-20b-3p	1.34	0.44	-
hsa-miR-3152-3p	1.29	0.25	-
hsa-miR-3154	1.28	0.40	-

C

miRNA	rpm
hsa-miR-25-3p	72426.16
hsa-let-7a-5p	71366.13
hsa-miR-19b-3p	56208.75
hsa-miR-148a-3p	49537.68
hsa-miR-92a-3p	49078.68
hsa-miR-21-5p	45015.38
hsa-miR-378a-3p	44045.69
hsa-miR-19a-3p	39872.16
hsa-miR-151a-3p	33499.15
hsa-miR-222-3p	29595.22
hsa-let-7f-5p	29335.59
hsa-miR-93-5p	25558.78
hsa-miR-221-3p	25409.08
hsa-miR-122-5p	25126.81
hsa-miR-7-5p	24497.39
hsa-miR-183-5p	20521.32
hsa-miR-17-5p	16220.11
hsa-miR-196b-5p	15418.23
hsa-miR-101-3p	13292.19
hsa-miR-106b-5p	12663.86
hsa-miR-103a-3p	11994.55
hsa-let-7c-5p	11500.92
hsa-miR-1269a	11264.55
hsa-miR-10b-5p	9983.73
hsa-miR-196a-5p	9771.34
hsa-miR-148b-3p	8913.09
hsa-miR-10a-5p	7894.93
hsa-miR-1269b	7726.05
hsa-miR-20a-5p	7384.74
hsa-miR-182-5p	7305.53

D



E

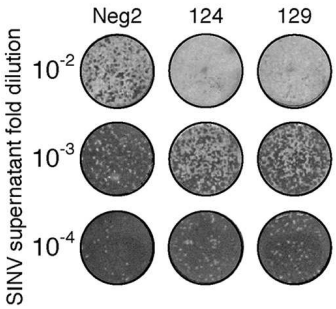


FIG 2 Identification of cellular miRNAs positively regulating SINV infection in Huh7.5.1 cells. (A) Dot plot representation of the strictly standardized mean difference (SSMDr) in fluorescence for each mimic or antimiRNA. Candidates with an absolute SSMDr equal to or higher than the threshold 1.28 were considered significant. In red, significant mimic candidates. (B) List of significant candidates identified in the mimic screen with their respective SSMDr and associated toxicity values. miRNA expression levels in Huh7.5.1 cells determined by small RNA sequencing (small RNA-seq) are indicated as reads per million miRNA reads (rpm). (C) Top 30 microRNAs expressed in Huh7.5.1 cells. Ranking was based on the reads normalized per million microRNA reads (rpm) in the small RNA-seq experiment. (D) Representative fluorescence images from the mimic screen of some of the top candidates compared to the controls (Neg2 and miR-137). (Left) GFP signals from infected cells; (right) merges of GFP signals and Hoechst staining of cell nuclei. Pictures taken at ×10 magnification. (E) Representative plaque assay image after crystal violet staining of SINV-infected Huh7.5.1 cells transfected with control or candidate mimics.

(Fig. 2B and C), which explains why transfection of the corresponding antimiRNAs showed no effect on GFP levels. Among the top hits, miR-124-3p and miR-129-5p, which are known to be expressed in neuronal cells (30, 31), had the most striking effect (SSMDr $>$ 2.20), suggesting a link between neuron-enriched miRNAs and positive regulation of the virus. To determine whether the positive effect of these miRNAs on SINV-GFP expression (Fig. 2D) implied an increase in viral production, we measured the effect of the overexpression of miR-124-3p and miR-129-5p on SINV titers compared to that for a negative miRNA mimic control (Fig. 2E). As observed from the measurement of GFP fluorescence, miR-124-3p and miR-129-5p also increased SINV-GFP viral titers in Huh7.5.1 cells, providing additional evidence for their involvement in the positive regulation of viral infection.

miR-124 positively regulates SINV by increasing viral structural protein synthesis and viral particle production in a seed-dependent manner. Because miR-124 was the top hit of our screen, the most expressed miRNA in neuronal tissues, and one of the most conserved microRNAs in metazoans, we decided to focus our efforts on the characterization of the mechanism underlying its effect on the SINV cycle. We first assessed the impact of miR-124-3p expression during SINV binding and internalization, the early steps of the viral cycle. We thus performed a SINV attachment and entry assay in cells transfected with a control or miR-124-3p mimic. At 72 h posttransfection, we detected a decrease rather than an increase of SINV attachment (Fig. 3A, attachment). Moreover, miR-124-3p overexpression did not affect the efficiency of SINV entry into Huh7.5.1 cells compared to that of cells transfected with control mimic (Fig. 3A, entry). Collectively, these data suggest that the positive effect of miR-124-3p on SINV is not due to increased viral binding or internalization.

We then analyzed the effect of miR-124-3p expression on SINV RNA replication by measuring viral genomic, subgenomic, and antigenomic RNA accumulation. Transfection of miR-124-3p mimic, which could readily be measured by reverse transcriptase quantitative PCR (RT-qPCR) (Fig. 3B), induced repression of a known miR-124 target, the cyclin-dependent kinase 4 (CDK4) (32). However, neither SINV genomic nor subgenomic RNA levels were significantly affected (Fig. 3C).

To better assess the miR-124 effect on SINV RNA replication, we performed semiquantitative strand-specific RT on the negative-strand antigenomic RNA. We observed that a similar amount of antigenome accumulated in Huh7.5.1 cells transfected with control or miR-124 mimic (Fig. 3D), suggesting that the positive effect of miR-124-3p on SINV is not due to an increased antigenome synthesis.

Finally, we analyzed the accumulation of either nonstructural (i.e., nsP2) or structural viral proteins (i.e., capsid) upon mimic transfection and SINV-GFP infection. The results showed an increase in the structural capsid protein but not in the nonstructural protein nsP2 upon mimic-124 transfection (Fig. 3E), strongly suggesting that positive regulation by miR-124 is due to an enhancement of viral structural proteins synthesis from the subgenomic RNA.

Among the top candidates showing a proviral effect in the screen (Fig. 2A and B), we found miR-506-3p, which belongs to the same family as miR-124 and shares similar physiological functions (33). This strongly suggests a possible implication of their seed region in the mechanism of viral regulation. In order to assess whether the proviral effect observed depended on the miRNA seed sequence, we transfected control mimics or mimics containing either the miR-124-3p wild-type seed sequence (mimic-124) or a miR-124-3p seed with three mutations at positions 4 to 6 (mimic-124mut) (Fig. 3F) in Huh7.5.1 cells before infecting them with SINV-GFP. Viral capsid protein production demonstrated that the positive effect of miR-124-3p expression was lost when cells were transfected with the seed mutant mimic (Fig. 3G). In addition, the same effect was observed on the viral titer quantification (Fig. 3H), strongly suggesting that miR-124-3p positively regulates SINV via its seed region, by enhancing viral structural protein translation and viral production.

Mutation of a miR-124-3p binding site within the SINV E1 coding region prevents miRNA-mediated regulation. As miRNA-mediated viral regulation can hap-

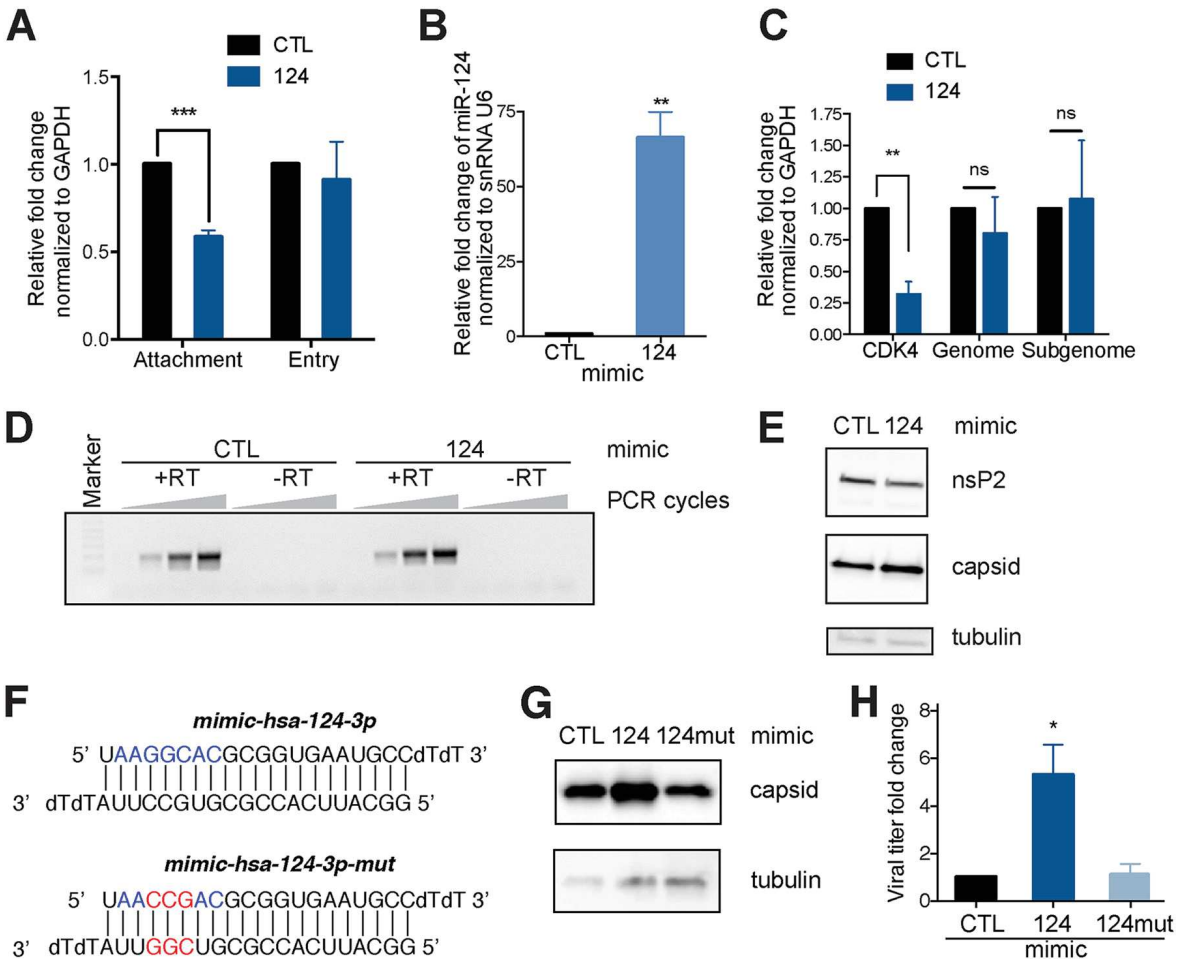
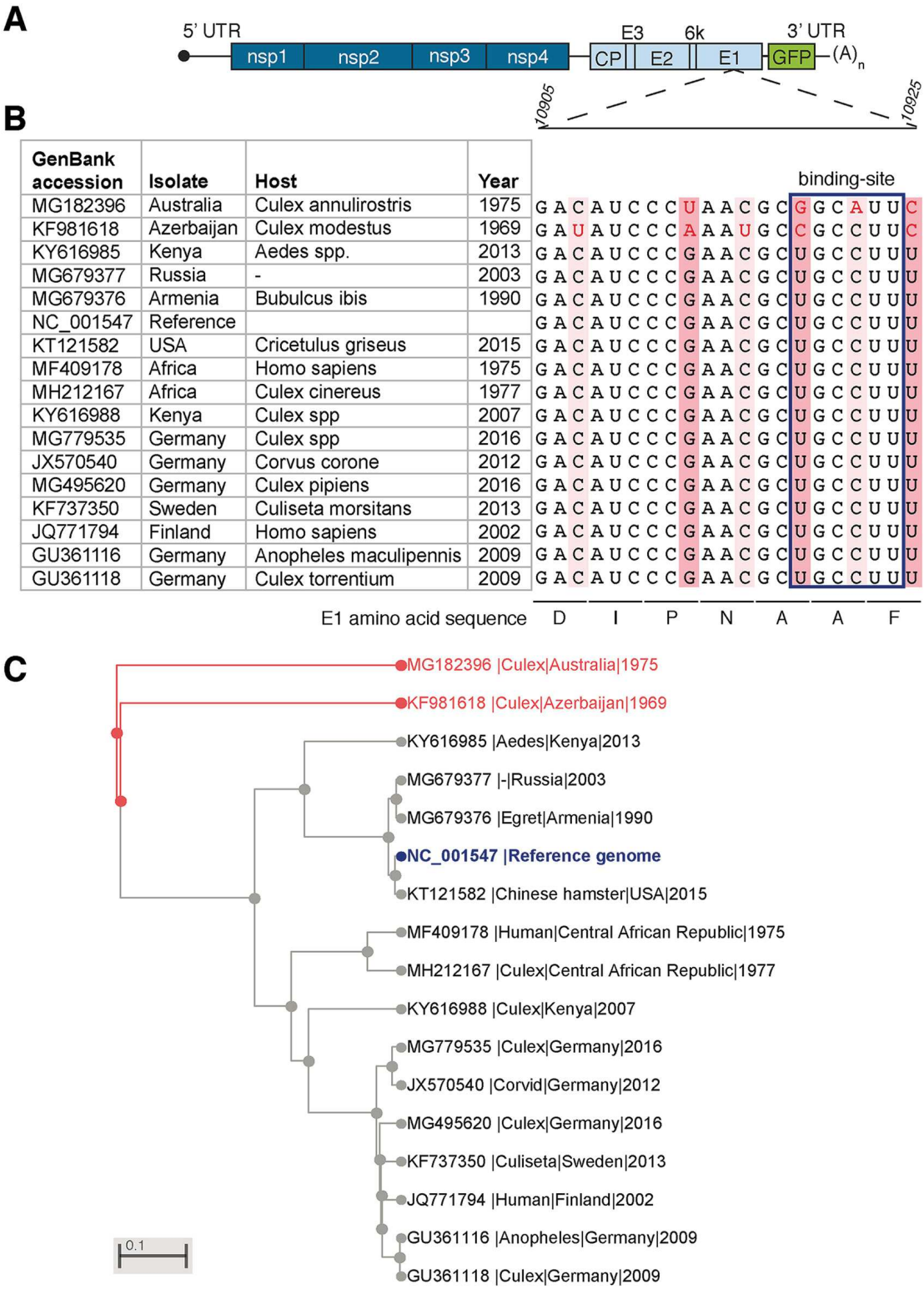


FIG 3 Importance and mechanism of miR-124-3p and its seed in the positive regulation of SINV infection. (A) Huh7.5.1 cells transfected with mimic CTL or 124 for 72 h were incubated with SINV-GFP (MOI of 5) at 4°C for 30 min and washed with PBS three times to remove unbound SINV particles. Total RNA was extracted and used for quantification of SINV genomic RNA by RT-qPCR. Data are presented as means ± SEMs from three independent experiments normalized to GAPDH. In parallel, transfected cells were incubated with SINV-GFP (MOI of 5) at 4°C for 30 min, washed with PBS, and then transferred to 37°C for 30 min to allow virus entry. Total RNA was extracted and used for quantification of SINV genomic RNA by RT-qPCR. Data are presented as means ± SEMs from three independent experiments. (B) miR-124 accumulation after mimic transfection quantified by RT-qPCR and relative to control mimic and normalized to snRNA U6. (C) RT-qPCR analysis of CDK4 expression and SINV genomic and subgenomic RNA accumulation in mimic-124-transfected cells following SINV infection at an MOI of 10^{-3} for 24 h relative to mimic CTL transfection and normalized to GAPDH. (D) Strand-specific RT-PCR on SINV antigenome produced in Huh7.5.1 cells transfected with mimic-124 or CTL and infected with SINV-GFP at an MOI of 10^{-3} for 24 h. PCR products from 15, 20, 25, and 30 cycles are shown. (E) Western blot analysis of viral nsP2 and capsid protein synthesis in Huh7.5.1 transfected cells with mimic-124 or CTL and infected with SINV at an MOI of 10^{-3} for 24 h. Tubulin is used as a loading control. (F) Diagram of miRNA mimic-124 and mimic-124-mut (seed mutant). Seed sequence is indicated in blue and mutations in red. Western blot analysis of viral capsid protein accumulation (G) and plaque assay quantification of SINV-GFP viral titers (H) after transfection of control mimic, mimic-124, or mimic-124-mut in Huh7.5.1 cells and SINV infection at an MOI of 10^{-3} for 24 h. The means ± SEMs from three independent experiments are presented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant, unpaired Student's *t* test.

pen by direct binding of the miRNA to the viral RNA (34), we bioinformatically searched for predicted miR-124-3p binding sites on the SINV genome. Using the prediction algorithm ViTa (35), we found three putative binding sites at positions 2454 to 2475, 3828 to 3853, and 10904 to 10926 in the viral genome. We further studied the latter, localized within the virus ORF2, and more specifically, in the glycoprotein E1 coding region (Fig. 4A), which possesses a canonical 6-nucleotide (nt) seed match (nucleotides 2 to 7) for miR-124-3p.

Using available SINV sequences in the NCBI virus database (36), we sought to determine the conservation of this region among SINV strains. Sequence alignment of the entire genomic sequences of 16 SINV strains isolated worldwide from various insects and vertebrates between 1969 and 2016 compared to the reference SINV genome (NC_001547) revealed that most of the strains (14 of 16) share the same



GenBank accession	Isolate	Host	Year
MG182396	Australia	Culex annulirostris	1975
KF981618	Azerbaijan	Culex modestus	1969
KY616985	Kenya	Aedes spp.	2013
MG679377	Russia	-	2003
MG679376	Armenia	Bubulcus ibis	1990
NC_001547	Reference		
KT121582	USA	Cricetulus griseus	2015
MF409178	Africa	Homo sapiens	1975
MH212167	Africa	Culex cinereus	1977
KY616988	Kenya	Culex spp	2007
MG779535	Germany	Culex spp	2016
JX570540	Germany	Corvus corone	2012
MG495620	Germany	Culex pipiens	2016
KF737350	Sweden	Culiseta morsitans	2013
JQ771794	Finland	Homo sapiens	2002
GU361116	Germany	Anopheles maculipennis	2009
GU361118	Germany	Culex torrentium	2009

FIG 4 Acquisition and conservation of miR-124-3p binding site in natural SINV isolates. (A) Schematic representation of SINV-GFP genomic structure with a diagram of miR-124-3p binding site within glycoprotein E1 coding region. (B) Alignment of 16 SINV complete genomic sequences from natural isolates corresponding to nucleotide positions 10905 to 10925 in the reference genome (GenBank accession no. NC_001547). Blue rectangle delimits predicted miR-124-3p binding site. Nonconserved nucleotides are highlighted in red. (C) Phylogenetic tree generated from NCBI virus sequence alignment of the entire genomic sequence of SINV strains in panel B. In blue, the SINV reference sequence; in red, the isolates without miR-124-3p binding site conservation. For each strain, the GenBank accession number, host, geographical region, and collection date are shown. Scale bar represents genetic distance.

nucleotide composition at the binding site, independently of the host, the year, or the geographical areas in which they were isolated (Fig. 4B and C). Moreover, two of the most ancient and phylogenetically distant strains (MG182396 and KF981618) display silent point mutations in the miRNA binding site (Fig. 4B).

With the aim of disrupting the putative binding site without interfering with the viral coding sequence, we introduced two silent mutations at positions 10919 and 10922 (Fig. 5A) in the viral genome. We first characterized the mutant virus (SINV-GFP-U10919C-C10922A, referred to as SINV-GFP-mut) by an infection kinetic study compared to the wild-type SINV-GFP in Huh7.5.1 cells. SINV-GFP-mut growth kinetics showed no significant differences in viral titers compared to those of the wild-type SINV-GFP (Fig. 5B). Furthermore, we assessed the release and spread of the mutant virus by verifying the plaque size phenotype, and we observed no difference in plaque size between the two viruses (Fig. 5C). We then verified that miRNA-mediated regulation depends on the binding of this particular site. We transfected Huh7.5.1 cells with a control mimic or with different doses of a miR-124 mutant mimic (124comp) containing compensatory mutations within its seed (Fig. 5D) followed by infection with either SINV-GFP or SINV-GFP-mut. Viral titer quantification by plaque assay shows that mimic 124comp significantly enhanced SINV-GFP-mut but did not affect SINV-GFP (Fig. 5D).

In order to measure the effect of miR-124-3p inhibition on the wild-type (WT) and mutant SINV, we established a Huh7.5.1 cell line stably expressing the human *mir-124-1* gene (LV124 cells). miR-124-3p levels were verified in LV124 cells and compared to the expression in differentiated human neuroblastoma SK-N-BE(2) cells, used as a positive control. Huh7.5.1 cells were used as a negative control. Northern blot analysis showed that miR-124 expression in the LV124 cells was similar to the expression level in differentiated neuroblastoma cells, known to express miR-124 (37) (Fig. 5E). Antisense oligonucleotides have been previously used as a strategy to sequester and block miRNAs (38). To test whether miR-124-3p inhibition reduced SINV infection, we transfected an antimiR-124 or a control antimiRNA into LV124 cells, which resulted in a complete depletion of the mature miRNA as assessed by Northern blotting (Fig. 5F). We then evaluated the effect of miR-124 inhibition in this setup on SINV-GFP and SINV-GFP-mut infection. After antimiR-124 transfection, SINV-GFP viral titers as well as the capsid viral protein synthesis were significantly reduced (Fig. 5G and H). In contrast, SINV-GFP-mut viral titers and protein levels remained unchanged upon miR-124-3p inhibition (Fig. 5G and H). These results show that inhibition of miR-124-3p negatively regulates SINV infection and that mutations introduced in the binding site on the viral RNA abolish the miRNA-mediated regulation. In addition, regulation of the mutant virus can be rescued by overexpression of a compensatory mutant mimic in a dose-dependent manner.

miR-124-3p inhibition in differentiated neuroblastoma cells restricts SINV-GFP infection. Since miR-124-3p is a neuron-specific microRNA, we turned to a system where the miRNA was expressed endogenously to verify both the miRNA effect on SINV infection and the effect of virus infection on miRNA expression. The human neuroblastoma SK-N-BE(2) cells can be differentiated into neuron-like cells by retinoic acid (RA) treatment (39). We first verified neurite outgrowth, which is a morphological hallmark of neuroblastoma cell differentiation *in vitro* (Fig. 6A). To confirm the correct differentiation, we verified by RT-qPCR analysis that the expression of the neuron proliferation marker *MYCN* decreased after RA treatment (40) (Fig. 6B). miR-124-3p endogenous expression increased at 6 days postdifferentiation compared to that in proliferating cells but was not affected by SINV infection (Fig. 6C). We assessed the effect of miR-124-3p inhibition on the production of SINV-GFP wild-type and mutant in these cells by transfecting miR-124-3p or control antimiR prior to differentiation and infection. Northern blot analysis of miR-124-3p expression confirmed the depletion of miR-124-3p under both mock and SINV-infected conditions (Fig. 6D). While SINV-GFP wild-type viral titers as well as viral capsid protein levels were reduced about 50% in antimiR-124-transfected cells compared to that in the control (Fig. 6E and F), the mutation in the viral RNA (SINV-GFP-mut) abolished the effect. These results show that

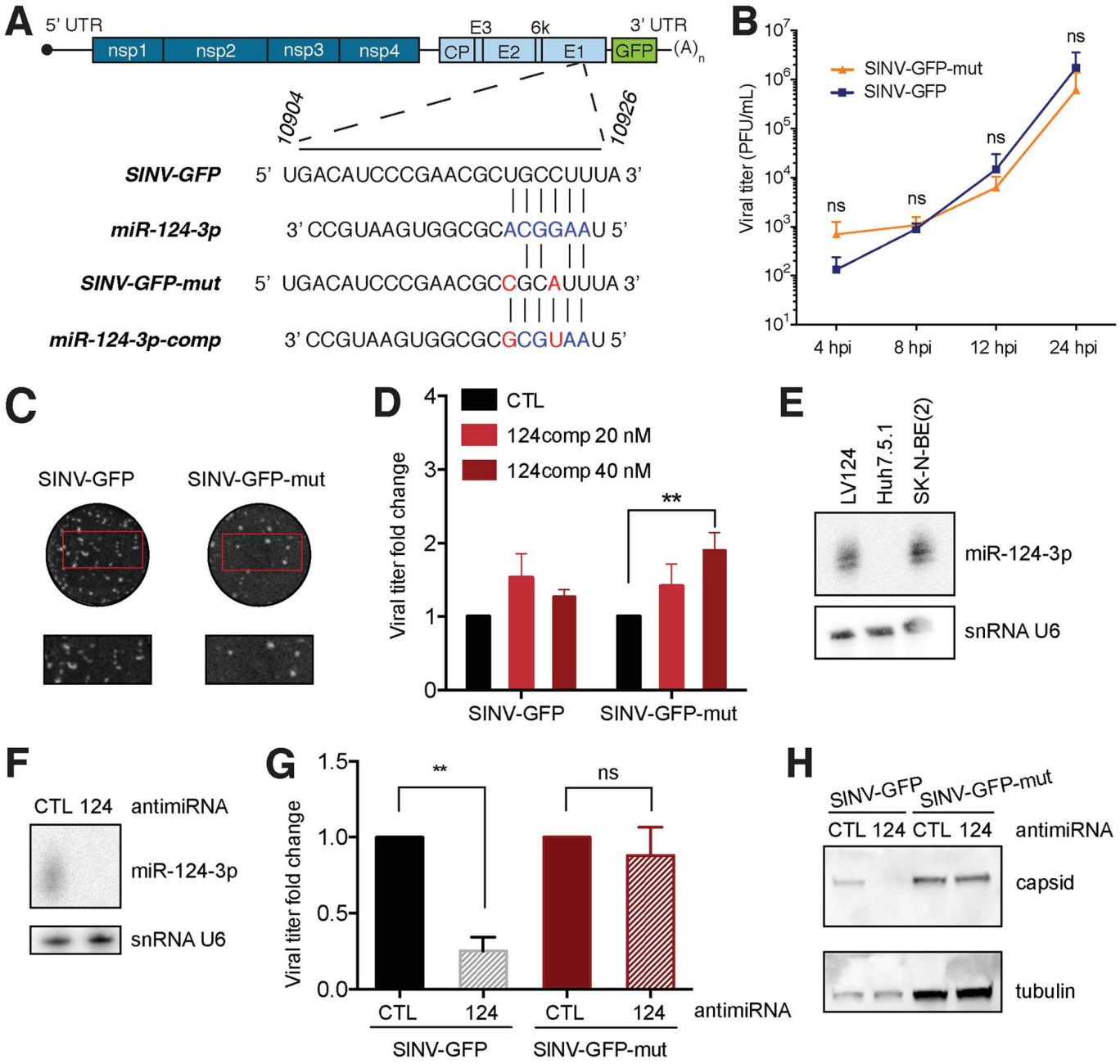


FIG 5 Mutation of one miR-124-3p binding site within SINV genomic RNA prevents regulation. (A) Schematic representation of SINV-GFP genomic structure with a diagram of miR-124-3p binding site within glycoprotein E1 coding region. The miRNA seed is indicated in blue; point mutations introduced in the mutant virus (SINV-GFP-mut) and in the compensatory mutant miR-124 mimic are indicated in red. (B) Plaque assay quantification of viral titers produced by SINV-GFP and SINV-GFP-mut on Huh7.5.1 cells at 4, 8, 12, or 24 hpi. Error bars represent means ± standard deviations (SDs). ns, nonsignificant, two-way analysis of variance (ANOVA) with Bonferroni's correction. (C) Plaques for SINV-GFP and SINV-GFP-mut visualized by crystal violet staining on infected Vero cells to assess plaque size phenotype. (D) Differential fold change in viral titers produced by SINV-GFP and SINV-GFP-mut in Huh7.5.1 cells after transfection of control mimic or mimic-124comp (compensating the viral binding site mutation). (E) miR-124-3p expression in lentiviral transduced Huh7.5.1 cells stably expressing miR-124-3p (LV124), wild-type Huh7.5.1 cells, and SK-N-BE(2) neuroblastoma cells. (F) Northern blot analysis of miR-124-3p expression in LV124 after antimiRNA transfection. snRNA U6 is used as a loading control. (G) Fold differences in viral titers produced by SINV-GFP or SINV-GFP-mut in LV124 after antimiRNA transfection. (H) Western blot analysis of viral capsid protein accumulation in LV124 after antimiR transfection. Tubulin is used as a loading control. Error bars represent means ± SEMs from three independent experiments. **, $P < 0.01$; ns, nonsignificant, unpaired Student's *t* test.

inhibition of miR-124-3p negatively affects SINV-GFP viral infection in human neuronal differentiated cells and that this regulation involves the miR-124 binding site at position 10904 to 10926 in the viral genome.

Inhibition of miR-124-3p restricts CHIKV infection. In order to test whether miR-124-3p could positively regulate other positive single-stranded RNA viruses, we

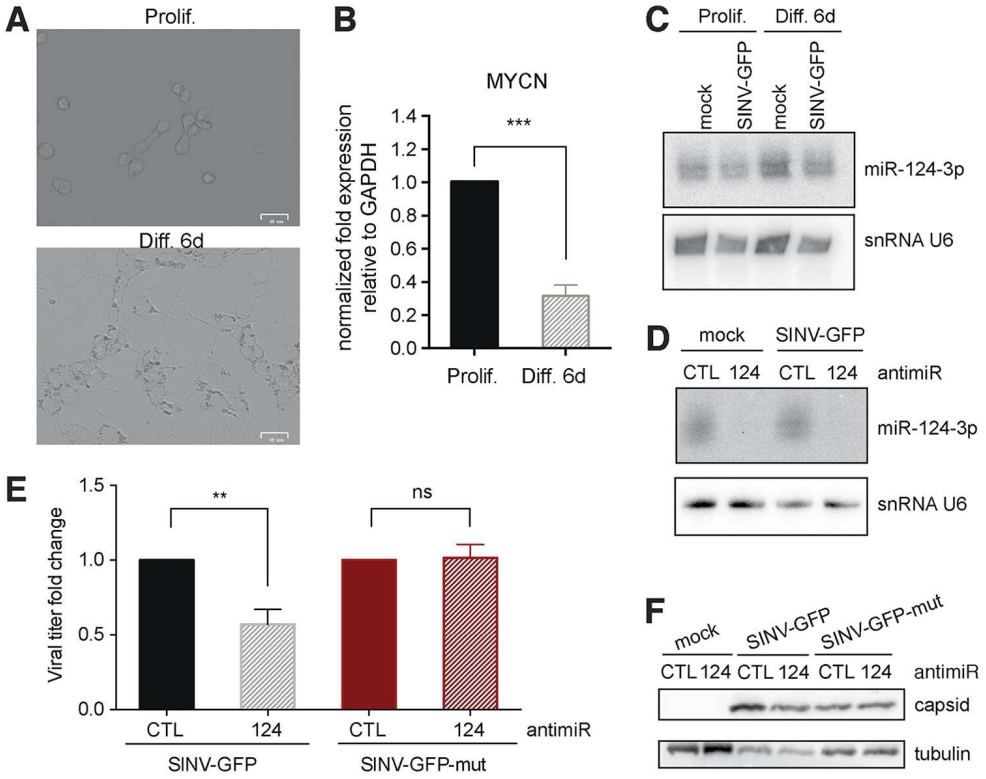


FIG 6 Sequestration of miR-124-3p in differentiated neuronal SK-N-BE(2) cells attenuates SINV-GFP infection. (A) Microscopy images of proliferating (top) or 6-day-differentiated (bottom) SK-N-BE(2) cells by 10 μM RA treatment. Bars, 25 μm. (B) RT-qPCR analysis of relative *MYCN* expression in proliferating or differentiated cells. (C) Northern blot analysis of miR-124-3p expression levels in proliferating or 6-day-differentiated cells, mock or SINV-GFP infected. (D) Northern blot analysis of miR-124-3p expression levels upon antimiRNA transfection in differentiated cells at 6 days post-RA treatment. Plaque assay quantification of viral titer production of SINV-GFP or SINV-GFP-mut, shown as relative fold change compared to that under the control condition (E), and Western blot analysis of viral capsid protein accumulation (F) after antimiRNA transfection and 6-day post-RA differentiation of SK-N-BE(2) cells. Tubulin is used as a loading control. Data are presented as means ± SEMs from three independent experiments. **, $P < 0.01$; ***, $P < 0.005$, unpaired Student's *t* test.

tested two different alphavirus CHIKV strains (CHIKV La Réunion and Caribbean strains) and two strains of the flavivirus Zika virus (ZIKV African and French Polynesia strains). CHIKV La Réunion viral production was significantly increased following miR-124-3p overexpression. Though to a lesser extent, the viral production from the Caribbean strain also followed the tendency of increased titers following miR-124-3-p overexpression. In contrast, no significant effect was observed on the two ZIKV strains tested (Fig. 7A). To verify whether miR-124-3p inhibition could reduce CHIKV infection, we transfected an antimiR-124 or a control antimiRNA in LV124 cells and we infected them with a CHIKV La Réunion strain expressing GFP (Fig. 7B). After antimiR-124 transfection, CHIKV-GFP viral titers were reduced about 50% (Fig. 7C). Moreover, antimiR-124 treatment significantly reduced CHIKV-GFP viral titers in differentiated neuroblastoma SK-N-BE(2) cells (Fig. 7D). Interestingly, sequence alignment of the CHIKV genomic sequence corresponding to the binding site present in the SINV genome revealed the presence of a suboptimal seed match with miR-124-3p, which could be compensated by 3′ pairing (Fig. 7E). These results extend the proviral role of miR-124-3p to another alphavirus and confirm that miR-124-3p inhibition might be a promising strategy to block these viruses.

DISCUSSION

In addition to their role as fine-tuners of cellular functions, microRNAs are emerging as important regulators of host-pathogen interactions (34). They can regulate viral infection either directly, by targeting RNAs of viral origin, or indirectly, by targeting host

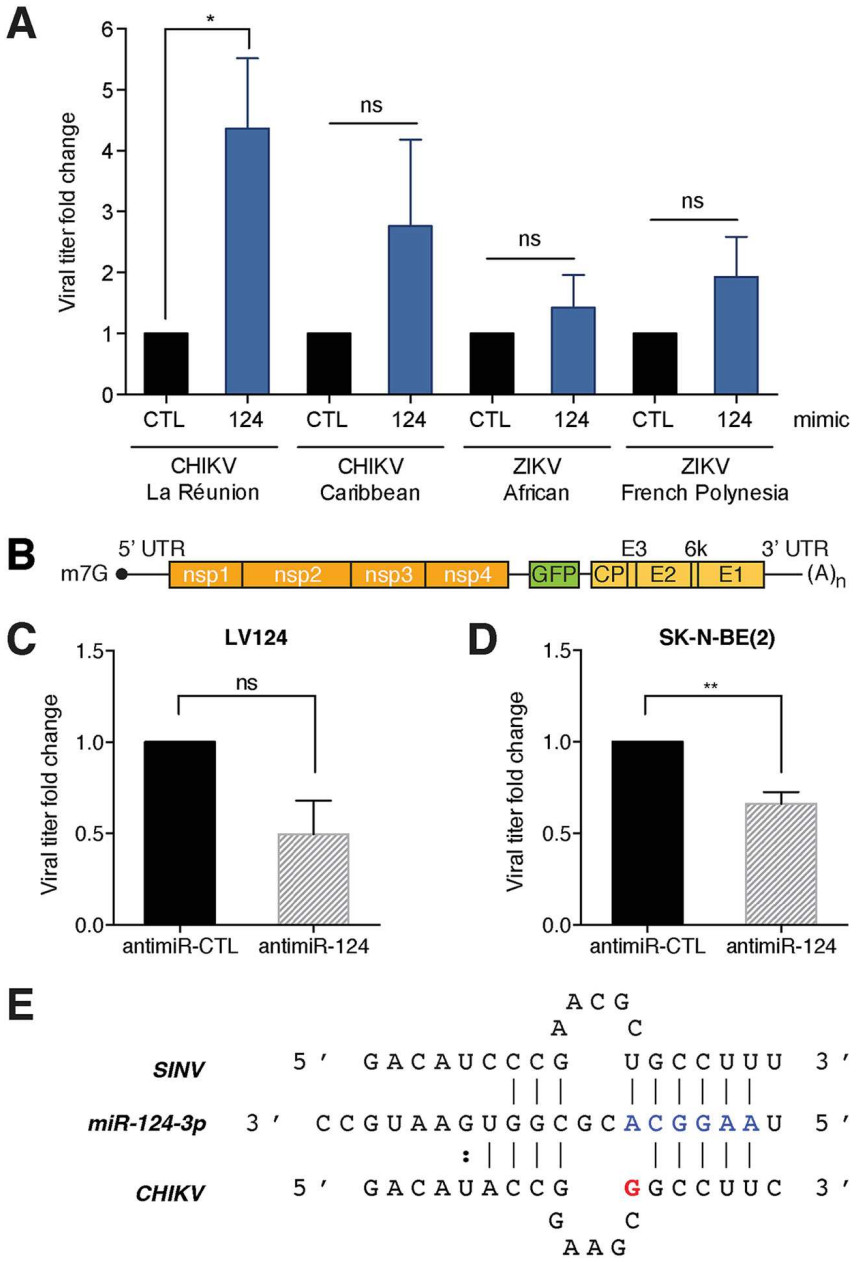


FIG 7 miR-124-3p positively regulates CHIKV infection. (A) Plaque assay quantification of viral titers, shown as relative fold change, produced by CHIKV La Réunion strain, CHIKV Caribbean strain, ZIKV African strain, and ZIKV French Polynesia strain after transfection of mimic-124-3p (blue) in Huh7.5.1 cells compared to that after transfection of control mimic (black). (B) Schematic representation of CHIKV-GFP genomic structure. Plaque assay quantification of viral titer production of CHIKV-GFP in LV124 cells (C) and 6-days post-RA differentiation in SK-N-BE(2) cells (D), shown as relative fold change of antimiR-124 compared to that under the control condition. Data are presented as means ± SEMs from three independent experiments. *, $P < 0.05$; **, $P < 0.01$, for two-way ANOVA with Bonferroni's correction (A) or unpaired Student's *t* test (C and D). (E) Sequence alignment of miR-124 binding site on SINV and CHIKV RNAs. The miRNA seed is indicated in blue, and the unpaired nucleotide in the seed match for miR-124 in the CHIKV genome is shown in red.

RNAs, with a positive or negative outcome for the virus. Given the cell specificity of certain miRNAs (41), the resulting interactions with viral RNAs can participate in determining the tissue tropism of viral pathogens. For instance, stable expression of the liver-specific miR-122 increases HCV replication in nonhepatic cells (42). Another example is the inhibition of the Eastern equine encephalitis virus (EEEV) cycle by miR-142-3p, which binds to the 3′ UTR of the virus in hematopoietic/myeloid cells (17).

SINV belongs to the *Alphavirus* genus, which includes viruses already emerged or with the potential to emerge as important human pathogens (8). Interestingly, as the alphavirus genome mimics the host mRNA and its replication takes place in the cytoplasm, the incoming viral RNA has the potential to directly interact with cellular miRNAs. In agreement, binding sites for cellular microRNAs were identified by Argonaute crosslinking immunoprecipitation (AGO-CLIP) within the alphavirus CHIKV, SINV, and Venezuelan equine encephalitis virus (VEEV) genomes (15).

In this study, we took advantage of a functional genome-wide screen approach (43, 44) to identify pro- and antiviral miRNA activity on SINV. The gain-of-function approach identified 16 miRNAs whose overexpression had a positive effect on the virus. Since we used GFP expressed from the virus as a proxy for measuring viral accumulation, it might be that we have enriched for miRNA regulating viral gene expression rather than just virus replication, but this allowed us to efficiently screen a large number of candidates. Surprisingly, we could not identify any miRNA mimic with an antiviral effect, which might reflect the fact that many viruses are refractory to inhibition by cellular miRNAs, as assessed by the depletion of Dicer (45). In contrast, our loss-of-function screen based on the use of antimicroRNAs was not suitable to identify any phenotypic effect. This could reflect the fact that Huh7.5.1 cells lack miRNAs that could play a role in SINV infection; hence, no miRNAs naturally expressed in those cells can have a pro- or antiviral effect on SINV. Among the hits, we uncovered hsa-miR-124-3p as a novel positive regulator of SINV, and we demonstrated that its overexpression increases SINV structural protein synthesis and viral production while mutations in its seed sequence abolish this effect. We identified and validated a binding site for hsa-miR-124-3p in the SINV genome within positions 10904 and 10926. We focused our efforts on this site for two reasons: first, it is conserved in several strains of SINV found in nature, and second, it lies in a position which corresponds at the same time to the 3′ UTR of the viral genomic RNA and to ORF2, which is only translated when expressed from the subgenomic RNA. Our results indicate that miR-124-3p binding more likely occurs on the viral subgenomic RNA, since it affects viral structural protein translation only.

As SINV may have a neuronal tropism (46), the binding of the cellular miRNA to the viral genome could provide an evolutionary advantage and could be considered a novel example of coevolution between the virus and its host. Indeed, we were able to show that miR-124 inhibitors reduced SINV infection in differentiated human neuroblastoma cells expressing miR-124-3p endogenously. The importance of this miRNA in alphavirus regulation is reinforced by our results obtained with CHIKV, which is also regulated by miR-124. The binding site that we validated in SINV is partially conserved in the CHIKV genome, with one mutation in the seed that could be compensated by extended pairing in the 3′ end of the miRNA.

While we were able to validate that miR-124 appears to regulate SINV by direct binding to the viral RNA, we do not formally exclude that the regulation of cellular RNAs by miR-124 could also participate in the process for both SINV and CHIKV. It has indeed been reported that cellular miRNAs can also regulate viral infections by targeting host mRNAs positively or negatively involved in the host response (34). In particular, this has already been observed for miR-124 regulation of measles virus (47), Japanese encephalitis virus (JEV) (48), and human immunodeficiency virus (HIV) (49) infections. In addition, miR-124 expression is modulated by different viruses, including ZIKV (50), enterovirus 71 (EV71) (51), human cytomegalovirus (HCMV) (52), and influenza H1N1 (53), suggesting a possible implication of the miRNA in the regulation of these infections as well.

CHIKV and SINV are classified as arthritogenic alphaviruses (5) due to the natural symptoms of the infection, which include arthritis and bone pathology (54). This suggests that prior to reaching the CNS, productive infection of cell types other than neurons must take place. Interestingly, in addition to its involvement in the CNS, miR-124 has more recently emerged as a critical modulator of immunity and inflammation (55) by preventing microglia activation (56) or by regulating the adaptive immune response through STAT3 regulation (57). Furthermore, it has also been linked

to bone pathology by playing a role in the regulation of osteoclast differentiation (58, 59). Thus, we cannot exclude a role of miR-124–SINV interaction in these cell types. It would be of interest to study miR-124 expression during alphavirus infection in synovial tissues and cartilage, the latter being more physiologically relevant cell types according to these viruses' tropism. Indeed, this could give more insight into a possible advantage of miR-124–SINV interaction in establishing alphavirus-induced arthritis rather than encephalitis.

We showed that miR-124 is involved in positively regulating viral mRNA translation and viral production, possibly by increasing the amount of structural proteins needed for encapsidation of the viral genome. However, further work will be needed to determine the underlying molecular mechanism. Although the usual outcome of miRNA-mediated regulation is negative, other examples of positive regulation upon miRNA binding have been reported, as it is the case for Bovine viral diarrhea virus and miR-17 (15) or HCV and miR-122 (60). In the former case, miR-17 also binds at the 3′ extremity of the viral genome, similar to our observation with miR-124 and SINV RNA. Nonetheless, our results indicate that miRNAs such as miR-124 could be a potential target for the development of therapeutic drugs to treat diseases associated with alphaviruses. Finally, given the extensive usage of alphaviruses as a vehicle for vaccine and gene therapy delivery (61), the identification of a positive regulation by miR-124 may have a strong impact on the development of more powerful biotechnologies based on the SINV genome.

MATERIALS AND METHODS

Viral stocks, cell culture, and virus infection. Plasmids carrying a green fluorescent protein (GFP)-SINV genomic sequence or a green fluorescent protein (GFP)-CHIKV La Réunion genomic sequence (kindly provided by Carla Saleh, Institut Pasteur, Paris, France) were linearized with XhoI or NotI, respectively, as in reference 62. They were used as a substrate for *in vitro* transcription using an mMESSAGE mMACHINE capped RNA transcription kit (Ambion, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. GFP expression is driven by duplication of the subgenomic promoter. SINV-GFP and CHIKV-GFP viral stocks were prepared in BHK21 baby hamster kidney cells, and titers were measured by plaque assay. The CHIKV strains used were La Réunion, 06-049 AM258994, and Caribbean (63). The ZIKV strains used were the African HD78788 and French Polynesia, PF-13.

The SINV-GFP-mut plasmid was generated by site-directed mutagenesis using forward (5′-CGCATT TATCAGGACATCAGATGCACCACTGGTCTCA-3′) and reverse (5′-ATGTCCTGATAAATGCGGCGTTCGGGATGT CAATAGA-3′) primers and the In-Fusion HD Cloning kit (TaKaRa) according to the manufacturer's instructions.

Cells were infected with all viruses at a multiplicity of infection (MOI) of 10^{-3}, and samples were harvested at 24 h postinfection (hpi) unless specified otherwise.

Huh7.5.1 cells were maintained in Dulbecco's modified eagle medium (DMEM) with 4.5 g/liter glucose (Gibco, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS; TaKaRa), 1% MEM nonessential amino acids solution (NEAA 100×; Gibco, Thermo Fisher Scientific Inc.), and gentamicin (50 μg/ml; Gibco, Thermo Fisher Scientific Inc.) in a humidified atmosphere of 5% CO_2 at 37°C. HEK293A (QBiogene) and Vero R (88020401; Sigma-Aldrich) cells were maintained in DMEM (Gibco, Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Clontech) in a humidified atmosphere of 5% CO_2 at 37°C.

SK-N-BE(2) cells (95011815; Sigma-Aldrich) were maintained in 1:1 medium composed of Ham's F12 medium (Gibco, Thermo Fisher Scientific Inc.) supplemented with 15% FBS and Eagle's minimal essential medium (ENEM) supplemented with 1% NEAA (Gibco, Thermo Fisher Scientific Inc.). For miR-124-3p inhibition experiments in SK-N-BE(2) cells, due to the low transfection efficiency of these cells once differentiated, they were first transfected with 75 nM antimiR specific to miR-124-3p or antimiR-CTL, and 6 h later, differentiation was induced by 10 μM RA treatment (R2625; Sigma-Aldrich).

Lentivirus production and generation of stable cell line. The human pre-miR-124a-1 gene in locus 8p23.1 flanked by about 200 nucleotides of its upstream and downstream genomic sequences was PCR amplified using the following primers: hsa-pri-miR-124 Fw, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT TCGAGCTGCGGCGGGGAGGATGC-3′; hsa-pri-miR-124 Rv, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCC CCCTGTCTGTCACAGGCTGC-3′.

The obtained insert was first cloned by the Gateway BP reaction (Invitrogen, Thermo Fisher Scientific Inc.) in pDONOR-221 and then by the Gateway LR reaction (Invitrogen, Thermo Fisher Scientific Inc.) in the lentiviral destination vector pLenti6.2-3×FLAG-V5-ccdB (87072; Addgene). Lentiviruses were produced by cotransfection of pLenti6.2-3×FLAG-V5-hsa-pri-miR-124 with packaging vectors encoding lentiviral Gag and Pol proteins, pPAX, and vesicular stomatitis virus (VSV) envelope glycoprotein pVSV-G. Twenty-four hours posttransfection, the supernatant was collected and used as the inoculum for transduction. Huh7.5.1 cells were transduced with lentiviruses in the presence of 4 μg/ml Polybrene (SC-134220; Santa Cruz Biotechnology) for 6 h. Afterwards, the inoculum was removed, and cells were incubated in complete medium. Selection pressure was applied by supplementing the complete medium

with 15 μg/ml of blasticidin (Invivogen). Surviving cells were maintained in culture in the presence of blasticidin as a polyclonal miR-124-3p-expressing cell line (LV124).

High-content miRNA-based phenotypic screening. For the miRNA screen, the miRIDIAN microRNA mimic (catalog number CS-001030) and inhibitor (catalog number IH-001030) libraries (19.0, human microRNAs) were purchased from Dharmacon (Thermo Fisher Scientific Inc.). For the mimic screen, 20 nM each mimic microRNA was transfected into Huh7.5.1 cells (cultured in DMEM with 4.5 g/liter glucose, 10% HyClone fetal calf serum [FCS], 50 μg/ml gentamicin) grown in Greiner μClear 96-well microplates using a high-throughput (HT) reverse chemical transfection with the INTERFERin HTS delivery reagent (Polyplus-transfection SA, Illkirch, France). For the inhibitor screen, 75 nM each inhibitor microRNA was transfected into Huh7.5.1 cells as described above. The HT transfection protocol was optimized for reaching 90% to 95% transfection efficiency with minimal toxicity on a TECAN Freedom EVO liquid handling workstation. The screens were performed in technical triplicates. To limit biological variability, the cell passage ($n = 3$ after thawing), serum batch, and transfection reagent batch were strictly determined. Internal controls such as positive (siRNA targeting SINV 3′ UTR at nt 12345 and nt 12437: 5′-AACUCGAUGUACUUCCGAGGAUU-3′; Integrated DNA Technologies) and negative siRNA controls (ON-TARGETplus nontargeting siRNA number [no.] 2; Horizon Discovery, Dharmacon), and transfection efficiency control ("PLK1" siRNA that leads to cell death) were added to each microplate to determine parameters for interplate and day-to-day variability. Three days posttransfection, the cells were subjected to SINV-GFP viral infection for 24 h before fixation and staining with Hoechst 3342 (labeling nuclear compartments [nuclei]). High-throughput cell imaging was carried out with the INCELL1000 HCS epifluorescence microscope to collect an average of ~6,000 cells analyzed per microwell (Hoechst 3342 and GFP channels).

Analysis of the high-content siRNA screening data. Hoechst 3342 and GFP signals were extracted for all individual cells using the Multi Target Analysis module of the INCELL1000 platform. These parameters describe the GFP^- noninfected and GFP^+ SINV-infected Huh7.5.1 cell populations for each miRNA treatment. The robust strictly standardized mean difference (SSMD* or SSMDr) value for each well was calculated as described below according to reference 64.

$$\mathrm{SSMDr} = \mathrm{median}(\mathrm{hit}) - \mathrm{median}(\mathrm{plate}) * 1/(\sqrt{2} * \mathrm{MAD}),$$

where MAD is the mean absolute deviation.

Small RNA library preparation, sequencing, and analysis. RNA was extracted from Huh7.5.1 cells, and a small RNA library was prepared from 25 μg of total RNA as previously described (62, 65, 66). Single-end sequencing was performed at the IGBMC Microarray and Sequencing platform, Illkirch, France, on an Illumina Genome Analyzer IIx machine with a read length of 36 bp. Deep-sequencing data analysis was performed as in reference 62 with slight modifications. Briefly, FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) was first applied to remove instances of the 3′ adaptor. Remaining reads between 18 and 32 nt in length were then mapped to the human genome (assembly version hg19; UCSC repository) using Bowtie 1.0.0 (67). Up to 2 mismatches in total with no more than 1 mismatch in the first 15 nucleotides of each read were permitted. In addition, only alignments from the lowest mismatch stratum were recorded, and reads that could map to more than 50 loci were discarded. Finally, expressed human miRNAs (miRBase Release 20 [68]) were identified and quantified using BEDTools 2.16.2 (69) by comparing their genomic coordinates to those of the aligned reads. During the quantification process, multiple mapped reads were weighted by the number of mapping sites in other miRNAs, and the final counts were normalized per million miRNA reads (RPM).

microRNA mimic and siRNA transfection. For reverse transfection in Huh7.5.1 or HEK293A cells, transfection complexes were prepared using mimics (miRIDIAN microRNA mimics; Dharmacon) or siRNAs (Dharmacon or Integrated DNA Technologies) at a final concentration of 30 nM unless specified differently in INTERFERin-HTS transfection reagent (Polyplus-transfection SA, Illkirch, France) and transfection medium according to the manufacturer's instructions. Transfection complexes were added to 1.5×10^4 cells in each well of 48-well cell culture plates. Transfected cells were subsequently incubated for 72 h before being infected with SINV-GFP at an MOI of 10^{-3} for 24 h. For the experiment with the mutant mimic of miR-124-3p, mimics for *Caenorhabditis elegans elegans* miR-67 (5′-UCACAACCUCCUAGAAAGA GdTdT-3′), miR-124-3p (5′-UAAGGCACGCGGUGAAUGCCdTdT-3′), and miR-124-3p mutated in the seed region (5′-UAACCGACGCGGUGAAUGCCdTdT-3′) were purchased from Integrated DNA Technologies.

miRNA inhibition with 2′*O*-methylated oligonucleotides. For inhibition of miRNAs with antimiRs, 1.5×10^4 LV124 cells were cultured in 48-well dishes, and SK-N-BE(2) cells were cultured in 6-well plates at a confluence of 2×10^4 cells/cm^2. Cells were transfected with the 2′*O*-methylated antimiRs against the endogenously expressed miR-124-3p (5′-GGCAUUCACCGCGUGCCUUA-3′) or with the control sequence of *C. elegans* cel-miR-67 (5′-UCACAACCUCCUAGAAAGAGUAGA-3′), using INTERFERin-HTS transfection reagent (Polyplus-transfection SA, Illkirch, France) or Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific Inc.). AntimiRs were used at a final concentration of 75 nM and transfections were performed according to the manufacturer's instructions. Forty-eight hours posttransfection cells were infected with SINV-GFP at an MOI of 10^{-3} for 24 h.

Standard plaque assay. Vero R cells seeded either in 96- or 24-well plates were infected with infection supernatants prepared in cascade 10-fold dilutions for 1 h. Afterwards, the inoculum was removed, and cells were cultured in 2.5% carboxymethyl cellulose for 72 h at 37°C in a humidified atmosphere of 5% CO_2. Plaques were counted manually under the microscope. For plaque visualization, the medium was removed, and cells were fixed with 4% formaldehyde for 20 min and stained with 1× crystal violet solution (2% crystal violet [Sigma-Aldrich], 20% ethanol, 4% formaldehyde).

Virus attachment and entry test. Huh7.5.1 cells were reverse transfected in 48-well plates with control or miR-124 mimic at a final concentration of 30 nM with Lipofectamine 2000 according to the manufacturer's instructions and incubated for 72 h. Prechilled cells (15 min at 4°C) were incubated with SINV-GFP at an MOI of 5 at 4°C for 30 min (attachment assay). Unbound SINV virions were removed by washing with phosphate-buffered saline (PBS) three times. Total RNA was extracted and used for quantification of SINV RNA by qRT-PCR. SINV entry test was carried out by incubating SINV with cells at 4°C for 30 min to allow viral binding, and then unbound SINV virions were washed rapidly three times with PBS. Finally, cells were shifted to 37°C for 30 min to allow virus internalization, as described previously (70).

Western blotting. Proteins were extracted by collecting cell lysates in RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitor). Lysates were cleared by centrifugation at 13,000 rpm for 30 min at 4°C to remove cell debris, and the supernatant was retained for Western blotting. Samples were loaded in a 10% acrylamide–bis-acrylamide gel, and proteins were separated by migration at 100 V in 1× Tris-glycine-SDS buffer. Proteins were transferred to a nitrocellulose membrane by wet transfer in 1× Tris-glycine and 20% ethanol buffer. Viral proteins were detected using primary polyclonal antibodies against SINV capsid protein (CP) and nsP2 (kind gift from Diane Griffin, Johns Hopkins University School of Medicine, Baltimore, MD) and a secondary antibody anti-rabbit-horseradish peroxidase (HRP) (NA9340; GE Healthcare, Thermo Fisher Scientific Inc.). The signal was revealed by incubating the membrane for 10 min with SuperSignal West Femto maximum sensitivity Substrate (Pierce, Thermo Fisher Scientific Inc.). Tubulin was detected with a primary monoclonal antibody (T6557; Sigma-Aldrich) and a secondary antibody anti-mouse-HRP (NXA931; GE Healthcare, Thermo Fisher Scientific Inc.).

Northern blotting. Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Five to ten micrograms of total RNA was loaded on a 17.5% acrylamide-urea 4 M gel and resolved by running in 1× Tris-borate-EDTA for isolation of small RNAs. Nucleic acids were transferred to a nylon membrane by semidry transfer. Small RNAs were cross-linked to the membrane by chemical cross-link using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich). Membrane was prehybridized for 20 min with PerfectHyb Plus hybridization buffer (Sigma-Aldrich). DNA oligonucleotides directed against hsa-miR-124-3p (5′-GGCATTCACCGCGTGCCTTA-3′) and snRNA U6 (5′-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3′) were radiolabeled with 2.5 μCi of γ-ATP by polynucleotide kinase (PNK). After removal of unbound γ-ATP by MicroSpin G-25 column (GE Healthcare, Thermo Fisher Scientific Inc.) purification, the probe was incubated with the membrane in hybridization buffer overnight at 50°C. Membranes were washed twice with SSC 4× solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C and exposed on an image plate in a cassette. Imaging of the signal was obtained with Typhoon FLA 7000 laser scanner (GE Healthcare Life Sciences).

RT-qPCR. For *MYCN* expression, DNase I (Invitrogen, Thermo Fisher Scientific Inc.) treatment was performed on 1 μg of RNA, which was retrotranscribed using a random nonameric primer and SuperScript IV reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. For miR-124-3p, SINV genomic and subgenomic RNA, and CDK4 expression, 250 ng of RNA was retrotranscribed using miScript II (Qiagen) according to the manufacturer's instructions. Quantitative PCR was performed on a 1/10 dilution of cDNA using SYBR green PCR master mix (Thermo Fisher Scientific Inc.) with the following primers: *MYCN* forward (5′-GAGCGATTCAGATGATGAAG-3′) and reverse (5′-TCGTTTGAGGATCAGCTC-3′) (40); SINVgenome forward (5′-CCACTACGCAAGCAGAGACG-3′) and reverse (5′-AGTGCCCAGGGCCTGTGTCCG-3′); SINVsubgenome forward (5′-CCACAGATACCGTATAAGGCA-3′) and reverse (5′-TGCAGGTAATGTACTCTTGG-3′); CDK4 forward (5′-CCGTGGTTGTTACACTCTGG-3′) and reverse (5′-ATTTTGCCCAACTGGTCGGC-3′); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward (5′-CTTTGGTATCGTGGAAGGACT-3′) and reverse (5′-CCAGTGAGCTTCCCGTTCAG-3′). For the miScript primer assay, mature hsa-miR-124-3p (MIMAT0000422; 5′-UAAGGCACGCGGUGAAUGCC-3′) or MS00033740 hs_RNU6-2_11 was amplified with a reverse 10× miScript universal primer (Qiagen).

Strand-specific RT-PCR. Infected cells were lysed with TRIzol, cellular RNA was extracted according to the manufacturer's instructions, and RNA pellets were resuspended in water. To detect the levels of SINV antigenomic RNA, negative-strand-specific reverse transcription was performed with a plus-sense primer annealing to the 5′ region of the SINV genome (nucleotides 1 to 42) (5′-ATTGACGGCGTAGTAC ACACTATTGAATCAAACAGCCGACCA-3′). The RT reaction mix was set up with 100 ng of RNA, 1 μl of 2 μM specific primer, 1 μl 10 mM deoxynucleoside triphosphates (dNTPs), and water to reach a final volume of 13 μl. Samples were mixed, incubated at 65°C for 5 min, and placed 2 min on ice. Reaction mix was completed with 4 μl 5× Superscript IV buffer (Invitrogen), 1 μl 0.1 M dithiothreitol (DTT), 1 μl RNase inhibitor, and either 1 μl Superscript IV enzyme (Invitrogen) or water for a negative control. Samples were mixed and incubated at 50°C for 10 min and 80°C for 10 min. One microliter of cDNA products was then amplified by PCR with specific antigenome forward (5′-CATTCTACGAGCCGGTGCGC-3′) and reverse (5′-TAGACGTAGACCCCCAGAGTC-3′) primers using the GoTaq DNA polymerase (Promega). PCR was run as follows: 94°C for 2 min, 15, 20, 25, or 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 5 min at 72°C. PCR products were loaded on a 1.5% agarose gel for analysis.

Data availability. The sequencing data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (71) and are accessible through GEO Series accession number GSE136740.

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S.P. and E.G. conceived the project. S.P., E.G., and P.L. designed the work and analyzed the results. P.L., E.G., A.W., B.C.M., P.K., and M.M. performed the experiments. E.G. and A.W. set up the high-throughput screen, A.W. performed the high-throughput screen and data acquisition, and A.K. performed the bioinformatic analysis of the screen. B.C.-W.-M. generated the screen dot plot and table. P.L. and B.C.-W.-M. analyzed the microscopy images with CellProfiler software of selected candidates. P.K. generated the Huh7.5.1 small RNA libraries, and B.C.-W.-M. performed the bioinformatic analysis. A.F. participated to the initial phase of the study for the candidate validation, and P.L. and E.G. validated the mimic candidates. P.L. established the miR-124 stable cell line, generated and characterized the SINV-GFP mutant virus, and produced CHIKV-GFP virus. P.L. and E.G. performed the miR inhibition assay with SINV-GFP and CHIKV-GFP in LV124 cells. B.C.M. performed the ZIKV and CHIKV infections in mimic-transfected Huh7.5.1 cells. E.G., P.L., B.C.-W.-M., and D.B.-B. identified miR-124 binding site. E.G. set up the SK-NB-E(2) differentiation experiments. P.L. and E.G. performed and analyzed SINV-GFP and CHIKV-GFP infections in differentiated SK-NB-E(2) cells, and M.M. contributed to the time course experiments and Western blot analysis. M.V. coordinated the work on ZIKV and CHIKV. L.B. coordinated the high-throughput screen and its bioinformatic analysis. P.L. and E.G. drafted the manuscript and designed the figures. P.L., E.G., and S.P. wrote the manuscript with input from the other authors. S.P. and E.G. coordinated the work. S.P. ensured funding. All authors reviewed the final manuscript.

REFERENCES

1. Griffin D. 2007. Alphaviruses, p 1023–1067. Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. Atkins GJ. 2013. The pathogenesis of alphaviruses. ISRN Virol 2013: 861912. https://doi.org/10.5402/2013/861912.
3. Adouchief S, Smura T, Sane J, Vapalahti O, Kurkela S. 2016. Sindbis virus as a human pathogen-epidemiology, clinical picture and pathogenesis. Rev Med Virol 26:221–241. https://doi.org/10.1002/rmv.1876.
4. Vernon PS, Griffin DE. 2005. Characterization of an *in vitro* model of alphavirus infection of immature and mature neurons. J Virol 79: 3438–3447. https://doi.org/10.1128/JVI.79.6.3438-3447.2005.
5. Suhrbier A, Jaffar-Bandjee M-C, Gasque P. 2012. Arthritogenic alphaviruses—an overview. Nat Rev Rheumatol 8:420–429. https://doi.org/10.1038/nrrheum.2012.64.
6. Das T, Jaffar-Bandjee MC, Hoarau JJ, Krejbich Trotot P, Denizot M, Lee-Pat-Yuen G, Sahoo R, Guiraud P, Ramful D, Robin S, Alessandri JL, Gauzere BA, Gasque P. 2010. Chikungunya fever: CNS infection and pathologies of a re-emerging arbovirus. Prog Neurobiol 91:121–129. https://doi.org/10.1016/j.pneurobio.2009.12.006.
7. Burt FJ, Chen W, Miner JJ, Lenschow DJ, Merits A, Schnettler E, Kohl A, Rudd PA, Taylor A, Herrero LJ, Zaid A, Ng LFP, Mahalingam S. 2017. Chikungunya virus: an update on the biology and pathogenesis of this emerging pathogen. Lancet Infect Dis 17:e107–e117. https://doi.org/10.1016/S1473-3099(16)30385-1.
8. Levi LI, Vignuzzi M. 2019. Arthritogenic alphaviruses: a worldwide emerging threat? Microorganisms 7:133. https://doi.org/10.3390/microorganisms7050133.
9. Bartel DP. 2018. Metazoan microRNAs. Cell 173:20–51. https://doi.org/10.1016/j.cell.2018.03.006.
10. Guo X.-k, Zhang Q, Gao L, Li N, Chen X.-x, Feng W.-h. 2013. Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol 87:1159–1171. https://doi.org/10.1128/JVI.02386-12.
11. Ingle H, Kumar S, Raut AA, Mishra A, Kulkarni DD, Kameyama T, Takaoka A, Akira S, Kumar H. 2015. The microRNA miR-485 targets host and influenza virus transcripts to regulate antiviral immunity and restrict viral replication. Sci Signal 8:ra126. https://doi.org/10.1126/scisignal.aab3183.
12. Li L, Gao F, Jiang Y, Yu L, Zhou Y, Zheng H, Tong W, Yang S, Xia T, Qu Z, Tong G. 2015. Cellular miR-130b inhibits replication of porcine reproductive and respiratory syndrome virus *in vitro* and *in vivo*. Sci Rep 5:17010. https://doi.org/10.1038/srep17010.
13. Otsuka M, Jing Q, Georgel P, New L, Chen J, Mols J, Kang YJ, Jiang Z, Du X, Cook R, Das SC, Pattnaik AK, Beutler B, Han J. 2007. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity 27:123–134. https://doi.org/10.1016/j.immuni.2007.05.014.
14. Peng S, Wang J, Wei S, Li C, Zhou K, Hu J, Ye X, Yan J, Liu W, Gao GF, Fang M, Meng S. 2018. Endogenous cellular microRNAs mediate antiviral defense against influenza A virus. Mol Ther Nucleic Acids 10:361–375. https://doi.org/10.1016/j.omtn.2017.12.016.
15. Scheel TKH, Luna JM, Liniger M, Nishiuchi E, Rozen-Gagnon K, Shlomai A, Auray G, Gerber M, Fak J, Keller I, Bruggmann R, Darnell RB, Ruggli N, Rice CM. 2016. A broad RNA virus survey reveals both miRNA depen-

dence and functional sequestration. Cell Host Microbe 19:409–423. https://doi.org/10.1016/j.chom.2016.02.007.
16. Song L, Liu H, Gao S, Jiang W, Huang W. 2010. Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells. J Virol 84:8849–8860. https://doi.org/10.1128/JVI.00456-10.
17. Trobaugh DW, Gardner CL, Sun C, Haddow AD, Wang E, Chapnik E, Mildner A, Weaver SC, Ryman KD, Klimstra WB. 2014. RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature 506:245–248. https://doi.org/10.1038/nature12869.
18. Lee C-T, Risom T, Strauss WM. 2007. Evolutionary conservation of microRNA regulatory circuits: an examination of microRNA gene complexity and conserved microRNA-target interactions through metazoan phylogeny. DNA Cell Biol 26:209–218. https://doi.org/10.1089/dna.2006.0545.
19. Jonas S, Izaurralde E. 2015. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet 16:421–433. https://doi.org/10.1038/nrg3965.
20. Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136:215–233. https://doi.org/10.1016/j.cell.2009.01.002.
21. Broughton JP, Lovci MT, Huang JL, Yeo GW, Pasquinelli AE. 2016. Pairing beyond the seed supports microRNA targeting specificity. Mol Cell 64:320–333. https://doi.org/10.1016/j.molcel.2016.09.004.
22. Grimson A, Farh KK-H, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27:91–105. https://doi.org/10.1016/j.molcel.2007.06.017.
23. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science 309:1577–1581. https://doi.org/10.1126/science.1113329.
24. Li Y, Yamane D, Lemon SM. 2015. Dissecting the roles of the 5′ exoribonucleases Xrn1 and Xrn2 in restricting hepatitis C virus replication. J Virol 89:4857–4865. https://doi.org/10.1128/JVI.03692-14.
25. Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, Barton DJ, Lemon SM. 2012. Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. Proc Natl Acad Sci U S A 109:941–946. https://doi.org/10.1073/pnas.1112263109.
26. van der Ree MH, de Vree JM, Stelma F, Willemse S, van der Valk M, Rietdijk S, Molenkamp R, Schinkel J, van Nuenen AC, Beuers U, Hadi S, Harbers M, van der Veer E, Liu K, Grundy J, Patick AK, Pavlicek A, Blem J, Huang M, Grint P, Neben S, Gibson NW, Kootstra NA, Reesink HW. 2017. Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. Lancet 389:709–717. https://doi.org/10.1016/S0140-6736(16)31715-9.
27. Sun AX, Crabtree GR, Yoo AS. 2013. MicroRNAs: regulators of neuronal fate. Curr Opin Cell Biol 25:215–221. https://doi.org/10.1016/j.ceb.2012.12.007.
28. Cheng L-C, Pastrana E, Tavazoie M, Doetsch F. 2009. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat Neurosci 12:399–408. https://doi.org/10.1038/nn.2294.
29. Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-Messer C, Dolmetsch RE, Tsien RW, Crabtree GR. 2011. MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476:228–231. https://doi.org/10.1038/nature10323.
30. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. Curr Biol 12:735–739. https://doi.org/10.1016/s0960-9822(02)00809-6.
31. Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Šestan N, Rakic P, Constantine-Paton M, Horvitz HR. 2004. Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol 5:R68. https://doi.org/10.1186/gb-2004-5-9-r68.
32. Deng X, Ma L, Wu M, Zhang G, Jin C, Guo Y, Liu R. 2013. miR-124 radiosensitizes human glioma cells by targeting CDK4. J Neurooncol 114:263–274. https://doi.org/10.1007/s11060-013-1179-2.
33. Zhao Z, Ma X, Hsiao T-H, Lin G, Kosti A, Yu X, Suresh U, Chen Y, Tomlinson GE, Pertsemlidis A, Du L. 2014. A high-content morphological screen identifies novel microRNAs that regulate neuroblastoma cell differentiation. Oncotarget 5:2499–25112. https://doi.org/10.18632/oncotarget.1703.
34. Girardi E, López P, Pfeffer S. 2018. On the importance of host microRNAs during viral infection. Front Genet 9:439. https://doi.org/10.3389/fgene.2018.00439.
35. Hsu P-C, Lin L-Z, Hsu S-D, Hsu J-K, Huang H-D. 2007. ViTa: prediction of host microRNAs targets on viruses. Nucleic Acids Res 35:D381–D385. https://doi.org/10.1093/nar/gkl1009.
36. Hatcher EL, Zhdanov SA, Bao Y, Blinkova O, Nawrocki EP, Ostapchuck Y, Schäffer AA, Brister JR. 2017. Virus variation resource – improved response to emergent viral outbreaks. Nucleic Acids Res 45:D482–D490. https://doi.org/10.1093/nar/gkw1065.
37. Laneve P, Di Marcotullio L, Gioia U, Fiori ME, Ferretti E, Gulino A, Bozzoni I, Caffarelli E. 2007. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. Proc Natl Acad Sci U S A 104:7957–7962. https://doi.org/10.1073/pnas.0700071104.
38. Meister G, Landthaler M, Dorsett Y, Tuschl T. 2004. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA 10:544–550. https://doi.org/10.1261/rna.5235104.
39. Thiele CJ, Reynolds CP, Israel MA. 1985. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313:404–406. https://doi.org/10.1038/313404a0.
40. Leotta CG, Federico C, Brundo MV, Tosi S, Saccone S. 2014. HLXB9 gene expression, and nuclear location during *in vitro* neuronal differentiation in the SK-N-BE neuroblastoma cell line. PLoS One 9:e105481. https://doi.org/10.1371/journal.pone.0105481.
41. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129:1401–1414. https://doi.org/10.1016/j.cell.2007.04.040.
42. Da Costa D, Turek M, Felmlee DJ, Girardi E, Pfeffer S, Long G, Bartenschlager R, Zeisel MB, Baumert TF. 2012. Reconstitution of the entire hepatitis C virus life cycle in nonhepatic cells. J Virol 86:11919–11925. https://doi.org/10.1128/JVI.01066-12.
43. Santhakumar D, Forster T, Laqtom NN, Fragkoudis R, Dickinson P, Abreu-Goodger C, Manakov SA, Choudhury NR, Griffiths SJ, Vermeulen A, Enright AJ, Dutia B, Kohl A, Ghazal P, Buck AH. 2010. Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. Proc Natl Acad Sci U S A 107:13830–13835. https://doi.org/10.1073/pnas.1008861107.
44. Smith JL, Jeng S, McWeeney SK, Hirsch AJ. 2017. A microRNA screen identifies the Wnt signaling pathway as a regulator of the interferon response during flavivirus infection. J Virol 91:e02388-16. https://doi.org/10.1128/JVI.02388-16.
45. Bogerd HP, Skalsky RL, Kennedy EM, Furuse Y, Whisnant AW, Flores O, Schultz KLW, Putnam N, Barrows NJ, Sherry B, Scholle F, Garcia-Blanco MA, Griffin DE, Cullen BR. 2014. Replication of many human viruses is refractory to inhibition by endogenous cellular microRNAs. J Virol 88:8065–8076. https://doi.org/10.1128/JVI.00985-14.
46. Jackson AC, Moench TR, Trapp BD, Griffin DE. 1988. Basis of neurovirulence in Sindbis virus encephalomyelitis of mice. Lab Invest 58:503–509.
47. Naaman H, Rall G, Matullo C, Veksler-Lublinsky I, Shemer-Avni Y, Gopas J. 2017. miRNA-124 is a link between measles virus persistent infection and cell division of human neuroblastoma cells. PLoS One 12:e0187077. https://doi.org/10.1371/journal.pone.0187077.
48. Yang S, Pei Y, Li X, Zhao S, Zhu M, Zhao A. 2016. miR-124 attenuates Japanese encephalitis virus replication by targeting DNM2. Virol J 13:105. https://doi.org/10.1186/s12985-016-0562-y.
49. Farberov L, Herzig E, Modai S, Isakov O, Hizi A, Shomron N. 2015. MicroRNA-mediated regulation of p21 and TASK1 cellular restriction factors enhances HIV-1 infection. J Cell Sci 128:1607–1616. https://doi.org/10.1242/jcs.167817.
50. Dang JW, Tiwari SK, Qin Y, Rana TM. 2019. Genome-wide integrative analysis of Zika-virus-infected neuronal stem cells reveals roles for microRNAs in cell cycle and stemness. Cell Rep 27:3618.e5–3628.e5. https://doi.org/10.1016/j.celrep.2019.05.059.
51. Bian L, Wang Y, Liu Q, Xia J, Long J-E. 2015. Prediction of signaling pathways involved in enterovirus 71 infection by algorithm analysis based on miRNA profiles and their target genes. Arch Virol 160:173–182. https://doi.org/10.1007/s00705-014-2249-2.
52. Fu M, Gao Y, Zhou Q, Zhang Q, Peng Y, Tian K, Wang J, Zheng X. 2014. Human cytomegalovirus latent infection alters the expression of cellular and viral microRNA. Gene 536:272–278. https://doi.org/10.1016/j.gene.2013.12.012.
53. Huang L, Ma J, Sun Y, Lv Y, Lin W, Liu M, Tu C, Zhou P, Gu W, Su S, Zhang

G. 2015. Altered splenic miRNA expression profile in H1N1 swine influenza. Arch Virol 160:979–985. https://doi.org/10.1007/s00705-015-2351-0.
54. Chen W, Foo S-S, Sims NA, Herrero LJ, Walsh NC, Mahalingam S. 2015. Arthritogenic alphaviruses: new insights into arthritis and bone pathology. Trends Microbiol 23:35–43. https://doi.org/10.1016/j.tim.2014.09.005.
55. Qin Z, Wang P-Y, Su D-F, Liu X. 2016. miRNA-124 in immune system and immune disorders. Front Immunol 7:406. https://doi.org/10.3389/fimmu.2016.00406.
56. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. 2011. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-α–PU.1 pathway. Nat Med 17:64–70. https://doi.org/10.1038/nm.2266.
57. Wei J, Wang F, Kong L-Y, Xu S, Doucette T, Ferguson SD, Yang Y, McEnery K, Jethwa K, Gjyshi O, Qiao W, Levine NB, Lang FF, Rao G, Fuller GN, Calin GA, Heimberger AB. 2013. miR-124 inhibits STAT3 signaling to enhance T cell-mediated immune clearance of glioma. Cancer Res 73:3913–3926. https://doi.org/10.1158/0008-5472.CAN-12-4318.
58. Lee Y, Kim HJ, Park CK, Kim Y-G, Lee H-J, Kim J-Y, Kim H-H. 2013. MicroRNA-124 regulates osteoclast differentiation. Bone 56:383–389. https://doi.org/10.1016/j.bone.2013.07.007.
59. Ohnuma K, Kasagi S, Uto K, Noguchi Y, Nakamachi Y, Saegusa J, Kawano S. 2019. MicroRNA-124 inhibits TNF-α- and IL-6-induced osteoclastogenesis. Rheumatol Int 39:689–695. https://doi.org/10.1007/s00296-018-4218-7.
60. Luna JM, Scheel TKH, Danino T, Shaw KS, Mele A, Fak JJ, Nishiuchi E, Takacs CN, Catanese MT, de Jong YP, Jacobson IM, Rice CM, Darnell RB. 2015. Hepatitis C virus RNA functionally sequesters miR-122. Cell 160:1099–1110. https://doi.org/10.1016/j.cell.2015.02.025.
61. Hewson R. 2000. RNA viruses: emerging vectors for vaccination and gene therapy. Mol Med Today 6:28–35. https://doi.org/10.1016/s1357-4310(99)01622-6.
62. Girardi E, Chane-Woon-Ming B, Messmer M, Kaukinen P, Pfeffer S. 2013. Identification of RNase L-dependent, 3′-end-modified, viral small RNAs in Sindbis virus-infected mammalian cells. mBio 4:e00698-13. https://doi.org/10.1128/mBio.00698-13.
63. Stapleford KA, Moratorio G, Henningsson R, Chen R, Matheus S, Enfissi A, Weissglas-Volkov D, Isakov O, Blanc H, Mounce BC, Dupont-Rouzeyrol M, Shomron N, Weaver S, Fontes M, Rousset D, Vignuzzi M. 2016. Whole-genome sequencing analysis from the chikungunya virus Caribbean outbreak reveals novel evolutionary genomic elements. PLoS Negl Trop Dis 10:e0004402. https://doi.org/10.1371/journal.pntd.0004402.
64. Zhang XD. 2011. Illustration of SSMD, z score, SSMD*, z* score, and t statistic for hit selection in RNAi high-throughput screens. J Biomol Screen 16:775–785. https://doi.org/10.1177/1087057111405851.
65. Pfeffer S. 2007. Identification of virally encoded microRNAs. Methods Enzymol 427:51–63. https://doi.org/10.1016/S0076-6879(07)27003-X.
66. Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grässer FA, van Dyk LF, Ho CK, Shuman S, Chien M, Russo JJ, Ju J, Randall G, Lindenbach BD, Rice CM, Simon V, Ho DD, Zavolan M, Tuschl T. 2005. Identification of microRNAs of the herpesvirus family. Nat Methods 2:269–276. https://doi.org/10.1038/nmeth746.
67. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25. https://doi.org/10.1186/gb-2009-10-3-r25.
68. Griffiths-Jones S. 2004. The microRNA registry. Nucleic Acids Res 32:D109–D111. https://doi.org/10.1093/nar/gkh023.
69. Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26:841–842. https://doi.org/10.1093/bioinformatics/btq033.
70. Niu J, Jiang Y, Xu H, Zhao C, Zhou G, Chen P, Cao R. 2018. TIM-1 promotes Japanese encephalitis virus entry and infection. Viruses 10:630. https://doi.org/10.3390/v10110630.
71. Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207–210. https://doi.org/10.1093/nar/30.1.207.
72. Taipale M, Tucker G, Peng J, Krykbaeva I, Lin Z-Y, Larsen B, Choi H, Berger B, Gingras A-C, Lindquist S. 2014. A quantitative chaperone interaction network reveals the architecture of cellular protein homeostasis pathways. Cell 158:434–448. https://doi.org/10.1016/j.cell.2014.05.039.

CHAPTER III. Study of the molecular mechanism of miR-124 -SINV regulation

As previously addressed in the introduction, miRNAs have to be loaded in Ago proteins in order to bind target RNAs and exert their function. For this reason, and to validate the interaction with the viral RNA, I used two techniques based on the isolation of Ago complexes loaded with miR-124. The objective was to verify whether SINV RNA could be found in complex with miR-124-loaded Ago. More importantly, these techniques would also serve for the direct identification of miR-124 other viral and/or cellular targets.

A. Isolation of Ago-miR-124 specific targets by TAP-Tar

Among the possible approaches described in the literature, an option to identify miRNA-specific mRNA targets is to use biotinylated mimics. This technique known as TAP-Tar (for Tandem Affinity Purification of miRNA Target mRNAs) relies on the immunoprecipitation of Ago coupled with a Biotin-Streptavidin pulldown to enrich a given miRNA specific targets by sequencing (Figure 10) (Orom and Lund, 2007; Nonne et al., 2010).

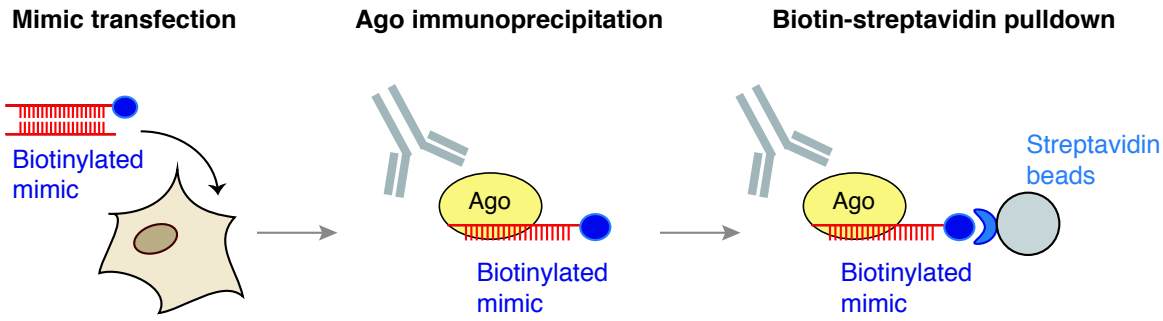

Figure IV.10. Schematic representation of the TAP-Tar approach.
The technique relies on the transfection of biotinylated mimics to the cells, followed by Ago immunoprecipitation and coupled to a biotin-streptavidin pulldown.

I sought to establish this technique in the laboratory and determine whether we could identify miR-124 targets by this approach. I used HEK293T cells that were transfected with classical mimics, a control (CTL) or a miR-124 mimic (124), or with biotinylated control (CTLB) or biotinylated miR-124 mimics (124B). Ago proteins were pulled down using the Ago-APP approach based on the pull-down of all Ago proteins using a Flag-GST-tagged TNRC6B derived peptide (WT) or a mutated peptide not able to bind Ago anymore (All Ala) as a control for IP (Hauptmann et al., 2015). In order to retrieve the Ago-loaded complex, I tested two elution methods, either a Flag peptide competition elution or release of the complex by GST-tag cleavage (Figure 11).

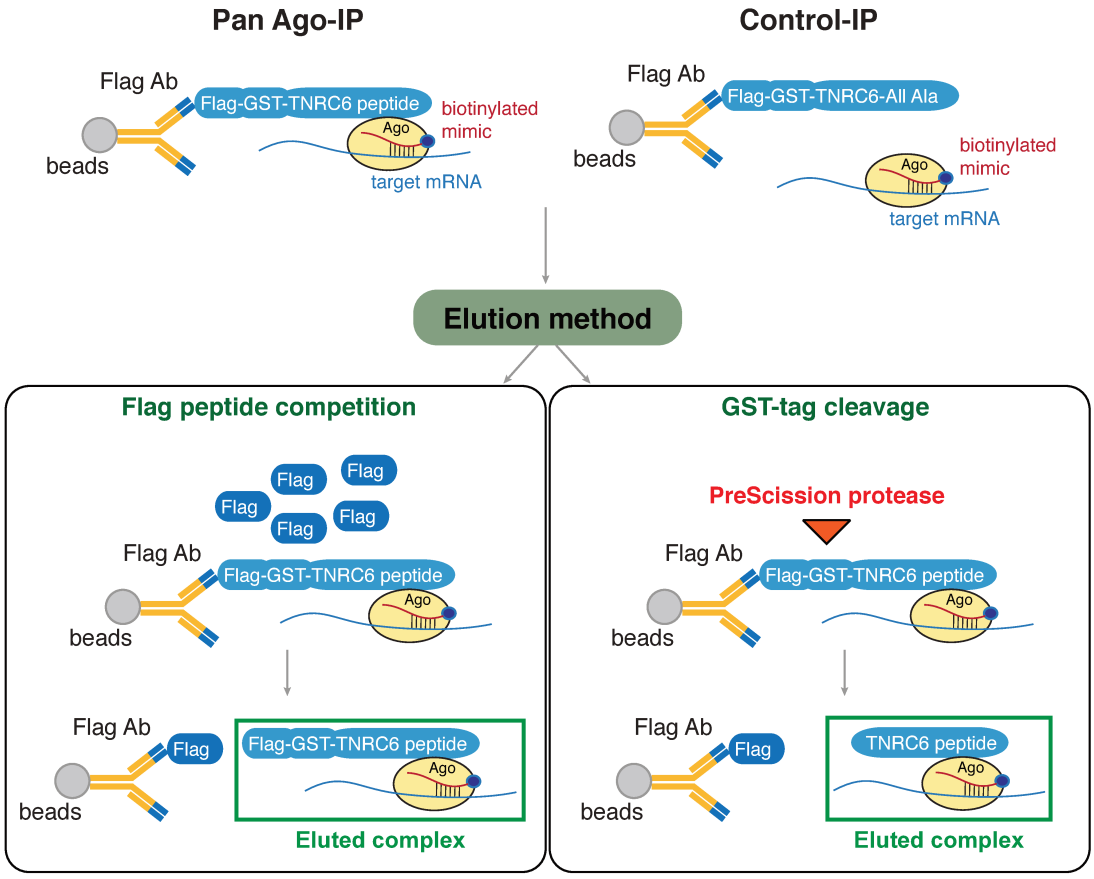

Figure IV.11. Schematic representation of an adapted version of the TAP-Tar approach.
Following transfection of biotinylated mimics, Ago proteins are isolated by Ago-APP and the complexes are eluted by two different elution methods.

I could detect both miR-124 mimics, 124 and 124B, in the inputs by northern blot analysis with a slight shift in 124B migration corresponding to the biotin moiety. In this experiment, I performed an elution by competition using an excess of Flag peptide. While I was not able to detect the miR-124 mimics in the eluate, I could retrieve them after TRIzol elution from the beads (Figure 12).

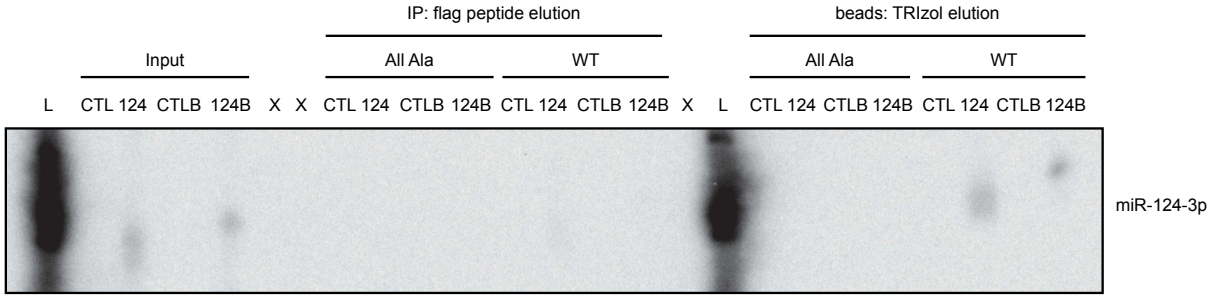

Figure IV.12. Pulldown of biotinylated mimics by Ago-APP.
Northern blot analysis of Ago-loaded biotinylated miR-124 mimic pulled down by Ago-APP using a TNRC6B peptide (WT) or a mutated peptide as a control (All Ala).

To improve the elution step, I repeated the same experiment using Huh7.5.1 cells and this time eluting the TNRC6-Ago complexes by digestion of the peptide using the PreScission protease, that cleaves specifically the GST tag. This time, I could detect both miR-124 mimics (124 and 124B) in the eluted complexes from WT peptide and not in the control All Ala by northern blot analysis. Nonetheless, elution was still not optimal since I could still detect some miR-124 mimics (both 124 and 124B) attached to the beads by performing a TRIzol extraction (Figure 13A).

I also verified the efficiency of the immunoprecipitation by detection of Ago2 by western blot analysis, which confirmed that Ago2 was immunoprecipitated with the WT, but not with the mutant peptide (Figure 13B). Finally, to investigate whether the biotinylated miR-124 mimic could still play a role in the regulation of SINV infection, I quantified viral production of transfected cells following SINV infection. Plaque assay analysis shows an increase of about 5-fold in viral titers in cells transfected with the 124B mimic compared to CTLB mimic (Figure 13C).

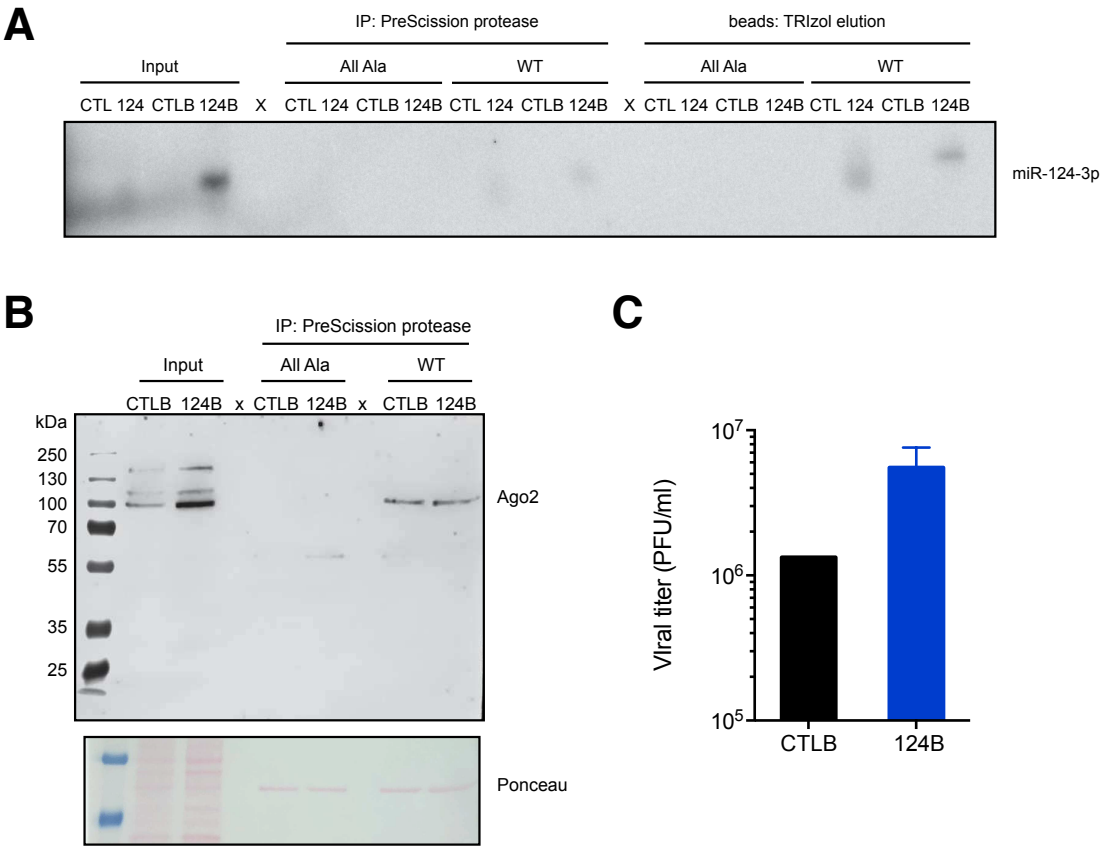

Figure IV.13. Biotinylated mimics are loaded on Ago.
(A) Northern blot analysis of Ago-loaded miR-124 biotinylated or classic mimic elution after Ago pulldown. (B) Western blot analysis Ago2 pulldown from cell lysates after transfection of biotinylated. Ponceau staining is used as loading control. (C) Viral titer quantification of SINV-GFP particle production of biotinylated mimic control (CTL) or miR-124 (124) transfected and infected Huh7.5.1 cells. Data presented as mean + SEM of technical replicates of a single experiment.

Altogether these results show that the biotin moiety does not impair miR-124 regulation of SINV infection. Furthermore, I could show that Ago2 can be efficiently immunoprecipitated by Ago-APP and I managed to detect miR-124 mimics, biotinylated or not loaded in Ago upon elution with the PreScission protease responsible of the GST tag cleavage. Nevertheless, an important amount of mimics remains attached to the beads, either loaded on Ago and not efficiently eluted or non-specifically bound to the beads.

Although the elution step still requires some optimization before the pulldown for isolation of miR-124-specific complexes, this approach seems very promising for future experiments aiming at the identification and validation of miR-124 targets.

B. Identification of miR-124 specific targets by CLEAR-CLIP

In our attempts to decipher the molecular mechanism of SINV positive regulation by cellular miRNAs, we turned to another technique based on Argonaute proteins cross-link and immunoprecipitation. To this end, we performed a CLEAR-CLIP (*Covalent Ligation of Endogenous Argonaute-bound RNAs – Cross-link Immunoprecipitation*) (Moore et al., 2014) analysis to identify miR-124 targets in the context of SINV infection. Briefly, this approach relies on the UV-crosslinking of Ago-bound miRNAs and their target mRNAs followed by an RNase treatment to reduce the size of mRNAs. After isolation of the ternary complex by Ago IP, chimeric RNA molecules are generated between the miRNA and the target. RNA tags are radiolabelled for size selection of the complex by SDS-PAGE, getting rid at this step of complexes formed of miRNA-only loaded in Ago or non-digested mRNA targets. Finally, libraries are prepared for high-throughput sequencing in order to identify miRNA specific targets (Figure 14).

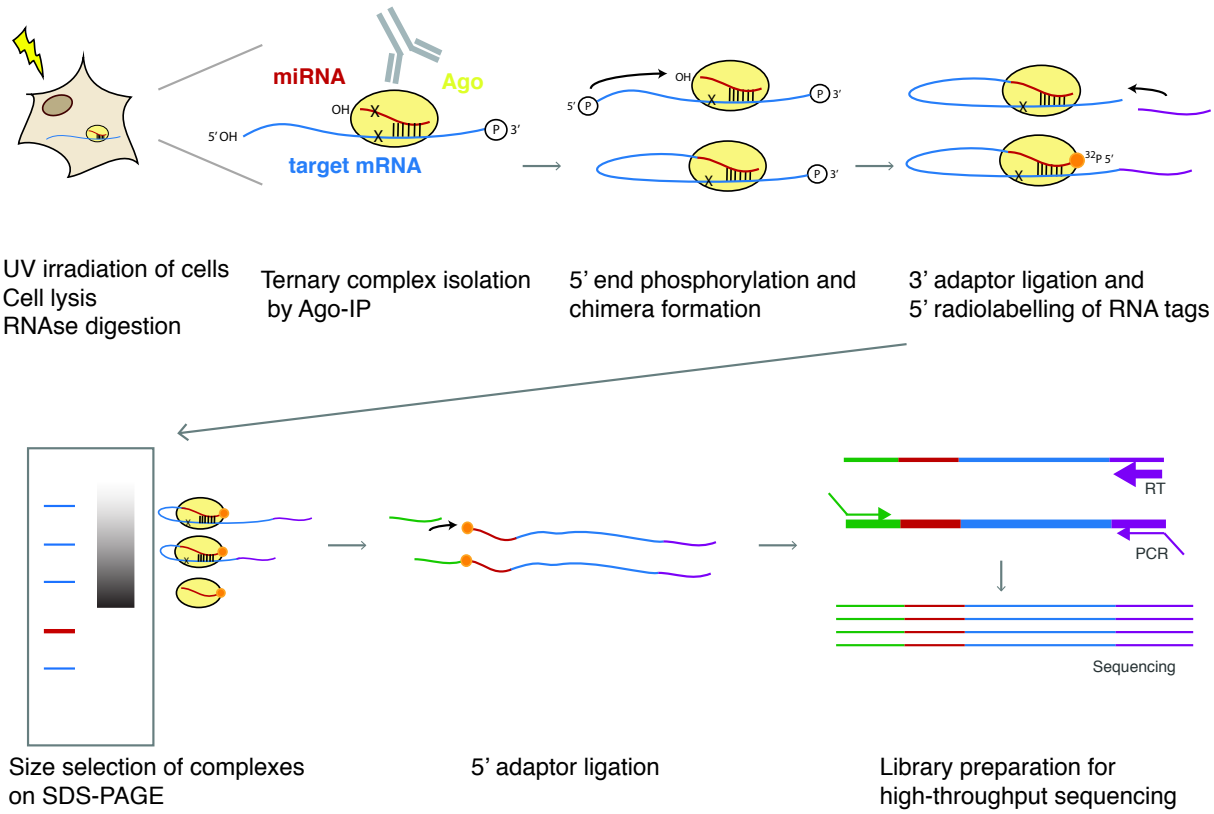

Figure IV.14. Schematic representation of the CLEAR-CLIP approach.
The main steps of the technique are represented in the diagram.

Compared to TAP-Tar, this approach has the advantage to precisely identify the miRNA binding site within a given target sequence. In addition, CLEAR-CLIP is an unbiased genome-wide approach that allows identification of all miRNA-target interactions happening at a specific moment in the cell providing us with a complete image of all cellular networks involved in the regulation of infection as well as the contribution of other miRNAs synergistically with miR-124.

For the purpose of this experiment, we used miR-124 expressing HEK293T cell line (124+), or wild-type HEK293T cells (WT) as a control, both treated with 1 μg/ml of Dox for 48h prior to mock or SINV infection. At 24 h of infection, we could observe an increased GFP signal in infected 124+ cells compared to the control cell line (Figure 15A). Following infection, cells were UV cross-linked at a wavelength of 254 nm and

collected in PBS to proceed to lysis. Cell lysates were subjected to a mild RNase A digestion prior to IP of ternary complexes. We performed a Pan-Ago IP using the Ago-APP approach previously described. We could efficiently immunoprecipitate Ago in all of our samples except in the All Ala control peptide condition (Figure 15B). In order to isolate the Ago:miRNA:mRNA ternary complex, RNAs were 3' radiolabeled with ^{32}P for size selection after SDS-PAGE and nitrocellulose membrane blotting (Figure 15C). According to the expected size of the complexes, two bands were excised from the membrane, the first of about 130 kDa containing our complex of interest Ago:miRNA:mRNA and the second one of about 110 kDa with Ago:miRNA complexes as a control. We also observed a less intense but similar profile in the control-IP sample (sample 1), suggesting the presence of radiolabelled contaminants migrating at the same positions (Figure 15C).

Finally, after extraction of the RNA from the membrane, we performed retrotranscription followed by different cycles of PCR amplification (Figure 15D). At 30 cycles of amplification we could observe a fragment of about 200 bp, corresponding to the expected size of chimeras, in Ago immunoprecipitated samples of WT of 124+ cells, mock or infected (samples 2-5), and not in the IP control using the AllAla peptide (sample 1) nor in the ligation and RT negative controls (sample 6 and 7) . Furthermore, we observed an amplification of about 150 bp in all samples except in the negative control of RT that could correspond to the expected size of miRNA-only amplicons or an unspecific amplification from the adaptor (sample 6).

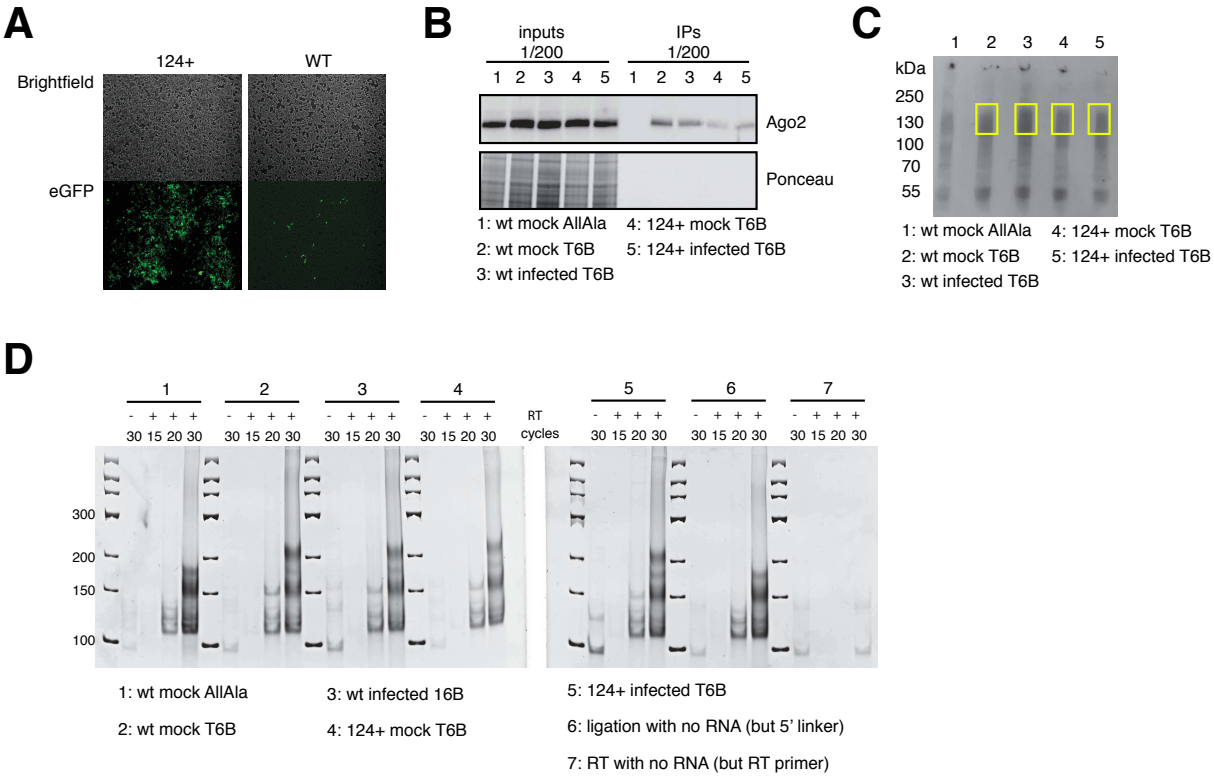

Figure IV.15. CLEAR-CLIP on miR-124 expressing cells and HEK293T cells during SINV infection.
(A) Microscopy images of SINV-GFP infected cells. (B) Western blot analysis of IP efficiency. (C) Autoradiogram of radiolabelled RNAs crosslinked to Ago proteins. Yellow squares delimit the region cut from the membrane where complexes were isolated from (D) PCR analysis on acrylamide gel for amplicon verification.

The bioinformatic analysis of the experiment after high-throughput sequencing was performed by Béatrice Chane-Woon-Ming, bioinformatician at the institute, following previously described protocols (Scheel et al., 2016). After initial processing of the reads in which PCR duplicates were collapsed, the total reads for analysis represent between 4.5 to 7 million, depending on the sample (Table 1). The first step in the analysis was to perform a reverse mapping of all human miRNAs in these reads. In all samples, about 30% of the reads contained miRNA sequences in the positive sense of the sequenced reads. For further analysis, the fragment of sequence downstream of the identified miRNA was extracted and aligned to SINV or human hg38 genomes. From

these extracted fragments, 1.5% reads reported at least one alignment, thus obtaining a total of about 0.5 % chimeras from the initial reads (Table 1)

Table IV.1. Identification of chimeras in HEK293T or 124+ cells SINV- or mock-infected.

	HEK293T				124+			
	mock		SINV		mock		SINV	
	nb	%	nb	%	nb	%	nb	%
Total preprocessed reads	**6744365**		**7058346**		**4356846**		**4570158**	
miRNA containing reads	2004673	29,72	2080851	29,48	1307065	30,00	1208219	26,44
Reads with at least one reported alignment (on SINV or human genomes)	33264	**0,49**	30845	**0,44**	22434	**0,51**	20235	**0,44**

In order to get more insight into the nature of the initial reads that did not contain miRNAs (nomiR) or the unaligned extracted fragments from the remaining chimeras, we looked for the 3' adaptor sequence. Indeed, this analysis showed that 35%-40% of the nomiR sequences contained the 3' adaptor sequence suggesting that mRNA sequences were not efficiently ligated to miRNAs. As for the unaligned fragments, 75-86% were made of 3'adapter ligated to RNA fragments impossible to map due to the presence of a too short target sequence (Table 2).

Table IV.2. Analysis of unexploited reads and identification of 3' adaptor sequences.

	HEK293T		124+	
	mock	SINV	mock	SINV
Total preprocessed reads	**6744365**	**7058346**	**4356846**	**4570158**
NomiR-refseqs	5014910	5257163	3228987	3523362
% of nomiR reads	74,36	74,48	74,11	77,09
Reads with 3' adapter	1987677	1917526	1312396	1232218
% of reads with 3´adapter on nomiR reads	39,64	36,47	40,64	34,97
Fragments that failed to align	1955939	2036300	1275352	1179958
Reads with 3' adapter	1587230	1515738	1104364	979502
% of reads with 3´adapter on extracted fragments	81,15	74,44	86,59	83,01

Among the identified miRNA-target chimeras, we determined the origin of the RNA fragments associated with our miRNA of interest miR-124-3p as well as the most abundantly expressed miRNAs, miR-16-5p and miR-92a-3p, in SINV infected 124+ cells.

First, we confirmed the previous observation that miR-92a-3p binds SINV RNAs (Scheel et al., 2016), as almost 50% of miR-92a chimeras are formed with viral RNA in our CLEAR-CLIP experiment. Moreover, miR-16 was found to target mainly cellular RNAs, with less than 20% of viral RNA targets. Finally, miR-124 chimeras were formed by 30% RNA targets of viral origin and 70% of cellular target RNAs (Figure 16A). We then analysed the distribution of miR-124-associated targets and we could find that the majority of the identified targets represent 3' UTRs (32%), introns (27%) and coding regions (21%). Finally, the other targets mapped to other types of regions such as exons, 5' UTRs or other genomic regions like intergenic or repeats (Figure 16B).

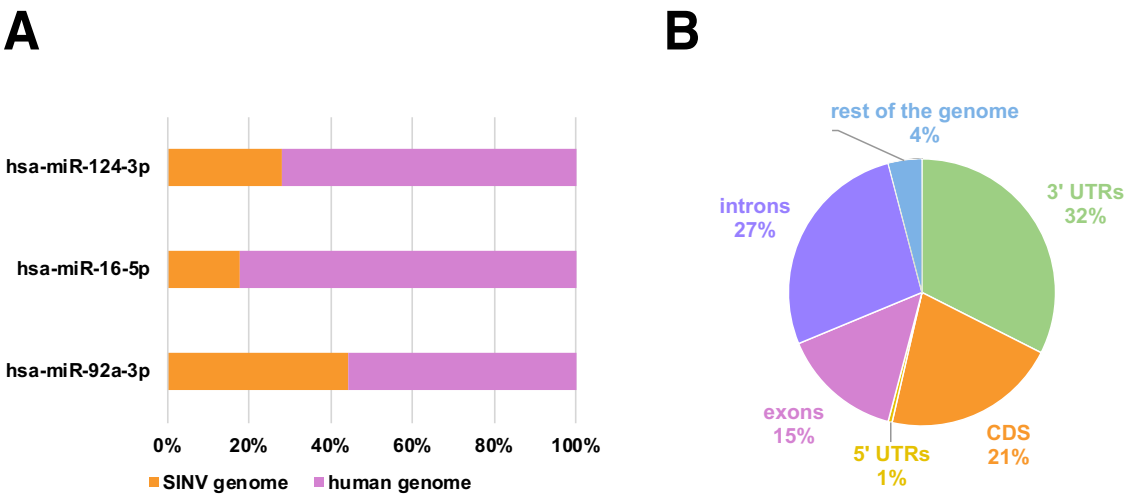

Figure IV.16. miRNA identified targets by CLEAR-CLIP.
(A) Bar chart representation of miR-124-3p, miR-16-5p and miR-92a-3p associated target RNA's origin. (B) Distribution of miR-124-associated fragments.

Although the low number of obtained chimeras did not allow us to perform a very deep analysis, among the identified cellular targets we did find some of the known and

validated miR-124 targets such as the cyclin-dependent kinase 4 (CDK4) (Deng et al., 2013).

Finally, we sought to identify the viral sequences found among miR-124 targets. After alignment of miR-124 chimeras on the viral genome, we identified several peaks corresponding to putative binding sites in the genome (Figure 17A). Three of them are located in the non-structural proteins coding region of the genome, including two within nsP2 and one within nsP4. The other three are in the structural proteins coding region, one within capsid protein and two within the glycoprotein E1. Among these sites, three of them have been bioinformatically predicted by the prediction tool ViTa (Hsu et al., 2007), namely the two in nsP2 and the 3' terminal site in E1.

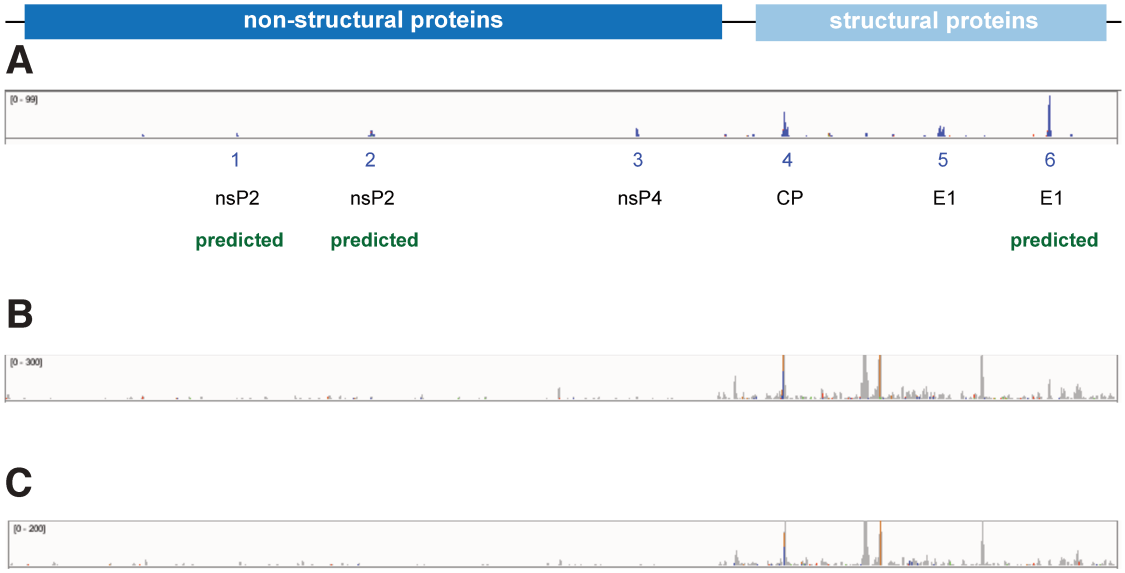

Figure IV.17. Peak distribution of miRNA specific targets mapping to the SINV genome.
(A) miR-124 specific viral target reads identified in 124+ cells. Global microRNA target reads mapping the viral genome in (B) 124+ cells or in (C) HEK293T cells.

We also looked at the overall binding of cellular miRNAs on the viral genome (Figure 17B and C) and we could observe that the majority of cellular miRNAs bind mostly the 3' extremity of the genome, possibly due to the high abundance of the subgenomic RNA. In addition, if we compare the peaks in WT or miR-124 expressing cells we observe similar profiles suggesting that miR-124 overexpression does not seem

to impair or displace binding of the other miRNAs on the viral genome (Figure 17B and C).

In conclusion, we successfully accomplished one replicate of CLEAR-CLIP analysis using the HEK293T cells expressing miR-124. The promising result validated our method since we were able to find known validated cellular miR-124 targets as well as several of the previously identified cellular miRNAs binding to SINV genome such as miR-92 (Scheel et al., 2016). In addition, we found several miR-124 binding sites in the viral genome, that have not been described previously, indicating a direct interaction between miR-124 and the viral RNA.

Improvement of certain important steps of the technique will allow to increase the number and abundance of cellular targets in order to have a better idea of the contribution of these mRNAs in the regulation. In addition, additional biological replicates will be needed in order to be confident about these newly identified interactions.

Finally, we have recently set up a collaboration with Dr Troels K. H. Scheel (University of Copenhagen, Denmark) to apply his expertise on CLEAR-CLIP and we are currently performing this experiment with his team. In sight of our recently published results, we will also compare miR-124 binding to genomic RNA in the context of infection by the two viruses SINV WT as well as our mutant virus lacking one binding site.

C. Luciferase reporters for miRNA functional validation

The CLIP data as well as the bioinformatically predicted binding sites identified several binding sites for miR-124 in the SINV genome. So far, I generated a single SINV mutant for just one of the binding sites, which is the one described in the paper. With the aim of validating this particular site, I used luciferase reporters, which are widely used tools to assess functional studies of miRNA activity and validation of predicted binding sites. They are used to measure translational repression or cleavage based on the introduction of a mRNA region suspected of being targeted by a miRNA in the 3'UTR of the *Firefly* luciferase (Fluc). With this tool, I sought to determine and validate the predicted miR-124 binding site found in SINV genome, more precisely the one present

within the glycoprotein E1 coding region and whose mutation impaired SINV regulation (Chapter II of Results).

I cloned a fragment of about 200 nucleotides of the viral genome containing this binding site in the 3' UTR of the *Firefly* luciferase (Fluc) reporter vector (site WT). I also used the same vector but carrying two mutations in the seed region of this site (site mut) as a control. The aim of introducing these particular mutations was to be able to compare the functional studies using these reporters to the validations made *in cellulo* with the WT and mutant viruses. Hence, the mutations in the mutant reporter site are the same silent mutations introduced in the mutant virus disrupting the binding site but still allowing correct viral protein synthesis. Furthermore, we used two control vectors to validate the assay: the positive control with a miR-124 imperfect match (124i), producing a bulge between nucleotides 8 to 10 in order to avoid Ago cleavage; and miR-124 seed mutant reporter (124m) as a negative control.

I measured the Fluc activity of each vector normalized to the *Renilla* luciferase (Rluc) activity in the presence of mimic-124 or a mimic control (mimic-CTL). I saw a reduction of about 40% in Fluc signal from 124i with mimic-124 compared to the control and no significant effect of mimic-124 in the 124m reporter (Figure 18). Furthermore, I did not observe any significant effect of mimic-124 in site WT or site mut reporters compared to mimic-CTL.

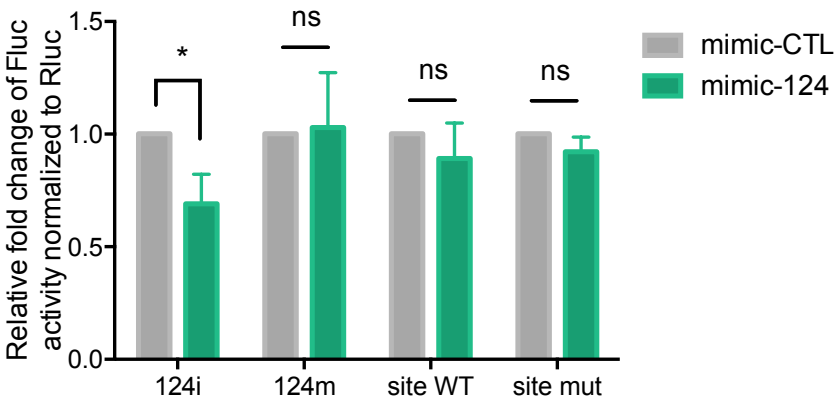

Figure IV.18. Functional validation of miR-124 on different target sites.
Reporter assay using HEKgrip cells cotransfected with *Firefly* luciferase (Fluc) constructs harboring different target sites within the 3' UTR and a mimic control (CTL)

or a mimic-124. *Renilla* luciferase (Rluc) activity is used to normalize Fluc. * $p < 0.05$, Two-way ANOVA with Bonferroni correction.

Therefore, I concluded that mimic-124 is functional and represses Fluc through an imperfect match binding site and this repression depends on the seed region. The low repression levels that I observed could be explained by the imperfect match of the target site.

On the other hand, this method was not suitable to validate miR-124 binding to the viral genome as no effect was measured on the "site WT" reporter in presence of the miRNA mimic. These results suggest that the positive regulation of miR-124 on SINV cannot be recapitulated with this system. One of the reasons could be that while luciferase reporters are commonly used for the study of mRNA repression by miRNA binding, the effect that we observe in SINV infection is a positive regulation. Thus, this approach might not represent the appropriate readout for the interaction that we are studying.

D. Other tools developed to study miR-124 effect on viral infection

1. SINV replicon

To overcome the technical limitations that I was facing with the use of luciferase reporters for the functional validation of the targeting of the viral genome, I decided to set up a suitable system allowing me to reproduce the positive regulation as opposed to the repression.

We obtained a SINV replicon from Dr Carla Saleh's laboratory (Institut Pasteur, Paris, France). The replicon encodes exclusively for the viral non-structural proteins allowing replication but lacks the structural proteins ORF thereby preventing viral production. It still contains the regulatory regions of the viral genome including the subgenomic promoter allowing the insertion of a reporter in lieu of the second ORF. This tool allows exclusive study of viral replication by uncoupling replication steps from structural protein synthesis and further viral particle production.

For the functional validation of miR-124, I designed a replicon-based system in which I cloned the GFP as a reporter protein, in fusion with the 3' extremity of glycoprotein E1 coding region, where miR-124 binding site is localized (Figure 19A). The objective would be to study the effect of miR-124 on non-structural protein production, replication and viral RNA stabilization. While I managed to obtain the designed replicon, so far I did not finish yet the optimization steps to efficiently transfect an optimal amount of replicon RNAs to conduct the subsequent experiments.

2. miR-124 sponge: Tough decoy inhibitor

In this study, I mainly used antisense oligonucleotides for inhibition experiments, which turned out to be extremely effective. Nonetheless, there are other situations where the use of such oligonucleotides could prove more difficult. Therefore, I also developed an alternative method to block miR-124 by using a miRNA tough decoy (Bak et al., 2013). This strategy is based on the expression from a vector of an RNA molecule able to fold on itself forming a hairpin structure with the complementary sequence to the miRNA on each of the strands forming the stem (Figure 19B). In addition, I cloned this structure in fusion with the mCherry fluorescent protein in order to visually track the cells expressing the sponge. I obtained the vector that is now available for testing, however due to lack of time I could not test it yet to assess its efficacy.

One of the advantages of this strategy is that the sponge can be expressed from any type of vector, notably lentiviral vectors. This way, transduction of the sponge could be a possibility for cells hard to transfect or primary neuronal cultures for which lipofection seems to be disadvantageous for subsequent viral infection as well as for future *in vivo* studies.

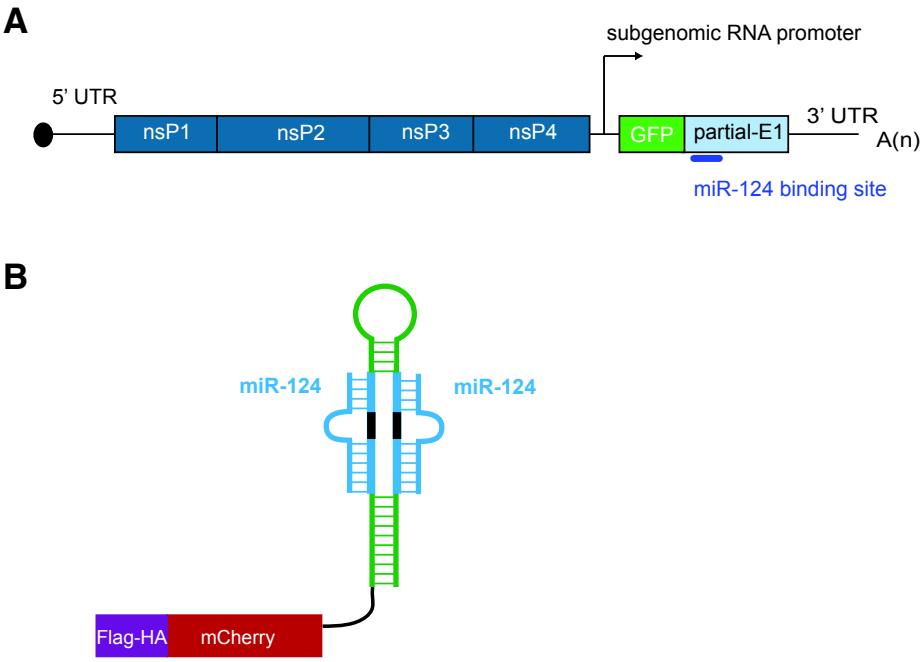

Figure IV.19. Tools developed for future studies.
(A) SINV replicon strategy to study miR-124 function on replication. N-terminal extremity of E1 glycoprotein sequence containing the miR-124 binding site was cloned in fusion with GFP. (B) miR-124 sponge for inhibition studies. Two miR-124 binding sites are placed in the stem of a hairpin RNA structure. Perfect pairing was avoided by introducing bulges between nucleotides at position 10-11 avoiding cleavage. The sponge was cloned in fusion with the fluorescent mCherry protein.

E. Study of other miRNA candidates affecting SINV

The initial miRNA screen led to the identification of a list of many interesting miRNAs with a great positive effect on SINV infection. Among the ones showing a strong phenotype on SINV were miR-129-5p and miR-485-3p in which we could observe that GFP fluorescence was strongly increased (Figure 20).

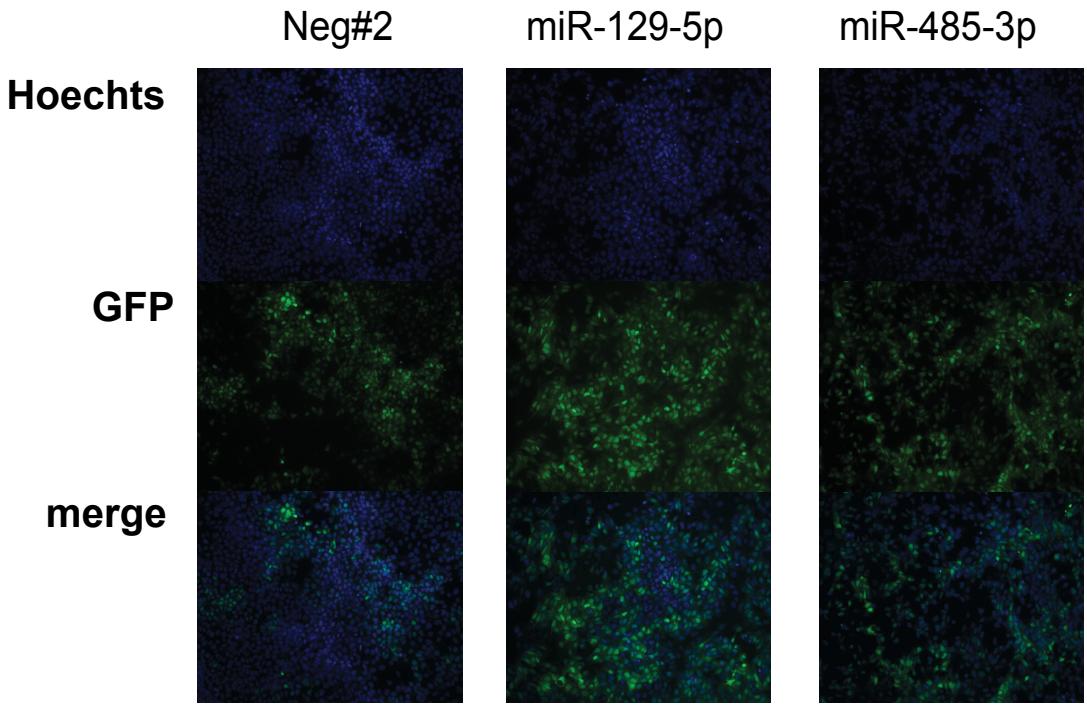

Figure IV.20. Representative fluorescence images from the screen.
Huh7.5.1 cells were transfected with candidate mimics or a mimic control (Neg#2) and infected with SINV-GFP.

For these two candidates, I also started to establish Huh7.5.1 cell lines stably expressing these miRNAs. I used the same strategy as for miR-124 described in Chapter II of Results. I cloned the corresponding pri-miRs in a lentiviral vector, I produced lentiviral particles and transduced Huh7.5.1 cells. I selected the polyclonal resulting cell line by antibiotic selection pressure. The expression of each of the candidates was verified by northern blot analysis using a total RNA sample extracted from these cells. The results show that miR-129-5p is correctly expressed, however there is no signal for miR-485 (Figure 20).

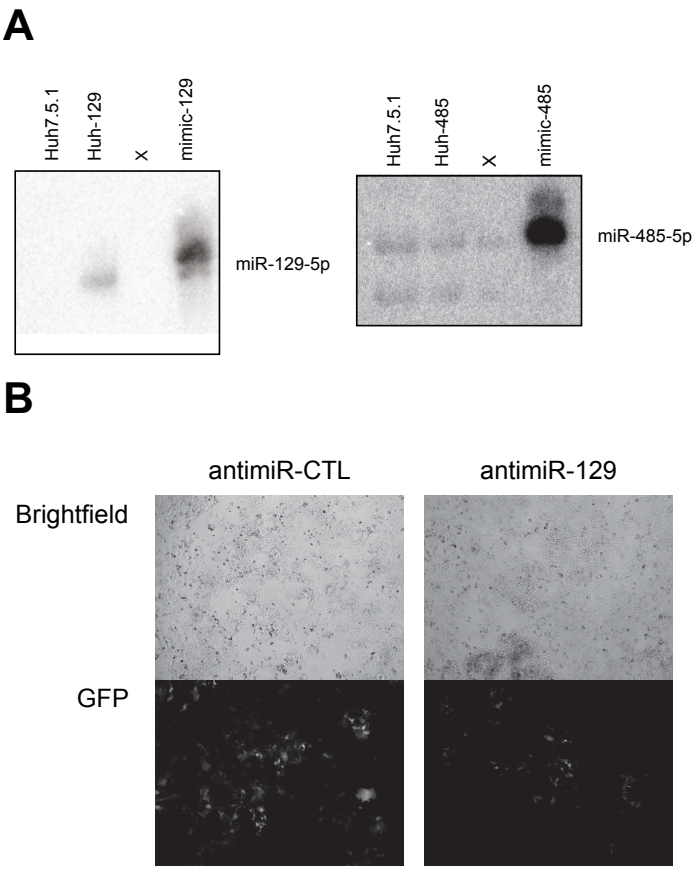

Figure IV.21. Study of the effect of miR-129-5p and miR-485-5p on SINV.
(A) Northern blot analysis of candidate miRNAs expression in Huh7.5.1 transduced cells with lentiviruses encoding the corresponding pri-miRNAs. Synthetic mimics were used as positive controls (B) GFP fluorescent signal of Huh-129 infected cells with SINV-GFP after transfection of miR-129 or control antimiR.

In conclusion, I successfully generated a transgenic cell line expressing miR-129. Furthermore, preliminary data on inhibition experiments using miR-129 antimiR before SINV infection showed decreased viral protein synthesis (Figure 21), suggesting that this miRNA might be a good candidate showing similar effects as miR-124.

Unfortunately, I did not achieve to obtain a stable cell line correctly expressing miR-485 impeding further studies of these candidates with this strategy.

CONCLUSIONS & PERSPECTIVES

V. CONCLUSIONS & PERSPECTIVES

In view of the recurring epidemics associated with viral infections, the need for deeper understanding of these viruses and their interactions with the host has become a major issue in order to develop effective treatments to fight them. As obligatory intracellular parasites, viruses depend on cellular machineries, structures and factors leading to tight interactions between the host and the pathogen. The regulatory pathway of post-transcriptional gene expression by miRNAs is no exception to these borrowed functions. As the majority of RNA viruses, alphaviruses have full cytoplasmic replication cycles. The presence and availability of miRNAs in the same compartment has led viruses to turn the function of these molecules into their advantage while replicating in the host cell. Conversely, these molecules can also instead interfere with the viral cycle by repressing viral gene expression directly or indirectly by regulating the expression of different host factors. This is one of many examples representing the evolutionary adaptation between the host and the virus.

In this thesis project, I focused specifically on the study of miRNAs that could have a pro-viral or antiviral effect on the regulation of alphaviruses. The initial studies were based on the findings from the genome-wide miRNA screen aiming to identify new candidates involved in the regulation of viral infection. First, the loss-of-function screen failed to identify any potential miRNAs involved in the processes, possibly because of the specific miRNA expression pattern in hepatocytes, the cell line used in the study. However, a list of interesting positive regulators of Sindbis virus infection was identified upon overexpression in the gain-of-function screen. The principal axis of research presented in this thesis involved the study of the candidate showing the strongest pro-viral effect on SINV, miR-124.

The wide conservation of this miRNA across different organisms reflects the importance of the original physiological functions as a brain-enriched miRNA. In addition, in the context of infection, this interaction seemed plausible as alphaviruses are able to establish infection in the CNS. Thus, here I have presented the work performed

with the aim of better understanding the role of miR-124 as a positive regulator of alphavirus infection.

As miR-124 is a tissue-specific miRNA, I first worked on setting up different cellular systems for this study. In the first chapter of Results, I recapitulated the observations and data obtained while evaluating different systems expressing our miRNA of interest, with the aim of defining the best tool to further investigate miRNA-virus interactions.

I used different neuronal models endogenously expressing miR-124. The neuroblastoma cell line SK-N-BE(2) turned out to be the best choice for the study providing an appropriate neuron-like background and in which I could efficiently achieve a full miR-124 inhibition without impairing the infection. Other alternatives included the use of human neural progenitor cells (hNPC) and primary cultures of rat cerebellar granule cells (GCs).

hNPC showed a high miR-124 expression upon 14 days of differentiation. In addition, we could show that they are infectable by SINV at a range of different MOIs and time points. It remains to be assessed the possible consequences of antimiR transfection and its effects on viral infection. Finally, the use of this system might evoke controversial concerns due to the ethical issues arising from the origin of these cells. Nonetheless, I believe they represent an interesting physiologically relevant model to use in the future for brain-specific miRNA studies upon alphavirus infection. As an alternative to circumvent the ethical constraints of the use of these cells, NPCs derived from inducible pluripotent stem cells (iPSC) could also be of interest.

Concerning the rat primary granule cell cultures, I was able to achieve a complete depletion of the miRNA in the inhibition studies despite the reduced cell transfectability. One of the major drawbacks of the GC primary cultures is that we have a limited amount of cells to work with and GCs are notably difficult to manipulate. Indeed, preparation of the cells and plating account for a big part in the success of the subsequent steps of the experiment. Moreover, the lipofection of antimiR molecules has the disadvantage of rendering the cells not infectable by SINV, possibly by impairing viral entry or leading

to unproductive infections. In the perspective of improving this approach, I developed the miR-124 sponge which we could make use for inhibiting miR-124. Delivery of the sponge by lentiviral transduction might be more efficient than lipofection of antimiRs.

This miR-124 sponge approach could be also used for other futures perspectives regarding this project notably to perform *in vivo* studies in order to assess the relevance of this regulation by taking into account the whole organism.

Although antisense RNA molecules are extremely efficient in cell culture, they show some disadvantages when used for *in vivo* treatments. Different chemical modifications need to be added in order to increase uptake during delivery as well as to increase their stability. For instance, *in vivo* delivery of antimiRs against miR-122 was proved to be very promising to treat HCV as it can efficiently reduce HCV viral RNA levels (van der Ree et al., 2017). However, tissue-specificity, stability and uptake are still challenging. The major difficulty faced when dealing with the CNS is to achieve correct delivery by trespassing the blood-brain barrier to reach the brain. This is why treatments based on using miRNA sponges by lentiviral vector delivery could be of great advantage, especially if using non-integrative versions of lentiviruses to avoid disrupting physiological functions of miR-124 in the long-term.

In this work, I also developed two engineered stable cell lines with the aim of performing deeper mechanistic studies in a cellular model exhibiting fewer constraints regarding its manipulation and that could be amplified fast to obtain an important amount of material as opposed to neuronal primary cell cultures or neural stem cells.

The first stable cell line was derived from human embryonic kidney cells in which we could induce miR-124 expression upon Dox treatment and the second derived from modified human hepatic cells that constitutively express miR-124. Unfortunately, I was not able to obtain clear results from the Dox-inducible cell line. I observed a leaky activity of the promoter reflected by miR-124 expression without Dox treatment. Because miRNAs are defined as fine-tuners of gene expression, this basal level of expression could already be exerting an effect on SINV virus infection, thus making it impossible to differentiate the effects on the infection between mock- or Dox-treated, or antimiR-CTL or antimir-124 leading to the important variability that we observed in these assays. In

addition, doxycycline is known to be an antiviral compound for different arboviruses, including the alphavirus CHIKV (Rothan et al., 2015)(Rothan et al., 2014). Indeed, we cannot exclude that treatment of the cells with such molecule prior to the infection might have undesired effects on the establishment and development of the infection. It would be thus needed to determine the toxicity of the treatment to the cells as well as the effect of doxycycline on the virus previous to assessing miRNA regulation.

In contrast, the stable cell line derived from human hepatocellular carcinoma cells resulted in an excellent model displaying a high ectopic expression of miR-124 that could be efficiently inhibited by antisense RNA molecules. In this regard, deeper characterization of this cell line could be considered to assess the influence of expressing miR-124 in these cells at the transcriptomic level as important changes in networks are observed in HeLa cells expressing miR-124 (Lim et al., 2005b). Furthermore, differentially expressed genes in cells in the presence or absence of miR-124 and infected or not might give a first view of potential targets involved in miR-124-mediated regulation of alphaviruses.

The main work done to describe the precise molecular functions of miR-124 and its modulation of the viral cycle was presented in the form of our article in Chapter II of Results. With these findings we could show the importance of the seed mediating the regulation and that this effect is not restricted to SINV, but that it can equally be observed in other alphaviruses like CHIKV. It is however not conserved in all RNA viruses as flaviviruses were not affected. Furthermore, we identified a genomic binding site on SINV and we showed its implication on the regulation since a mutant virus is no longer affected. Interestingly, the genome of CHIKV lacks an optimal binding site, suggesting that miR-124 might participate in the process by other means, possibly involving regulation of cellular functions by repressing cellular targets. The research done to get more insight into this possibility was presented in Chapter III of Results.

Based on the fact that miRNAs have to be loaded into Ago proteins to exert their function, we opted for the isolation of miRNA-loaded Ago proteins in complex with the miRNA targets by using either biotinylated mimics or by CLEAR-CLIP.

In our efforts to identify miR-124 targets in the context of infection, we began to establish an adapted version of the previously described TAP-Tar technique (Nonne et al., 2010), a two-step process involving an Ago IP and specific miRNA-target isolation by biotin-streptavidin pulldown of biotinylated mimics. This is a quite controversial method since part of the scientific community argues that the biotin moiety of the mimic does not allow a correct loading within Ago (Guo and Steitz, 2014). In our case, although our elution method based on GST tag cleavage by PreScission protease treatment requires more optimization, we were able to retrieve biotinylated mimic-124 loaded in Ago. Indeed, loading might be less efficient with these modified mimics, but it can possibly still be used for the identification of high abundant mRNA targets, as can be viral RNAs present in the cells during viral replication. Although this technique does not require UV irradiation of cells for crosslinking of mRNA targets to RISC, it could be considered to avoid losing in the process the weakest interactions thus identify the maximum of targets using this approach.

Instead, the CLEAR-CLIP approach is a well-characterized method previously used for this type of studies that allowed us to obtain very encouraging results. We could show molecular interactions between miR-124 and SINV viral genome in predicted sites as well as report new binding sites in other genomic regions. We will still need to study the biological importance of these sites and to validate whether they are all indeed functional and involved in the regulation of viral infection. However, there is room for improvement of the technique in this particular cell system to increase the overall number of chimeras. There are some key steps of the experiment that will need to be improved, for example reducing the RNase treatment in order to obtain longer target sequences that can be aligned uniquely, testing other Ago-IP methods that could yield a higher amount of isolated complexes or improving the ligation step between miRNAs and target mRNAs. This way we will be able to have a global view of the contribution of cellular targets potentially participating in these regulatory mechanisms.

This information, coupled with the transcriptomic analysis of the cell line, will be crucial to understand the main regulatory networks modulated by miR-124 in the context of alphavirus infection in order to propose a potential mechanism of regulation for CHIKV, who lacks a genomic binding site, and that can eventually also be important in

SINV infection. In addition, performing the CLEAR-CLIP with CHIKV infected cells will elucidate whether there might be 3' supplementary pairing of miR-124 in the identified non-canonical binding site or the identification of other potential binding sites. Finally, a CLEAR-CLIP approach done in neuronal cells would in addition give more insight into other neuronal miRNAs acting synergistically.

Finally, we could show that miR-124-mediated regulation of alphaviruses led to an increase in translation and viral production. According to already described examples of miRNA positive regulation of viral infection, there are several possibilities for the mechanism upon which miR-124 regulates alphaviruses. Interaction of the miRNA with the viral genome could have a beneficial effect by stabilizing viral RNAs or directly enhance translation of viral proteins. Another possibility is that the viral RNAs could act as a sponge to miR-124 and induce derepression of otherwise silenced cellular targets. Further investigation will be needed in order to elucidate the precise mechanism leading to the positive effect on alphavirus infection.

While the main work presented in this thesis was centered on miR-124, the gain-of-function screen revealed the presence of other interesting miRNAs with pro-viral properties during SINV infection. Very interestingly, many of them are also miRNAs expressed in the central nervous system, suggesting an involvement of miRNAs possibly sharing common regulatory networks in the regulation of infection, particularly in the CNS.

An interesting line of research for future work would be to get a deeper understanding of the implication of these miRNAs and see whether they share similar regulatory mechanisms of the infection. Also, it will be interesting to study the possible cumulative effects of these miRNAs and whether their functions are individual, or they can complement each other. As for these other potential candidates I focused on miR-129-5p and miR-485-3p. Besides being among the top candidates positively regulating SINV infection, these miRNAs share several characteristics with miR-124 supporting their pro-viral implications and possible similar mechanisms.

First, miR-129 is also a conserved miRNA in vertebrates and is strongly expressed in the CNS, however this is not a tissue-specific miRNA since it is also expressed in lungs, heart and kidney (Wu et al., 2010). Among its functions, it has been described as a tumor suppressor, in part by regulating cell proliferation through targeting CDK6 and thus blocking cell cycle (Wu et al., 2010). In our study, miR-129 showed a great pro-viral potential translated by a strong effect in GFP fluorescence.

Concerning miR-485, it participates in important neuronal functions and like miR-124 and miR-129, it is also considered a tumor suppressor which expression is dysregulated in several types of cancers (Cohen et al., 2011; Lou et al., 2016). This candidate showed great potential on alphavirus infection upon mimic transfection and we could imagine its involvement in the control of the antiviral response in the case of alphavirus infection in a similar manner. However, in my efforts to obtain a cellular system to analyse this interaction I could not establish a cellular system correctly expressing miR-485. Given the fact that miR-485 is expressed from the large miRNA cluster miR-379/miR-410 (Winter, 2015), it is possible that the presence of other miRNAs of the cluster is needed for its correct processing as it is the case for miR-144 influencing processing of miR-451 (Fang and Bartel, 2020; Shang et al., 2020). Bioinformatic analysis of predicted binding sites of these two miRNA candidates on the viral genome using ViTa (Hsu et al., 2007) showed that there are four predicted sites for miR-129 and one for miR-485. Thus, it would be interesting to study the possible cooperative effects of these candidates in the positive regulation of SINV.

Altogether, the work presented in this thesis uncovers a new RNA-based interaction between viruses and the host implicating cellular miRNAs. This work reveals that there is still a lot of interesting possibilities to further investigate to fully understand the complex regulatory interactions happening in the cell during viral infection and leading to important outcomes at the level of the whole organism. This thesis contributes to the deeper understanding of these interactions providing useful information that can help to the development of miRNA-based therapies as well as biotechnological applications for the development of vaccines and alphavirus vectors.

BIBLIOGRAPHY

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Abdelnabi, R., Jacobs, S., Delang, L., and Neyts, J. (2020). Antiviral drug discovery against arthritogenic alphaviruses: Tools and molecular targets. *Biochem. Pharmacol.* 174, 113777. doi:10.1016/j.bcp.2019.113777.

Abu Bakar, F., and Ng, L. (2018). Nonstructural Proteins of Alphavirus—Potential Targets for Drug Development. *Viruses* 10, 71. doi:10.3390/v10020071.

Adouchief, S., Smura, T., Sane, J., Vapalahti, O., and Kurkela, S. (2016). Sindbis virus as a human pathogen-epidemiology, clinical picture and pathogenesis: Sindbis virus as a human pathogen. *Rev. Med. Virol.* 26, 221–241. doi:10.1002/rmv.1876.

Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., et al. (2003). A uniform system for microRNA annotation. *RNA* 9, 277–9.

Andersson, M. G., Haasnoot, P. C. J., Xu, N., Berenjian, S., Berkhout, B., and Akusjärvi, G. (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J. Virol.* 79, 9556–9565. doi:10.1128/JVI.79.15.9556-9565.2005.

Atkins, G. J., and Sheahan, B. J. (2016). Molecular determinants of alphavirus neuropathogenesis in mice. *J. Gen. Virol.* 97, 1283–1296. doi:10.1099/jgv.0.000467.

Azouz, F., Arora, K., Krause, K., Nerurkar, V., and Kumar, M. (2019). Integrated MicroRNA and mRNA Profiling in Zika Virus-Infected Neurons. *Viruses* 11, 162. doi:10.3390/v11020162.

Babiarz, J. E., Ruby, J. G., Wang, Y., Bartel, D. P., and Blelloch, R. (2008). Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev* 22, 2773–85.

Bail, S., Swerdel, M., Liu, H., Jiao, X., Goff, L. A., Hart, R. P., et al. (2010). Differential regulation of microRNA stability. *RNA N. Y. N* 16, 1032–1039. doi:10.1261/rna.1851510.

Bak, R. O., Hollensen, A. K., Primo, M. N., Sorensen, C. D., and Mikkelsen, J. G. (2013). Potent microRNA suppression by RNA Pol II-transcribed “Tough Decoy”

inhibitors. *RNA* 19, 280–293. doi:10.1261/rna.034850.112.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–33.

Bartel, D. P. (2018). Metazoan MicroRNAs. *Cell* 173, 20–51. doi:10.1016/j.cell.2018.03.006.

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nat. Rev. Genet.* 12, 846–860. doi:10.1038/nrg3079.

Berezikov, E., Chung, W. J., Willis, J., Cuppen, E., and Lai, E. C. (2007). Mammalian mirtron genes. *Mol Cell* 28, 328–36.

Béthune, J., Artus-Revel, C. G., and Filipowicz, W. (2012). Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells. *EMBO Rep.* 13, 716–723. doi:10.1038/embor.2012.82.

Bogerd, H. P., Karnowski, H. W., Cai, X., Shin, J., Pohlers, M., and Cullen, B. R. (2010). A mammalian herpesvirus uses noncanonical expression and processing mechanisms to generate viral MicroRNAs. *Mol. Cell* 37, 135–142. doi:10.1016/j.molcel.2009.12.016.

Bohnsack, M. T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* 10, 185–91.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljeström, P., Fazakerley, J., et al. (2007). Semliki Forest Virus Nonstructural Protein 2 Is Involved in Suppression of the Type I Interferon Response. *J. Virol.* 81, 8677–8684. doi:10.1128/JVI.02411-06.

Brnic, D., Stevanovic, V., Cochet, M., Agier, C., Richardson, J., Montero-Menei, C. N., et al. (2012). Borna Disease Virus Infects Human Neural Progenitor Cells and Impairs Neurogenesis. *J. Virol.* 86, 2512–2522. doi:10.1128/JVI.05663-11.

Broderick, J. A., Salomon, W. E., Ryder, S. P., Aronin, N., and Zamore, P. D. (2011). Argonaute protein identity and pairing geometry determine cooperativity in

mammalian RNA silencing. *RNA* 17, 1858–1869. doi:10.1261/rna.2778911.

Chandradoss, S. D., Schirle, N. T., Szczepaniak, M., MacRae, I. J., and Joo, C. (2015). A Dynamic Search Process Underlies MicroRNA Targeting. *Cell* 162, 96–107. doi:10.1016/j.cell.2015.06.032.

Cheloufi, S., Dos Santos, C. O., Chong, M. M. W., and Hannon, G. J. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* 465, 584–589. doi:10.1038/nature09092.

Chen, R., Mukhopadhyay, S., Merits, A., Bolling, B., Nasar, F., Coffey, L. L., et al. (2018). ICTV Virus Taxonomy Profile: Togaviridae. *J. Gen. Virol.* 99, 761–762. doi:10.1099/jgv.0.001072.

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., et al. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436, 740–4.

Chi, S. W., Hannon, G. J., and Darnell, R. B. (2012). An alternative mode of microRNA target recognition. *Nat. Struct. Mol. Biol.* 19, 321–327. doi:10.1038/nsmb.2230.

Chi, S. W., Zang, J. B., Mele, A., and Darnell, R. B. (2009). Argonaute HITS-CLIP decodes microRNA–mRNA interaction maps. *Nature* 460, 479–486. doi:10.1038/nature08170.

Cifuentes, D., Xue, H., Taylor, D. W., Patnode, H., Mishima, Y., Cheloufi, S., et al. (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* 328, 1694–1698. doi:10.1126/science.1190809.

Clark, A. M., Goldstein, L. D., Tevlin, M., Tavaré, S., Shaham, S., and Miska, E. A. (2010). The microRNA miR-124 controls gene expression in the sensory nervous system of Caenorhabditis elegans. *Nucleic Acids Res.* 38, 3780–3793. doi:10.1093/nar/gkq083.

Cohen, J. E., Lee, P. R., Chen, S., Li, W., and Fields, R. D. (2011). MicroRNA regulation of homeostatic synaptic plasticity. *Proc. Natl. Acad. Sci.* 108, 11650–11655. doi:10.1073/pnas.1017576108.

Conaco, C., Otto, S., Han, J.-J., and Mandel, G. (2006). Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc. Natl. Acad. Sci.* 103, 2422–2427. doi:10.1073/pnas.0511041103.

Contestabile, A. (2002). Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. *Cerebellum Lond. Engl.* 1, 41–55. doi:10.1080/147342202753203087.

Deng, X., Ma, L., Wu, M., Zhang, G., Jin, C., Guo, Y., et al. (2013). miR-124 radiosensitizes human glioma cells by targeting CDK4. *J. Neurooncol.* 114, 263–274. doi:10.1007/s11060-013-1179-2.

Deperasińska, I., Schulz, P., and Siwicki, A. K. (2018). Salmonid alphavirus (SAV). *J. Vet. Res.* 62, 1–6. doi:10.2478/jvetres-2018-0001.

Dubuisson, J., Lustig, S., Ruggli, N., Akov, Y., and Rice, C. M. (1997). Genetic determinants of Sindbis virus neuroinvasiveness. *J. Virol.* 71, 2636–2646.

Eichhorn, S. W., Guo, H., McGeary, S. E., Rodriguez-Mias, R. A., Shin, C., Baek, D., et al. (2014). mRNA Destabilization Is the Dominant Effect of Mammalian MicroRNAs by the Time Substantial Repression Ensues. *Mol. Cell* 56, 104–115. doi:10.1016/j.molcel.2014.08.028.

Eiden, M., Ziegler, U., Keller, M., Müller, K., Granzow, H., Jöst, H., et al. (2014). Isolation of sindbis virus from a hooded crow in Germany. *Vector Borne Zoonotic Dis. Larchmt. N* 14, 220–222. doi:10.1089/vbz.2013.1354.

Ender, C., Krek, A., Friedlander, M. R., Beitzinger, M., Weinmann, L., Chen, W., et al. (2008). A human snoRNA with microRNA-like functions. *Mol Cell* 32, 519–28.

Esquela-Kerscher, A., and Slack, F. J. (2006). Oncomirs — microRNAs with a role in cancer. *Nat. Rev. Cancer* 6, 259–269. doi:10.1038/nrc1840.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007a). P bodies: at the crossroads of post-transcriptional pathways. *Nat. Rev. Mol. Cell Biol.* 8, 9–22. doi:10.1038/nrm2080.

Eulalio, A., Behm-Ansmant, I., Schweizer, D., and Izaurralde, E. (2007b). P-body

formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol* 27, 3970–81.

Fang, W., and Bartel, D. P. (2020). MicroRNA Clustering Assists Processing of Suboptimal MicroRNA Hairpins through the Action of the ERH Protein. *Mol. Cell* 78, 289-302.e6. doi:10.1016/j.molcel.2020.01.026.

Fares, M., Cochet-Bernoin, M., Gonzalez, G., Montero-Menei, C. N., Blanchet, O., Benchoua, A., et al. (2020). Pathological modeling of TBEV infection reveals differential innate immune responses in human neurons and astrocytes that correlate with their susceptibility to infection. *J. Neuroinflammation* 17, 76. doi:10.1186/s12974-020-01756-x.

Firth, A. E., Chung, B. Y., Fleeton, M. N., and Atkins, J. F. (2008). Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. *Virol. J.* 5, 108. doi:10.1186/1743-422X-5-108.

Forrester, N. L., Palacios, G., Tesh, R. B., Savji, N., Guzman, H., Sherman, M., et al. (2012). Genome-Scale Phylogeny of the Alphavirus Genus Suggests a Marine Origin. *J. Virol.* 86, 2729–2738. doi:10.1128/JVI.05591-11.

Francy, D. B., Jaenson, T. G., Lundström, J. O., Schildt, E. B., Espmark, A., Henriksson, B., et al. (1989). Ecologic studies of mosquitoes and birds as hosts of Ockelbo virus in Sweden and isolation of Inkoo and Batai viruses from mosquitoes. *Am. J. Trop. Med. Hyg.* 41, 355–363.

Frank, F., Sonenberg, N., and Nagar, B. (2010). Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* 465, 818–822. doi:10.1038/nature09039.

Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19, 92–105. doi:gr.082701.108 [pii] 10.1101/gr.082701.108.

Frolov, I., Hardy, R., and Rice, C. M. (2001). Cis-acting RNA elements at the 5′ end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis. *RNA* 7, 1638–1651. doi:10.1017/S135583820101010X.

Frolova, E. I., Fayzulin, R. Z., Cook, S. H., Griffin, D. E., Rice, C. M., and Frolov, I. (2002). Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. *J. Virol.* 76, 11254–11264.

Frolova, E. I., Gorchakov, R., Pereboeva, L., Atasheva, S., and Frolov, I. (2010). Functional Sindbis Virus Replicative Complexes Are Formed at the Plasma Membrane. *J. Virol.* 84, 11679–11695. doi:10.1128/JVI.01441-10.

Garneau, N. L., Sokoloski, K. J., Opyrchal, M., Neff, C. P., Wilusz, C. J., and Wilusz, J. (2008). The 3' Untranslated Region of Sindbis Virus Represses Deadenylation of Viral Transcripts in Mosquito and Mammalian Cells. *J. Virol.* 82, 880–892. doi:10.1128/JVI.01205-07.

Gaudet, A. D., Fonken, L. K., Watkins, L. R., Nelson, R. J., and Popovich, P. G. (2018). MicroRNAs: Roles in Regulating Neuroinflammation. *The Neuroscientist* 24, 221–245. doi:10.1177/1073858417721150.

Gérardin, P., Couderc, T., Bintner, M., Tournebize, P., Renouil, M., Lémant, J., et al. (2016). Chikungunya virus–associated encephalitis: A cohort study on La Réunion Island, 2005–2009. *Neurology* 86, 94–102. doi:10.1212/WNL.0000000000002234.

Gonza´lez, M. E., and Carrasco, L. (2001). Human Immunodeficiency Virus Type 1 VPU Protein Affects Sindbis Virus Glycoprotein Processing and Enhances Membrane Permeabilization. *Virology* 279, 201–209. doi:10.1006/viro.2000.0708.

Gorchakov, R., Frolova, E., and Frolov, I. (2005). Inhibition of Transcription and Translation in Sindbis Virus-Infected Cells. *J. Virol.* 79, 9397–9409. doi:10.1128/JVI.79.15.9397-9409.2005.

Götte, B., Liu, L., and McInerney, G. (2018). The Enigmatic Alphavirus Non-Structural Protein 3 (nsP3) Revealing Its Secrets at Last. *Viruses* 10, 105. doi:10.3390/v10030105.

Gregory, R. I., Yan, K., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., et al. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432, 235–240. doi:10.1038/nature03120.

Grimson, A., Farh, K. K.-H., Johnston, W. K., Garrett-Engele, P., Lim, L. P., and Bartel,

D. P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell* 27, 91–105. doi:10.1016/j.molcel.2007.06.017.

Gruol, D. L., Koibuchi, N., Manto, M., Molinari, M., Schmahmann, J. D., and Shen, Y. eds. (2016). *Essentials of Cerebellum and Cerebellar Disorders*. Cham: Springer International Publishing doi:10.1007/978-3-319-24551-5.

Guo, Y. E., and Steitz, J. A. (2014). 3'-Biotin-tagged microRNA-27 does not associate with Argonaute proteins in cells. *RNA* 20, 985–988. doi:10.1261/rna.045054.114.

Ha, M., and Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* 15, 509–524. doi:10.1038/nrm3838.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., et al. (2010). Transcriptome-wide Identification of RNA-Binding Protein and MicroRNA Target Sites by PAR-CLIP. *Cell* 141, 129–141. doi:10.1016/j.cell.2010.03.009.

Han, S. M., Na, H. Y., Ham, O., Choi, W., Sohn, M., Ryu, S. H., et al. (2016). TCF4-Targeting miR-124 is Differentially Expressed amongst Dendritic Cell Subsets. *Immune Netw.* 16, 61. doi:10.4110/in.2016.16.1.61.

Hardy, R. W. (2006). The role of the 3′ terminus of the Sindbis virus genome in minus-strand initiation site selection. *Virology* 345, 520–531. doi:10.1016/j.virol.2005.10.018.

Hauptmann, J., Schraivogel, D., Bruckmann, A., Manickavel, S., Jakob, L., Eichner, N., et al. (2015). Biochemical isolation of Argonaute protein complexes by Ago-APP. *Proc. Natl. Acad. Sci.* 112, 11841–11845. doi:10.1073/pnas.1506116112.

Hefti, E., Bishop, D. H. L., Dubin, D. T., and Stollar, V. (1976). 5′ Nucleotide Sequence of Sindbis Viral RNA. *J. Virol.* 17, 149–159. doi:10.1128/JVI.17.1.149-159.1976.

Hermanns, K., Marklewitz, M., Zirkel, F., Overheul, G. J., Page, R. A., Loaiza, J. R., et al. (2020). Agua Salud alphavirus defines a novel lineage of insect-specific alphaviruses discovered in the New World. *J. Gen. Virol.* 101, 96–104. doi:10.1099/jgv.0.001344.

Hermanns, K., Zirkel, F., Kopp, A., Marklewitz, M., Rwego, I. B., Estrada, A., et al. (2017). Discovery of a novel alphavirus related to Eilat virus. *J. Gen. Virol.* 98, 43–49. doi:10.1099/jgv.0.000694.

Hesson, J. C., Verner-Carlsson, J., Larsson, A., Ahmed, R., Lundkvist, Å., and Lundström, J. O. (2015). Culex torrentium Mosquito Role as Major Enzootic Vector Defined by Rate of Sindbis Virus Infection, Sweden, 2009. *Emerg. Infect. Dis.* 21, 875–878. doi:10.3201/eid2105.141577.

Hou, Q., Ruan, H., Gilbert, J., Wang, G., Ma, Q., Yao, W.-D., et al. (2015). MicroRNA miR124 is required for the expression of homeostatic synaptic plasticity. *Nat. Commun.* 6, 10045. doi:10.1038/ncomms10045.

Hsu, P. W.-C., Lin, L.-Z., Hsu, S.-D., Hsu, J. B.-K., and Huang, H.-D. (2007). ViTa: prediction of host microRNAs targets on viruses. *Nucleic Acids Res.* 35, D381–D385. doi:10.1093/nar/gkl1009.

Hutvágner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293, 834–8.

Hyde, J. L., Chen, R., Trobaugh, D. W., Diamond, M. S., Weaver, S. C., Klimstra, W. B., et al. (2015). The 5′ and 3′ ends of alphavirus RNAs – Non-coding is not non-functional. *Virus Res.* 206, 99–107. doi:10.1016/j.virusres.2015.01.016.

Hyde, J. L., Gardner, C. L., Kimura, T., White, J. P., Liu, G., Trobaugh, D. W., et al. (2014). A Viral RNA Structural Element Alters Host Recognition of Nonself RNA. *Science* 343, 783–787. doi:10.1126/science.1248465.

Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., et al. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell* 39, 292–299. doi:10.1016/j.molcel.2010.05.015.

Jalava, K., Sane, J., Ollgren, J., Ruuhela, R., Rätti, O., Kurkela, S., et al. (2013). Climatic, ecological and socioeconomic factors as predictors of Sindbis virus infections in Finland. *Epidemiol. Infect.* 141, 1857–1866. doi:10.1017/S095026881200249X.

Jia, X., Wang, X., Guo, X., Ji, J., Lou, G., Zhao, J., et al. (2019). MicroRNA-124: An emerging therapeutic target in cancer. *Cancer Med.* 8, 5638–5650. doi:10.1002/cam4.2489.

Johnson, R. T., McFarland, H. F., and Levy, S. E. (1972). Age-dependent Resistance to Viral Encephalitis: Studies of Infections Due to Sindbis Virus in Mice. *J. Infect. Dis.* 125, 257–262. doi:10.1093/infdis/125.3.257.

Jolene Ramsey, and Suchetana Mukhopadhyay (2017). Disentangling the Frames, the State of Research on the Alphavirus 6K and TF Proteins. *Viruses* 9, 228. doi:10.3390/v9080228.

Jonas, S., and Izaurralde, E. (2015). Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* 16, 421–433. doi:10.1038/nrg3965.

Karra, D., and Dahm, R. (2010). Transfection Techniques for Neuronal Cells. *J. Neurosci.* 30, 6171–6177. doi:10.1523/JNEUROSCI.0183-10.2010.

Khvorova, A., Reynolds, A., and Jayasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115, 209–16.

Kielian, M., Chanel-Vos, C., and Liao, M. (2010). Alphavirus Entry and Membrane Fusion. *Viruses* 2, 796–825. doi:10.3390/v2040796.

Kim, D. Y., Firth, A. E., Atasheva, S., Frolova, E. I., and Frolov, I. (2011). Conservation of a Packaging Signal and the Viral Genome RNA Packaging Mechanism in Alphavirus Evolution. *J. Virol.* 85, 8022–8036. doi:10.1128/JVI.00644-11.

Knipe, D. M., and Howley, P. M. eds. (2013). *Fields virology*. 6th ed. Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Krichevsky, A. M., Sonntag, K.-C., Isacson, O., and Kosik, K. S. (2006). Specific MicroRNAs Modulate Embryonic Stem Cell-Derived Neurogenesis. *STEM CELLS* 24, 857–864. doi:10.1634/stemcells.2005-0441.

Kutsche, L. K., Gysi, D. M., Fallmann, J., Lenk, K., Petri, R., Swiersy, A., et al. (2018). Combined Experimental and System-Level Analyses Reveal the Complex Regulatory Network of miR-124 during Human Neurogenesis. *Cell Syst.* 7, 438-

452.e8. doi:10.1016/j.cels.2018.08.011.

La Linn, M., Gardner, J., Warrilow, D., Darnell, G. A., McMahon, C. R., Field, I., et al. (2001). Arbovirus of Marine Mammals: a New Alphavirus Isolated from the Elephant Seal Louse, Lepidophthirus macrorhini. *J. Virol.* 75, 4103–4109. doi:10.1128/JVI.75.9.4103-4109.2001.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* 294, 853–8.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. *Curr Biol* 12, 735–9.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129, 1401–14. doi:S0092-8674(07)00604-6 [pii] 10.1016/j.cell.2007.04.040.

Laneve, P., Di Marcotullio, L., Gioia, U., Fiori, M. E., Ferretti, E., Gulino, A., et al. (2007). The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. *Proc. Natl. Acad. Sci.* 104, 7957–7962. doi:10.1073/pnas.0700071104.

Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* 294, 858–62.

Lee, P., Knight, R., Smit, J. M., Wilschut, J., and Griffin, D. E. (2002). A Single Mutation in the E2 Glycoprotein Important for Neurovirulence Influences Binding of Sindbis Virus to Neuroblastoma Cells. *J. Virol.* 76, 6302–6310. doi:10.1128/JVI.76.12.6302-631-.2002.

Lee, R. C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. *Science* 294, 862–4.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843–54.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419. doi:10.1038/nature01957.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *Embo J* 23, 4051–60.

Lemm, J. A., Rümenapf, T., Strauss, E. G., Strauss, J. H., and Rice, C. M. (1994). Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J.* 13, 2925–2934. doi:10.1002/j.1460-2075.1994.tb06587.x.

Leung, J. Y.-S., Ng, M. M.-L., and Chu, J. J. H. (2011). Replication of Alphaviruses: A Review on the Entry Process of Alphaviruses into Cells. *Adv. Virol.* 2011, 1–9. doi:10.1155/2011/249640.

Levi, and Vignuzzi (2019). Arthritogenic Alphaviruses: A Worldwide Emerging Threat? *Microorganisms* 7, 133. doi:10.3390/microorganisms7050133.

Levis, R., Schlesinger, S., and Huang, H. V. (1990). Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* 64, 1726–1733. doi:10.1128/JVI.64.4.1726-1733.1990.

Lewis, B. P., Shih, I., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of Mammalian MicroRNA Targets. *Cell* 115, 787–798. doi:10.1016/S0092-8674(03)01018-3.

Lewis, J., Wesselingh, S. L., Griffin, D. E., and Hardwick, J. M. (1996). Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. *J. Virol.* 70, 1828–1835.

Lian, S. L., Li, S., Abadal, G. X., Pauley, B. A., Fritzler, M. J., and Chan, E. K. L. (2009). The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. *RNA* 15, 804–813. doi:10.1261/rna.1229409.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., et al. (2005a). Microarray analysis shows that some microRNAs downregulate large

numbers of target mRNAs. *Nature* 433, 769–73.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., et al. (2005b). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. doi:10.1038/nature03315.

Lin, S., and Gregory, R. I. (2015). MicroRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* 15, 321–333. doi:10.1038/nrc3932.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., et al. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305, 1437–41.

López, P., Girardi, E., Mounce, B. C., Weiss, A., Chane-Woon-Ming, B., Messmer, M., et al. (2020). High-throughput fluorescence-based screen identifies the neuronal microRNA miR-124 as a positive regulator of alphavirus infection. *J. Virol.*, JVI.02145-19, jvi;JVI.02145-19v1. doi:10.1128/JVI.02145-19.

Lou, C., Xiao, M., Cheng, S., Lu, X., Jia, S., Ren, Y., et al. (2016). MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1α expression. *Cell Death Dis.* 7, e2159–e2159. doi:10.1038/cddis.2016.27.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004). Nuclear export of microRNA precursors. *Science* 303, 95–8.

Lundström, J. O., Hesson, J. C., Schäfer, M. L., Östman, Ö., Semmler, T., Bekaert, M., et al. (2019). Sindbis virus polyarthritis outbreak signalled by virus prevalence in the mosquito vectors. *PLoS Negl. Trop. Dis.* 13, e0007702. doi:10.1371/journal.pntd.0007702.

Lundström, J. O., Lindström, K. M., Olsen, B., Dufva, R., and Krakower, D. S. (2001). Prevalence of Sindbis Virus Neutralizing Antibodies Among Swedish Passerines Indicates that Thrushes are the Main Amplifying Hosts. *J. Med. Entomol.* 38, 289–297. doi:10.1603/0022-2585-38.2.289.

Lustig, S., Jackson, A. C., Hahn, C. S., Griffin, D. E., Strauss, E. G., and Strauss, J. H. (1988). Molecular basis of Sindbis virus neurovirulence in mice. *J. Virol.* 62, 2329–2336.

Makeyev, E. V., Zhang, J., Carrasco, M. A., and Maniatis, T. (2007). The MicroRNA miR-124 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA Splicing. *Mol. Cell* 27, 435–448. doi:10.1016/j.molcel.2007.07.015.

Malherbe, H., Strickland-Cholmley, M., and Jackson, A. L. (1963). Sindbis virus infection in man. Report of a case with recovery of virus from skin lesions. *South Afr. Med. J. Suid-Afr. Tydskr. Vir Geneeskd.* 37, 547–552.

McGeary, S. E., Lin, K. S., Shi, C. Y., Pham, T. M., Bisaria, N., Kelley, G. M., et al. (2019). The biochemical basis of microRNA targeting efficacy. *Science* 366, eaav1741. doi:10.1126/science.aav1741.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 15, 185–197.

Mendes, A., and Kuhn, R. (2018). Alphavirus Nucleocapsid Packaging and Assembly. *Viruses* 10, 138. doi:10.3390/v10030138.

Miska, E. A., Alvarez-Saavedra, E., Abbott, A. L., Lau, N. C., Hellman, A. B., McGonagle, S. M., et al. (2007). Most Caenorhabditis elegans microRNAs Are Individually Not Essential for Development or Viability. *PLoS Genet.* 3, e215. doi:10.1371/journal.pgen.0030215.

Moore, M. J., Zhang, C., Gantman, E. C., Mele, A., Darnell, J. C., and Darnell, R. B. (2014). Mapping Argonaute and conventional RNA-binding protein interactions with RNA at single-nucleotide resolution using HITS-CLIP and CIMS analysis. *Nat. Protoc.* 9, 263–293. doi:10.1038/nprot.2014.012.

Mukherjee, N., Corcoran, D. L., Nusbaum, J. D., Reid, D. W., Georgiev, S., Hafner, M., et al. (2011). Integrative Regulatory Mapping Indicates that the RNA-Binding Protein HuR Couples Pre-mRNA Processing and mRNA Stability. *Mol. Cell* 43, 327–339. doi:10.1016/j.molcel.2011.06.007.

Nasar, F., Palacios, G., Gorchakov, R. V., Guzman, H., Da Rosa, A. P. T., Savji, N., et al. (2012). Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc. Natl. Acad. Sci.* 109, 14622–14627.

doi:10.1073/pnas.1204787109.

Nguyen, T. A., Jo, M. H., Choi, Y.-G., Park, J., Kwon, S. C., Hohng, S., et al. (2015). Functional Anatomy of the Human Microprocessor. *Cell* 161, 1374–1387. doi:10.1016/j.cell.2015.05.010.

Nielsen, C. B., Shomron, N., Sandberg, R., Hornstein, E., Kitzman, J., and Burge, C. B. (2007). Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. *RNA* 13, 1894–1910. doi:10.1261/rna.768207.

Nonne, N., Ameyar-Zazoua, M., Souidi, M., and Harel-Bellan, A. (2010). Tandem affinity purification of miRNA target mRNAs (TAP-Tar). *Nucleic Acids Res.* 38, e20–e20. doi:10.1093/nar/gkp1100.

Orom, U. A., and Lund, A. H. (2007). Isolation of microRNA targets using biotinylated synthetic microRNAs. *Methods San Diego Calif* 43, 162–165. doi:10.1016/j.ymeth.2007.04.007.

Ou, J. H., Rice, C. M., Dalgarno, L., Strauss, E. G., and Strauss, J. H. (1982). Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. *Proc. Natl. Acad. Sci.* 79, 5235–5239. doi:10.1073/pnas.79.17.5235.

Parrott, M. M., Sitarski, S. A., Arnold, R. J., Picton, L. K., Hill, R. B., and Mukhopadhyay, S. (2009). Role of Conserved Cysteines in the Alphavirus E3 Protein. *J. Virol.* 83, 2584–2591. doi:10.1128/JVI.02158-08.

Pasquinelli, A. E. (2012). MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat. Rev. Genet.* 13, 271–282. doi:10.1038/nrg3162.

Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., et al. (2000). Conservation of the sequence of let-7 heterochronic regulatory RNA. *Nature* 408, 86–89.

Passoni, G., Langevin, C., Palha, N., Mounce, B. C., Briolat, V., Affaticati, P., et al. (2017). Imaging of viral neuroinvasion in the zebrafish reveals that Sindbis and chikungunya viruses favour different entry routes. *Dis. Model. Mech.* 10, 847–857.

doi:10.1242/dmm.029231.

Periyasamy, P., Thangaraj, A., Guo, M.-L., Hu, G., Callen, S., and Buch, S. (2018). Epigenetic Promoter DNA Methylation of miR-124 Promotes HIV-1 Tat-Mediated Microglial Activation via MECP2-STAT3 Axis. *J. Neurosci.* 38, 5367–5383. doi:10.1523/JNEUROSCI.3474-17.2018.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grässer, F. A., et al. (2005). Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2, 269–276. doi:10.1038/nmeth746.

Pierson, J., Hostager, B., Fan, R., and Vibhakar, R. (2008). Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. *J. Neurooncol.* 90, 1–7. doi:10.1007/s11060-008-9624-3.

Ponomarev, E. D., Veremeyko, T., Barteneva, N., Krichevsky, A. M., and Weiner, H. L. (2011). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-α–PU.1 pathway. *Nat. Med.* 17, 64–70. doi:10.1038/nm.2266.

Qin, Z., Wang, P.-Y., Su, D.-F., and Liu, X. (2016). miRNA-124 in Immune System and Immune Disorders. *Front. Immunol.* 7. doi:10.3389/fimmu.2016.00406.

Ramsey, J., Renzi, E. C., Arnold, R. J., Trinidad, J. C., and Mukhopadhyay, S. (2017). Palmitoylation of Sindbis Virus TF Protein Regulates Its Plasma Membrane Localization and Subsequent Incorporation into Virions. *J. Virol.* 91, e02000-16, /jvi/91/3/e02000-16.atom. doi:10.1128/JVI.02000-16.

Reeves, W. C., Hammon, W. Mcd., Furman, D. P., Mcclure, H. E., and Brookman, B. (1947). Recovery of Western Equine Encephalomyelitis Virus From Wild Bird Mites (Liponyssus sylviarum) in Kern County, California. *Science* 105, 411–412. doi:10.1126/science.105.2729.411.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403, 901–6.

Ronca, S. E., Dineley, K. T., and Paessler, S. (2016). Neurological Sequelae Resulting from

Encephalitic Alphavirus Infection. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.00959.

Rose, P. P., Hanna, S. L., Spiridigliozzi, A., Wannissorn, N., Beiting, D. P., Ross, S. R., et al. (2011). Natural Resistance-Associated Macrophage Protein Is a Cellular Receptor for Sindbis Virus in Both Insect and Mammalian Hosts. *Cell Host Microbe* 10, 97–104. doi:10.1016/j.chom.2011.06.009.

Rothan, H. A., Bahrani, H., Mohamed, Z., Teoh, T. C., Shankar, E. M., Rahman, N. A., et al. (2015). A Combination of Doxycycline and Ribavirin Alleviated Chikungunya Infection. *PLOS ONE* 10, e0126360. doi:10.1371/journal.pone.0126360.

Rothan, H. A., Mohamed, Z., Paydar, M., Rahman, N. A., and Yusof, R. (2014). Inhibitory effect of doxycycline against dengue virus replication in vitro. *Arch. Virol.* 159, 711–718. doi:10.1007/s00705-013-1880-7.

Rubach, J. K., Wasik, B. R., Rupp, J. C., Kuhn, R. J., Hardy, R. W., and Smith, J. L. (2009). Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology* 384, 201–208. doi:10.1016/j.virol.2008.10.030.

Ruby, J. G., Jan, C. H., and Bartel, D. P. (2007). Intronic microRNA precursors that bypass Drosha processing. *Nature* 448, 83–6.

Rupaimoole, R., and Slack, F. J. (2017). MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat. Rev. Drug Discov.* 16, 203–222. doi:10.1038/nrd.2016.246.

Rupp, J. C., Sokoloski, K. J., Gebhart, N. N., and Hardy, R. W. (2015). Alphavirus RNA synthesis and non-structural protein functions. *J. Gen. Virol.* 96, 2483–2500. doi:10.1099/jgv.0.000249.

Saetrom, P., Heale, B. S. E., Snøve, O., Aagaard, L., Alluin, J., and Rossi, J. J. (2007). Distance constraints between microRNA target sites dictate efficacy and cooperativity. *Nucleic Acids Res.* 35, 2333–2342. doi:10.1093/nar/gkm133.

Salimi, H., Cain, M. D., Jiang, X., Roth, R. A., Beatty, W. L., Sun, C., et al. (2020). Encephalitic Alphaviruses Exploit Caveola-Mediated Transcytosis at the Blood-Brain Barrier for Central Nervous System Entry. *mBio* 11, e02731-19,

/mbio/11/1/mBio.02731-19.atom. doi:10.1128/mBio.02731-19.

Santos, M. C. T., Tegge, A. N., Correa, B. R., Mahesula, S., Kohnke, L. Q., Qiao, M., et al. (2016). miR-124, -128, and -137 Orchestrate Neural Differentiation by Acting on Overlapping Gene Sets Containing a Highly Connected Transcription Factor Network: miRNA-Transcription Factor Networks in Neural Differentiation. *STEM CELLS* 34, 220–232. doi:10.1002/stem.2204.

Sanuki, R., Onishi, A., Koike, C., Muramatsu, R., Watanabe, S., Muranishi, Y., et al. (2011). miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nat. Neurosci.* 14, 1125–1134. doi:10.1038/nn.2897.

Schafer, A., Brooke, C. B., Whitmore, A. C., and Johnston, R. E. (2011). The Role of the Blood-Brain Barrier during Venezuelan Equine Encephalitis Virus Infection. *J. Virol.* 85, 10682–10690. doi:10.1128/JVI.05032-11.

Scheel, T. K. H., Luna, J. M., Liniger, M., Nishiuchi, E., Rozen-Gagnon, K., Shlomai, A., et al. (2016). A Broad RNA Virus Survey Reveals Both miRNA Dependence and Functional Sequestration. *Cell Host Microbe* 19, 409–423. doi:10.1016/j.chom.2016.02.007.

Schnall-Levin, M., Zhao, Y., Perrimon, N., and Berger, B. (2010). Conserved microRNA targeting in Drosophila is as widespread in coding regions as in 3'UTRs. *Proc. Natl. Acad. Sci.* 107, 15751–15756. doi:10.1073/pnas.1006172107.

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199-208.

Shang, R., Baek, S. C., Kim, K., Kim, B., Kim, V. N., and Lai, E. C. (2020). Genomic Clustering Facilitates Nuclear Processing of Suboptimal Pri-miRNA Loci. *Mol. Cell* 78, 303-316.e4. doi:10.1016/j.molcel.2020.02.009.

Shin, C., Nam, J.-W., Farh, K. K.-H., Chiang, H. R., Shkumatava, A., and Bartel, D. P. (2010). Expanding the microRNA targeting code: functional sites with centered pairing. *Mol. Cell* 38, 789–802. doi:10.1016/j.molcel.2010.06.005.

Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R., and Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7

regulatory RNA and the LIN-29 transcription factor. *Mol Cell* 5, 659–69.

Smibert, P., Yang, J.-S., Azzam, G., Liu, J.-L., and Lai, E. C. (2013). Homeostatic control of Argonaute stability by microRNA availability. *Nat. Struct. Mol. Biol.* 20, 789–795. doi:10.1038/nsmb.2606.

Smith, P., Al Hashimi, A., Girard, J., Delay, C., and Hébert, S. S. (2011). In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs: APP splicing is regulated by microRNAs. *J. Neurochem.* 116, 240–247. doi:10.1111/j.1471-4159.2010.07097.x.

Snyder, J. E., Kulcsar, K. A., Schultz, K. L. W., Riley, C. P., Neary, J. T., Marr, S., et al. (2013). Functional Characterization of the Alphavirus TF Protein. *J. Virol.* 87, 8511–8523. doi:10.1128/JVI.00449-13.

Sokoloski, K. J., Dickson, A. M., Chaskey, E. L., Garneau, N. L., Wilusz, C. J., and Wilusz, J. (2010). Sindbis Virus Usurps the Cellular HuR Protein to Stabilize Its Transcripts and Promote Productive Infections in Mammalian and Mosquito Cells. *Cell Host Microbe* 8, 196–207. doi:10.1016/j.chom.2010.07.003.

Song, J. J., Smith, S. K., Hannon, G. J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* 305, 1434–7.

Sonntag, K.-C. (2010). MicroRNAs and deregulated gene expression networks in neurodegeneration. *Brain Res.* 1338, 48–57. doi:10.1016/j.brainres.2010.03.106.

Soreq, H., and Wolf, Y. (2011). NeurimmiRs: microRNAs in the neuroimmune interface. *Trends Mol. Med.* 17, 548–555. doi:10.1016/j.molmed.2011.06.009.

Spuul, P., Salonen, A., Merits, A., Jokitalo, E., Kääriäinen, L., and Ahola, T. (2007). Role of the Amphipathic Peptide of Semliki Forest Virus Replicase Protein nsP1 in Membrane Association and Virus Replication. *J. Virol.* 81, 872–883. doi:10.1128/JVI.01785-06.

Standart, N., and Weil, D. (2018). P-Bodies: Cytosolic Droplets for Coordinated mRNA Storage. *Trends Genet.* 34, 612–626. doi:10.1016/j.tig.2018.05.005.

Stark, A., Brennecke, J., Bushati, N., Russell, R. B., and Cohen, S. M. (2005). Animal

MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell* 123, 1133–46.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* 58, 491–562.

Sun, K., Westholm, J. O., Tsurudome, K., Hagen, J. W., Lu, Y., Kohwi, M., et al. (2012). Neurophysiological Defects and Neuronal Gene Deregulation in Drosophila mir-124 Mutants. *PLoS Genet.* 8, e1002515. doi:10.1371/journal.pgen.1002515.

Sun, Y., Li, Q., Gui, H., Xu, D.-P., Yang, Y.-L., Su, D.-F., et al. (2013). MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. *Cell Res.* 23, 1270–1283. doi:10.1038/cr.2013.116.

Sun, Y., Luo, Z.-M., Guo, X.-M., Su, D.-F., and Liu, X. (2015). An updated role of microRNA-124 in central nervous system disorders: a review. *Front. Cell. Neurosci.* 9. doi:10.3389/fncel.2015.00193.

Suthar, M. S., Shabman, R., Madric, K., Lambeth, C., and Heise, M. T. (2005). Identification of adult mouse neurovirulence determinants of the Sindbis virus strain AR86. *J. Virol.* 79, 4219–4228. doi:10.1128/JVI.79.7.4219-4228.2005.

Taksdal, T., Bang Jensen, B., Böckerman, I., McLoughlin, M. F., Hjortaas, M. J., Ramstad, A., et al. (2015). Mortality and weight loss of Atlantic salmon, *Salmon salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. *J. Fish Dis.* 38, 1047–1061. doi:10.1111/jfd.12312.

Tang, J., Jose, J., Chipman, P., Zhang, W., Kuhn, R. J., and Baker, T. S. (2011). Molecular Links between the E2 Envelope Glycoprotein and Nucleocapsid Core in Sindbis Virus. *J. Mol. Biol.* 414, 442–459. doi:10.1016/j.jmb.2011.09.045.

Taylor, R. M., Hurlbut, H. S., Work, T. H., Kingston, J. R., and Frothingham, T. E. (1955). Sindbis Virus: A Newly Recognized Arthropod-Transmitted Virus 1. *Am. J. Trop. Med. Hyg.* 4, 844–862. doi:10.4269/ajtmh.1955.4.844.

Torii, S., Orba, Y., Hang'ombe, B. M., Mweene, A. S., Wada, Y., Anindita, P. D., et al. (2018). Discovery of Mwinilunga alphavirus: A novel alphavirus in Culex

mosquitoes in Zambia. *Virus Res.* 250, 31–36. doi:10.1016/j.virusres.2018.04.005.

Treiber, T., Treiber, N., and Meister, G. (2019). Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. *Nat. Rev. Mol. Cell Biol.* 20, 5–20. doi:10.1038/s41580-018-0059-1.

Trent, D. W., Butenko, A. M., Vladimirtseva, E. A., Lvov, D. K., Calisher, C. H., and Karabatsos, N. (1988). Identity of Karelian Fever and Ockelbo Viruses Determined by Serum Dilution-Plaque Reduction Neutralization Tests and Oligonucleotide Mapping. *Am. J. Trop. Med. Hyg.* 39, 607–610. doi:10.4269/ajtmh.1988.39.607.

Trobaugh, D. W., Gardner, C. L., Sun, C., Haddow, A. D., Wang, E., Chapnik, E., et al. (2014). RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. *Nature* 506, 245–248. doi:10.1038/nature12869.

Tucker, P. C., Strauss, E. G., Kuhn, R. J., Strauss, J. H., and Griffin, D. E. (1993). Viral determinants of age-dependent virulence of Sindbis virus for mice. *J. Virol.* 67, 4605–4610.

van der Ree, M. H., de Vree, J. M., Stelma, F., Willemse, S., van der Valk, M., Rietdijk, S., et al. (2017). Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. *The Lancet* 389, 709–717. doi:10.1016/S0140-6736(16)31715-9.

van Rooij, E., Sutherland, L. B., Qi, X., Richardson, J. A., Hill, J., and Olson, E. N. (2007). Control of Stress-Dependent Cardiac Growth and Gene Expression by a MicroRNA. *Science* 316, 575–579. doi:10.1126/science.1139089.

Vasiljeva, L., Valmu, L., Kääriäinen, L., and Merits, A. (2001). Site-specific Protease Activity of the Carboxyl-terminal Domain of Semliki Forest Virus Replicase Protein nsP2. *J. Biol. Chem.* 276, 30786–30793. doi:10.1074/jbc.M104786200.

Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., et al. (2007). Two Chikungunya Isolates from the Outbreak of La Reunion (Indian Ocean) Exhibit Different Patterns of Infection in the Mosquito, Aedes albopictus. *PLoS ONE* 2, e1168. doi:10.1371/journal.pone.0001168.

Vénien-Bryan, C., and Fuller, S. D. (1994). The Organization of the Spike Complex of

Semliki Forest Virus. *J. Mol. Biol.* 236, 572–583. doi:10.1006/jmbi.1994.1166.

Ventoso, I. (2012). Adaptive changes in alphavirus mRNA translation allowed colonization of vertebrate hosts. *J. Virol.* 86, 9484–9494. doi:10.1128/JVI.01114-12.

Vieira, M. S., Santos, A. K., Vasconcellos, R., Goulart, V. A. M., Parreira, R. C., Kihara, A. H., et al. (2018). Neural stem cell differentiation into mature neurons: Mechanisms of regulation and biotechnological applications. *Biotechnol. Adv.* 36, 1946–1970. doi:10.1016/j.biotechadv.2018.08.002.

Vogel, P., Kell, W. M., Fritz, D. L., Parker, M. D., and Schoepp, R. J. (2005). Early Events in the Pathogenesis of Eastern Equine Encephalitis Virus in Mice. *Am. J. Pathol.* 166, 159–171. doi:10.1016/S0002-9440(10)62241-9.

Weaver, S. C., Chen, R., and Diallo, M. (2020). Chikungunya Virus: Role of Vectors in Emergence from Enzootic Cycles. *Annu. Rev. Entomol.* 65, 313–332. doi:10.1146/annurev-ento-011019-025207.

Weaver, S. C., Hagenbaugh, A., Bellew, L. A., Netesov, S. V., Volchkov, V. E., Chang, G.-J. J., et al. (1993). A Comparison of the Nucleotide Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related RNA Viruses. *Virology* 197, 375–390. doi:10.1006/viro.1993.1599.

Wei, J., Wang, F., Kong, L.-Y., Xu, S., Doucette, T., Ferguson, S. D., et al. (2013). miR-124 Inhibits STAT3 Signaling to Enhance T Cell–Mediated Immune Clearance of Glioma. *Cancer Res.* 73, 3913–3926. doi:10.1158/0008-5472.CAN-12-4318.

Weiss, B., Nitschko, H., Ghattas, I., Wright, R., and Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J. Virol.* 63, 5310–5318. doi:10.1128/JVI.63.12.5310-5318.1989.

Wengler, G., Würkner, D., and Wengler, G. (1992). Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. *Virology* 191, 880–888. doi:10.1016/0042-6822(92)90263-O.

Winter, J. (2015). MicroRNAs of the miR379-410 cluster: New players in embryonic

neurogenesis and regulators of neuronal function. *Neurogenesis Austin Tex* 2, e1004970. doi:10.1080/23262133.2015.1004970.

Winter, J., and Diederichs, S. (2011). Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. *RNA Biol.* 8, 1149–1157. doi:10.4161/rna.8.6.17665.

Wu, J., Qian, J., Li, C., Kwok, L., Cheng, F., Liu, P., et al. (2010). miR-129 regulates cell proliferation by downregulating Cdk6 expression. *Cell Cycle* 9, 1809–1818. doi:10.4161/cc.9.9.11535.

Yang, J.-S., Maurin, T., Robine, N., Rasmussen, K. D., Jeffrey, K. L., Chandwani, R., et al. (2010). Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proc. Natl. Acad. Sci.* 107, 15163–15168. doi:10.1073/pnas.1006432107.

Yekta, S., Shih, I. H., and Bartel, D. P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 304, 594–6.

Yeom, K.-H., Mitchell, S., Linares, A. J., Zheng, S., Lin, C.-H., Wang, X.-J., et al. (2018). Polypyrimidine tract-binding protein blocks miRNA-124 biogenesis to enforce its neuronal-specific expression in the mouse. *Proc. Natl. Acad. Sci.* 115, E11061–E11070. doi:10.1073/pnas.1809609115.

Yoda, M., Cifuentes, D., Izumi, N., Sakaguchi, Y., Suzuki, T., Giraldez, A. J., et al. (2013). Poly(A)-Specific Ribonuclease Mediates 3′-End Trimming of Argonaute2-Cleaved Precursor MicroRNAs. *Cell Rep.* 5, 715–726. doi:10.1016/j.celrep.2013.09.029.

Zacks, M. A., and Paessler, S. (2010). Encephalitic alphaviruses. *Vet. Microbiol.* 140, 281–286. doi:10.1016/j.vetmic.2009.08.023.

Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E., and Filipowicz, W. (2004). Single Processing Center Models for Human Dicer and Bacterial RNase III. *Cell* 118, 57–68. doi:10.1016/j.cell.2004.06.017.

Zhang, R., Kim, A. S., Fox, J. M., Nair, S., Basore, K., Klimstra, W. B., et al. (2018). Mxra8 is a receptor for multiple arthritogenic alphaviruses. *Nature* 557, 570–574. doi:10.1038/s41586-018-0121-3.

Zhou, Z., Lv, J., Wang, J., Yu, H., Lu, H., Yuan, B., et al. (2019). Role of MicroRNA-124 as a Prognostic Factor in Multiple Neoplasms: A Meta-Analysis. *Dis. Markers* 2019, 1–12. doi:10.1155/2019/1654780.

Résumé de thèse en français

Les microARN (miARN) sont des petits ARN non-codants qui servent de guides aux protéines de la famille Argonaute pour cibler des ARN messagers et ainsi réguler leur expression en inhibant leur traduction et en induisant leur déstabilisation. Les miARN sont impliqués dans la régulation d'un grand nombre de processus cellulaires et ils peuvent également participer aux interactions hôte-pathogène.

Ce projet de recherche s'intéresse à la régulation de virus par des miARN cellulaires. Le virus choisi comme modèle d'étude est le virus Sindbis (SINV), appartenant au genre des alphavirus. SINV possède un génome d'ARN simple brin de polarité positive structuré avec deux ORF, le premier codant pour les protéines non structurales impliquées dans la réplication virale et expression d'ARN viraux, et le deuxième codant pour les protéines structurales formant les particules virales nouvellement produites. SINV est transmis par les moustiques aux hôtes vertébrés y compris l'homme. En termes de tropisme, chez l'homme ce virus infecte principalement les cellules de la peau, muscles ou articulations alors que chez la souris, il est capable d'atteindre le système nerveux et ainsi causer des troubles neurologiques comme des encéphalites.

Afin d'identifier les miARN impliqués dans la régulation de l'infection par SINV, un crible à haut débit a été réalisé en surexprimant des miARN cellulaires à l'aide de mimiques ou bien en les bloquant à l'aide d'oligonucléotides antisens dans des cellules de mammifère infectées par une souche de SINV modifiée génétiquement pour exprimer la protéine fluorescente GFP. Nous avons ainsi pu mettre en évidence la régulation du virus par certains miARN, dont le miR-124, un miARN exprimé spécifiquement dans le système nerveux. De manière intéressante, ce miARN semble favoriser la production de virus lors de l'infection par SINV. Le but du projet de thèse a été de caractériser les mécanismes moléculaires impliqués afin de comprendre comment s'effectue la régulation du cycle viral par les miARN cellulaires.

Premièrement, nous avons pu valider l'effet positif sur SINV du premier candidat identifié sur le crible, le miARN neuronal miR-124, en quantifiant la production de particules virales après surexpression par des mimiques dans des cellules Huh7.5.1. De plus, cet effet a également été vérifié sur un autre membre des alphavirus, le virus Chikungunya. Les mêmes analyses ont été menées sur deux souches du virus Zika, appartenant à une autre famille virale, sans observation du même phénotype indiquant qu'il s'agit d'un effet spécifique aux alphavirus.

Ensuite, nous avons focalisé nos efforts sur l'étude des étapes du cycle viral régulées par miR-124 à l'aide de transfection de mimiques dans des cellules Huh7.5.1. Nous avons pu observer que ni l'entrée, ni la réplication virale ou l'expression des ARN viraux n'étaient affectés. Par contre, en présence de miR-124 nous avons observé une augmentation de la synthèse des protéines virales ainsi qu'une augmentation de la production virale.

Afin de s'affranchir de l'étape de transfection pour surexprimer le miARN pour la suite des expériences, nous avons généré et mis en place différentes lignées cellulaires stables exprimant le miARN sauvage. Premièrement, nous avons généré des cellules HEK293T qui expriment miR-124 à partir d'un promoteur inductible sous contrôle de doxycycline, pour lequel on a pu confirmer l'expression de miR-124 de façon doxycycline dose-dépendante. Cependant, nous avons rencontré des soucis de reproductibilité avec les expériences d'infection dans ce système, liés probablement à l'effet de la doxycycline comme molécule antivirale. Nous avons donc testé d'autres systèmes ou miR-124 est exprimé de façon endogène afin d'utiliser les oligonucléotides antisens pour inhiber le miARN, notamment des cultures primaires de cellules granulaires de cervelet de rat ou la lignée cellulaire humaine de neuroblastome SK-N-BE(2).

Nous avons pu bloquer efficacement miR-124 dans des cultures primaires de cellules en grain de cervelet de rat mais l'infection de ces cellules par SINV n'était pas productive. De plus, ces cellules sont plus difficiles à manipuler et ces expériences requièrent encore des mises au point pour avoir une infection optimale. La lignée cellulaire de neuroblastome humain, SK-N-BE(2), peut être différenciée en neurones suite au traitement à l'acide rétinoïque. Nous avons ainsi réussi à différencier cette lignée

avec succès ce qui nous a permis par la suite de valider l'effet de miR-124 sur SINV. L'inhibition du miARN dans cette lignée par un oligonucléotide antisens a résulté dans l'inhibition du virus, observé par une diminution de la production de protéines structurales ainsi que de particules virales.

Enfin, afin de mieux étudier le mécanisme moléculaire entrainant cette régulation, nous avons généré par transduction lentivirale une lignée stable Huh7.5.1 exprimant miR-124 de façon constitutive (Huh124). Sur cette lignée, plus facile à manipuler que la lignée de neuroblastome, nous avons validé un des sites ciblés par miR-124 sur le génome viral prédit bioinformatiquement. Il s'agit d'une séquence au sein du deuxième ORF du génome viral et sur la phase codante des ARN subgénomiques, correspondant à la glycoprotéine de surface E1. Cette région est assez conservée parmi les alphavirus arthritogéniques, et nous avons pu trouver la même séquence cible de miR-124 partiellement présente dans le génome de CHIKV.

Pour valider ce site nous avons produit un virus SINV mutant (SINV-mut) en introduisant des mutations silencieuses pour modifier la séquence complémentaire au miRNA sur le génome sans perturber la séquence codante des protéines virales. Des expériences de cinétique nous ont permis de montrer que SINV-mut se réplique correctement comparé à SINV. L'inhibition de miR-124 avec l'oligonucléotide antisens sur la lignée Huh124 résulte en une diminution du titre des particules virales et de l'accumulation des protéines virales produites par SINV. Cependant, SINV-mut n'est pas affecté, les titres viraux des particules produites et accumulation de protéines virales restent inchangés. Des expériences similaires menées avec CHIKV nous ont permis valider également l'effet de miR-124 sur ce virus. Finalement, nous avons pu montrer l'importance de ce site par compensation avec une mimique de miR-124 mutant pouvant se lier à la séquence virale de SINV-mut qui restaure de façon dose-dépendante la régulation par le miARN.

Dans le but d'identifier d'autres ARN cibles de miR-124, cellulaires ou virales, pouvant être également impliqués dans cette régulation, nous avons mis au point au

laboratoire la technique CLEAR-CLIP (*covalent ligation of endogenous Argonaute-bound RNAs–crosslink immunoprecipitation*). Cette technique est basée sur l'immunoprécipitation des protéines Argonaute chargées avec tous les miARN exprimés dans les cellules et leurs cibles, et l'identification de ces dernières par séquençage à haut débit. Afin d'identifier les cibles spécifiques de miR-124 en contexte d'infection par SINV nous avons utilisé la lignée HEK293T exprimant miR-124 suite au traitement par doxycycline. Les résultats de cette expérience sont très prometteurs, en effet nous avons pu valider notre méthode grâce à l'identification d'ARNm déjà décrits comme cibles de miR-124. De plus, d'autres séquences correspondantes au génome viral ont été identifiées, indiquant une possible régulation de façon directe sur les ARN viraux, y compris les différents sites identifiés bioinformatiquement. Cependant, il s'agit d'une expérience qui nécessite des réplicats biologiques pour confirmer nos premières observations et pouvoir étudier également les cibles cellulaires de miR-124, potentiellement impliquées dans la régulation positive du cycle viral de SINV.

En conclusion, cette thèse a permis de mettre en évidence d'une part l'implication de miR-124 dans la régulation de l'infection par les alphavirus CHIKV et SINV, et d'autre part l'identification et validation d'un site de fixation de miR-124 sur le génome viral de SINV. Ainsi, ces travaux ont pu apporter des nouvelles connaissances qui permettront de mieux comprendre le rôle des miARN dans la régulation des infections virales. L'étude du mécanisme mis en place suite à la fixation du miARN sur les ARN viraux et cellulaires permettra d'éclaircir les connaissances sur l'infection du système nerveux par les alphavirus dans le contexte physiologique afin de pouvoir envisager des nouvelles approches antivirales, plus particulièrement en utilisant miR-124 comme potentielle cible thérapeutique pour proposer des nouvelles stratégies de lutte antivirale.

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Étude du mécanisme de régulation des alphavirus par les microARN cellulaires

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Résumé

Les microARN (miARN) sont des petits ARN non-codants qui agissent comme des régulateurs post-transcriptionnels de l'expression de gènes en ciblant les ARN messagers. Ils sont impliqués dans la régulation d'un grand nombre de processus cellulaires et peuvent également participer aux interactions hôte-pathogène. Ce projet de recherche s'intéresse à la régulation par des miARN cellulaires de virus, plus particulièrement les alphavirus comme les virus de Sindbis ou chikungunya. Au cours de cette thèse j'ai pu mettre en évidence l'implication de miR-124 comme régulateur positif de l'infection. Différentes approches et outils ont été mis en place et utilisés afin d'élucider les mécanismes moléculaires impliqués dans cette régulation du cycle viral. Ainsi, j'ai pu identifier une régulation directe par ciblage des ARN viraux. Finalement cette thèse apportera des nouvelles connaissances qui permettront de mieux comprendre le rôle des miARN dans la régulation des infections virales.

Mots clés : microARN, miR-124, interaction hôte-pathogène, infection virale, alphavirus, virus de Sindbis

Résumé en anglais

MicroRNAs (miRNA) are small non-coding RNAs that act as post-transcriptional regulators of gene expression. They are involved in a wide range of cellular processes including host-pathogen interactions. This project focuses on the study of miRNA regulation of viral infection, particularly of alphaviruses like Sindbis virus or chikungunya virus. During this thesis project, I identified miR-124 as a positive regulator of viral infection in different systems expressing the miRNA. In addition, I established and applied different approaches with the aim of identifying the molecular mechanisms behind this regulation. We thus described a novel role for miR-124 in alphavirus infection by targeting viral RNAs. Finally, this thesis will provide new insights into the role of microRNAs in the regulation of viral infection.

Keywords : microRNA, miR-124, host-pathogen interaction, viral infection, alphaviruses, Sindbis virus