# Circular RNAs: from host RNA molecules to novel broad-spectrum antivirals

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*A la iaia Conxita,*

*Vida i més vida*
*convertida en pregunta*
*que cal respondre*
*fent l'esforç de no perdre*
*ni el goig ni l'esperança*

*Miquel Martí i Pol*

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# ABSTRACT

The clinical importance of the mosquito-borne viruses, such as dengue virus (DENV), zika virus (ZIKV) chikungunya virus (CHIKV) and West Nile virus (WNV), has dramatically increased over the last years, resulting in a global health problem. Currently, there are no available treatments or effective vaccines to treat these infections. All these viruses produce acute infections that require to be treated early after the onset of the symptoms for drugs to be effective. However, an early diagnosis remains still as an unsolved challenge. This brings to the spotlight the need to uncover novel fundamental virus-cell interactionsthat could be targeted and to develop efficient broad-spectrum antiviral therapies that could be administered before an accurate diagnosis is achieved. In this thesis we addressed these two major concerns with a focus in circular RNAs (circRNAs).

CircRNAs are a class of RNAs generated from linear RNA progenitors by an alternative splicing mechanism termed back splicing. They are highly stable relative to their linear spliced counterparts due to exonuclease resistance. Currently, cellular circRNAs are described to be involved in viral infections. However, their precise role is mainly unknown. The first chapter of the thesis addresses this intriguing issue using HCV as a model system and analyzing the effect of the identified circRNAs in mosquito-borne viruses that belong to the same viral group. By RNA-Seq analyses we identified 73 HCV-differentially expressed circRNAs whose changes could not be explained by parallel changes in linear mRNAs. Silencing of five selected HCV-induced up-regulated circRNAs altered viral infectivity, acting either as anti-viral or pro-viral molecules. Further characterization of one of the selected circRNAs, cPSD3, show, that it also impaired DENV infections.

The second chapter focuses on the generation of a novel circRNA-based platform that is versatile, hampers the emergence of resistant mutants, and allows developing broad-spectrum antivirals. In contrast to other RNA-based therapies, circRNAs are highly stable molecules, a trait that will simplify their therapeutic use. The designed synthetic circRNAs contain long sequences that hybridize to multiple target sequences in the viral RNA genome involved in forming RNA structures essential for virus survival. As a proof of concept, we

have successfully validated circRNAs that inhibit HCV, DENV, CHIKV or WNV. Furthermore, we have generated circRNAs with broad-spectrum antiviral capacity and optimized the production *in vitro* of these molecules to obtain high amounts at low price.

In conclusion, our results (i) emphasize the complexity of the interaction between cellular circRNAs and viruses and (ii) uncover the great potential of artificial circRNAs as novel platforms for drug development.

La rellevància clínica dels virus transmesos per mosquits, com el virus del dengue (DENV), el virus del zika (ZIKV), el virus del chikungunya (CHIKV) i el virus del Nil Occidental (WNV), ha augmentat dràsticament durant els darrers anys, provocant un problema de salut global. Actualment, no hi ha cap tractament disponible ni cap vacuna efectiva per tractar aquestes infeccions. Tots aquests virus causen infeccions agudes que han de ser tractades ràpidament després de l'aparició dels símptomes inicials perquè els medicaments siguin efectius. Tot i això, el diagnòstic precoç continua sent un repte no resolt. Això evidencia la necessitat de descobrir noves interaccions essencials entre el virus i la cèl·lula que podrien ser utilitzades com a noves dianes terapèutiques; i la necessitat de desenvolupar teràpies antivirals d'ampli espectre, eficients, que puguin ser administrades abans que s'aconsegueixi un diagnòstic precís. En aquesta tesi hem abordat aquests dos grans problemes centrant-nos en els ARNs circulars (circRNAs).

Els circRNAs són una classe d'ARN generats a partir de progenitors lineals d'ARN mitjançant un mecanisme alternatiu de *splicing* anomenat *back-splicing*. En comparació amb els seus homòlegs lineals, els circRNAs són molt estables a causa de la seva resistència a les exonucleases. Actualment, s'ha descrit la implicació dels circRNAs en les infeccions virals, tanmateix, no es coneix el seu rol precís. El primer capítol de la tesi intenta respondre a aquest buit de coneixement utilitzant el virus de l'hepatitis C (HCV) com a sistema model i analitza l'efecte dels circRNAs identificats en altres virus de la mateixa família, en concret, els virus transmesos per mosquits. Mitjançant anàlisis de seqüenciació d'ARN, hem identificat 73 circRNAs cel·lulars induïts pel HCV. Aquest canvi en l'expressió dels circRNAs no pot ser explicat a través de canvis paral·lels en els ARNs lineals. A més a més, hem identificat que el silenciament de cinc d'aquests circRNAs provoca canvis en la infectivitat viral, actuant com a molècules pro- o anti- virals. Un d'aquests circRNAs, cPSD3, és clau per a la infectivitat del virus del dengue.

El segon capítol de la tesi se centra en desenvolupament d'una nova plataforma basada en circRNAs que sigui versàtil, dificulti l'aparició de mutants resistents i permeti desenvolupar antivirals d'ampli espectre. En contrast amb

altres teràpies basades en ARN, els circRNAs són molècules altament estables, una característica que simplificarà el seu ús terapèutic. Els circRNAs sintètics dissenyats contenen seqüències llargues que s'hibriden a regions del genoma viral implicades en formar estructures d'RNA essencials per a la supervivència del virus. Com a prova de concepte, hem validat amb èxit circRNAs que inhibeixen el HCV, el DENV, el CHIKV o el WNV. A més, hem generat circRNAs amb capacitat antiviral d'ampli espectre i hem optimitzat la producció *in vitro* d'aquestes molècules per obtenir quantitats elevades a baix preu.

En conclusió, els nostres resultats (i) emfatitzen la complexitat de la interacció entre els circRNAs cel·lulars i els virus i (ii) descobreixen el gran potencial dels circRNAs artificials com a noves plataformes per al desenvolupament de fàrmacs.

# PREFACE

The clinical importance of emerging and re-emerging viruses, including West-Nile and dengue viruses has increased over the last years, resulting in a global health problem. Due to the rapid spread of mosquito vectors across the world, globalization and climate change, the WHO has estimated that in the upcoming years nearly 40% of the total population will be in risk of suffering one of these infections. Currently, there are no available treatments or effective vaccines and the costs associated with differential diagnostic are high because these viral infections share similar initial symptoms, geographic areas and vectors. This brings to the spotlight the need to find a cure for these infections. A new strategy to treat viral infection is based on the fact that viruses rely on host-cell factors to multiply. Host-targeting drugs inhibiting cellular factors required for different viruses may simplify the treatment and result in broad-spectrum antivirals. Understanding how viruses behave inside the cell and deciphering the molecular mechanisms that underly the complex relation between the virus and their hosts is crucial for the development of new antivirals or vaccines.

In this thesis we have addressed these major concerns following two different approaches. First, thanks to the collaboration with two prestigious groups at Stanford University and The Hebrew University of Jerusalem, we have studied the role of circular RNAs (circRNAs), a class of single-stranded covalently-closed RNAs, in viral infections. CircRNAs have been described to be involved in different diseases, however their role in viral infections has not yet been elucidated. We have seen and characterized differentially expressed circRNAs under the infection of hepatitis C virus (HCV), a well-studied model for (+) strand RNA virus infection. These differentially expressed circRNAs are important for HCV infectivity. Moreover, we have seen the importance of these circRNAs in other viral infection processes.

Second, we have gone a step further and taken a translational approach to apply our knowledge of circRNAs to develop a broad-spectrum antiviral platform. Broad-spectrum antivirals are a proposed solution to treat emerging viral infections because they can be administered even before a complete diagnose without knowing the exact emerging virus responsible for infection.

Here, we have designed, tested, produced and patented a new antiviral based in artificial circRNAs. With this innovative approach, we propose to disrupt crucial viral RNA structures of different viruses at the same time impairing their propagation. This technology might have the potential not only to treat viral infections but also to treat other diseases with aberrant expression of RNAs. Excitingly, we have optimized the production of *in vitro* circRNAs, really expensive and limited in the market, to a cost of 3€/μg of circRNA.

Overall, this PhD opens a new door not only in the characterization of viral infections and the role of circRNAs but giving the *proof-of-concept* for a new therapeutic platform.

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# INTRODUCTION

# CIRCULAR RNAs

## A. GENERAL FEATURES

Circular RNAs (circRNAs) are single-stranded covalently-closed RNA molecules produced in the cell during splicing. During three decades they were considered artefacts of the splicing reaction with no functional value (1–3). It was not until 2012 that they came back into the spotlight when the circRNA CDR1as, also known as CiRS-7, was described to act as microRNA (miRNA) sponge and showed to be crucial in brain development (4,5).

CircRNAs are generally produced from protein-coding genes and their median length ranges around 500 base-pairs. The same gene can produce multiple circRNAs, usually between 2 to 10 isoforms and the majority of circRNAs comprise typically between 1 to 5 exons. Therefore, not all the possible combinations between exons are produced (6). Due to their circular nature, circRNAs do not present neither 5' capping structures nor 3' poly(A) tail (1,2). This confers them resistance to exonuclease degradation and a higher stability than other linear RNA molecules. Recently, Enuka et al. showed that the half-life of circRNAs extend to approximately 24h whereas their linear RNA species exhibit half-lives of approximately 8h (7).

CircRNAs are present among the eukaryotic tree of life: from animals to plants, protists and fungi. Moreover, we find differentially expressed circRNAs across organisms (8), cell types, tissues and development stages (9–11). Although most circRNAs are expressed at low levels, they are highly abundant in some cell types. In humans, for example, neural tissue has high levels of circRNAs while liver tissue contains low levels (9). This is likely explained by differences in the proliferative capacity of cell types as there is a strong anti-correlation between the amount of circRNAs and cell replication rates (12). Assuming that circRNAs behave as other messenger RNAs (mRNAs) in the cell, in every division the total amount of circRNAs will be divided between daughter cells.

Thus, in non-proliferative cells, like neurons, circRNAs will highly accumulate. However, in other non-proliferative tissues like cardiomyocytes, where less alternative splicing events occur, there is no circRNA accumulation (10,13).This suggest that the observeddifferential expression of circRNAs throughout cell types and organisms is caused by a combination of proliferation and splicing rates.

Since their re-discovery a few years ago, an ever-increasing number of manuscripts on circRNA biology have been published. However, although it is now well established that circRNA expression changes in response to different pathological conditions, including cancer and viral infections, it remains mostly unclear (i) how these changes are caused and (ii) whether they contribute to the pathological condition.I will next summarize the current knowledge and future perspectives of circRNA biology.

## B. BIOGENESIS OF circRNAs

In cells, after transcription, the resulting RNAsare processed to generate the mRNAs. This processing comprises post-transcriptional modifications such as capping and polyadenylation. Once modified, if the RNA molecule contains splicing signals, it will be spliced by the splicing machinery (14,15).

Canonical splicing is an essential step in mRNA maturation where introns are processed and generally removed from the pre-mRNA molecule to generate a linear mRNA with 5'-3' polarity (**Figure 1**). Splicing is a two-step process mediated by the spliceosome: first, the 5'-splice site (acceptor site) is nucleophilic attacked by the 2'-OH group of the adenine nucleotide (branching point) forming a lariat structure with a 2'-5'-phosphodiester bond and a free 3'-OH group at the upstream exon (**Figure 1.1**). Then, this 3'-OH group from the 5'-exon will attack the 3'-splice site (donor sequence) generating a 3'-5' bond between exons and releasing the intronic lariat structure (**Figure 1.2**). This process ends with the linear mRNA form with ordered exons (16). Next, thanks

to debranching enzymes, the resulting intronic lariat is cleaved and degraded by exonucleases (17).

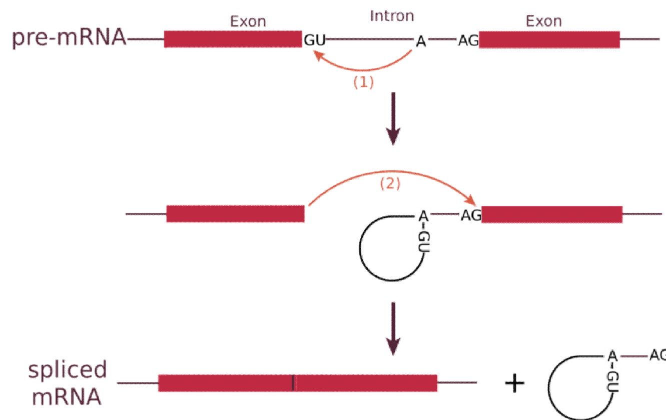


***Figure 1. Canonical splicing****. In canonical splicing, the introns of the pre-mRNA are removed generating a splice mRNA molecule and an intron lariat. (**1**) The adenine from the branch point attacks the 5' splice site (donor) forming a lariat structure. (**2**) The 5' splice site attacks the 3' splice site (acceptor) releasing the intronic lariat. Adapted from BCSteve (creative commons).*

In addition to canonical splicing, there are alternative and non-canonical splicing mechanisms that occur in the cell generating alternative spliced mRNAs (**Figure 2**). One example of alternative splicing is exon-skipping where some exons are excluded, and the resulting mRNA has less exons than predicted (**Figure 2a**). The skipped exons will be comprised in the intronic lariat (6). Alternative splicing is a highly regulated process that expands the coding potential of individual genes andits missregulation can lead to several pathologies (15). In this context, as circRNAs are produced during splicing in a non-canonical manner, some groups have reported circRNA biogenesis as non-canonical splicing mechanism. CircRNAs are generated in a process called back-splicing or head-to-tail splicing (**Figure 2b**). In this case, we find exons spliced in a non-canonical order. Here, the downstream 5' splice site acting as a donor attacks to an upstream 3' splice site that acts as an acceptor. This results in a new junction, called back-spliced junction (BSJ), unique for circRNAs, which will generate a new molecule with scrambled exon's order compared to the linear mRNA (18) (**Figure 3**).

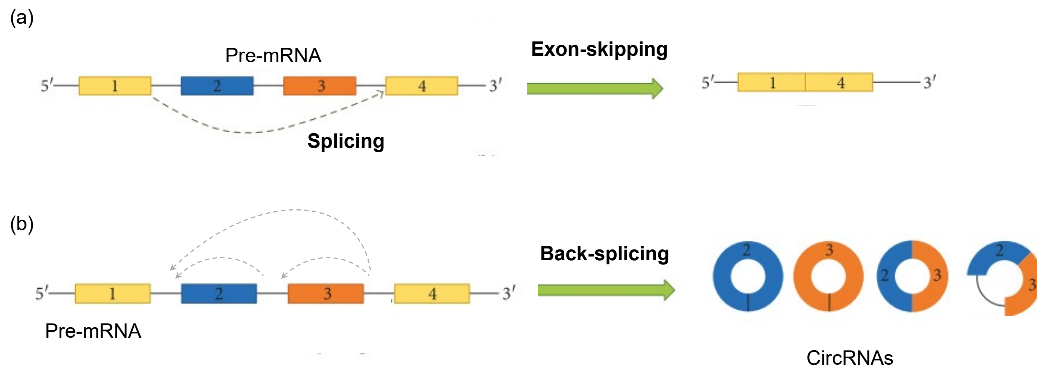


***Figure 2. Non-canonical splicing****. Some pre-mRNAs undergo alternative or non-canonical splicing mechanisms. (****a****)* ***Exon skipping****: Some exons can be skipped from splicing generating a new isoform with less exons. (****b****)* ***Back-splicing****: Some exons can undergo to back-splicing generating circular molecules. The generated circRNAs can be derived from one exon or several exons and can retain some introns. Adapted from Bolha, Int J Genomics 2017.*

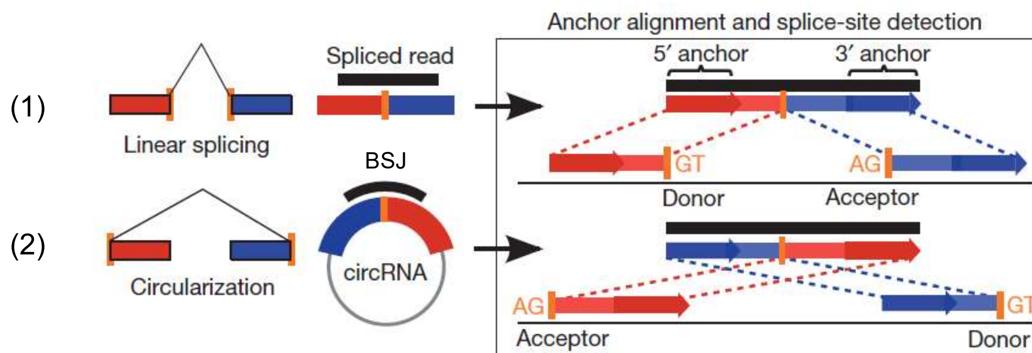


***Figure 3. Comparison between linear splicing and Back-splicing****. (****1****) In linear splicing the upstream exon gives the donor sequence to the acceptor site generating a splice read with the canonical order. (****2****) In the circularization process, the downstream exon acts as a donor and the upstream as acceptor generating the back-spliced junction (BSJ) and a circular molecule with scrambled order. Adapted from Memczak, Nature 2013.*

The mechanism by which the splicing machinery decides the fate of the splicing reaction is still unknown. However, there are some studies that have elucidated key features and steps for back-splicing to occur and to promote circRNA biogenesis. Back-splicing requires canonical spliceosome and canonical splice signals (19). As both canonical and non-canonical splicing depend on the same sequence features and machinery, it was postulated that circRNAs could

compete with linear RNAs for their generation (20). Nevertheless, the expression levels of circRNAs do not always anticorrelate with those of their linear mRNAs counterparts. Some circRNAs accumulate at higher, lower or comparable levels than their linear counterparts (21). However, in general terms, non-canonical splicing is less efficient than canonical splicing. This might be explained by an unfavourable assembly of the spliceosome, generally assembled for canonical splicing, and a less efficiently catalysed reaction in the back-splicing process (22). Then, for back-splicing to efficiently occur, it would needto be facilitated by*cis*-acting elements or by *trans* factors. These factors wouldhave to overcome the long distance between the donor and the acceptor sequence by bringing the circRNA flanking introns in close proximity to allow back-splicing.

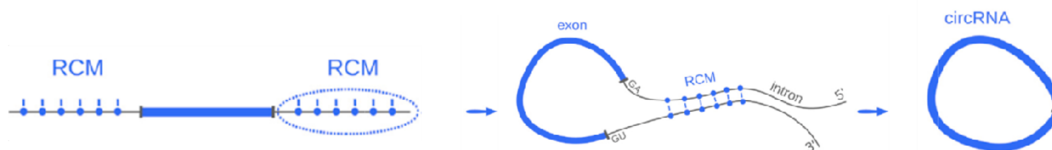


***Figure 4. Cis acting elements****. Circularizing exons have flanking introns with repetitive complementary motifs (RCM). These motifs can complementary bind shortening the distance between the splice sites and promoting back-splicing and the formation of a circRNA. Adapted from Ivanov, Cell Reports 2015.*

Some flanking or "cis-acting" sequence elements that promote back-splicing have been identified. While analysing the surrounding sequences, an enrichment of long introns with reverse complementary motifs was detected (RCMs). These elements comprise flanking intronic reversely complementary sequences that can pair together. The pairing of the intronic sequences shortens the distance between the 3' splice donor and the 5' splice acceptor and promotes back-splicing (23) (**Figure 4**). Further bioinformatic analyses were carried out to characterize RCMs and their presence throughout the genome. The most frequent elements in flanking introns are canonical ALU repeats (24). These ALU elements are 300 base-pairs short interspersed elements derived from retrotransposons that are present throughout the genome. In their study, Jeck et colleagues, observed that flanking introns of

circularizing exons contained an enrichment in ALU elements with inverted orientations (24). The location and direction of these elements determine that the mRNA undergoes to canonical splicing, if they locate in the same intron, or to back-splicing, if they locate in different introns (23). In addition to ALU elements, other flanking elements have been described. These sequences must contain at least 40 nucleotides and are sufficient to facilitate back-splicing (25,26). Importantly, not all exons containing long flanking introns with complementary repeats are circularized. Moreover, in many cases, it is not possible to identify the complementary sequences in the introns, suggesting that RNA pairing is not indispensable to generate circRNAs. For instance, in fibroblasts around 40% of circRNAs come from genes with ALU elements, but only 20% of them are complementing (24,27). In fact, in lower eukaryotes ALU elements are not common, suggesting other sequences or motifs being crucial for circRNAs biogenesis. Thus, circularization cannot be explained solely by cis factors (6,28).

Multiple *trans* factors that regulate back-splicing have been identified. Some of them promote back-splicing whereas others inhibit it. They are RNA Binding Proteins (RBPs) that bind flanking introns either bringing exons in close proximity allowing the formation of circRNAs (positive regulators) or breaking the RNA pairing inhibiting the circularization (negative regulators). Generally, their mode of action requires the dimerization of RBPs (6) (**Figure 5**). Examples of positive regulators include the Quacking (QKI) (**Figure 5A**), the MBL (20) (**Figure 5B**), the FUS (29), the ESRP1 (30), the hnRNPs, the SR (31) and the NF90/110 (32) which promote circularization of some transcripts.From these, the QKI is one of the most characterized. QKI binds to QKI-intronic motifs and dimerizes promoting a shorter distance between the exon splice sites needed for the circularization. With the proximity between splice sites, the efficiency of back-splicing is increased (**Figure 5A**). QKI is a key factor in the epithelial to mesenchymal transition (EMT). The expression of QKI increases with this transition causing a parallel increase of circRNAs (33). Examples of negative regulators of the biogenesis of circRNAs include the Adenosine deaminase 1

acting on RNA (ADAR1) and the RNA helicase DHX9. The ADAR1 catalyses the deamination of adenosine to inosine in double stranded RNAs (dsRNAs). This change breaks the complementarity between intronic sequences removing the RNA pairing that helps to generate circRNAS (28) (**Figure 5C**). The RNA helicase DHX9 disrupts secondary structures, similar to the generated by ALU elements, impairing intron-intron interactions (34). Interestingly, DHX9 and ADAR1 can interact disrupting RNA secondary structures.

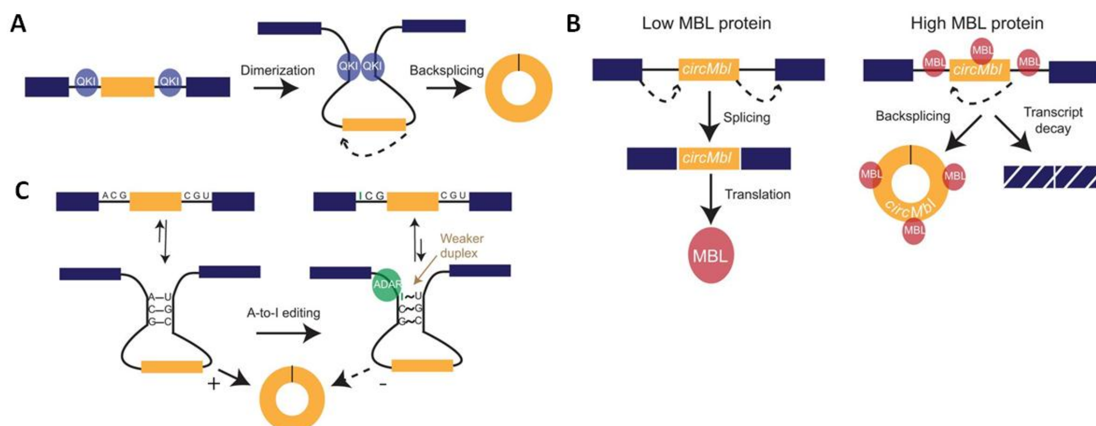


***Figure 5. Trans factors****. Several RBPs can regulate the formation of circRNAs. There are two kinds of regulators: positive (****A*** *and* ***B****) and negative (****C****). (****A****) The QKI binds flanking intronic motifs, dimerizes and promotes back-splicing. (****B****) The MBL protein controls its levels by promoting the formation of circRNA and the decay of its transcript. (****C****) ADAR1 edits A-to-I generating weaker RNA duplex and impeding the formation of circRNAs. Adapted from Barrett, Development 2016.*

### C. CLASSES OF circRNAs

CircRNAs can be generated from different parts of the pre-mRNAs (exons, introns or both). Depending on their composition, circRNAs can be classified in three groups: exonic circRNAs (ecircRNAs); exonic-intronic circRNAs (EIciRNA); and intronic circRNAs (ciRNAs) (**Figure 6**).

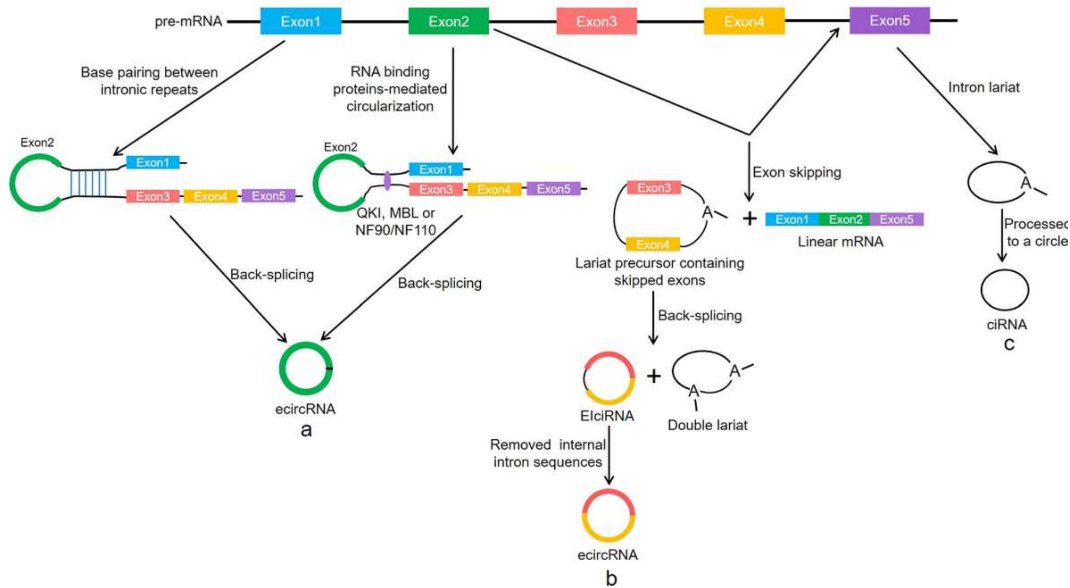


***Figure 6. Classes of circRNA**. There are three classes of circRNAs: exonic circRNAs (ecircRNA), exonic-intronic circRNAs (EIciRNA) and intronic circRNAs (ciRNA). (**a**) With the help of cis-elements or trans factors (such as QKI, MBL or NF90/110), the flanking introns of an exon can be complementary bound and promote the back-splicing of the exon, generating an ecircRNA. (**b**) In exon skipping, there are several exons that can be skipped generating an alternative-spliced linear mRNA, and an intronic lariat with the skipped exons. The intronic lariat can undergo back-splicing again generating EIciRNA (if some intron is retained) or ecircRNA (if an intron is removed). (**c**) In all splicing events, anintronic lariat structure is generated, in some cases it can be processed and can form an intronic circRNA (ciRNA). Adapted from Zhou, Cell Death and Disease 2019.*

EcircRNAs comprise the majority of circRNAs in cells. They are mainly generated by direct back-splicing. However, in some cases the back-splicing can occur after an exon-skipping event where exons are retained in the lariat structure. Then, a second splicing reaction will generate the ecircRNA and remove the introns. This class of circRNAs mainly localizes in the cytoplasm. The transport mechanism by which ecircRNAs are exported from the nucleus to the cytoplasm is unclear. However, some research groups have proposed that they might recruit mRNA export factors and be transported via the nuclear export system. Indeed, several factors have been recently described to transport circRNAs from nucleus to the cytoplasm in a length-dependent manner. In *Drosophila,* the helicase Hel25E mediates the export of circRNAs

longer than 800 nucleotides. In addition, its human homologs URH49/UAP56 export circRNAs in a length-dependent manner (35) (**Figure 6a**).

EIciRNAs are produced by an exon-skipping event where some exons are included in the intronic lariat structure. This lariat comprising exons can undergo a secondary splicing event where they generate an ecircRNA or, if an intron is retained, an EIciRNA (**Figure 6b**). Finally, ciRNAs are produced by canonical splicing with the formation of the intronic lariat structure. They have a 2'-5' phosphodiester bond and lack the linear part stretching from the branch site to the end of the intron. To avoid the debranching of the 2'-5' bond, several conserved sequences are needed: a 7 nucleotide GU-rich motif around the 5' splice site and a 11-nucleotide C-rich motif close to the branch site (36) (**Figure 6c**). In contrast to ecircRNAs, ciRNAs and EIciRNAs mainly localize in the nucleus. Interestingly, some studies have showed a role of these circRNAs in gene transcription. In fact, circEIF3J is suggested to regulate the transcription of its linear counterpart. Accordingly, specific silencing of the circRNA decreased the levels of the EIF3J mRNA (37).

### D. DEGRADATION OF circRNAs

Currently it is unclear how circRNAs are degraded. The absence of 5' and 3' ends protects these molecules from the traditional RNA decay pathways that rely on 5' or 3' exonucleases. Although there are several indications of how circRNAs can be degraded, further studies are needed to confirm them. These include endonucleolytic cleavage and the AGO2-mediated degradation (38). Endonucleolytic cleavage will render linear molecules sensitive to exonucleases. Indeed, some studies recently showed the activation of RNAse L and the posterior degradation of circRNAs (39). Moreover, the modification N6-methylation of adenosine (m6A) in the circRNAs can recruit endonucleases with the potential to degrade circRNAs (40). In the AGO2-mediated circRNA degradation, binding of fully complementary miRNA to RNA molecules would trigger the AGO2/RISC complex and promote degradation. There is only one

example described of this circRNA degradation mode. The binding of miR-671 to circRNA CDR1as triggers the AGO2-complex and subsequent circRNA degradation (5,41). Degradation is not the only way to eliminate circRNAs. Some reports showed the presence of circRNAs in exosomes (42–44) suggesting that secretion of circRNAs might contribute to decrease their intracellular levels.

## E. MOLECULAR FUNCTIONS OF circRNAs / MODE OF ACTION

Although a main effort has been made in the last years, the precise molecular function role of circRNAs remains elusive. Only a few number of circRNAs have an assigned mode of action. These include miRNA sponges, RBP sponges, transcriptional regulators and alternative splicing regulators, among others.

### 1. miRNA sponges

Hansen and colleagues were the pioneers in the molecular characterization of circRNAs through the discovery of CDR1as. This circRNA is produced from an antisense transcript of CDR1 and expressed mainly in mice neuronal tissue and in human brain. CDR1as has 73 seed-binding sites for miR-7. These binding sites are not completely complementary allowing the binding of miR-7 without activating the miRNA/AGO-2 mediated cleavage. Therefore, this circRNA acts as a competing endogenous RNA (ceRNA) with the miRNA-7-targeted-mRNAs. As miR-7 has an important role regulating the expression of mRNAs affecting brain development, CDR1as circRNA overexpression impairs midbrain development (4,5). There are other examples of circRNAs that interact with miRNAs. The testis-specific circSry produced from the sex determining region Y has 16 binding sites for miR-138. However, its physiological importance has not been yet established (5,45). Further characterization of other circRNAs have shown single or very few miRNA binding sites in these molecules suggesting that most circRNAs do not bind extensively to miRNAs (46).

## 2. Protein sponges, transporters or scaffolds

CircRNAs have been postulated to work as protein sponges, protein transporters and protein scaffolds.

### a) Protein sponges

CircRNAs might help to control the amount of free proteins in the cell by sponging the excess of it. As a result, many functional proteins would be hijacked, and their function inhibited. Thus, circRNAs would act as "protein reservoirs". One example of circRNA acting as protein sponge is circMBL, a circRNA derived from the *muscleblind* (MBL) transcript that contains multiple binding sites for MBL protein. When there are high amounts of MBL, circMBL sponges out the excess of protein regulating its levels (20). Another way to control protein expression is to regulate proteins that promote translation. For example, the circPABPN1 binds HuR, an RBP that binds to PABPN1 mRNA and promotes its translation. The binding of HuR to the circRNA, reduces thus translation of the PABPN1 mRNA (47). A similar example is circANRIL that binds PES1, an essential protein for ribosomal RNA maturation. CircANRIL-PES1 binding results in an inhibition of ribosomal RNA maturation (48).Other circRNAs described to bind and inhibit the function of proteins includes the circ-FOXO3 that interact with several antisenescence and antistress factors (ID-1, E2F1, FAK and HIF1-$\alpha$) (49) and the circPOLR2A and circDHX34 that are proposed to act as NF90/110 reservoirs in non-infected cells prior to an infection. The accumulation of NF90/110 allows the cell to have a faster response to infections (32,39).

### b) Protein transporters

Rather than sponging RBPs, some circRNAs have been described to facilitate the translocation of functional proteins from different cellular compartments, mainly from the cytoplasm to the nucleus.One example is circ-Amotl1 that binds several proteins and translocate them into the nucleus. It has been described that circ-Amotl1 binds and transports to the nucleus (i) PDK1 and AKT1 in

cardiopathies (50); (ii) c-myc in tumour cells,inducing the transcription of genes (51) and (iii) STAT3 in wound repair, enhancingthe expression of Dnmt3a, a DNA methyltransferase that represses the expression of miRNAs and enhance the expression of fibronectin (52).

c) Protein scaffolds

CircRNAs can also promote the interplay of several RBPs modulating the protein-protein interaction. This is the case of circFOXO3, a circRNA that enhances the interaction between p21 and CDK2. By this mechanism the phosphorylation of downstream cyclins is avoided and cell cycle progression is inhibited (53).

### 3. Transcription regulators

Although the majority of circRNAs are in the cytoplasm, intron-containing circRNAs (EIciRNA and ciRNA) can be found in the nucleus and act as transcription regulators. Some ciRNAs, like ci-ankrd52 and ci-sirt7, regulate transcription by interacting with the RNA polymerase II (RNA Pol II) elongation complex. The depletion of the intronic circles leads to a decrease in the transcription of their linear transcripts (36). Other EIciRNAs can interact with RNA Pol II and U1 snRNP promoting the transcription of their parental genes (37). These examples provide insights of nuclear circRNAs as transcription regulators acting in cis.

### 4. Alternative splicing regulators

CircRNAs are produced mainly from exons of protein-coding genes. This can generate a competition between canonical splicing and circRNA circularization due to the usage of the same splice sites (20). This competition can result in two different scenarios: the splicing process results in lower levels of the linear mRNA containing the exons that will be circularized or the back-splicing process leads to the removal of internal exons generating an alternative spliced linear mRNA (54). In the latter, the circRNA acts as a “mRNA trap” sequestering

crucial exons for the linear molecule. This is the case of Fmn ecircRNA where the exons comprised in the circRNA contain the translation start site impeding the truncated linear RNA molecule to be translated (55).

### 5. Encoding capacity

CircRNAs have always been considered non-coding RNA molecules because of the lack of capping structures and poly A tail in their ends. However, it is under study whether they can have translation capacity via non-cap-dependent mechanisms. Indeed, *in vitro* studies showed that circRNAswith IRES bind ribosomes and translate (56,57). Surprisingly, researchers found that circRNAs lacking IRES are translated, as well (58). Further studies showed that circRNAs containing N6-methiladenosine (m6A) motifs are sufficient to drive translation initiation (59). Other studies have shown that circRNAs are not associated with polyribosomes (21,24,46). However, there is no need of polyribosomes to synthesize small proteins or micropeptides (60). Currently, there are only fivemolecules characterized as protein-coding circRNAs: circ-ZNF609 (61), circ-FBXW7 (62), circ-SHRHP (63), CircPINTexon2 (64) and circMbl3 (58).

## F. CircRNAs IN NORMAL HOMEOSTASIS

Expression of certain circRNAs are required for fundamental physiological processes such as development, cell cycle, immune responses or insulin secretion (65).

### 1. circRNAs in development

CircRNAs are differentially expressed across the organisms in a temporal and spatial manner (4). This suggests a role of these molecules during development. In fact, some circRNAs change their expression in different steps of the development. For example, circRNAs generated from the Albumin gene (ALB) elicit differentially levels of expression when comparing an adult liver with a developing foetal one (66).

As mentioned above CiRS-7, one of the best-characterized circRNAs, changes its expression during cerebellum development, being highly expressed at late stages of development compared to earlier ones (E115 vs E60) (27). Moreover, the transgenic expression of CiRS-7 can induce alterations in the midbrain development mimicking the knock-down of miR-7 where brains had smaller sizes (67). These results suggest a fine-tune regulation between the expression of circRNAs and miR-7 during brain development. CiRS-7-miR-7 also plays a major role in pancreatic cells where it prevents the proliferation of ß-cells and induces insulin secretion. Thus, the expression of CiRS-7, can regulate the metabolism of insulin by sponging miR-7 and promoting the secretion of insulin (68).

### 2. circRNAs in cell cycle

Cell cycle is a highly regulated process controlled by several check-points. In each check-point different factors, mainly kinases, have to be activated by external stimulus to allow transition progression. CircRNAs can regulate the availability of some of these factors acting as a protein scaffold. This is the case of circFOXO3 that forms a complex with p21 and CDK2 preventing their function. As p21 and CDK2 will not interact with their partners, there will be a block in the cell cycle progression (53).

### 3. circRNAs in immune system

The effect of circRNAs in immune responses is controversial. Chen et al., while trying to elucidate the role of *in vitro* generated circRNAs, realized that artificial-produced-circRNAs are recognized by innate immune sensor RIG-I. RIG-I is a cytoplasmatic helicase enzyme that recognizes dsRNAs, ssRNAs and 5' triphosphate motifs and activates a signalling cascade that leads to the production of type I interferon (IFN) and the secretion of cytokines. The activation of RIG-I is one of the first mechanisms to fight against viral infections. In contrast, endogenous circRNAs are able to bind cellular RBPs not activating RIG-I pathway and the subsequent antiviral pathways. Nevertheless, some questions remain unanswered regarding the recognition by RBPs of self-/non-

self- circRNAs (69). Later, Wesselhoeft et al, showed that the activation of RIG-I pathway was not due to the artificial-circRNAs but toaproblem with their purification. When purified by RNAse R and phosphatases treatment plus HPLC no activation of the innate sensors was detected (70).

Interestingly, another study showed the involvement of two immune response factors in circRNA biogenesis. NF90/110 are two factors encoded by the Interleukin enhancer binding factor 3 (ILF3) present in the nucleus that can stabilize intronic complementary sequences. This function helps the circularization of distant exons allowing the formation of circRNAs. As they are immune factors, after infection with the vesicular stomatitis virus (VSV), NF90/110 are translocated to the cytoplasm where they bind the double-stranded viral RNA impairing viral replication. This implies a decrease in the nuclear fraction of NF90/110 that helps the circRNA biogenesis and consequently a decrease in circRNA production. The authors suggest that the overexpression of circRNAs will compete for the binding of NF90/110 with viral RNAs favouring viral infectivity (32). Moreover, some circRNAs present dsRNA structures that inhibit PKR, a double-stranded RNA dependent kinase that recognize dsRNAs and activates the phosphorylation of eiF2$\alpha$ inhibiting protein synthesis and the transcription of interferon stimulated genes (ISGs). Upon encephalomyocarditis virus (ECMV) infection the endonuclease RNAse L is activated and degrades circRNAs. Thus, PKR is no longer bound to circRNAs and can be activated (39).

In summary, the dysregulation of circRNA expression in autoimmune diseases and the cell-specific expression of circRNAs in the different subtypes of blood cells suggest an important role of circRNAs in the control of the immune system (39,71–74). However, their mode of action is still controversial.

## G. CircRNAs IN PATHOLOGIES

Currently most of the studies addressing the involvement of circRNAs in pathological conditions are descriptive. These studies have shown correlations between differential expression of circRNAs and several pathologies. However, whether these changes are the cause or the consequence remains a major open question. Next, I will summarize the state of the art of these studies.

### 1. Non-infectious diseases

Changes in circRNA expression levels have been reported in multiple non-infectious diseases like cancer, neurological, cardiovascular or autoimmune diseases. For example, circHIPK3 expression is involved in cell growth and upregulated in liver cancer. Silencing of this circRNA results in a decrease of the tumorigenic capacity of the cells (75). Another tumour up-regulated circRNA is circ-Amotl1. Exogenous expression of the circ-Amotl1 revealed the formation of tumours and its tumorigenic capacity via interaction and translocation of c-myc to the nucleus (51). In contrast, tumour suppression circRNAs can be down-regulated in tumours due to miRNA-induced cleavage or due to a down-regulation of the parental transcription. CircITCH is a circRNA down-regulated in several tumours like lung (76), colorectal cancer (77) and oesophageal squamous carcinoma (78). Overexpression of circITCH supresses tumour growth capacity suggesting an antiproliferative role of the circRNA. Excitingly, some *de novo* generated circRNAs have been described as a result of genomic instability events. Guanerio et collegues showed that genomic translocation of PML and RAR genes in promyelocytic leukemia results in the formation of a fusion oncogenic circRNA (f-circRNA) (79).

In the brain and neural tissues, circRNAs accumulate with aging. Due to the higher levels of circRNAs several studies have shown the involvement of circRNAs in the memory process (80,81), epilepsy (82), depression (67) and Alzheimer's Disease (AD). In neurological disorders such as AD and Parkinson there is a decrease of CiRS-7, increasing the availability of miR-7 and therefore a down-regulation of miR-7 targets. Some examples of down-regulated targets

are ubiquitin protein ligase A, a key enzyme in the clearance of ß-amyloid aggregates responsible for AD (83) or α-synuclein, implicated in Parkinson Disease (5,84). Furthermore, the depletion of proteins such as QKI and MBL, crucial for circRNAs production, leads to neurological disorders suggesting a new layer of regulation in neurological diseases with circRNAs (29,85,86).

2. **Infectious diseases**

In gram negative bacteria Lipopolysaccharide (LPS)-stimulated macrophages elicit the expression of the mcircRasGEF1B circRNA, a circRNA that is in charge of the fine-tune of the immune system (87,88). In contrast, not much is known about the effect of viruses in host circRNA expression levels. These include the grass carp reovirus (GCRV), a dsRNA virus that infects grass carp (89); the avian leukosis virus (ALV-J), a retrovirus that infects chickens (90); the transmissible gastroenteritis virus (TGEV), a positive-stranded RNA virus that infects pigs (91); the Orf virus (ORFV), a dsDNA virus that infects sheeps and goats (92); the simian virus 40 (SV40), a dsDNA that infects macaques (93) and the human Herpes Virus Simplex (HSV-1) infection. In the latter, the differentially expressed circRNAs are derived from genes implicated in cellular immune responses and viral pathogenesis, including inflammatory pathways or cell cycle progression (94). Excitingly, several reports done in Epstein-Barr virus (EBV) infected cells have uncovered the presence of viral circRNAs. EBV is a dsDNA virus from the herpes virus family and one of the most common virus in humans. EBV has a lytic phase that results with the production of virions and three latent phases where the viral DNA genome is copied by the human polymerase. The three phases will depend on the viral protein expression pattern. Three studies have characterized the different expression of viral circRNAs in each phase of the viral cycle suggesting a role of circRNAs in herpes virus infection (95–97). Indeed, another report with Kaposi Sarcoma Herpes Virus (KSHV) showed the presence of viral circRNAs finding one viral circRNA crucial for the lytic phase of the infection (98).

Overall, the putative role of circRNAs in viral infections is mainly unknown. Its characterization will be of great interest in virus biology and also may lead to discover new features from these exciting molecules.

# FLAVIVIRUSES

## A. GENERAL FEATURES

Positive-strand RNA ((+) RNA) viruses constitute one third of all virus genera and include a wide range of pathogens with clinical and economical importance. One of the most relevant family of human and animal pathogens within this group is the *Flaviridae* family. The *Flaviviridae* family is divided in two main genera: *Flavivirus* and *Hepacivirus* (99).

### *1. Flavivirus*

In the last decade an increasing number of viral outbreaks have affected both developed and undeveloped countries. This has been mainly caused by the globalization, that favours movement of travellers, animals and merchandises; and by the climate change, that favoursthe expansion of mosquitoes from their endemic areas to other geographical regions now with milder temperatures (**Figure 7**) (99). Some of the most important mosquito-transmitted viral outbreaks, because of the number of infections and clinical relevance, have been caused by members of the *Flavivirus* genus. These include dengue virus (DENV), zika virus (ZIKV) and west Nile virus (WNV). The level of alarm over the spread of these viruses has intensified asit is estimated that in the forthcoming years more than 40% of the global population will be at risk. This will represent more than 120 countries affected, including the warmer regions of Europe, where mosquito vectors have expanded and caused already outbreaks (100).

All these viruses produce acute infections and require to be treated early after the onset of the symptoms for drugs to be effective. Until now, there are no treatments or effective vaccines available in the market. Moreover, differential diagnostic is an unsolved challenge because they (i) share initial common symptoms such as fatigue, fever, rash, joint paint, myalgia and arthralgia; (ii)

share mosquito vectors and areas of geographical distribution (101); and (iii) cannot be properly diagnosed due to antibody cross-reactivity (102). A definitive diagnosis then requires costly PCR test not routinely available in most of the countries affected. Therefore, conventional virus-specific drugs targeting viral enzymes do not seem the best approach to treat these emerging viruses. A possible solution to overcome the problem is the development of broad-spectrum antivirals (BSAs). BSAs can be a very effective alternative to treat the different infections. They could be administered before an accurate diagnosis, increasing the likelihood of viral control (103).

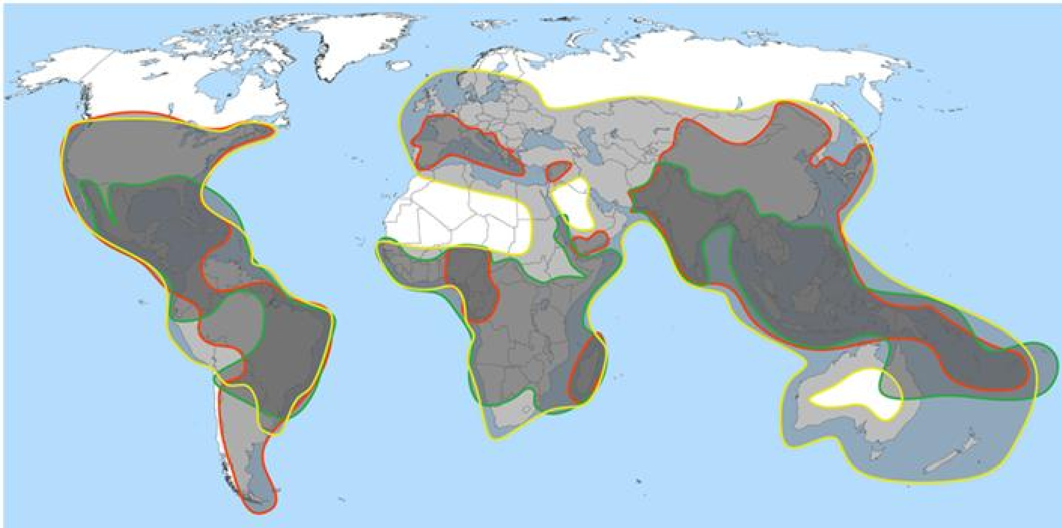

***Figure7. Global distribution of mosquito vectors and affected areas.*** *Current distribution of the Aedes Albopictus (in red) and Aedes Aegypti (in green) mosquitoes, which serve as vectors of the dengue, chikunguya and zika viruses, and Culex Spp (in yellow) mosquito, which represents the most common vector of the West Nile Virus.*

Next, I will summarize the most important features of the epidemiology and clinical symptoms of DENV, ZIKV and WNV viruses. DENV has become in the last years a major health problem worldwide, more than 100 countries have endemic cases with 390 million people being infected by DENV and around 96 million of new infections every year. Due to the rapid expansion of the mosquito vector, around 3 to 6 billion people are at risk of disease transmission (104). DENV can produce different outcomes, from a self-limiting illness to sever diseases. The majority of infections (75%) are asymptomatic or subclinical.

However,around 5% progress to a sever disease characterized by a febrile phase, hypovolemic shock and organ impairment (105). It has been reported that there are symptoms such arthralgia or fatigue that can persist up to 2 years in half of the patients (106). Moreover, after a primary DENV infection the risk for developing severe outcomes in a secondary infection highly increases even 20 years after the primary one. This is due to the immune responses to the DENV infection. After a first infection with DENV, memory T-cells can react against other dengue serotypes due to serotype cross reactivity. This generates a positive feedback loop with the binding of antibodies to the immune cells and the secretion of high levels of cytokines (such as IFN-g or TNF-a) without a proper feedback regulation to control the inflammatory response. Thus, the incontrollable inflammatory response causes the deregulation of the haemocoagulation system and the disruption of vascular endothelial cells provoking plasma leakage, shock and haemorrhagic manifestations (107).

ZIKV was described 5 decades ago. It was not until the large outbreak occurred in 2016 in Brazil, that affected around 1.5 million people, that ZIKV threat wasconsidered by the public health offices. The majority of the ZIKV cases (80%) are asymptomatic and the disease is mild and self-limiting. The initial clinical symptoms are similar to DENV and other emerging viruses. The most problematic outcome for ZIKV infection is the cause of severe developmental defects in the human brain such as microcephaly or the Guillain-Barré syndrome to the new-born. In pregnant infected patients, ZIKV can be transmitted vertically and infect the foetus (108). Then, ZIKV can enter to neural progenitor cells by the AXL receptor altering signalling pathways and immune responses. In consequence, cells undergo apoptosis and there is a down-regulation of neurogenesis that leads to cell cycle arrest and death of developing neurons. This can result in neurodevelopmental disorders with the reduction in brain size due to an impairment of cell proliferation and death of cortical progenitors (109).

WNVis a neurotropic virus wide spread all over the world. In the USA there was a huge outbreak from 1999 to 2010 that highlighted the danger of emerging viruses outside their current habitat representing a serious danger to the world (110). The majority of the WNV infections are asymptomatic, however those that present symptoms (20%) can develop severe neurological manifestations with sequelae and death. After the initial period of incubation (2-14 days), the typical symptoms are fever, myalgia, arthralgia, headache, fatigue… The worst cases, estimated in 1 every 150 infections, result in chronic kidney diseases, paralysis, meningitis, encephalitis or other neurological disorders (111). The development of encephalitis depends on the capacity to neuroinvade the central nervous system (CNS). WNV can pass through the blood-brain barrier (BBB) thanks to the pro-inflammatory response and the increase of the permeabilization. Then, once in the CNS, WNV can infect several neuronal tissues. Epidemiological studies showed that immunocompromised or elderly people are highly susceptible to develop severe neurological diseases. The mortality rate after neuroinvasive infection is around 10% whereas more than the 50% have long-term sequelae (110).

### 2. *Hepacivirus*

HCV is mainly transmitted through exposure to HCV-contaminated blood. Most infections (around 80%) remain persistent, summing up to an estimated 150 million chronic HCV carriers worldwide (112). As persistent HCV infection frequently causes chronic hepatitis that can progress to liver cirrhosis and liver cell carcinoma, it is a major threat to human health. Treatment options for chronically infected individuals have dramatically improved over the last few years. This has been due to the development of highly potent directing antivirals (DAAs) that increased sustained response rates to over 90% (103,113). As these treatments are quite expensive, nowadays the main challenge to stop the pandemic is the access to treatment. HCV is the most characterized member of the *Flaviviridae* family and in this thesis we used it for some experiments as a model system.

## B. VIRION COMPOSITION

The members of the *Flaviridae* family have single-stranded, positive-sense RNA genomes. Their viral genomes range between 9 to 11 kilo-bases with a single, long ORF flanked by highly structured 5' and 3' untranslated regions (UTRs).

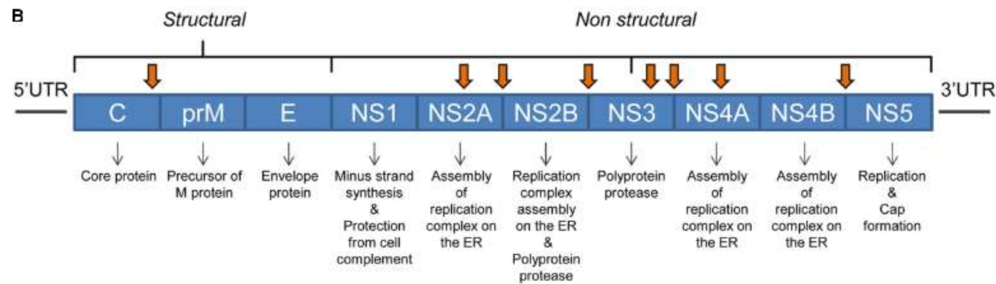


***Figure 8. Flavivirus genome organization.*** *Genetic organization of Flavivirus genomes. They have a cap structure and no poly (A) tails. Their genome is divided in the different structural (SP) and non-structuralproteins (NSPs). Adapted from Fernández-Sanlés, Front Microbiol 2017.*

The different viral genomes encode for single polyproteins that are later post-translationally cleaved into the different viral proteins. These include structural proteins (SPs), important for the formation of the virion, and non-structural proteins (NSPs), required for viral RNA replication (**Figure 8** & **Figure9**). SPs are located in the N-terminal region of the viral polyprotein and include the Capsid protein (C), the envelope protein (E) and for flavivirus, the pre-membrane protein (prM). The different NS proteins needed for the replication are homologous among the different *Flaviviridae* members. They contain conserved motifs and are encoded at similar locations in the genome. The NSPs of members of the family *Flaviviridae* include the RNA-dependent RNA polymerase (RdRp), proteases, helicases and other proteins involved in the replication or in the escape of the immune responses. In general, members of Flavivirus genus have cap structures in the 5'-UTR that allow the cap-dependent translation (**Figure 8**) whereas members of the Hepacivirus such as HCV, contain IRES (**Figure 9**). The genome 3'-UTR ends do not contain poly A tails. Instead, they harbour hairpin structures that stabilize the genome (114–116). Regarding the virion structure, *Flaviviridae* viruses have a spherical enveloped virion with a diameter of about 30 to 60 nm. The structural capsid-

protein assembles to form the capsid that protects the viral RNA and is covered by different glycoproteins associated with the envelope. Thanks to this enveloped structure, virions are fused to the plasma membrane and evade the host immune system (114,116).

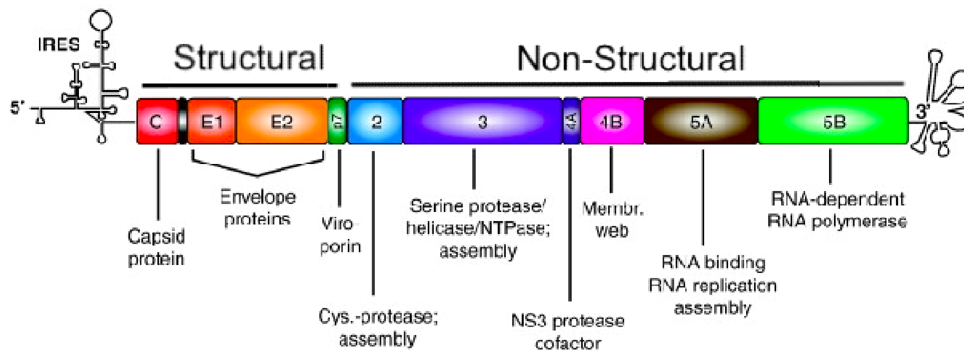


***Figure 9. HCV genome organization and characterization of the viral proteins function.*** *Genetic organisation of HCV genome. It contains an IRES in the 5'-UTR and no poly (A) tail. HCV genome encodes for a polyprotein that contains the different structural (SP) and non-structural proteins (NSP). Adapted from Bartenschlager, Trends Microbiol 2011.*

Besides the information contained in the primary sequence, viral RNA genomes have an additional layer of information contained in their secondary and tertiary structures. Viral RNA genomes are highly structured molecules not only in the UTRs but also in the coding region (117,118). Long-range structures in the 5' and 3' terminal regions have been described to be crucial for translation and replication of viruses. Moreover, the majority of these features are conserved among the flavivirus family indicating a high degree of selective pressure to maintain them and suggesting an important role. Until now the best characterized viral RNA structures are present in the terminal regions and some local structures throughout the genome are being computationally predicted. Thanks to the application of structural techniques like SHAPE (Selective 2′Hydroxyl Acylation analyzed by Primer Extension) into viral genomes, now it is possible to better characterize those predictions and decipher internal RNA secondary and tertiary structures within the RNA genome.

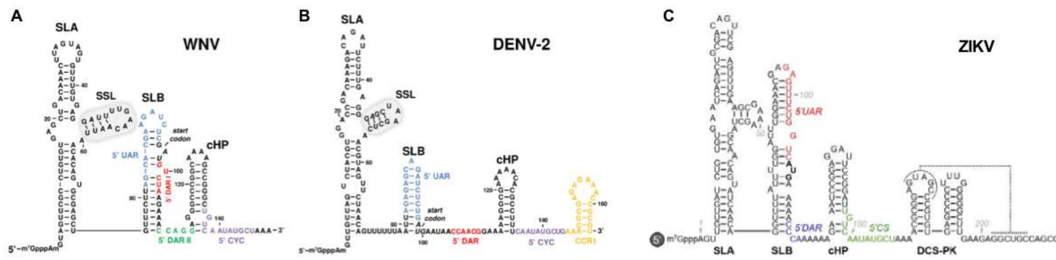


***Figure 10. Secondary structure of the 5'UTR of flavivirus genomes.*** *The figure represents the 5' UTR end of WNV (**A**), DENV (**B**) and ZIKV (**C**) including the 5'-cap, the large stem loop (SLA), the short stem loop (SLB) and cHP (capsid region hairpin). There is a conservation of the structures within the three viruses. Adapted from Fernández-Sanlés, Front Microbiol, 2017 and Yun, J Microbiol 2017.*

The 5'-UTR contains critical structures to control translation, replication and stability. In flavivirus, there are two conserved structural elements, the large stem loop (SLA) and the short stem loop (SLB). SLA is folded into a Y-shaped structure with a side stem loop and a top loop. Filomatori et colleagues showed that it is crucial for DENV replication and its mutation impairs the synthesis of viral RNA (119). SLB has been described to be involved in the interaction between the two terminal UTRs, critical for the viral cyclisation and the proper viral replication. These elements are conserved in different Flavivirus, suggesting a conservation of their functional role (**Figure 10**). Hepacivirus such as HCV containan IRES structure in the 5'-UTR. The IRES comprise several stem-loop (SL) domains: II, III and IV. The starting codon AUG locates within the SL IV. In addition, the core region contains three stem loops (SL V, VI, 588) essentials for viral RNA translation (112). In the other SL domains from the IRES we find essential structures for the translation initiation. Besides, their role in translation, most of the stem-loops in the 5'-UTR have been described to have a role in viral RNA replication (118) (**Figure 11**).

The 3'-UTR contains RNA structuresimportant to facilitate viral RNA replication, interaction with the 5'-UTR and protection from degradation. Three main RNA structures have been defined in the 3´UTR of flavivirus, the SL I-IV, the DB1-2 and the CRE elements. The SL II and SLIV elements contribute to nuclease resistance. The DB1-2 element is formed by two pseudo-repeats crucial for

translation. The most characterized structure within the 3'UTR is the Cis-acting replication element (CRE or 3'SL), presented in some viruses by a pseudoknot structure (sHP), crucial for the replication process (120) (**Figure 12**). The 3'-UTR of HCV contains three main structures, a variable region (VR), described to contribute to viral replication but not essential; a poly (U/C) tract, that interacts with the 5'-UTR; and the 3' X-trail, a highly structured region formed by three stem loops that is essential for replication and RNA packaging. In HCV the CRE element is located in the coding region of NS5B protein, 90 nucleotides upstream the 3'-UTR, and forms a kissing-loop with the 5'-UTR SL IV and the 3'-UTR(112,121) (**Figure 11**).

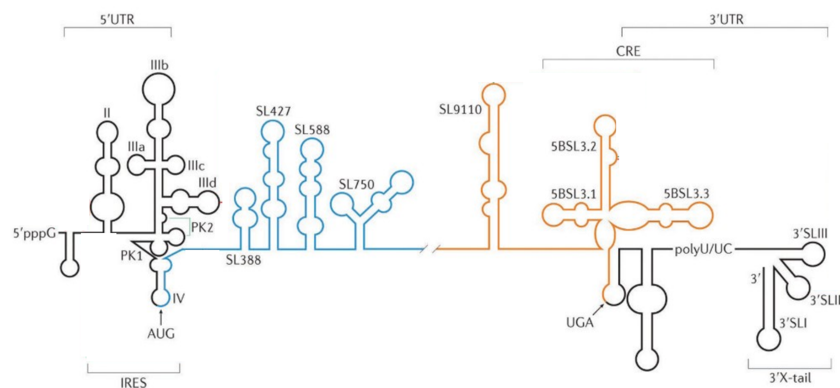


***Figure 11. Secondary structure of the HCV genome.*** *Representative structure of the HCV genome including 5'-UTR, IRES, coding region and 3'-UTR. Adapted from Li, Nat Rev Microbiol, 2015.*

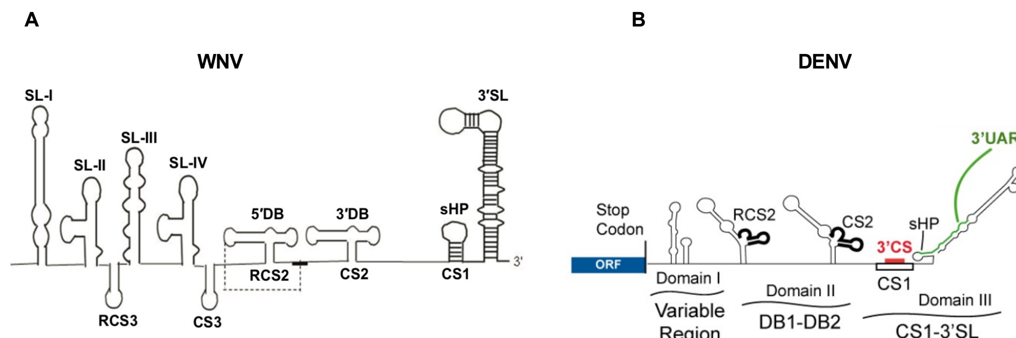


***Figure 12. Secondary structure of the 3'UTR of flavivirus genomes.*** *Representative figure of the 3' UTR end of Flavivirus: WNV (**A**) and DENV (**B**) including the stem loop structures (SLI-IV), dumbbell regions (DB) and the CRE (3'SL) element. Adapted figure from Gebhard, Viruses, 2011 and Brinton, Viruses 2014.*

The coding region of viruses from the *Flaviviridae* family contains multiple RNA structures (112), most of them of unknown function. One example is the conserved hairpin (cHP) structure found in several flavivirus downstream of the start codon (122).

## C. INFECTION CYCLE

All flavivirus share some common features in their infection cycle. After the binding to specific-receptors in the host cell, the virions enter to the cell by endocytosis. The fusion between the viral envelope and the cell membrane is pH-dependent. A low pH allows the rearrangement of the conformation of the envelope protein to an unfolded structure that drives their insertion into the endosome membrane. Then, the viral RNA genome is released from the endosome to the cytoplasm where the entire life cycle occurs. As the virion does not contain the viral polymerases, the first step for the viral RNA genome is to act as an mRNA to translate the viral polyprotein. For this, the RNA genome mimics the host mRNA to be efficiently recognized by the host translation machinery. Next, host and viral proteases cleave the polyprotein to generate the different viral proteins. Some of the polyprotein products remain in the cytosol while others will cross the ER. The second step for the viral RNA genome is to act as template for the replication. This process occurs in the replication complex, a membrane multicomplex within the ER formed by host membranes, the viral RNA, the viral NS proteins (proteases, helicases and the RNA-dependent RNA polymerase), lipid droplets and host factors. This localized replication increases the efficiency of the process, protects the genome from viral degradation and eliminates the competition from other processes (123). First, the (+) RNA genome generates a negative-strand RNA intermediate that acts as a template for the generation of (+) RNA synthesis. Thus, new generated genomes have three different fates: undergo translation, RNA replication or the formation of virus particles. The last step in the viral cycle is the formation of the virion that occurs in the cytoplasm when the viral genomes assemble with the capsid proteins. Then, immature virions interact

with viral glycoproteins displayed in the cytoplasmic side of the ER and the capsid enters to the ER-lumen by budding. A pre-requisite for the maturation of the virions is the sequential trimming of glucose residues from the glycoprotein surface by host glucosidases. This process occurs in the ER under the supervision of host chaperons. Then, viruses undergo final maturation in the trans-Golgi network following the secretory pathway. There, thanks to a decrease in the pH, the envelop protein (E) change its conformation and the prM, present in flavivirus, is cleaved. prM is essential for the control of the proper folding of the viral envelop proteins and prevents virion maturation before the release of the virion. Finally, virions egress from the cell via exocytosis (**Figure 13**) (103,114).

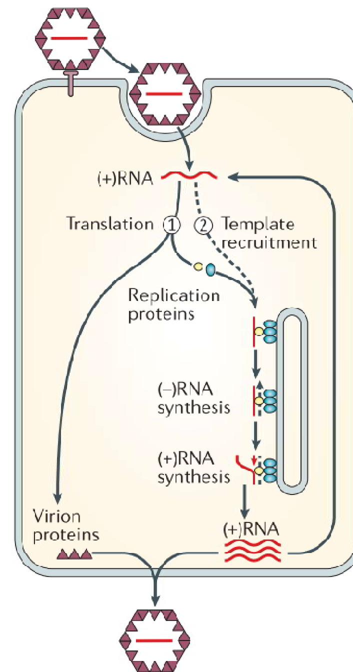


***Figure 13. Positive strand RNA virus life cycle.*** *Once the virus enters by endocytosis to the cell, the (+) RNA molecule is released and translated to produce the viral proteins. This (+) RNA molecule will also later serve as a template for replication, which occurs in association of cellular membranes. Finally, the viral RNA progeny is encapsidated and exit the cell. Adapted from Ahlquist, Nature 2007.*

## D. VIRAL VARIATIONS

Members of the Flaviviridae family, as all (+) RNA viruses, encode viral RNA polymerases with low fidelity because they lack the proofreading function. This has a major effect in their biology and treatment. The high mutation rates (estimated around 1 mutation each $10^4$-$10^5$ nucleotides) generates such a genetic variation that in viral RNA progenies each individual is closely related to others but not identical. This structure of viral populations is named

quasiespecies (124). It provides a great capacity of adaptation to changing environments, such as the presence of antiviral or immune responses, because mutation/s required to overcome them are likely already present in the viral population. This is the paramount importance for the development of efficient antiviral treatments as resistant mutants might be easily selected (125).

## ANTIVIRALS

There is undoubtedly an urgent need to develop efficient treatments against some of the most virulent emerging viruses such as chikungunya virus (CHIKV), DENV, WNV and ZIKV (103). Currently, there are no known treatments available in the market for these emerging viruses, and the only feasible containment measure currently available is to control the mosquitoes that transmit them with insecticides, which have shown very poor performance. Consequently, the Word Health Organization stated that the control of these emerging viruses remains as key challenge for the foreseeable future.

DENV, CHIKV, ZIKV and WNV viruses produce acute infections and require to be treated early after the onset of the symptoms for drugs to be effective. However, an early diagnosis of these infections remains as an unsolved challenge due to the fact that there are not accurate serological test and costly molecular techniques are needed for an accurate diagnosis. Moreover, these viruses are spread through identical geographical areas, share the same mosquito vectors and produce similar initial symptoms (101,126). Therefore, in contrast to the traditional one virus-one drug strategy, the best option to treat these infections would be broad-spectrum antiviral drugs (BSAs) (103,126). They would allow treating patients early after the onset of initial symptoms even before a proper diagnosis has been achieved. How to develop such BSA drugs?

The classical approach to develop antiviral treatments focuses on protein-based drugs that directly target viral proteins (directed acting antivirals, DAAs) or cellular proteins used by viruses to complete their viral lifecycle or by the immune system to improve the response to the viral infection (host-acting antivirals, HAAs). Lately, recent RNA-based approaches are revolutionizing the pharma market. This novel strategy might provide new schemes to develop BSAs.

## A. PROTEIN-BASED ANTIVIRALS

Nowadays the majority of available antivirals in the market are DAAs against viral polymerases or proteases. Nevertheless, antivirals against other crucial viral proteins have also been designed to prevent viral entry and exit or chromosomal integration (127). In spite to their success to treat some viral infections, DAAs present three major drawbacks. First, DAAs target one specific viral protein and structure. Consequently, they have a narrow-spectrum of action. Some of them are solely active against one specific viral serotype. Second, most viral human pathogens contain RNA genomes replicated by low fidelity viral RNA polymerases. The high viral RNA replication rates combined with this low fidelity result in frequent selection of scaping mutants. Third, generating molecules that specifically block the function of the viral protein is an expensive and time-consuming process that includes structural studies and inhibitor screenings. Development of broad-spectrum DAAs antivirals would require focusing on region of viral enzymes conserved throughout different viruses. This high conservation implies an essential viral function and thus it would be predicted mutations in this region to be deleterious and eliminated from the viral population. Some of the most promising viral targets for BSA development are viral proteases and viral polymerases (103). For example, inhibitors of the HIV retrotranscriptase are also active for the hepatitis B retrotranscriptase. However, in HBV patients these drugs are not as efficient as in HIV patients (128).

Development of broad-spectrum antiviral HAAs would conceptually solve some of the drawbacks of DAAs as host proteins are highly conserved and genetically stable. Moreover, some host factors are used by multiple viruses. However, viruses tend to depend on essential proteins and targeting them might lead to serious toxic effects (129). Currently, there are several HAAs in clinical trials but none of them act as a BSA. Another approach to obtain BSA drugs would be to boost the immune responses against viral infections. A common strategy has been to administer interferon (IFN) to increase the activity of interferon stimulated genes (ISGs) with antiviral functions (113). For example, IFN

treatment was used in the early days of HCV treatment when there were no other therapeutic options. However, IFN causes serious secondary effects and it is not effective in multiple virus infections.

## B. RNA-BASED THERAPEUTICS

The discovery of different classes of regulatory RNAs opened the possibility of using RNA molecules to treat human diseases that could not be treated by conventional protein-based therapies. These novel RNA-based therapeutics can mimic or antagonize the function of host or viral RNAs and thus it should be possible to manipulate gene expression to treat viral infections. In comparison to the costly, time-consuming and target limited protein-based therapies, the versatility of RNA molecules offers the attractive possibility to overcome these long-standing problems increasing the likelihood of targeting from single point mutations to genome fragments. Moreover, as RNA-based therapies target nucleic acids, almost every gene in the genome is a potential target of these therapies giving the opportunity to treat previously untreatable diseases. RNA-based drugs are chemically designed and *in vitro* produced reducing the production costs and timing and avoiding variability of biologics. This allows a more controllable and more convenient process because it allows chemical modifications of the drug. Therefore, in contrast with therapeutic antibodies, which are generated *in vivo*, RNA-based drugs have less variability, less immunogenicity, and are more stable.

It is then not surprising that RNA therapeutics are in the spotlight of pharma industries. In 2018 there were 400 companies with RNA-based therapeutics in clinical development or clinical trials to treat a wide range of diseases from cancer, viral infections or genetic disorders. These RNA-based approaches are mainly based (i) on the binding of complementarity molecules to different transcripts to regulate their expression or splicing and (ii) on the transfection of synthetic mRNAs. They include antisense mechanisms to inhibit gene expression, genetic editing, mRNA-based overexpression and aptamers

(**Figure 14**). Next, we will first summarize the main characteristics of each of these strategies and then their putative use to treat viral infections (130,131).

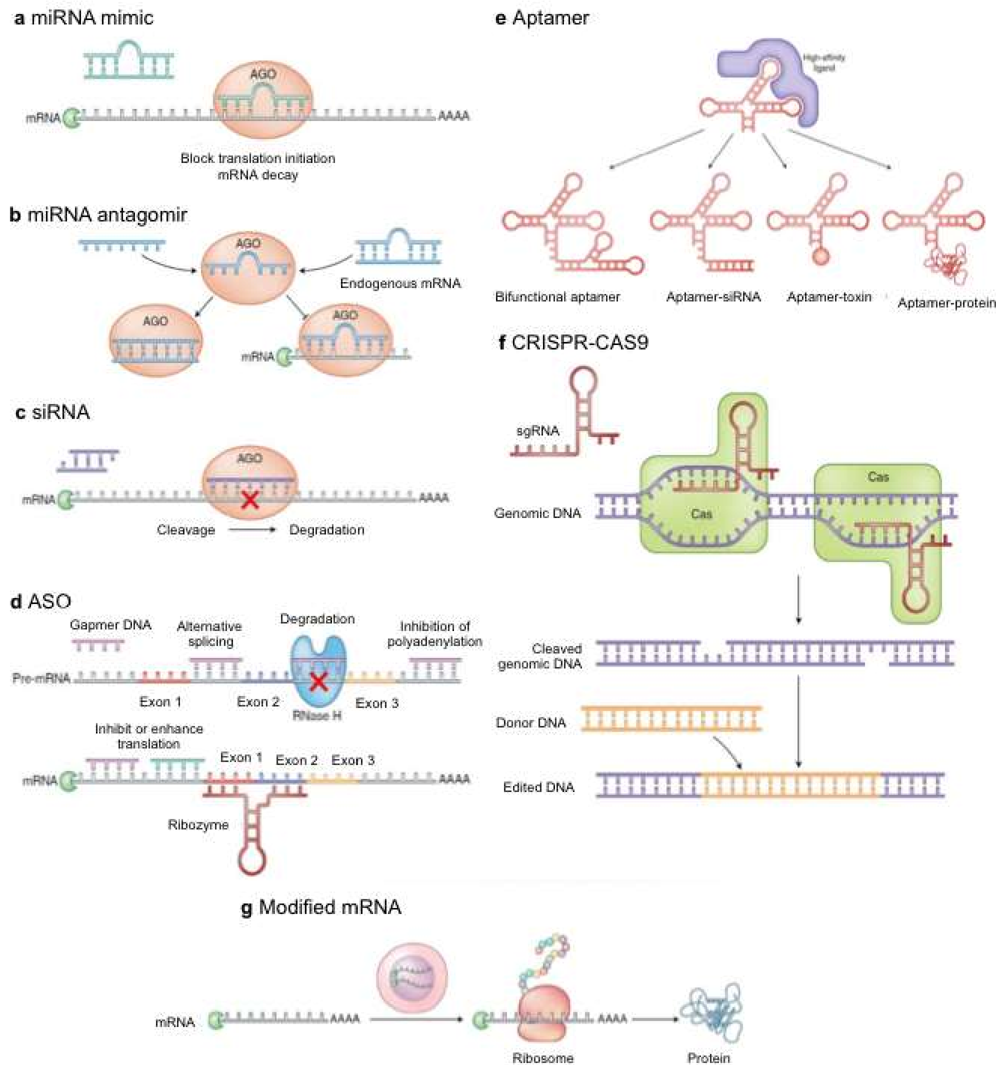


***Figure 14. RNA-based therapeutic strategies***. *(**a-d**) Antisense mechanisms. (**e**) Aptamers. (**f-g**) gene editing mechanisms. (**a**) ASOs can bind pre-mRNA promoting alternative splicing, degradation mediated by RNAse H or inhibit the polyadenylation (above). They can also bind to mRNAs inhibiting or enhancing translation (below) (**b**) siRNAs can bind mRNAs triggering the degradation of the molecule by AGO complex. (**c**) miRNA mimic imitates the role of host miRNA and can block translation initiation or promote mRNA decay. (**d**) AntagomiR will bind host miRNA inhibiting the degradation of miRNA-target mRNAs. (**e**) Aptamers can bind with high affinity the desired ligands. (**f**) In the CRISPR-Cas9 approach, sgRNAs provoke a cleavage in the genomic DNA that can be edited and add donor sequences. (**g**) Modified mRNAs can be transfected in the cell to translate the proteins of interest. Adapted from Lieberman NatStruct Mol Biol, 2018*

## 1. Antisense mechanisms to inhibit gene expression

The RNA-based antisense therapeutics are based on cellular complementary antisense mechanisms that trigger mRNA decay and include micro RNAs (miRNAs), small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs). These small non-coding RNAs bind to cellular transcripts and promote their degradation through the activation of the Ago2/RISC silencing complex.

There are two main miRNA-based strategies: miRNA mimics and miRNA inhibitors. miRNA mimics are synthetic double-stranded oligonucleotides that mimic the function of miRNAs in the cell degrading targeted mRNAs or inhibiting their expression (**Figure 14a**). These different outcomes depend on the complementarity between the miRNA sequence and the target sequence. When the complementarity between them is partial, miRNAs repress translation whereas if it is perfect, it drives the degradation of the target mRNA. In contrast, miRNA inhibitors are antisense single-stranded oligonucleotides that bind miRNAs to inhibit their function. Consequently, the targeted mRNAs will be up-regulated. Depending on the length of the synthetic molecule we find: antagomirs, which are chemically modified oligonucleotides (21-23 nucleotides) with complementarity sequences beyond the seed region (**Figure 14b**); and Locked-Nucleic Acids (LNA), shorter single-stranded oligonucleotides with a methylene group that bind the seed region of target miRNAs. LNAs block more rapidly, efficiently and with higher stability the resulting duplex (132).

The siRNAs are double-stranded short-interfering RNA molecules (21-23 nucleotides) that induce mRNA degradation. The different strains of the siRNA will bring the target mRNA and the Ago2/RISC complex which will cleave the specific mRNA. This process can be carried out repetitively by the same siRNAs (133) (**Figure 14c**). Finally, ASOs are single-stranded short oligodeoxynucleotides that can alter mRNA and decrease, restore or modify protein expression (102). Thus, if the ASO binds the mRNA generating a DNA-RNA duplex, RNAse H can recognize the duplex and cleave it, degrading the mRNA (134). Alternatively, ASOs may target translational start sites or block

key regions for RBPs or ribosomes ultimately leading to translation repression (**Figure 14d**) (135).

## 2. Genetic editing

In the last years, the possibility to edit genomes has come into the spotlight after the characterization and application of the CRISPR/Cas9 technique (**Figure 14f**). This technique is based in an essential mechanism of the adaptative immunity in bacteria and archaea. In brief, CRISPR/Cas9 is based on the cleavage of specific double-stranded DNA sequence and the activation of the double-strand break (DSB) repair mechanism. This process is mediated by a synthetic single guide RNA (sgRNA) which targets a specific DNA site. Then, the RNA-guided DNA endonuclease Cas9 cleaves the DNA generating DSB. Depending on the presence or absence of homologous repair template, there are two different possible scenarios: non-homologous end joining, which results in insertions or deletions (indels) of the targeted zone, or homologous direct repair, which results in precise mutations and knock-ins depending on the template (136). Currently, CRISPR/Cas9 editing has been widely adapted in multiple cell lines and organisms, from plants to animals, to correct mutations via indels or the knock-out of entire genes (137).

An additional mechanism to edit gene expression is the use of ASOs to modulate splicing, binding splice sites in order to include or exclude exons or introns (**Figure 14d**) (138,139).

## 3. Overexpression mechanisms to up-regulate transcript levels

Two main RNA-based approaches are also used to alter protein levels in the cell. They include: (i) the transfection of synthetic modified mRNAs to supply complete, mutated or truncated transcripts (140) (**Figure 14g**) and (ii) ASOs to up-regulate the expression of some transcripts via binding the 5'-UTR to block translation initiation of upstream ORFs (uORFs) and promote the translation of canonical ORFs, or via disrupting inhibitory structures allowing translational initiator factors to bind (141).

### 4. Aptamers

Aptamers are single stranded DNA or RNA molecules of 20 to 100 nucleotides in length with defined structures. They complementary pair with themselves forming three-dimensional (3D) structures that specifically bind to targets. The 3D interactions are essential for binding and affinity to the different targets. Aptamers mimic the mode of action of therapeutic antibodies that recognize specific antigens. Moreover, aptamers present several advantages in comparison with antibodies (i) they have smaller and more flexible structures that allow the recognition of small targets, closely related molecules or hidden domains inaccessible to therapeutic antibodies; (ii) they are generated using *in vitro* platforms decreasing the manufacturing time and cost; (iii) the potential targets of antibodies are immunogenic molecules whereas aptamers have a wide range of potential targets; and (iv) they are more stable molecules than antibodies that can be lyophilized for their transport at room temperature. Currently, aptamers have been proposed to be used instead of therapeutic antibodies not only as treatment but as a way to deliver different drugs. Aptamers can be conjugated with toxins, siRNAs or proteins (142) (**Figure 14e**).

## C. DRAWBACKS OF RNA-BASED ANTIVIRAL THERAPIES

There are three main drawbacks in the development of RNA-based antiviral strategies. First, RNA molecules are very unstable and degraded by the abundant intracellular ribonucleases. To avoid degradation, they need to be modified. Typically, RNA modifications are performed at the level of the phosphodiester bond or the 2'-OH of the ribose sugar. These modifications provide more stability and resistance to phosphodiesterases. Moreover, modifications of the 2'-OH reduce the immune system recognition as well as avoid off-target effects. Second, RNA delivery into the cell should be improved. The lipid bilayer allows small, neutral and quite hydrophobic molecules to pass through. Instead, RNAs are large, charged molecules with high molecular weight. In order to overcome this problem, several delivery vehicles are under

study. Theyare viral agents (adeno-associated viruses, AAV) and non-viral agents (nanoparticles, naked or conjugated RNA) (143). Inside the non-viral agents, we find two main approaches, (i) the use of lipidic or polymeric nanoparticles that increase the cellular uptake and protect the RNA from degradation; and (ii) the conjugation of the RNA molecule with a specific ligand that allows the binding to specific receptors (144). There are some companies that commercialize *invivo* transfection reagents based mainly on the first approach. Polyplus®, for example, is a company that has developed and commercialized jetPEI®, a polymeric nanoparticle composed by polyethilenemine (PEI) that allows the transfection of nucleic acids highly efficiently and avoids their rapid degradation. Third, activation of the innate immune system should be taken into account. Cells have internal nucleic acid sensors like PKR, RIG-I and MDA5 that can detect foreign RNAs and activate the IFN response. This activation may lead to rapid degradation of the transfected RNA molecule. Last, some RNA-based strategies can present unintended off-targets. Despite these challenges, some modifications to avoid those drawbacks have been done and others are under study. Chemical modifications have shown to increase RNA stability and decrease immunogenicity (131,144–146).

## D. RNA-THERAPEUTICS TO TREAT VIRAL INFECTIONS

Nowadays the applicability of RNA therapeutics in the antiviral market is starting to be exploited. Although they are a promising approach to solve some of the problems derived by protein-based antivirals, their development is in an initial phase and all approved and commercialized RNA-based therapeutics are for non-infectious diseases. These include:pegaptanib, one RNA aptamer anti-VEGF to treat ocular vascular diseases; eteplirsen and nusinersen, two ASOs that alters gene splicing to treat Duchenne's Muscular Distrophy (DMD) and Spinal Muscular Atrophy (SMA); and patisiran, one siRNA against the 3'-UTR of TTR, the accumulation of which causes polyneuropathy of hereditary TTR-mediated amyloidosis (hATTR).

In the early 2000, Fomivirsen, an ASO-based antiviral against Cytomegalovirus (CMV) mRNA, was approved. This drug was an effective treatment against CMV-retinitis in patients with AIDS unresponsive to other treatments (147).However, the low number of AIDS patients with this complication made no rentable the commercialization of this drug and the RNA therapeutic treatment is no longer available (145). Nowadays, there are multiple RNA-based therapies under clinical trials. Some examples are a phase II LNA against miR-122, an essential miRNA for HCV life cycle (148–150); siRNAs against Respiratory Syncytial Virus (151), Ebola virus (EBOV) (133), Hepatitis B virus (HBV) and HIV (133). Clinical trials with aptamers are being developed, as well. There are two main aptamers designed to fight against viral infections, one against NS5B protein of HCV and another against HIV. The latter consists on an aptamer that combines two main functions, to block the interaction between HIV gp120 and CD4 receptor, and to cell-specific deliver a siRNA against HIV (142).

Nevertheless, although multiple efforts are made to develop RNA-based antivirals to target viral genomes, it is a challenging endeavour. The main drawback is mainly due to the rapid emergence and selection of mutations in RNA genomes that inhibit the interaction with their potential targets. Moreover, there is a poor translation of the cell culture results to the clinical use. However, the advantages in front of other protein-based therapies and the possibility from RNAs to be modified in order to increase their stability or decrease their immunogenicity encourage further investigation.

Overall, there is an increasing interest for new RNA-based antiviral therapies but the majority of them are in initial phases. More research has to be done but the actual direction is quite promising.

# OBJECTIVES

**From the cell…**

Viruses are obligate intracellular parasites that rely completely in the cell host-machinery to multiply. CircRNAs have been described to be differentially expressed in viral infections. However, it is not known whether (+) strand RNA viruses are relying on these molecules to perform its cycle and what is their role in this process. Understanding the interplay between circRNAs and viruses will help not only to better understand the biology of circRNAs but also elucidate whether circRNAs can be a good target for antiviral drugs.

The first aim of this thesis is to characterize the interplay between host circRNAs and flaviviruses using HCV as a model system. The specific objectives are:

1. To study whether HCV infection alters the circRNA expression landscape in the host cell
2. To determine whether differentially expressed circRNAs affect HCV infection
3. To determine whether different members of the *Flaviviridae* family can be affected by similar circRNAs

**… To the market**

Antiviral development is facing two main problems: the urgency to develop new BSAs against emerging diseases and the need to find effective drugs to the emergence of resistant mutants to therapeutics. In the last years, RNA therapeutics are in the spotlight because they can be directed against targets that were undruggable for conventional drugs and are relatively simple to produce and manipulate. A major drawback of RNA therapeutics is the intrinsic instability of RNA molecules. In this context, the second aim of this thesis is the use of stable circRNAs as novel broad-spectrum antiviral platform to treat emerging infections. For this, we will synthesise circRNA molecules designed to disrupt essential and conserved RNA structures in the viral RNA genomes.

The specific aims of this section are:

1. To identify essential RNA structures in the viral RNA genomes that can be disrupted by binding of complementary RNA sequences
2. To synthesise circRNAs containing the selected complementary RNA sequences
3. To test the effect of the synthesised circRNAon HCV, DENV and WNV as model systems.
4. To generate a BSA-circRNA

# RESULTS

# Identification of Host-derived Circular RNAs that Display Pro- and Anti-Viral Activities in Flavivirus-Infected Cells

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# CircRNAs-based broad spectrum therapy for viral infections

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## Abstract

Synthetic RNA biology is poised to be one of the most explosive fields of the 21st century. In this context, aided by state-of-the-art Artificial Intelligence based software, we have developed synthetic circular RNAs with the potential to inactivate functional structural regions of multiple viral RNA genomes,with the final goal of developing the long-pursued broad-spectrum antivirals. These circRNA-based broad-spectrum antivirals (i) are intrinsically stable molecules (ii) canbe administered before an accurate diagnosis, dramatically increasing the likelihood of viral control, (ii) are less likely to suffer from mutational escape and (iii) enormously reduce the cost and time required to develop virus-specific specific drugs.As a proof of concept here we develop circRNAs targeting the chronic hepatitis C virus and the acute dengue, chikungunya and west Nile viruses. Our developed circRNA-based platform is ready for being applied to other virus infectious and non-infectious diseases.

## Introduction

RNA viruses comprise some of the most virulent emerging viruses such as Dengue virus (DENV)[1,2], chikungunya virus (CHIKV)[3], west Nile virus (WNV)[4], zika virus (ZIKV)[5]and new viruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV)[6]. In the last decade the number of cases has increased due to climate change and the expansion of their mosquito vectors. Nowadays, there are no treatments or effective vaccines against them. All these viruses produce acute infections and require to be treated early after the onset of the symptoms for drugs to be effective. However, an early diagnosis of these infections still remains an unsolved challenge due to the fact that there are not accurate serological tests and all of these viruses are spread through identical geographical areas, share the same mosquito vectors and produce similar initial symptoms[7]. Moreover, costly molecular techniques are needed for an accurate

diagnosis[2]. Therefore, conventional virus-specific drugs targeting viral enzymes are not an efficient approach to treat these viral infections[8]. In order to overcome these problems, the generation of a broad-spectrum antiviral (BSA) arises as an effective approach to treat all the infections even before an accurate diagnose is provided[9].

Synthetic RNA biology is a growing research field with ground-breaking repercussions in clinical settings both in diagnostics and in therapy. From the emergence of RNA switches (riboregulators) around 15 years ago[10] to its role in CRISPR-Cas technology[11,12], the potential of the field has barely been tapped. With its first approved clinical applications around the corner[13], synthetic RNA biology is poised to be the most explosive field of the 21st century. The design of synthetic RNAs has been performed "manually"[14], with experimental techniques such as SELEX for aptamers[15] or, more recently, using specialized software tools[16]. Designs have included siRNAs[17], miRNAs[18,19], riboswitches[20], RNA thermometers[21,22], ribozymes[23,24], etc. even logic AND/OR circuits[25,26].

Circular RNAs (circRNAs) are a novel class of RNAs generated from linear RNA progenitors by an alternative splicing mechanism termed back splicing[27]. CircRNAs are highly stable relative to their linear spliced counterparts due to exonuclease resistance[28]. Hundreds of unique circRNAs are expressed in mammals, often in a tissue-specific manner. Currently, there are few known cellular functions of circRNAs, although a handful appears to act as miRNA sponges[29].

In this paper, we introduce, to the best of our knowledge, the first artificial circRNAs, designed using a specialized software tool called circaDesign, to inhibit viral RNA replication and/or translation. Our artificial circRNAs target relevant secondary structures of the viral genomes of (+) strand RNA viruses, yielding conformational changes that impede essential functions of the life cycle of these viruses. The paper shows a journey from a proof-of-concept HCV antiviral to a proof-of-concept BSA, passing through successful antivirals for

several emerging viruses. The development of BSA will reduce enormously the cost and time required for the development of specific drugs or vaccines. With our approach we are not only targeting several viruses at the same time but also developing an RNA-based platform ready for being applied to other viruses or diseases.

## Results

### Circular RNAs designed to target viral RNA structures

Approaches to target RNA viral genomes in order to disrupt their life cycles are not new. These include, to name a few, aptamers[30], trans-cleaving ribozymes[31] and siRNAs[32].However, these molecules (i) are rapidly degraded and (ii) share with protein- and antibodies-based therapies the emergence of drug resistance by the high tendency of viruses to mutate. Our approach aims at mitigating these two drawbacksby using circRNAs to target viral RNA genomes. First, circRNAs are highly stable molecules. As they have no ends, 5' or 3' exonucleases, responsible for most of the RNA degradation pathways, cannot carry out their function.Second, the designed artificial circRNAs are unlikely to suffer from mutational scape as they target several different long structural regions in the RNA viral genome (**Figure 1.1**). Viral RNA genomes contain highly structured regions within both untranslated regions (5'-UTR or 3'-UTR) and the coding sequence (CDS) that provide a new layer of information contained insecondary and tertiary structures. These structures are essentialfor multiple functions of the viral RNA and its infectivity, such as translation, replication, localization or/and encapsidation and consequently highly conserved. Moreover, to minimize further the risk of drug resistance, hybridization regions in the designed circRNAs (**Fig. 1.1**) that target the same viral region are all different as we included G-U pairing. In contrast to DNA where complementarity is required, G-U pairing is a valid hybridization pair in RNA.

We have generated a circRNA-based platform designed to hybridize selected regions of an RNA viral genome. The mechanism of action is not just the hybridization, but the structural changes triggered upon hybridization that will disrupt key features of the viral RNA structural motifs, ultimately affecting its function (**Fig. 1.2**). Thus, a key issue is to select vital structures in the viral RNA genome that contains a single stranded region in order to start the hybridization,similarly as in the concept of toehold switches[33]. Given the complexity of the design of such artificial circRNAs, we have developed a Constraint Programming based tool to automate the process. This allows us to streamline the design phase and produce highly probably successful candidate molecules to test in a wet-lab setting. Ultimately, the success of the generated circRNAs will depend on the proper designed and folding of the circRNAs and the relevance of the target structure in the viral life cycle.

**HCV: proof of concept**

For our proof of concept we decided to use the hepatitis C virus (HCV) as it is an excellent model system of positive-strand RNA virus biology whose RNA structure has been resolved[34]. **Figure 2A** depicts a schematic view of the HCV genome along with the chosen target regions. Our six artificial circRNAs (circHCV1-6) target such regions in the following manner: circHCV1-2 target regions of the IRES structure and circHCV3, circHCV5 and circHCV6 target regions of the CDS. We have designed an additional circRNA that combines circHCV1-3 targeting a region of the IRES and one of the CDS at the same time (circHCV4). Regarding circRNAs targeting the IRES structure, circHCV1 contains 7 hybridization sites of length 33 nucleotides (nt) and circHCV2 contains 11 hybridization sites of length 24 nt. Regarding circRNAs that target the coding region, circHCV3 contains 8 hybridization sites with a length of 28 nt, circHCV5 contains 12 hybridization sites of 11 nt-length and circ HCV6 contains 13 hybridization sites of 18 nt. For circHCV4, we have combined 3 hybridization sites per target region (circHCV1-3). The target regions in the HCV genome are

depicted in figure 2 and sequences and target regions for each circRNA are detailed in **Supplementary Table 1.**

To evaluate the effect of our designed-circRNAs on the viral infectivity, we took advantage of a circRNA-expression plasmid (pCV)[29]. The pCV containstwo complementary sequences and two splice sites flanking the insert that promote the back-splicing of the desired sequence and its circularization. Different HCV candidate sequences were cloned into pCV and human hepatoma Huh7/Scr cells were transfected with theresulting plasmids. Twenty-four hours later, cells were infected with HCV. To easily quantify effects on HCV infectivity we used a virus carrying a luciferase reporter (HCVJc1-luc). Finally, 48hours post-infection, we measured luciferase levels (Protocol in **Fig.2B**). Of note, as transfection rates of Huh7/Scr cells with circRNAs were only 40-50%, any decrease of luciferase levels equal or higher than2-fold were considered highly significant. Compared to the empty plasmid (negative control), three of the tested circRNAs (circHCV1, -4 and -5) caused a major decrease in viral infectivity (**Fig.2C**). From these, for further evaluation we focused on circHCV5 that targets a region in the HCV CDS involved in a tertiary interaction required for viral RNA replication[34]. The other two circRNAs contain sequences targeting the IRES element required for translation initiation. While very efficient, the IRES elements are solely present in some (+)RNA viruses and thus, this strategy could not be scaled to a general platform.

Then, we tested whether, besides initiation of infection, circHCV5 may inhibit infections already established. For this, Huh7/Scr cells were infected with HCVJc1-luc and 48 hours later transfected with circHCV5. Luciferase values were measured 24 hours post-transfection.Importantly, circRNAHCV5 inhibited infectivity with similar efficiency as when cells were expressing the circRNAHCV5 before infection (**Fig. 2D**). Next, weexamine whether the circHCV5 is indeed inhibiting the function described for the target sequence, viral RNA replication. For this, we used HCV RNA replicons that harbour a luciferase reporter gene but not the viral structural genes required for

encapsidation. Thus, these replicons allow efficient translation and replication of the viral RNA genome but not virion production. Huh7/Scr cells were transfectedwith the circHCV5 or the corresponding empty plasmid and the next day, transfected with the HCV replicon. Luciferase values were measured at 4 hours and 48 hours post-HCV replicon transfection. These times were selected because already established kinetics[35,36] prove that luciferase production derive at 4 hours solely from HCV RNA translation and at 48 hours from both translation and replication. Indeed, a decrease in viral infectivity was observed at 48 but not at 4 hours post-transfection (**Fig.2E**) indicating that circHCV5 impairs viral RNA replication.

**Emerging viruses: a practical application**

Given the success of our proof of concept, we extended our study to the emerging Dengue (DENV), West-Nile (WNV) and Chikungunya (CHIKV) viruses, for whom there are no efficient therapies. To test effects on infectivity of these viruses we use, as for HCV, virus derivatives carrying a luciferase reporter gene. All Infections were carried out in HEK293 cells. Eight circRNAs (circDENV1-8) were designed to target the DENV RNA genome with 7 hybridization regions of 32 nts. CircDENV1-3 target RNA structures in the 5'UTR, circDENV4-6 in the 3'-UTR and circDENV7-8 in the CDS. Six circRNAs (circCHIKV1-6) were designed to target the CHIKV RNA genome. CircCHIKV1 target RNA structures in the 5'UTR, circCHIKV2-3 in the 3' UTR and circCHIKV4-6 in the CDS. All six circRNAs contain 6 hybridization regions that target the corresponding regions in the CHIKV genome. Finally, seven circRNAs (circWNV1-7) were designed to target the WNV RNA genome. CircWNV1-2 target the cHP structure, circWNV3-5 the sHP and circWNV6-7 the stem-loop III (SL-III) of the 3'-UTR. All candidates contain 7 hybridization regions of 31 nts. All sequences and target regions are detailed in **Sup. Table 2-4**.

Effects on infectivity of all designed circRNAs were tested as outlined in **Fig. 2B** except that for CHIKV infections luciferase values were measured 16 hours post infection. Two of the designed circRNAs for each virus highly inhibited infectivity (**Fig. 3**). ThecircDENV1 and circDENV7 target structural regions mainly involved in replication[37,38], one in the 3'UTRand the other a hairpin element (cHP) in the capsid coding sequence (**Sup. Fig. 1**). Moreover, **Figure 3D** shows how it is indeed replication what is being impaired by both circRNAs.For CHIKV, we seepromising results for both circCHIKV1 and circCHIKV6, which target, respectively, a structured region of the 5'UTRand a region of the CDS known as Recoding Element (**Sup. Fig. 2**) in the CHIKV genome. There are 2 types of recoding elements: the Termination Codon Readthrough (TCR) and the Programmed -1 Ribosomal Frameshift (-1 PRF). In this case, our circRNA targets a PRF signal located towards the 3' end of the CHIKV 6K gene[39]. Finally, for WNV, we see that circWNV6 and circWNV7 show exciting results, lowering the infectivity by around 50% with respect to the negative control. In this case, both circRNAs target a region in the 3'UTR of WNV genome named stem-loop III (**Sup. Fig. 3**). Stem-loop III seems to be involved in translation initiation [40].

**The Holy Grail: a Broad-spectrum Antiviral**

There is an urgent need to develop new antivirals against emerging viruses. Viruses like DENV, WNV and CHIKV share initial symptoms and they have a very difficult and costly differential diagnose. To overcome this problem and attack all viruses at the same time, one possible solution is the design of broad-spectrum antivirals. The search of BSAs has been for a long time like the search of the Holy Grail, theoretically possible but difficult to achieve. We have designed, as a proof of concept, one circRNA combining the best target regions of HCV and DENV. The circRNA circDENV7-HCV5 contains 3 hybridization regions against DENV genome and 3 against HCV genome (**Sup. Table 5**).

We transfected the new designed candidate circDENV7-HCV5 and, as a control, both circHCV5 and circDENV7. Then, we infected the cells with DENV-Rluc or HCV-Fluc. Infectivity results are shown in **Figure 4**. As expected, neither the negative control nor the circ HCV5 significantly impaired DENV infectivity. We see similar results with HCV infectivity where neither the empty plasmid nor circ DENV7 show an effect. However, circ DENV7 and the new broad-spectrum candidate, circDENV7-HCV5 did show a significant decrease and the same results can be seen in circHCV5 and circDENV7-HCV5, for HCV infection. Excitingly, these results show the potential of circRNAs as broad-spectrum antivirals.

## Discussion

In this paper we show the journey towards developing the holy grail of antiviral therapies: a broad-spectrum antiviral. Through this journey, several relevant contributions arise: (i) The novel idea to use circRNAs as therapeutic agents, exploiting their greater stability, (ii) the targeting of structured regions in RNA viruses in order to disrupt them and impede essential functions of the viral genome, (iii) the development of an AI-based software to design circRNAs to target such regions, (iv) the concept and development of a broad-spectrum antiviral against emerging viruses and (v) the optimization ofa protocol for the production of in vitro circRNAs to reach high yields at low cost (see **Appendix 1**).

On the one hand, circRNAs are covalently-closed molecules without endings. This lack of 5' or 3' ends confers them resistance to exonucleases. Exonucleases are key enzymes in the RNA degradation pathway that cleave nucleotides from either 5' or 3' ends. As circRNAs do not have entry points, this confers them a higher stability than other linear RNA molecules[41],providing an advantage of circRNAs to the rapid degradation of other RNA-therapeutics.

On the other hand, it is well known that certain regions in the viral genomes are relevant due to their structure, in many cases because they either bind to RNA binding proteins (RBPs) or because they provide the adequate structural context for nearby regions to bind to RBPs. In our approach, the novelty lies in the fact that we target those structures in order to disrupt them, thus impeding the binding of RBPs and therefore jeopardizing the viral life cycle step they are involved in.

The results of our approach in terms of decrease in infectivity are clear. However, the fact that this decrease is indeed provoked by the mentioned mechanism might be subject to scepticism. We have shown, however, that the life cycle step impaired by our circRNAs is indeed the step in which the target structure is involved in (see **Fig.2D** and **Fig.3D**). In order to further prove the mechanism of action, we plan on doing MS2-immuno precipitation[42], which will show that our circRNA and the viral genome hybridize *in-vivo*. Moreover, we plan on doing *in-vitro* SHAPE experiments to prove both that the hybridization occurs and that such hybridization yields a structural change in the target region of the viral genome in-vitro.

Through our journey we have developed successful circRNAs against all regions: 3'UTR, CDS and 5'UTR; and structures both involved in replication and translation. A by-product of our approach is that our circRNAs are very robust against mutational escape by the viruses. This follows from a combination of several factors: (i) the length (and number) of the hybridization regions, which is typically around 30 nts, (ii) the strength of the hybridization, which typically supports several mismatches and allows for GU hybridization base pairs, (iii) and the low mutation capacity of the target regions. Let us expand on the last 2 factors. Consider, for instance, a target region in the CDS of a viral genome. This region needs to both maintain a specific structure to remain functional and to code for the same amino-acid sequence, or at least similar enough that the coded protein can perform the same function. Due to the high relevance of the selected regions for the virus, the number of potential mutations is really low,

being the majority of them deleterious. This, along with the use of GU hybridization base pairs, explains why very few mutations of such region can escape the targeting of a hybridization region of our circRNAs. It is important to recall that each of our circRNAs contains several different hybridization regions against the same viral structure, which renders a potential mutational escape almost non-existent. In this regard, it is important to acknowledge that in future designs, target regions will be chosen so that they are conserved across serotypes and so that the mutational escape potential (which can be theoretically calculated) is minimal. Moreover, the final goal is to design a broad-spectrum candidate against WNV-DENV, two of the most important emerging viruses.

Finally, we plan on doing *in-vivo* experiments in mice in order to test whether the efficacy shown in cell cultures translates into *in-vivo* settings, therefore paving the way towards a near-future real clinical application. Such in-vivo protocol has been already planned and commissioned, and results will be forthcoming. It consists in intravenous injections of a nanoparticle with the circRNA and the subsequent infection. Due to the rapid degradation of naked RNAs and the lack of proper information about the distribution, we decided to use jetPEI technology based on nanoparticles (in vivo-jetPEI®, Polyplus Transfection). They increase the stability of the RNA molecules in serum and facilitate endocytosis and the uptake from the cell. In order to know the success of our treatment, several aspects of the viral response and infectivity will be monitorized during the experiment.

To conclude, throughout this paper we have shown how our ground-breaking approach is capable of greatly reducing infectivity of emerging viruses, by harnessing the power of RNA synthetic biology applied to design and develop circRNAs that target relevant structures of the viral genomes. Infectivity decrease range from 80% to 50% in all cases, which is extraordinary given that, in all our experiments, we only reached 40-65% transfection efficiency. Although these results were measured with a GFP-plasmid, we expect similar

efficiencies for our constructs. Note that these viruses produce acute infections, this means that we only need to reduce infectivity below a symptomatic threshold in order to both allow the body to cure itself and stop infection propagation (which is typically non-existent among non-symptomatic individuals).

## Material and Methods

### Plasmids

The circoVIR (CV) plasmid was constructed by deleting 1466nt (1910–3375) from pcDNA3-CiRSplasmid (gently provided by Thomas B. Hansen) and inserting unique restriction sites between the splicing and circularization signals. Customized gBlocks (Integrated DNA Technologies), flanked by BamHI and EcoRI restriction sites, were amplified and then cloned into a BamHI-EcoRI linearized circoVIR plasmid. All constructs were verified by sequencing.

The Plasmid to generate HCV (pFK-Luc-Jc1) carrying the Firefly luciferase reporter gene has been previously described[43]. The plasmid to generate DENV (pFK-DVs-R2A) carrying the Renilla luciferase reporter gene and the plasmid to generate the subgenomic replicon (pFK-sgDVsR2A) have been previously described and gently provided by Ralph Bartenschlager[44]. The plasmid to generate WNV (pCCI-KUNV NanoLuc) carrying the reporter gene of NanoLuc luciferase and the plasmid to generate CHIKV (pSP6-CHIK-2SG-GLuc) carrying the reporter gene of Gaussian luciferase were kindly provided by Dr. Merits (University of Tartu, Estonia). The pCHIKV-GLuc is based directly on the viral sequence isolated from a human patient from La Reunion (isolate LR2006_OPY1 (DQ443544)). The HCV subgenomic replicon plasmid (pFL-J6/JFH1EMCVIRESaRlucNeo) was kindly provided by Apath, LLC (St. Louis, MO) and has been previously described[45].

### Cell cultures

The human hepatocarcinoma cell line Huh7/Scr, human hepatocarcinoma cell line Huh7.5.1 Cl2, kidney epithelial monkey cells VERO and the human embryonic kidney cell line HEK293 were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated foetal bovine serum (FBS) and 10% non-essential amino acids.

The baby hamster kidney cells BHK21 were maintained in Glasgow's modified Eagle's medium (GMEM, Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated foetal bovine serum (FBS) and 10% Triptose Phosphate Buffer (TPB). Cells were grown in an incubator with 5% $CO_2$ at 37 ºC. BHK21, VERO and Huh7.5.1 Cl2 were used for the viral production. Huh7/Scr were used in all HCV experiments whereas HEK293 were used for DENV, CHIKV and WNV infections.

**Viral production**

The pFK-Luc-Jc1 was linearized with the *MluI* enzyme (FastDigest) while the HCV subgenomic replicon (pFL-J6/JFH1EMCVIRESaRlucNeo) was linearized with the *XbaI* enzyme. Linearized plasmids were purified with a Phenol: Chloroform: Isoamyl alcohol extraction. Purified DNAs were *in vitro* transcribed with the MEGAscript T7 Transcription Kit (ThermoFisher) according to the manufacturer's protocol. RNA from the *in vitro* transcription was purified with RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). pFK-DVs-R2A and pFK-sgDVsR2A were linearized and *in* vitro transcribed as previously described[46]. pWNV was linearized with BshT1 enzyme (FastDigest) and after the purification was*in vitro* transcribed from the SP6 promoter using mMESSAGE mMACHINE kit (Ambion-Life Technologies). To generate HCV-Fluc, the purified RNA from the *in vitro* transcription was electroporated to suspensions of Huh7.5.1 Cl2 cells as described previously[47]. To generate DENV-Rluc, the purified RNA was electroporated to BHK21 cells as described before and the supernatant was harvested 48 hours post-infection[46]. To generate WNV-NLuc, purified RNA was transfected to VERO cells using lipofectamine (following manufacturer's instructions) and the harvest was done 10-12 days post-infection. To generate CHIKV-GLuc, we infected BHK21 cells with CHIKV-GLuc (gently provided by Andres Merits) at a MOI of 0.1 for 1 hour. Then the inocule was replaced for fresh media and the harvest was done 24 hours post-infection. For all the viruses, during the harvest, the supernatant was cleared by centrifugation (5

minutes at 1200 rpm). All stocks were aliquoted and stored at -80ºC. Titers of viral stocks were determined using $TCID_{50}$ or plaque assay.

**Plasmid Transfection and luciferase reporter assays**

$4 \cdot 10^4$Huh7/Scr cells/well or $10^5$ HEK293 cells/well were seeded in 24-well plates the day before transfection. 2 micrograms of each plasmid containing the circRNAs or the empty plasmid were transfected using Lipofectamine (Invitrogen) following the manufacturer's instructions. A GFP-plasmid control was also transfected to control efficiency of transfection 24 hours later with FACS analysis. The following day, DMEM was removed and cells were washed with 1x PBS before the infection. Infections were carried out with luciferase-reporter viruses. For HCV, DENV and WNV, cells were inoculated with the viruses for 4h at 37ºC. For CHIKV, cells were inoculated with the virus 1h at 37ºC. Finally, the inocule was replaced with fresh media. Sixteen hours post infection, luciferase activity was assayed for CHIKV samples; forty-eight hours post infection, luciferase activity was assayed for HCV, DENV and WNV. Cells were washed with 1X PBS, and lysed. For DENV, WNV and CHIKV, viruses carrying Renilla luciferase, cells were lysed in 150 µl of Renilla lysis buffer (1X) and frozen at -80ºC. Upon thawing, lysates were resuspended by pipetting. 4 µl of the lysates were mixed with 20 µl of Renila Luciferase Assay Buffer and 1/200 of substrate from the Renilla Luciferase assay system (Promega) and measured immediately in a luminometer for 2 s. For HCV-Firefly experiments, cells were lysed in 150 µl of Passive Lysis Buffer (1X). Upon thawing, 50 µl of the lysates were incubated 5 minutes with 25 µl of Reporter Lysis Buffer and measured in a luminometer for 2 s. Mean relative light units (RLU) were plotted as percentages relative to control infections (cells transfected with the circoVIR plasmid).

**Replicon experiment**

$4 \cdot 10^4$Huh7/Scr cells/well or $10^5$ HEK293 cells/well were seeded in 24-well plates the day before transfection. 2 micrograms of each plasmid containing the cirRNAs or the empty plasmid were transfected using Lipofectamine (Invitrogen) following the manufacturer's instructions. The following day, DMEM was removed and cells were washed with 1x PBS. 400ul of OptiMEM (Gibco) were added in each well for 30 minutes. 300ng of HCV-replicon RNA carrying Renilla luciferase were transfected using lipofectamine following the manufacturer's instructions. 5 hours and 48 hours post-transfection, RNA and protein were extracted. 300ng of DENV RNA replicon carrying Renilla luciferase (sgDVR2A) were transfected using lipofectamine (Invitrogen) following manufacturer's instructions. 8h and 48h post-transfection, RNA and protein were extracted. From protein lysates, luciferase values were measured with Renilla Luciferase Assay (as explained before). Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's instructions. After TURBO DNAse (ThermoFisher) treatment, quantitative PCRs (qPCR) against viral RNAs were performed using qScript XLT 1-Step Tough Mix (Qantabio) (following manufacturer's protocol). Luciferase values were normalized with the corresponding RNA.

**circRNA design software**

Our circRNA design software is programmed in C++ using the Constraint Programming library OR-TOOLS (https://developers.google.com/optimization/) by Google. The code is available upon demand.

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**Figure 1:**

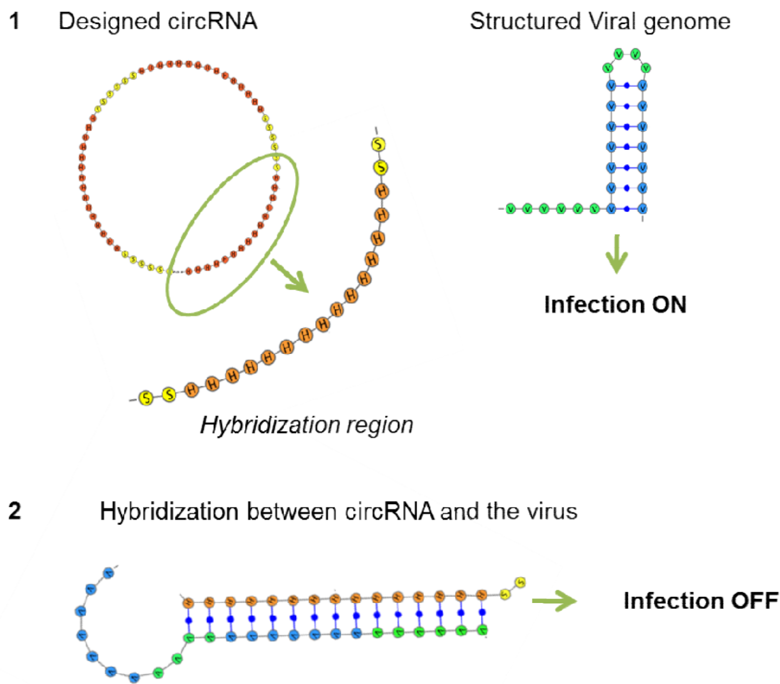


**Fig 1. Artificial circRNA structure and mode of action.1)** CircRNAs contain several different hybridization (brown regions H) and separation sequences (yellow regions S). Hybridization sequences target the viral RNA genome (blue regions) and separation sequences allow for structural flexibility and physical separation among the hybridization ones. Note that all H and S sequences are different among themselves.**2**) Hybridization regions are designed to target and disrupt a particular viral RNA genome structure, leading to a decrease in infectivity.The hybridization starts in a single stranded region, an external or hairpin loop or a pseudoknot and finish within an helix, so that the helix is disrupted.

**Figure 2:**

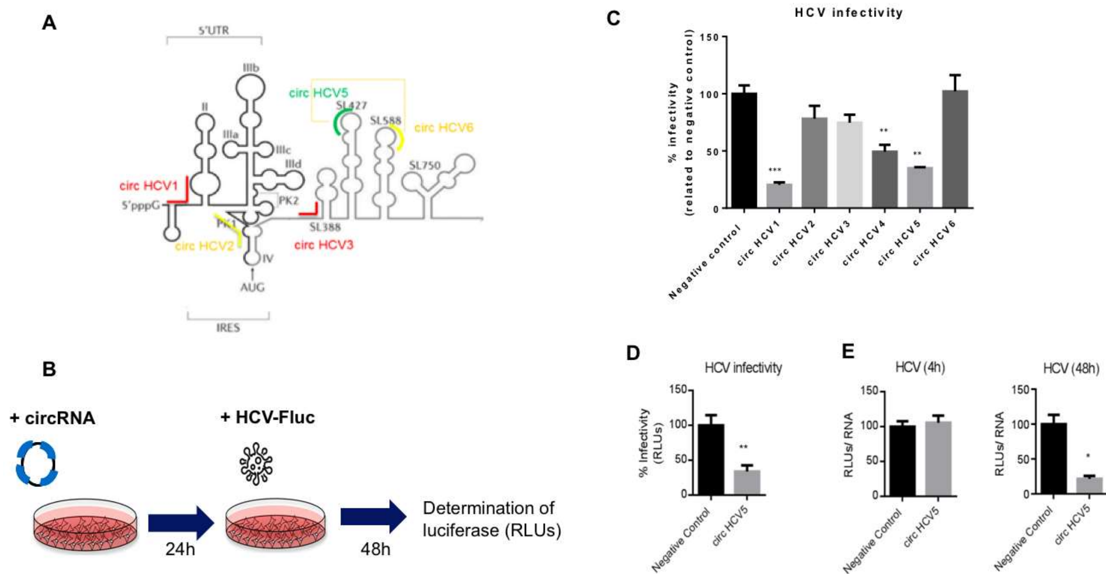


**Fig. 2. HCV proof of concept.A)** Targeted regions in the HCV genome. They include external loops (in red), a hairpin loop (in green) and two pseudoknots (in yellow). **B)** Experimental strategy to test antiviral activity of designed circRNAs. Plasmids expressing circRNAs are first transfected into Huh7/Scr cells. Next, cells are infected with an HCV derivative expressing luciferase (HCV-Fluc). Effects on infectivity are determined by changes in luciferase expression levels. **C)** Effect on designed circRNAs on HCV infectivity. Numbers of the circRNAs indicate the target regions indicated in A. Results are expressed as mean RLU values of three biological replicates (±SEM) relative to negative control. **D)** CircRNAs inhibit infectivity in HCV chronically infected cells.Huh7/Scr cells were infected with HCV and 48hpi transfected with circHCV5. Two days later luciferase values were measured. Viral infectivity was expressed as in B. **E)** CircHCV2, which targets a region required for HCV RNA replication, inhibits HCV RNA replication. CircHCV2 inhibited luciferase levels 48 hours post infection when HCV RNA is translated and replicated but not at 4 hours post infection when HCV RNA is solely translated. Viral infectivity was expressed as in B. Statistical significance was calculated using a T-test (*represents*p*-value < 0.05).

**Figure 3:**

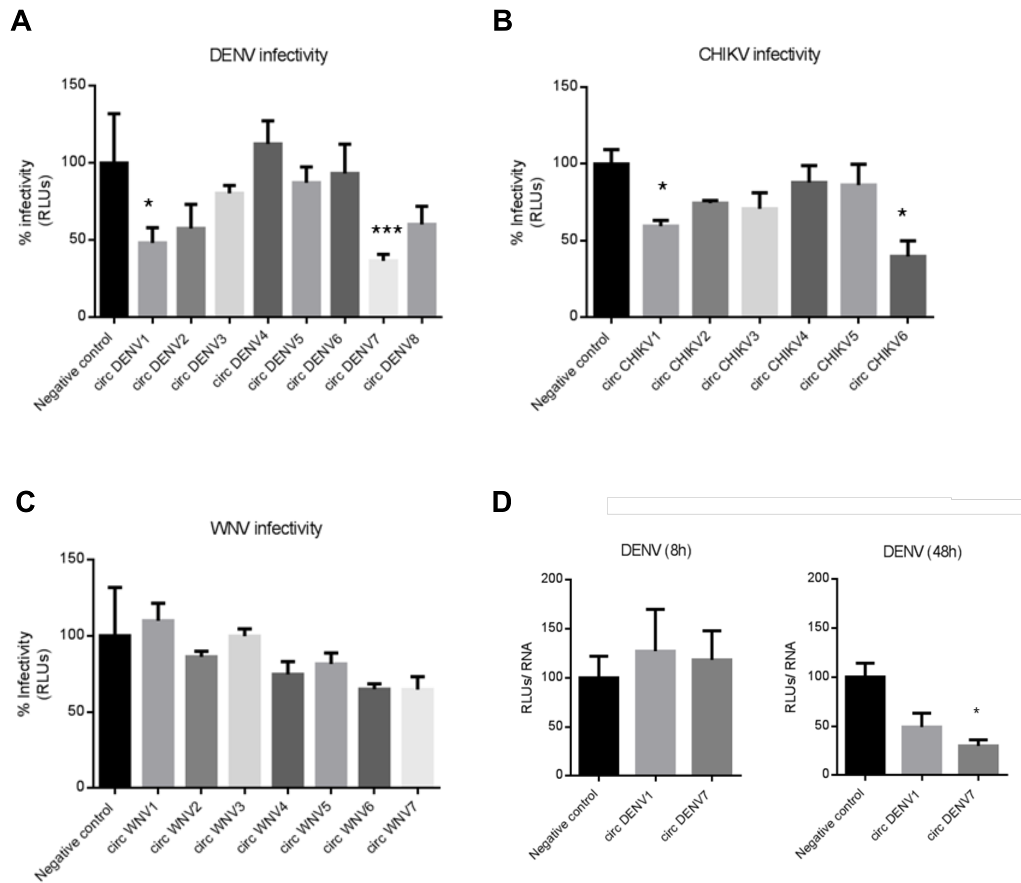


**Fig. 3. CircRNAs against emerging viruses decrease their infectivity.** CircRNAs were designed to target structures in the RNA genomes of **A)** DENV, **B)** CHIKV and **C)** WNV that contain the luciferase reporter gene. **D**) CircDENV1 and -7,designed to target structures within the DENV RNA genome directing RNA replication, inhibit DENV RNA replication. The CircDENV1 and -7 inhibit luciferase expression levels at 48 hours when the RNA genome is translated and replicated but not at 8 hours when is solely translated. All results were obtained from at least three biological replicates and plotted as in Fig 2. Statistical significance was calculated using a T-test (*represents *p*-value < 0.05).

**Figure 4:**

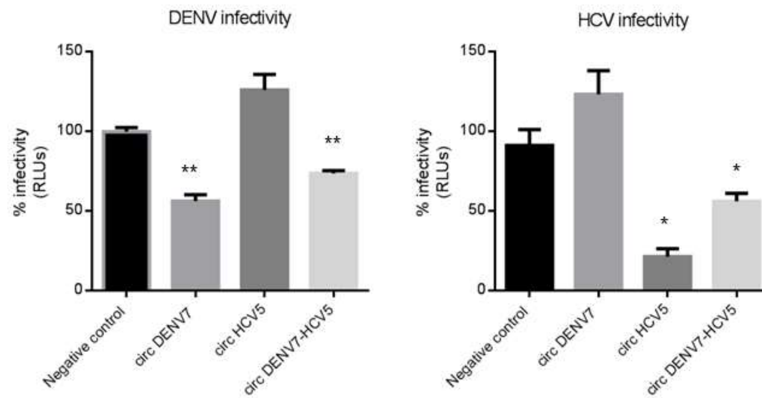


**Fig. 4. Designed broad-spectrum circRNAs impairs both DENV and HCV infectivity**. Cells were transfected with the circDENV7, the circHCV5 or the broad-spectrum circRNA (circ DENV7-HCV5) containing hybridization sequences from circDENV7 and circHCV5. Next, cells were infected either with DENV or HCV harbouring the luciferase reporter gene and 48h later the infectivity was measured. All Results were obtained from at least three biological replicates and plotted as in Fig 2. Statistical significance was calculated using a T-test (*represents $p$-value < 0.05).

## Supplementary Figure 1:

**Sup Fig 1. DENV structures used for the design of circDENV:** Representation of the target regions of the DENV genome. In red the hybridization regions used for the design of the circ DENV. [Adapted from Alvarez et al., 2008[48]].

## Supplementary Figure 2:

**Sup Fig 2. Schematic CHIKV structures used for the design of circCHIKV:** Representation of the predicted 5'-UTR (A), RSE (B) and Recording Element (C). In A and B all the structure is targeted by the circRNA in C, the hybridization region is depicted in red. UTR: untranslated region; RSE: repetitive sequence elements. [Adapted from Kendra et al., 2018 [39]; and from Khan et al., 2002 [49]].

## Supplementary Figure 3:

**Sup Fig 3. WNV structures used for the design of circWNV:** Representation of the 5'-UTR (above) and 3'-UTR (below) from WNV genome. In red the hybridization regions used for the design of the circ WNV. UTR: untranslated region [Adapted from Fernandez-Sanlés et al., 2017 [50]]

## Supplementary Table 1:

| Name | Target Region | Target sequence | Sequence | Hybridization sites |
|---|---|---|---|---|
| Circ HCV1 | IRES | CTCCGCCATG<br>AATCACTCCCC<br>TGTGAGGAACT<br>A | TAGTTCCTCACGGGGGAGTGGTTCGTGGTGGAGCGG<br>CGCCAATGGTTTTTTGCGGGGGAGTGGTTCGTGGCG<br>GGGGTAACCCCTTAGTTCTTCATGGGGGGGTGGTTC<br>ATGGTGGGGAAACTACCGTAGTTTTTCACAGGGGGG<br>TGGTTCATGGCGGAGAGAAGCGCTTAGTTTCTTATGG<br>GGGAGTGATTCATGGCGGAGTGTGGGGCATGGTTTT<br>TCGCAGGGGAGTGATTCGTGGTGGGGAGAGTTTTGT<br>GGTTCTTTGTAGGGGGGTGATTCGTGGTGGGGACAT<br>CATTG | 7 |
| Circ HCV2 | IRES | TCTCGTAGACC<br>GTGCACCATG<br>AGC | TGCCGAAAATGCTTATGGTGCACGGTTTGCGAGAGC<br>AAGAAGAGCTTGTGGTGCATGGTTTGCGGGGACAGT<br>AAAGGCTCATGGTGCATGGTCTACGAGGTGAGATAG<br>CGTTTATGGTGTACGGTCTGTGGGAAATTCCGGTGTT<br>CGTGGTGTGCGGTCTGTGGGATATTAGATCGTTCATG<br>GTGTACGGTTTGTGGGGATAATAGCGGCTTATGGTG<br>CACGGTCTATGGGACGCGAGAAGGCTCATGGTGTGT<br>GGTTTGTGAGGCAGTGTGCGGTTCATGGTGCACGGT<br>CTGCGAGACACCAGGCGGCTCGTGGTGCACGGTCTA<br>TGGGGTGTCGTCGTGCTTGTGGTGTGCGGTCTATGA<br>GGGCGGCTGAA | 11 |
| Circ HCV3 | cHP | CCAAAAGAAAC<br>ACCAACCGTC<br>GCCCAGA | CTGCACTGGGTTTGGGCGACGGTTGGTGTTTTTTTG<br>GACGAGATTTCTTGCTCTGGGCGACGGTTGGTGTTTC<br>TTTTGGGTGTTAGTGCGTACTCTGGGCGATGGTTGGT<br>GTTTCTTTTGGTAATTATTACTTTCTTTGGGCGATGGT<br>TGGTGTTTTTTTTGGTGGGGTGAAAAGAGTTTGGGCG<br>GTGGTTGGTGTTTCTTTTGGCGTGGTGAGTAACATTT<br>GGGTGGCGGTTGGTGTTTTTTTTGGCTGCAACGCAC<br>CGGTTTGGGCGACGGTTGGTGTTTCTTTTGGGGGTC<br>AACCGGGAATCTGGGTGGCGGTTGGTGTTTTTTTTGG<br>TGATGGCCGAGGTA | 8 |
| Circ HCV4 | IRES-CDS | Combination circHCV 1-3 | GCGCCAAGTATAGTTCCTCACAGGGGAGTGATTTATG<br>GTGGAGACCCCTAAACGCTTGTGGTGCACGGTCTAC<br>GGGGTACCGAGAAGTTTGGGCGGTGGTTGGTGTTTT<br>TTTTGGCGCTTGTGGGTGGTTTTTTGCAGGGGAGTGA<br>TTTATGGTGGAGGCAAGAGTTTGCTTATGGTGTACGG<br>TCTGTGAGATGACATCAGGTTTGGGCGGCGGTTGGT<br>GTTTTTTTTGGATGAGGAACCTAGTTCTTCATAGGGG<br>AGTGATTTGTGGTGGAGACAGCGAGTTGTTTGTGGT<br>GCACGGTTTACGAGAAGAATGAAGATCTGGGTGGCG<br>GTTGGTGTTTCTTTTGGTAGCACATGT | 3xtarget |
| Circ HCV5 | CDS | GGGGCCCCAG<br>GTTGGG | CCCCCTGGGGCTCTGATGAGGAACCTCTCTGGGGTC<br>CCCACAGCGAGTCTCCTTGGGGCCCCTAGAATGAAG<br>TTCTTTGGGGTTTCATAGCACGGTTCTCCTGGGGTTT<br>TCATACGATTGTCTCTTGGGGTCTTGCGTGTTTACCC<br>TTTTGGGGTCCTGCTAAGGGGGCTTTCTGGGGCCTT<br>TCGAATAAGTCTTTTTGGGGCCTCGTTTTATCACTCTT<br>CTGGGGTTCCCAGCCTTTCCCTTCTTGGGGCTCCTC<br>CGACCATGCCCCTTGGGGTTCTACCCTGTATG | 12 |
| Circ HCV6 | CDS | GCCCCTCCTG<br>GGGCCCCA | AGACATTGATTGGGGCTTTAGGAGGGGTTGTCGGCA<br>AATGGGGTTTTAGGGGGGGCTGACGTATACTGGGGC<br>TTTAGGGGGGGTCTTGGAAATATGGGGTTTTAGGGG<br>GGGTGCTCGGATCATGGGGTCTCGGGAGGGGTCTTA<br>CGTGAATGGGGTCCTAGGGGGGGTCTGCCACGTTTG<br>GGGCTTTAGGGGGGGCGAACCCGCTTTGGGGCTCT<br>GGGAGGGGTACGGGTCGCATGGGGCTCCAGGAGGG<br>GTGCAATGGTGATGGGGTCCTAGGAGGGGCCGTTTC<br>AAAGTGGGGCTCTAGGAGGGGTGATTGGTAAATGGG<br>GTCTCAGGAGGGGCACGTTATCAATGGGGCCTCAGG<br>AGGGGTTAGAAGTAAC | 13 |

**Sup Table 1. CircRNAs designed against HCV:** The different circRNAs designed against HCV (circ HCV1-6) are classified in the table with information of the region where they target, the sequence, the sequence of the whole circRNA and the number of hybridization sites present in each candidate. IRES: internal ribosomal entry site CDS: coding sequence cHP: capsid hairpin.

## Supplementary Table 2:

| Name | Target Region | Target sequence | Sequence | Hybridization sites |
|---|---|---|---|---|
| Circ DENV1 | 3' UTR | AACAGCAT ATTGACGC TGGGAAAG ACCAGAGA | GCGCCGTCTTTGGTCTTTTTTGGCGTCAGTATGTTGTTT TTTAACCATCTTTGGTTTTTCCTGGCGTCAGTGTGCTGT TATAGTATGGTCTCTGGTTTTTTTTAGCGTTAGTGTGCT GTTAAAGCTTTCTCTCTGGTCTTTTCCAGTGTCAATATG CTGTTTAGTGTGCTTCTTTGGTTTTTCTTGGTGTTGGTG TGCTGTTTCAGCAAGTTCTCTGGTCTTTTCTGGCGTTGA TGTGTTGTTCGCTGGCGATCTTTGGTCTTTCTCGGTGTC GATATGTTGTTAGTGGCCCA | 7 |
| Circ DENV2 | 3' UTR | AACAGCAT ATT GACG CTGGGAAA GACCAGA GA | GCGCCGTTTTTGGTCTTTTTTAGTGTCGATATGTTGTTC GTCAAGCCTCTTTGGTTTTTCCCAGCGTCAGTGTGTTGT TATAGTAACGTCTTTGGTTTTTTTCAGCGTTAATATGTTG TTAAAGCCCTCTCTCTGGTTTTTCCCGGTGTCAGTATGC TGTTTAGTGTACTTCTTTGGTTTTTCCTGGCGTTGATGT GCTGTTTCAATAAGGTCTTTGGTCTTTCCTGGCGTTGGT GTGTTGTTCTCAGACGATCTTTGGTCTTTTTTGGCGTCA ATGTGCTGTTATTCGCCTG | 7 |
| Circ DENV3 | 3' UTR | AACAGCAT ATT GACG CTGGGAG ACCAGAGA | GCGCCGTCTCTGGTTTTTTTCAGCGTTAGTATGTTGTTA AGTCTCACAAGGTCTCTTTGGTCTTTTTCGGCGTCGGT GTGTTGTTCCTGGCCCTGCATTCTCTTTGGTCTTTCCTA GCGTTGGTGTGCTGTTGTGTGCTGTATTAAGTCTCTGG TTTTTTTTAGCGTTAGTGTGTTGTTAGTTATTGTTGTTCC TCTCTGGTCTTTTCCAGCGTCAATGTGCTGTTAACCACC ACTGGACTTCTCTGGTCTTTTCTGGCGTTGATGTGTTGT TCGCGCTACATAAGTT | 7 |
| Circ DENV4 | 5’ UTR | UUUUUUAA UUAGAGA GCAGAUC UCUGAUG AAUA | GCGCCGTATTCGTCGGGGGTTTGCTTTTTGATTGAAAA ATCGGGGCTATATTCATTAGGGATCTGTTCTTTAGTTGA AAAATCGAGGCTTTATTCATTGGGGATTTGCTCTTTGGT TGAAAAACTGCGCGAGTATTCGTTAGAGATCTGCTTTTT GGTTGAAAAATCACCGAGGTATTCATCAGGGATCTGCT CTCTAGTTGAAAAAGTTATGCTCTATTCATCGGAGGTCT GTTTTCTGGTTAAAAAATAGGTCCATTATTCGTTGGAGA TCTGCTCTCTGGTTAAAAAACGTCGTTTA | 7 |
| Circ DENV5 | 5’ UTR | UUUUUUAA UUAGAGA GCAGAUC UCUGAUG AAUA | GCGCCGTGTTCGTTAGGGATTTGCTCTTTAATTGGAAG GGTGTGGCCTTGTTCATTAGAGATTTGCTTTCTAATTGG GGAGATCAGTCGTTATTTGTCGGGGATTTGCTCTTTAGT TGGAGAAGTGCTGAAATATTTGTTGGGGGTTTGCTCTTT GGTTGAAAAATGTGTTGGGTGTTTATCAGAGATCTGCTC TCTAATTGAAAAGTCCGCAATGTGTTCGTCGGGGATCT GCTCTTTGGTTAGAAAGAATGAGCCTTGTTTATTGGAGA TCTGCTCTTTGATTAGAGAGCGCCCCGCA | 7 |
| Circ DENV6 | 5’ UTR | UUUUUUAA UUAGAGA GCAGAUC UCUGAUG AAUA | GCGCCGTATTCGTCGGGGATTTGCTCTCTGGTTAAAAA AGTTATAAACCTCCCGTATTCATCAGAGATCTGTTCTTT GGTTAAAAAAAGAAGGTAATAGAAGTATTCGTTAGAGGT CTGTTCTTTAGTTGAAAAACGACTCCCCCTTCTGTATTC ATCAGGGATCTGCTTTTTGGTTAAAAAAAGTGCACAGCC TCCATATTCATTGGAGATTTGCTCTCTAGTTGAAAAAGG CACTGCGCCCCCGTATTCATCGGAGATTTGCTCTTTGA TTGAAAAACGTCAAAGTACTGTA | 7 |
| Circ DENV7 | cHP | ACGGAAAA AGGCGAAA AACACGCC TTTCAATA T | GCGCCGATGTTGGGGGGTGTGTTTTTTGTCTTTTTTCGT TGGGGTGTAATGTTGAGAGGCGTGTTTTTTGCTTTTTTT TGTGCACCTTATATATTGGGAGGTGTGTTTTTCGCTTTT TTCCGTCTGGCTGGCATATTGGGGGGCGTGTTTTTCGT TTTTTTTCGTTACAAGCTAATATTGAAGGGTGTGTTTTTC GCCTTTTTCCGTGTTAAAGATATGTTGAAAGGCGTGTTT TTCGTTTTTTTCCGTAAGTTTTAAATATTGGAGGGCGTG TTTTTCGCTTTTTTCTGTAGACCGGAA | 7 |
| Circ DENV8 | cHP | ACGGAAAA AGGCGAAA AACACGCC TTTCAATA T | GCGCCGATGTTGAGAGGTGTGTTTTTTGCTTTTTTTCGT TTATTACTCCAACAGATATTGAGAGGCGTGTTTTTCGCT TTTTTTCGTAGAAGGTAATAGAAAATATTGGGGGGCGT GTTTTTCGTCTTTTTTCGTGACTCTTACGAGTTTATGTTG AAAGGTGTGTTTTTCGCTTTTTTTTGTAGGGAACAGTCG CAAATATTGGGGGGTGTGTTTTTCGCCTTTTTTCGTGGC ACCGTAATCCGCATGTTGAAAGGTGTGTTTTTCGTTTTT TTCCGTCAACTCGTTCTTATA | 7 |

**Sup Table 2. CircRNAs designed against DENV:** The different circRNAs designed against DENV (circ DENV1-8) are classified in the table with information of the region where they target, the sequence, the sequence of the whole circRNA and the number of hybridization sites present in each candidate. UTR: untranslated region cHP: capsid region hairpin.

## Supplementary Table 3:

| Name | Target Region | Target sequence | Sequence | Hybridization sites |
|---|---|---|---|---|
| Circ CHIKV1 | 5' UTR | ACACACGT<br>AGCCTACC<br>AGTTTCTT<br>ACTGCTCT<br>ACT | GCGCCGAGTAGAGCAGTGAGGAACTGGTGGG<br>TTGCGTGTGTTTTGTCACTACGTAGTAGAGTGG<br>TAGGAAATTGGTGGGCTGCGTGTGTGGCTTCC<br>AGTGCCAGTAGAGCGGTGAGAGATTGGTAGGT<br>TATGTGTGTGTTCTCGCCCGAAAGTAGAGTGG<br>TGAGGGGTTGGTAGGTTACGTGTGTTCAAATA<br>TGACGGAGTAGAGTAGTGGGGGGCTGGTGGG<br>TTGTGTGTGTTCGGAGACCAGACAGTAGAGTA<br>GTAAGAGGTTGGTGGGCTACGTGTGTGCCACA<br>CCACCCG | 6 |
| Circ CHIKV2 | 5' UTR | ACACACGT<br>AGCCTACC<br>AGTTTCTT<br>ACTGCTCT<br>ACT | GCGCCGAGTAGAGTGGTGGGGGGTTGGTAGG<br>TTGCGTGTGTCTTAGCGCCAGTAGAGCAGTAA<br>GGGACTGGTAGGCTGCGTGTGTGGTACTTACA<br>GTAGAGCGGTGAGAGACTGGTGGGCTATGTGT<br>GTGCCATCCTTAGTAGAGTGGTAGGGAGTTGG<br>TGGGCTGTGTGTGTGGGGCCTATAGTAGAGTA<br>GTGGGAGATTGGTAGGTTACGTGTGTAGCTGC<br>ACGAGTAGAGCAGTGGGAAGTTGGTGGGTTGT<br>GTGTGTCTCCGACGAAGTAGAGTAGTAAGAAA<br>TTGGTGGGTTATGTGTGTACCTGAGTC | 7 |
| Circ CHIKV3 | Repetitive Sequence Element (3' UTR) | AGCAAATA<br>ATCTATAG<br>ATCAAAGG<br>GCTACGCA<br>A | GCGCCGTTGCGTGGCTCTTTGGTCTATAGATT<br>ATTTGTTTGCGGGTTGCCCTCTTGCGTGGTTCT<br>TTGGTCTGTGGATTGTTTGCTTGCCCGCGAGC<br>AGGTTGTGTAGTCCTTTGATCTGTAGGTTGTTT<br>GTTGTTCGTAACACTTCTTGCGTAGCTCTTTGA<br>TCTATAGGTTATTTGCTCTAGGTTCGAACCGTT<br>GCGTGGTCTTTTGATCTATGGGTTGTTTGTTCA<br>TCAAAACCTTTCTTGTGTGGCCTTTTGGTCTGT<br>AGATTGTTTGTTTCCAACGACGTACT | 6 |
| Circ CHIKV4 | CDS | GCTGCTGT<br>AAAACGTT<br>GGCTTTTT<br>TAGCCGTA<br>AT | GCGCCGATTGCGGCTGAGAAGGTCAGCGTTTT<br>GTGGTAGTTGCGGGTTGCCCTCATTGTGGTTG<br>GAAGAGTCAATGTTTTATAGCGGTTGCCCGCG<br>AGCAGGGTTGTGGTTAAGGGAGCCGGTGTTTT<br>ATAGTAGCGTTCGTAACACTTCATTATGGCTAG<br>AAGGGTCAGTGTTTTATGGCAGTCTAGGTTCG<br>AACCGGTTACGGCTGAAGGGGTCGGCGTTTTG<br>CGGCGGTCATCAAAACCTTTCATTGCGGCTAG<br>GGGGGCCAATGTTTTACGGTAGCTCCAACGAC<br>GTACT | 6 |
| Circ CHIKV5 | CDS | GCTGCTGT<br>AAAACGTT<br>GGCTTTTT<br>TAGCCGTA<br>AT | GCGCCGATTATGGTTAAAGAAGTCGGCGTTTT<br>ATGGCAGCATGTGTGCATTATGGTTGGAAGAG<br>TCAATGTTTTGTGGTAGCGAAAATATATTACGG<br>CTGGGAAAGTTGGTGTTTTGTGGCAGCACAGT<br>TCCATTATGGCTGGAAAGGTCGATGTTTTATAG<br>CAGCGCAGGTTCATTATGGTTAGAAAGGTTAG<br>CGTTTTGCAGTAGCGCCCATGCATTACGGTTG<br>GAAGGGTTGACGTTTTATGGTAGCCGCGTCTA<br>ATTACGGTTGGGAGAGTCAGTGTTTTACAGCA<br>GCGCTCCACA | 7 |
| Circ CHIKV6 | CDS | GCTGCTGT<br>AAAACGTT<br>GGCTTTTT<br>TAGCCGTA<br>AT | GCGCCGATTATGGCTAAAAGAGTCAACGTTTT<br>GTAGTAGCTGCGGGTTGCCCTCATTACGGTTA<br>AGAGGGTTAATGTTTTATAGCAGCTGCCCGCG<br>AGCAGGATTATGGTTGGAAAGGTTAACGTTTTG<br>CAGCAGCGTTCGTAACACTTCATTATGGCTAAG<br>GAAGCCAACGTTTTGTGGTAGCCTAGGTTCGA<br>ACCGATTATGGCTAGAGGGGTCAGCGTTTTAT<br>GGTAGCCATCAAAACCTTTCATTACGGCTGAG<br>AGAGCCAATGTTTTACAGTAGCTCCAACGACG<br>TACT | 6 |

**Sup Table 3. CircRNAs designed against CHIKV:** The different circRNAs designed against CHIKV (circ CHIKV1-6) are classified in the table with information of the region where they target, the sequence, the sequence of the whole circRNA and the number of hybridization sites present in each candidate. UTR: untranslated region CDS: coding sequence.

## Supplementary Table 4:

| Name | Target Region | Target sequence | Sequence | Hybridization sites |
|---|---|---|---|---|
| Circ WNV1 | cHP | CAGGAG<br>GGCCCG<br>GCAAGA<br>GCCGGG<br>CUGUCA<br>A | TGCAGTTAGATATTGACAGTTTGGCTTTTGCTGG<br>GCTCTCCTGGTCTCCTATTTTGACGGTTTGGCTC<br>TTGCTGGGTCCTCCTGCGTGTAACTATTGACAGT<br>CCGGTTCTTGTCGGGTTCTCCTGTCTGCCCCAAT<br>TGACAGCCTGGCTTTTGTCGGGTCTTCCTGCAG<br>GGTAACCTTGACGGCTCGGTTCTTGTTGGGCTTT<br>CCTGTCCTGGCAGATTGACAGCTTGGTTCTTGCT<br>GGGCCTTCCTGACGTGCCTATTTGACGGTCCGG<br>CTTTTGTTGGGTTTTCCTGGAAAGAAGCC | 7 |
| Circ WNV2 | cHP | CAGGAG<br>GGCCCG<br>GCAAGA<br>GCCGGG<br>CUGUCA<br>A | TGCAGTTAGATATTGACAGCTCGGCTCTTGCCGG<br>GTTCTTTTGAGGGGTTATATTGATAGTCTGGCTC<br>TTGTTGGGTTCTTTTGGCATTACGACTTGATGGT<br>CTGGCTCTTGCTGGGTTCTTCTGCCTCCGCAAAT<br>TGATAGCCCGGTTCTTGTTGGGCTCTCCTGAATA<br>CATTCTTTGACGGTTCGGTTCTTGTCGGGTCCTC<br>CTGGCTTCGGAGATTGGTAGTTTGGTTCTTGCTG<br>GGCCTTCTTGTTCCAGGTACTTGGTAGTCTGGTT<br>CTTGCCGGGTCCTCTTGGTGAATAGGC | 7 |
| Circ WNV3 | sHP (3-UTR) | AACAGC<br>AUAUUG<br>ACACCU<br>GGGAUA<br>GACUAG<br>G | TGCAGTTAGATACCTAGTTTATTTCGGGTGTCGG<br>TGTGTTGTTTGCACGTATACCTAGTCTATTCCAG<br>GTGTTGGTATGTTGTTGCATTACGCCCCTAGTCT<br>GTTCTAGGTGTCGATGTGTTGTTTCTCCGCAAAC<br>CTAGTTTGTCCCGGGTGTTAGTGTGCTGTTGATT<br>GAAACTCCTAGTTTATCTTGGGTGTTGATATGCT<br>GTTTCCTCGGAGACCTAGTCTGTCTCAGGTGTTA<br>ATGTGTTGTTTTGCAGCTACCCTAGTTTGTTTTAG<br>GTGTTAATATGTTGTTGTGCGACGGC | 7 |
| Circ WNV4 | sHP (3-UTR) | AACAGC<br>AUAUUG<br>ACACCU<br>GGGAUA<br>GACUAG<br>G | TGCAGTTAGATATCTGGTTTGTCCCAGGTGTTGG<br>TGTGCTGTTTGTTCTTATTTCTAGTTTATTTCAGG<br>TGTCGGTGTGTTGTTGCATTAAGGTCCTGGTCTA<br>TCTCAGGTGTTGATGTGTTGTTGATCCGCAAATT<br>TAGTCTGTCTCAGGTGTCAGTGTGCTGTTGAGCG<br>TATCGTTTGGTTTATCCTAGGTGTTAATGTGTTGT<br>TGACTCGGACATTTGGTCTATCTTAGGTGTTGGT<br>ATGTTGTTTTGCAGGTACTCTGGTCTATCCCAGG<br>TGTCGATGTGCTGTTGTGCATAGGC | 7 |
| Circ WNV5 | sHP (3-UTR) | AACAGC<br>AUAUUG<br>ACACCU<br>GGGAUA<br>GACUAG<br>G | TGCAGTTAGATATTTGGTCTGTTCTAGGTGTTGG<br>TATGTTGTTTGCACGTATATTTAGTCTGTCTCAGG<br>TGTTAATATGTTGTTGCATTACGCCCTTGGTTTGT<br>CTTAGGTGTCGGTGTGTTGTTTCTCCGCAAACCT<br>GGTCTATCTTAGGTGTTAATGTGCTGTTGATTGA<br>AACTTCTGGTTTATTCCAGGTGTTAGTATGCTGTT<br>TCCTCGGAGACTTAGTCTATCTCAGGTGTCAATG<br>TGTTGTTTTGCAGCTACTCTAGTTTATTTTAGGTG<br>TCAATATGCTGTTGTGCGACGGC | 7 |
| Circ WNV6 | SL III<br>3' UTR | UUUUGA<br>GGAGAA<br>AGUCAG<br>GCCGGG<br>AAGUU | TGCAGTTAGATAAACTTTCTGGTTTGGTTTTCTCC<br>TCAAAAGACAGTTCTTCGAACTTCCCGGTTTGAT<br>TTTCTCTTTAAAAATGGGGCCCCTCAACTTCTTG<br>GTCTGACTTTCTCTTCAAAAGGCTATACGATCAA<br>CTTCTCGGCCTGATTTTCTTTTCAAAAAGCTATCA<br>TCGCAACTTCCTGGTCTGGTTTTCTTCTCAAAAC<br>CCTCGGTAACCAACTTTCCGGCTTGACTTTCTTC<br>TTAAAACGTTTTGCGACAAACTTTTCGGCTTGGC<br>TTTCTCCTTAAAATATGTAAACAGC | 7 |
| Circ WNV7 | SL III<br>3' UTR | UUUUGA<br>GGAGAA<br>AGUCAG<br>GCCGGG<br>AAGUU | TGCAGTTAGATAAACTTTCCGGTTTGATTTTCTCT<br>TCAAAAGACAGTTCTTCGAACTTCCCGGCTTGGT<br>TTTCTTTTCAAAAATGGGGCCCCTCAACTTCCTG<br>GTCTGGTTTTCTCCTTAAAAGGCTATACGATCAA<br>CTTTCTGGCCTGACTTTCTTCTTAAAAAGCTATCA<br>TCGCAACTTCTCGGCTTGACTTTCTCTTTAAAACC<br>CTCGGTAACCAACTTTTCGGCCTGATTTTCTTCTC<br>AAAAGTTTTGGCGACTAACTTCTTGGTTTGGCTTT<br>CTCCTCAAAAAATATAAACAGC | 7 |

**Sup Table 4. CircRNAs designed against WNV:** The different circRNAs designed against WNV (circ WNV1-7) are classified in the table with information of the region where they target, the sequence, the sequence of the complete circRNA and the number of hybridization sites present in each candidate. SLIII: Stem Loop III. UTR: untranslated region cHP: capsid region hairpin sHP: short stem loop.

## Supplementary Table 5:

| Name | Target Region | Target sequence | Sequence | Hybridization sites |
|---|---|---|---|---|
| Circ DENV7_ HCV5 | DENV cHP HCV CDS | ACGGAAAAA GGCGAAAAA CACGCCTTT CAATAT<br><br>GGGGCCCC AGGTTGGG | GCGCCGATGTTGAAAGGTGTGTTTTTCGTTTTT TTCTGTCAAGTGTAGGATCTTCTGGGGTCTCG GGCCCCACGACCAATGTTGGGGGGCGTGTTTT TCGCTTTTTTCTGTCAAAATAATGTTGACCTGG GGTTCTTTACTGAACTCATTATGTTGGGAGGCG TGTTTTTTGTCTTTTTTTGTTTTATCTACACCCG TCTGGGGTTTTGTCGGCGCCACGACATGTTGG GGGGTGTGTTTTTTGCCTTTTTCCGTCTCAGTG CGGCGCCCTTGGGGTTCCCTTTCGCATCACGT | 4 x target |

**Sup Table 5. Broad-spectrum DENV-HCV circRNA.** Information for circDENV7-HCV5of the two target regions (DENV cHP and HCV CDS), the target sequences, the sequence of the complete circRNA and the number of hybridization sites. cHP: capsid region hairpin CDS: coding sequence.

**APPENDIX 1: Production of *in vitro* circRNAs**

## *In vitro production of* circRNAS

The possibility of using different classes of RNAs as new therapeutics is attracting a growing interest. There are companies that synthesize short RNAs from DNA transcription (in vitro or in vivo) or chemically. Chemical synthesis is based in the addition of nucleotides repeatedly without using enzymes. With this method, short RNA molecules are generated with the desired sequence and length. However, the production of large molecules of RNA is quite expensive. As far as we know there is only cone company that offer to generate custom circRNAs but at a very high cost (around 2000€/µg) and with a limited size (around 100 nucleotides). Because of this we decided to design a novel method to produce circ RNAs at low cost and of longer sizes. For this, we took advantage of self-processing ribozymes to produce circRNAs of 300 nucleotides length at a cost of 3 euro/ µg.

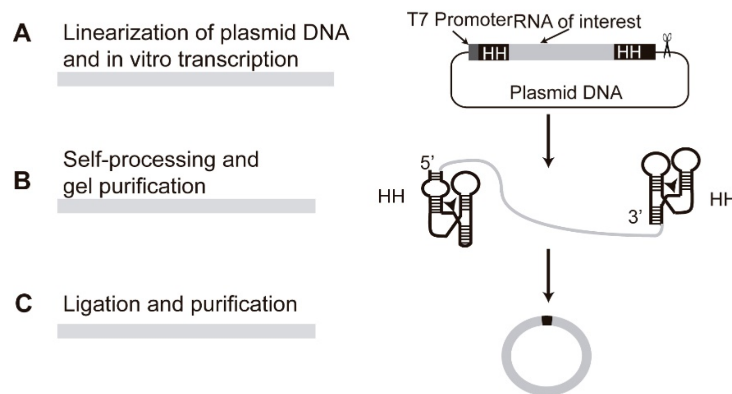


**Figure 1:** Synthesis and circularization of RNA. Workflow composed of three major steps: (A) production of T7 promoter-flanked templates (B) Self- processing of ribozyme- flanked RNAs. (C) Ligation and purification of in vitro circularized RNAs. The overall production cost is 3 euros per µg of circRNA. HH stands for hammerhead.

### 1. CLONING

The desired fragment to be circularized is inserted into a plasmid with the flanking Hammerheads (HH-coral-fragment of interest-HH ELVd) and a T7 promoter. The plasmid contains EcoRI/BamHI restriction sites for the insertion of any fragment with these ends.

## 2. PLASMID PURIFICATION

The resulting plasmid is isolated using Maxiprep Kit (Macherey Nagel) to avoid impurities that might interfere with the self-processing reaction.

## 3. PLASMID DIGESTION

### a. Digestion and confirmation by agarose gel

At least 10 μg of plasmid DNA is digested using Hind III (Fast Digest)at **37ºC for 60 minutes**. Then inactivate the enzyme by heating for 10 minutes at 80ºC and the digestions checked on a 1% agarose gel.

**Scaling up Plasmid DNA Digestion Reaction**

| | | | | |
|---|---|---|---|---|
| DNA | 1 µg | 2 µg | 3 µg | 4 µ |
| FastDigest enzyme | 1 µL | 2 µL | 3 µL | 4 µl |
| 10X FastDigest or 10X FastDigest Green Buffer | 2 µL | 2 µL | 3 µL | 4 µl |

### b. Extraction and ethanol precipitation of DNA

1. Adjust sample to a final volume of 200 µl with $H_2O$ (Sigma).
2. Add an equal volume of PCI (phenol/chloroform/isoamyl alcohol) to the digested DNA solution to be purified.
3. Vortex for 30 seconds to mix well and form an emulsion
4. Centrifuge at RT for 5- 10 min at Vmax
5. Pipette off and keep the top aqueous phase – you MUST avoid taking any precipitated material from the interphase or any phenol.
6. Add 2 µl of glycogen and mix well
7. Add 1/10th volume 3M sodium acetate pH 5.5 and mix thoroughly
8. Add 2 volumes of cold 100% ethanol mix thoroughly and incubate either at –20°C overnight (16 hr) or 30 minutes at -80ºC.
9. Centrifuge at 4ºC 13000 rpm for 30 minutes
10. Remove the supernatant
11. Wash the pellet with 200 µL of cold 70% EtOH
12. Centrifuge at 4ºC 13000 rpm for 5 minutes
13. Remove the supernatant. Dry the pellet at room temp. Never allow the DNA pellet over-dry, otherwise it will be very difficult to dissolve.
14. Resuspend the digested plasmid in 20μl of $H_2O$ (Sigma)

## 4. *IN VITRO* TRANSCRIPTION REACTION

1. Combine the following reaction components at room temperature in the order given:

X μl $H_2O$ (Sigma) (up to 50 μl)
X μl of digested plasmid (4μg)[1]
5 μl 10X Reaction Buffer
5 μl DTT 50 mM
5 μl NTPs 10mM
2.5 μl RNase OUT (Invitrogen)
2.5 μl T7 RNA Polymerase (Takara distrib. by Clontech).

2. Incubate the reaction at 37ºC for 6h.

Note: A successful transcription usually yields a white precipitate after some time. This is Mg-pyrophosphate which forms as a result of NTP hydrolysis. It could be resuspended always without any problem.

3. Add 50 μl of 2X Stop buffer/ formamide loading buffer (8M Urea; 50% formamide, 50 mM EDTA) and keep the transcribed RNAs at -20ºC.

## 5. *RNA PURIFICATION BY PREPARATIVE POLYACRLYLAMIDE GEL ELECTROPHORESIS*

### a. Solutions and buffers: 10X TBE

| Component | Final concentration | Amount |
|---|---|---|
| Tris base | 890 mM | 108 g |
| EDTA | 20 mM | 7.4 g |
| Boric acid | 890 mM | 55 g |
| milliQ $H_2O$ | | Up to 1L |

### b. Preparing the gel

1. Prepare gel plates. Wash plates thoroughly with detergent, rinse copiously with distilled water, rinse with ethanol, and dry with absorbent tissue. Wash spacers and comb the same way.
2. Assemble the gel plates with 1.5 mm spacers
3. Prepare the denaturing gel mix

[1] 4μg or 1 μg of HindIII- digested plasmid is used for preparative or analytical reactions, respectively.

**Denaturing gel mix**

| Component | Stock | Amount |
|---|---|---|
| TBE | 10X | 8 ml |
| Acrylamide/bis-acrylamide (37.5:1) | 40% | 10 ml |
| Urea | | 30.7 g |
| Water (Autoclaved MiliQ) | | 37 ml |
| Ammonium persulfate | 10% | 600µl |

4. Add 32 µl of TEMED to the denaturing gel mix and pour the gel. Insert the comb and allow the gel to polymerize.
5. Mount the gel plates onto the gel running apparatus. Add 1X TBE (100 ml of 10X TBE + 900 ml of autoclaved milliQ H2O) to both the upper and lower reservoirs. Remove the comb and rinse the wells with 1X TBE using a syringe.
6. Pre-run for 30 minutes at 200 V to equilibrate and preheat the gel.

## c. Running the gel

1. Load on the gel also 1-2µl of RNA Century-Plus Marker (1-2µl of RNA Marker + 20µl formamide loading buffer).
2. Heat the in vitro transcribed RNAs and the RNA Marker at 95ºC for 2 minutes and then cooling them on ice for at least 5 min.
3. Rinse the wells extensively with 1X TBE using a syringe. Load the samples into the wells.
4. Run the gel at 200V. Use the mobility of the tracking dyes on the gel to determine when to stop running the gel. (It will take approximately 4h). It is important to avoid overheating of the gel during electrophoresis, since this may result in aberrant migration of the RNA as well as plate cracking.
5. The RNA molecules will be visualized by UV shadowing.
6. Carefully flip the gel plate and soak the gel in 100 ml of autoclaved milliQ water + 2 drops of EtBr for 10 minutes.
7. Rinse the gel in autoclaved milliQ water
8. Cut out the band of interest and keep it at -20ºC.

**Dye Migration in Denaturing Polyacrylamide Gels**

| % Acrylamide | Xylene Cyanol (nucleotides) | Bromophenol Blue (nucleotides) |
|---|---|---|
| 5 | 130 | 35 |
| 6 | 106 | 26 |
| 8 | 75 | 19 |
| 10 | 55 | 12 |
| 12 | 28 | 8 |

**Fig. 3**: *Self-processing* of CDS2 circRNA transcribed from linearized plasmid. The transcription reaction generates a full-length transcript (1) as well as two shorter RNA entities (2-3). The 300 nt band (4) is the one which have to be cut off and eluted from the gel for the *in vitro* circularization assays.

## 6. *IN VITRO* CIRCULARIZATION REACTION

### a. Elution of lineal RNAs

1. Crush the gel fragment using a pestle
2. Wash the pestle by adding 150 μl of chloroform isoamyl-alcohol
3. Repeat the washing step by adding again 150 μl of chloroform isoamyl-alcohol
4. Repeat the washing step by adding again 150 μl of extraction buffer (Tris-HCl 10 mM pH 7.5, EDTA 1mM, SDS 0.1%)
5. Mix vigorously by hand and keep the tube on ice while purifying the other bands.
6. Vortex for 1 minute and then cooling them on ice for 1 minute. Repeat this step for at least 3 times.
7. Centrifuge the tube at maximum speed for 5 minutes at r.t and draw off and save the supernatant in a clean microcentrifuge tube. This supernatant should contain ~ 70% of the RNA.
8. Re-extract the RNA by adding 150 μl of extraction buffer to the tube containing the crushed acrylamide. Mix it again first by hand and subsequently by vortexing.
9. Centrifuge the tube at maximum speed for 5 minutes at r.t and draw off and combine both the supernatants.
10. Add 1/10th volume 3M sodium acetate pH 5.5 and mix thoroughly.

11. Add 2 volumes of cold 100% ethanol mix thoroughly and incubate either at -80ºC for 30 minutes.

Note: Purification yields ~ 9μg of purified 300 nt linear RNA from 4μg digested plasmid.

**b. RNA circularization reaction**

Important: Freshly purified RNAs

1. Set up the *in vitro* circularization reaction
   X μl of $H_2O$ (Sigma) (up to 250 μl)
   X μl of purified linear RNA (8-9 μg)
   25 μl of Reaction Buffer 10X
   25 μl 1mM GTP
   25 μl 10 mM $MnCl_2$
   5μl RNase OUT
   3.5 μl RtcB
2. Incubate the reaction at 37ºC for 1h.
3. Add an equal volume of chloroform/isoamyl alcohol
4. Vortex for 30 seconds to mix well and form an emulsion
5. Centrifuge at RT for 5- 10 min at Vmax
6. Pipette off and keep the top aqueous phase
7. Add 1/10th volume 3M sodium acetate pH 5.5 and mix thoroughly
8. Add 3 volumes of cold 100% ethanol mix thoroughly and incubate either at –20°C overnight (16 hr) or 30 minutes at -80ºC.
9. Centrifuge at 4ºC 13000 rpm for 30 minutes
10. Remove the supernatant
11. Wash the pellet with 200 μL of cold 70% EtOH
12. Centrifuge at 4ºC 13000 rpm for 5 minutes
13. Remove the supernatant. Dry the pellet at room temp. Never allow the RNA pellet over-dry, otherwise it will be very difficult to dissolve.
14. Resuspend the in 10μl of water by pipetting.

c. **Analysis of *in vitro* circularization reaction by denaturing PAGE gel**

Prepare the denaturing PAGE as previously explained

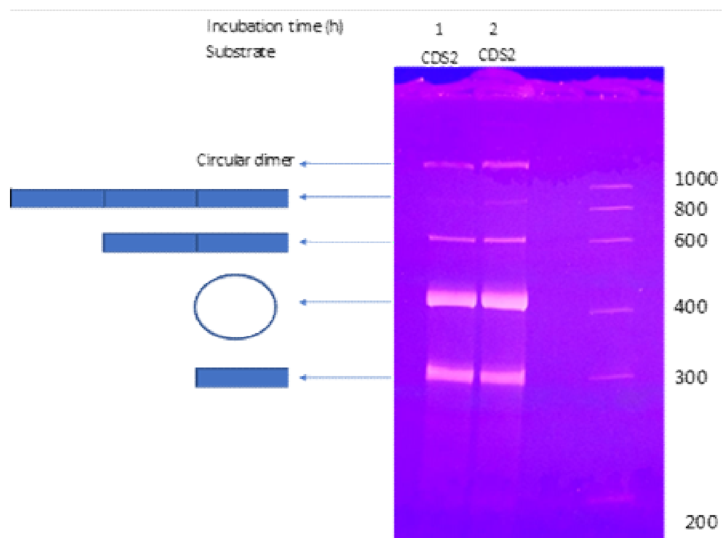


**Fig. 4**: Analysis of *in vitro* circularized RNAs

## 7. <u>ELUTION OF *IN VITRO* CIRCULARIZED RNAs</u>

Proceed as previously explained in Section 5a

Note: Purification yield of ~4μg of circular RNA from 8-9μg of linear RNA

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# DISCUSSION

This thesis focuses in two aspects of circRNA molecules, their cellular function under infection condition and their potential use as artificial designed molecules to develop broad-spectrum antivirals. Our main findings (i) emphasize the complexity of the interplay between viruses and cellular circRNAs during viral RNA infection, and (ii) point out the great potential of circRNAs as novel platform for drug development

## HCV ELICITS DIFFERENTIALLY EXPRESSION OF circRNAS CRUCIAL FOR VIRAL INFECTIONS

Viruses elicit changes in the host cell to achieve their goal: to multiply. For this, they rely on the host machinery using not only host proteins but also non-coding RNAs (158–160). The use of non-coding RNAs by viruses is not something new, however, the role of circRNAs in the viral-host interplay is not well-understood. Although some reports have found an enrichment of genes implicated with the immune or inflammatory response, it is not yet clear whether the function of the producing gene is important for the role of its circRNA (94, 96). Moreover, most of the studies are focused in the miRNA-sponge role, even though it is known already that not all circRNAs act as miRNA sponges (49). In this thesis, we showed that: (i) HCV changes actively the production of circRNAs, decreasing the production of some and increasing the production of others; (ii) the silencing of some of the up-regulated circRNAs affect HCV infectivity (iii) the circRNA cPSD3 is essential for viral replication and contributes to the inhibition of the nonsense-mediated decay pathway (NMD); and (iv) the depletion of cPSD3 impairs the infectivity of other (+) strand RNA viruses.

### Identification of circRNAs with altered expression levels under HCV infection conditions

In our work we faced several difficulties in the identification of circRNAs. First, circRNAs are produced from pre-mRNAs and the only difference between them and their linear counterpart is the presence of a back-spliced junction (BSJ).

This hampers the identification of circRNAs from total RNA-Seq samples because the majority of reads are not properly classified within the circular or linear molecule. In addition, there is not a consensus in the pipelines used for identifying circRNAs and most of them are focused in differences at the level of reads. This difference in the way of identifying and analysing sequencing data generates that depending on the tool used the results can differ significantly. Nowadays, most of the circRNA screenings are based in the difference on the number of reads for each condition. Here, we have applied more stringent steps (checking three pipelines, generating our own annotation and quantifying the counts relatively to the linear isoforms) that decreased dramatically the number of possible candidates (8176 initial circRNAs to 73 final candidates). The number of circRNAs detected in our analysis fits with the knowledge that liver is one of the organs with lower levels of circRNAs (9). CircRNAs range from 5000 to 25000 molecules/cell depending of cell type, development stage or condition (4,8). Although we successfully validate the different circRNAs of our screening, it was difficult to overlap between our analysis and the ones from our collaborators in Stanford. This implies that even being more stringent, sometimes we lose part of the big picture. However, we confirm the differential expression of circRNAs in independent experiments in the two laboratories.

From the 73 differentially expressed circRNAs, 10 were up-regulated and 63 down-regulated. There are several factors described to down regulate circRNA levels in some viral infections. Some examples are (i) NF90/110, two nuclear positive regulators of circRNA biogenesis that facilitates the base-pairing of flanking intronic repeats, that under VSV they translocate to the cytoplasm where they bind viral dsRNA (33); (ii) RNAse L, an endonuclease activated by ECMV, cleaves circRNAs allowing exonucleases to degrade them (42); and (iii) ADAR1, an editing enzyme that edits A-to-I changes promoting the destabilization of dsRNA structures (24). Interestingly, all these factors are activated by HCV. ADAR1 is responsible to supress viral replication of RNA viruses by editing the viral genome (161) and RNAse L has also an antiviral role cleaving HCV RNA and further activating RIG-I (162). In contrast, NF90 has

been described to be crucial for HCV replication (163,164). Alternatively, some of these changes might be explained by putative direct effects of the virus on the splicing reaction. Interestingly, HCV protein NS5 shuttles to the nucleus, and affects transcription (165). Excitingly, the NS5A protein of DENV, a virus in the same viral group as HCV and with similar viral proteins, interacts with the cellular splicing apparatus and subsequently alters canonical splicing of multiple pre-mRNAs (166). An exciting area for future studies would be thus to elucidate how RNA viruses induce changes in circRNA levels

**Characterization of up-regulated or down-regulated circRNAs**

Silencing of the selected up-regulated candidates identified pro-viral circRNAs, cEXOSC1 and cPSD3, whose silencing decrease HCV infectivity; one antiviral circRNAs, cMCM10, whose silencing increased HCV infectivity; and cGALK2 and cENAH, that have no effect on HCV infection, stressing the specificity of the observed effects. Together, these results support the hypothesis that HCV hijack specific circRNAs to perform its cycle and the cell others to respond to the infection. Notably, when we checked whether the pro-viral circRNA cPSD3 was used by other viruses (DENV, ZIKV, CHIKV) we determined that (i) cPSD3 was weakly overexpress in the DENV and CHIKV infections; (ii) silencing of cPSD3 impaired DENV infectivity, favoured ZIKV infectivity and had no effect on CHIKV. DENV, ZIKV and HCV belong to the flavivirus group while CHIKV belong to the Togavirus one. Thus, it was not surprise of us the lack of effect on CHIKV. More studies are needed to characterize why ZIKV behaved differently from DENV and HCV.

In the same direction of our results, Tagawa et collegues, showed that KHSV induced the up-regulation of one host circRNA (has_circ_0001400). Further investigation revealed that the circRNA, whose linear counterpart was not changed by the infection, was inhibiting the expression of viral genes and may serve as antiviral molecules (100).

## Mode of action of circRNAs affecting HCV infection

The characterization of the mode of action of circRNAs is not obvious. In cEXOSC1 and cMCM10 we do not find miRNA binding sites, RBP-binding sites, or ORF. Regarding their parental genes, EXOSC1 is a core component of the RNA exosome complex and interacts with DDX60 acting as antiviral molecule described to inhibit the infectivity of VSV (167). MCM10 is important for cellular DNA replication and it is upregulated in hepatocellular carcinoma (168). In our analyses, the linear forms of cEXOSC1 and cMCM10 were down regulated. A plausible possibility would be that the effect we observed in viral infections is not caused by the upregulation of the cirRNAs but by the down regulation of the corresponding linear form. However, this seems unlikely as in our silencing experiments we downregulate the circular form without significantly downregulate the linear one. To explore further the mode of action of the identified circRNAs it would be interesting to establish pull-down assays to identify their interactors.

The cPSD3 contains 6 binding sites for eIF4A3. Computational studies have shown that many circRNAs could potentially be associated with this factor although experimental assays do not agree with the computational predictions.. The initiation factor eIF4A3 targets BSJ at higher frequency than to other regions (169). Moreover, eiF4A3 has been described to regulate the expression of circRNAs (170) and to have a pro-viral role in Influenza virus and HCMV (171,172). In this context, it would be interesting to characterize whether cPSD3 acts an eIF4A3-protein sponge sequestering the eiF4A3 and co-orquestrating with the viral core protein the inhibition of the NMD or cPSD3 interacts with eiF4A3 to perform another function.

## What are the advantages for viruses to induce circRNAs?

But, in an evolutionary vision, what are the advantages for viruses to induce circRNAs? And for the cell to respond to viral infections? CircRNAs present

some advantages over other molecules or RNA species. First, they are more resistant to degradation (7,82); second, because of their length they may target multiple factors at the same time whereas other short RNAs may not; third, they bind molecules in a sequence dependent manner presenting more specificity than other innate immune sensors; and fourth, circRNAs are found in exosomes suggesting a communication role to neighbouring cells (46,47,100). Secretion of antiviral circRNAs could act as a SOS signal for the immune system or as an alert to the neighbouring cells. It would be interesting to study in single cell models whether the incubation of naïve cells with exosomes containing circRNAs produce any change in the cell in terms of antiviral response.

**Are host-induced circRNAs a good therapeutic target?**

The virus-induced up-regulated circRNAs are better targets for antiviral development than the down-regulated ones because they can be easily silenced with siRNAs while the overexpression of the downregulated ones would be very complicated to achieve. Delivery of siRNAs is rapidly improving because of the growing interest on RNA-based drugs. Thus, the use of siRNAs to target a circRNA required for the virus to multiply is not out of question. One putative advantage is the possibility to generate siRNAs that target multiple viruses. Our results strongly suggest that different viruses would use some common circRNAs. Thus, if we would generate global landscapes of circRNA expression under different viral infections we should be able to identify those shared ones and target them to develop broad-spectrum antivirals. However, this option, in comparison with current DAA drugs or with our designed circRNA targeting structures of the viral RNA genomes, has a main disadvantage. Symptoms of many serious acute infections such as those produced by DENV or CHIKV are the result of an overstimulated immune response to the extremely high titters of virus in the infected individual. To treat these patients successfully a rapid decrease of viral titters is needed. It seems more likely that treatments that target directly the virus, as the DAA that target viral proteins or our designed circRNAs that target the viral RNA, would do so more efficiently than

treatments that indirectly affect the virus by target one cellular factor. Moreover, the possibility of toxic off-target effects is higher.

CircRNAs, instead, might act as excellent biomarkers for diseases. It would be interesting to have a universal marker for viral infections as in clinical often is not possible to distinguish between viral and bacterial infections with the consequent effect on treatment options. CircRNAs are exported from the cell by exocytosis and they are present in exosomes, blood and saliva. Thus, the detection of circRNAs in body fluids could provide a new insight in the diagnosis or prognosis from diseases. This hypothesis has been widely study in cancer. In KRAS mutant colon cancer cells, for example, there are more circRNAs in the exosomes than in the cell (173).

## ARTIFICIAL circRNAS AS THERAPEUTIC PLATFORM

As a proof of concept, we have (i) designed a new antiviral circRNA-based platform based on the disruption of essential viral structures; (ii) successfully tested and selected candidates from the different designed circRNAs against HCV, DENV, CHIKV and WNV; (iii) proved that single circRNA molecules inhibit more than one virus at the same time by developing a circRNA with sequences against HCV and DENV, demonstrating the broad spectrum capabilities of the technology; and (iv) developed an in vitro method to produce circRNAs at low cost.

Our innovative approach offers fundamental advantages over other approaches to fight viral infections: first, circRNAs are extremely stable molecules due to the lack of 5’ and 3’ ends, required for exonucleases to degrade RNA molecules. Enuka et al. showed that the half-life of circRNAs extend to approximately 24h whereas their linear RNA species exhibit half-lives of approximately 8h (7). Linear molecules, in contrast, have less resistance to degradation and they require chemical modifications to increase their stability (151,174); second, our designed-circRNAs contain long and multiple sequences hybridizing to multiple

regions in the viral RNA genome hampering the emergence of resistant mutants. Due to the short sequences of the current RNA-based drugs, single point mutations in viral genomes produce the generation and selection of escaping mutants; third, our approach provides a versatile platform that allows the optimization of future designs to include sequences from different viruses or different serotypes to achieve the long-pursued goal to develop a broad-spectrum antiviral. For a long time, viruses were considered to have highly structures ends but nobody studied what was occurring on the coding sequence. Development of novel methods that allows to solve structures of complete genomes uncover that the CDSs of positive strand RNA viruses contain also multiple and functional structures (175–177). These studies are being expanded to multiple viruses what will provide us with numerous target possibilities and to improve our designs significantly. As an example, HCV RNA structure is determined in quite detailed while this is not the case of ZIKV. All designed circRNAs to target HCV were successful while all designed to target ZIKV were not.

To test the different candidate sequences, we used a plasmid-based approach to generate circRNAs. Our results are influenced by the efficiency of transfection of the plasmids. A GFP-containing plasmid with similar size to our CV plasmid was used in all experiments to determine the efficiency of transfections. In comparison to transfection rates (40-65%), the achieved inhibition of viral (50-80% in some candidates) is impressive. Our next step is to test our designed circRNAs in *in vivo* models. However, before moving to *in vivo* experiments we plan to solve some pending questions. First, we need to prove the mode of action. We claim that the designed circRNAs are hybridizing with the virus and disrupting the target structure. Indeed we have demonstrated that the designed circRNA inhibit the step of the viral life cycle modulated by the targeted viral RNA structure. This quite encouraging but to confirm this we will carry out *in vivo* immunoprecipitation with an MS2-tagged virus (178) and *in vitro* SHAPE. Moreover, we plan to optimize the number of hybridization

sequences in the designed circRNAs for maximum effectivity with minimum size.

In this thesis we have also generated a new protocol based on ribozymes to effectively generate *in vitro* circRNAs at low cost. There are few companies that develop circRNAs and the cost is extremely high (costumed circRNAs cost 1000€/μg). We now produce circRNA at a cost of 3€/μg. While doing so another group published a protocol to synthesize circRNAs using ribozymes, as well. However, their hammerhead sequences leave huge tails in the circRNA whereas our technology is circularizing just the fragment of interest (179). The problem with leaving longer tails of their method is that these tails may interfere with our sequence and form additional structures impairing the hybridization of our designed circRNA with the viral genome. Currently, we are optimizing the hammerheads to get the most effective hammerhead sequences in both extremes so that cleavage and ligation are maximized.

Our next step after these improvements is to generate a BSA circRNA against DENV and WNV, two of the most clinically relevant emerging viruses. The final goal is to into include CHIKV and ZIKV as well. However, once we obtained the DENV-WNV one we will start our *in vivo* assays in mice models, for which the laboratory had already obtained funding and I will be involved. One of the biggest problems with RNA therapies is their delivery. RNAs are large, charged molecules with a high molecular weight that impedes the crossing of the lipid bilayer. Moreover, naked circRNAs have a half-life of 15 seconds in plasma (180). Thus, it is important to find a vehicle that allows the cellular entry at the same time that protects the circRNA. For our *in vivo* purposes, we will use nanoparticles (*in vivo* JetPEI©). This technology allows global distribution of the nanoparticles and has been successfully used in other *in vivo* trials (181). These in vivo experiments will provide more information about the toxicity, pharmacokinetics and distribution of the circRNA that will be extremely useful to move our product to the next level of clinical trials. As there are no treatments available and the only vaccine commercialized (DengVaxia®, Sanofi Pasteur)

has safety problems and a lack of efficacy for some genotypes, we can face a difficulty to compare our results with a goal standard treatment.

**Can exogenous circRNAs boost the immune system?**

The role of artificial circRNAs in the cell has been under debate over the last years with some controversy. Chen et al., suggested that artificial circRNAs could activate RIG-I, boosting the innate immune response. They proposed that endogenous circRNAs could bind RBPs whereas exogen ones do not, activating the cell's immune sensors (72). Then, Wesselhoeft et al., showed that the activation of the immune response was due to a poor purification of the circRNAs. Thus, when the circRNA was purified by RNAse R, phosphatases and HPLC, this activation was not occurring (73). Interestingly, this month Chen et al., published that circRNAs produced in the cell contain m6A recognized by reader proteins, like YTHDF2, and suppress immunity. In contrast, exogenous circRNAs, like the ones that we are designing, do not have m6A, and may activate RIG-I and the expression of antiviral genes (182). Although there is some controversy, this activation of the immune response by exogenous circRNAs might be useful to boost the immune response and to help with the clearance of the virus. Most of the infections caused by emerging viruses produce acute infections, with the circRNA treatment we could reduce the infectivity below a symptomatic threshold and boost the immune system to stop the infection propagation.

**Social and economical implications of the project**

Public Health Offices have pointed out that emerging viruses are being the global threat of the 21$^{st}$ century. The WHO has estimated that in the forthcoming years the number of people at risk will be around 40% of the global population. Alarmingly, there are no treatments or vaccines available in the market. Our project intends to fill the gap in the treatment market providing one solution: a broad-spectrum antiviral to fight these infections. Right now, governments,

international organizations, private health insurances and pharmaceutical companies are racing to invest in controlling these viral infections. With our approach, several investment funds and organizations showed interest in the product. We have already filed a patent and received several grants to pursue our goal to achieve a BSA against emerging viruses. The in vivo experiments will be crucial for investors. If the obtained results are promising our next step will be to fund a start-up in which I will be deeply involved. The design of antivirals and vaccines is one of the main targets of pharmaceutical industries and the testing market is valued to increase in the next years opening a chance for our success.

**Our circRNA-based platform can be applied to other diseases**

Interestingly, our approach can be simply optimized to treat other diseases where upregulated mRNAs or misfolded RNA structures within the mRNA are the cause of the pathology. Secondary structures in mRNA are considered new layers of genetic information being implicated in different processes of the mRNA biology. They are important for translation where stable structures at the 5’ end may suppress translational initiation (183) or structures in 3’ can impede the accessibility of miRNAs interfering in translational repression (184). Moreover, highly structured regions in the coding sequence could interfere with the movement of ribosomes impairing translation elongation (185,186), alter RNA editing (187) and splicing (188). The alteration on the mRNA structure can result in a dysregulation of the protein folding generating protein aggregates (189). Point mutations, sequence deletions and expansions can result in the misfolding of secondary structures that can promote diseases like myotonic dystrophy caused by the formation of two hairpins (190). This kind of diseases might be a good target for our platform where we could try to design circRNAs against these hairpins.

# CONCLUSIONS

In this thesis we have characterized the role of host-induced circRNAs in viral infections and we have developed a novel antiviral strategy to generate broad-spectrum antivirals. The main milestones achieved are:

**From the cell…**

1. HCV changes the expression profile of host circRNAs
2. We have identified 73 circRNAs whose production is altered after HCV infection. Ten of them were up-regulated and 63 down-regulated.
3. All tested circRNAs have been validated in independent experiments.
4. Up-regulated circRNAs display anti-HCV and pro-HCV functions, indicating the complexity of the circRNA-virus interaction.
5. cPSD3 acts as a pro-viral circRNA for HCV and DENV whereas it acts as anti-viral circRNA for ZIKV.
6. Virus-induced circRNAs may provide novel targets or biomarkers of infection.

**To the market…**

1. We have developed circRNA-based antiviral platform based on the disruption of essential viral RNA structures.
2. We have generated circRNA that inhibit HCV, DENV, CHIKV or WNV
3. The designed circRNAs impairs the step of the viral life cycle promoted by the targeted RNA structure.
4. We have generated as a proof-of-concept a broad-spectrum circRNA that inhibit DENV and HCV.
5. We have optimized a low cost *in vitro* protocol to generate circRNAs
6. Our platform might be optimized to treat non-infectious diseasewhere upregulated mRNAs with defined strucuresare the main cause of the pathologies.

# ABBREVIATIONS

| | |
|---|---|
| **(+) RNA** | Positive-strand RNA |
| **3D** | Three-Dimensional |
| **AD** | Alzheimer's Disease |
| **ADAR1** | Adenosine Deaminase 1 Acting on RNA |
| **AGO2** | Argonaute 2 |
| **AGO2/RISC** | Argonaute 2/RNA-induced silencing complex |
| **AIDS** | Acquired Immune Deficiency Syndrome |
| **AKT1** | AKT serine-threonine kinase1 |
| **ALB** | Albumin |
| **ALU** | Arthrobacter luteus element |
| **ALV-J** | Avian Leukosis Virus subgroup J |
| **Amotl1** | Angiomotin-like protein 1 |
| **ANRIL** | Antisense RNA in the INK4 Locus |
| **ASO** | Antisense Oligonucleotide |
| **AXL** | Anexelekto receptor tyrosine kinase |
| **BBB** | Blood-Brain Barrier |
| **BSA** | Broad-Spectrum Antiviral |
| **BSJ** | Back-Spliced Junction |
| **C** | Capsid protein |
| **CD** | Cluster of Differentiation |
| **CDK2** | Cyclin-Dependent Kinase 2 |
| **CDR1as** | Cerebellar Degeneration-Related protein1 Antisense RNA |
| **ceRNA** | Competing endogenous RNA |
| **CHIKV** | Chikungunya Virus |
| **cHP** | Capsid-coding region Hairpin element |
| **ciRNAs** | Intronic circRNA |
| **CiRS-7** | Circular RNA sponge for miR-7 |
| **CMV** | Cytomegalovirus |
| **CNS** | Central Nervous System |

**CRE** Cis-acting Replication Element

**CRISPR/Cas9** Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9

**DAA** Directed Acting Antiviral

**DB** Dumbbells

**DENV** Dengue Virus

**DHX** DEAH Box helicase

**DMD** Duchenne's Muscular Distrophy

**DNA** Deoxyribonucleic Acid

**DSB** Double-Strand Break

**dsDNA** Double-stranded DNA

**dsRNA** Double-stranded RNA

**E** Envelope protein

**E2F1** E2F transcription Factor 1

**EBOV** Ebola Virus

**EBV** Epstein-Barr Virus

**ecircRNA** Exonic circRNA

**ECMV** Encephalomyocarditis Virus

**EIciRNA** Exonic-Intronic circRNA

**eiF2α** Eukaryotic initiation Factor 2 alfa

**EIF3J** Eukaryotic translation initiation factor 3 subunit J

**EMT** Epithelial to Mesenchymal Transition

**ER** Endoplasmatic Reticulum

**ESRP1** Ephitelial Splicing Regulatory Protein 1

**FAK** Focal Adhesion Kinase

**FBXW7** F-Box/WD repeat-domain containing 7 gene

**f-circRNA** Fusion circRNA

**FOXO3** Forkhead Box O3

**FUS** Fused in Sarcoma

| | |
|---|---|
| **GCRV** | Grass Carp Reovirus |
| **HAA** | Host-Acting Antiviral |
| **hATTR** | Hereditary Transthyretin Mediated Amyloidosis |
| **HBV** | Hepatitis B Virus |
| **HCV** | Hepatitis C Virus |
| **HIF1α** | Hypoxia-Inducible Factor1 Alfa |
| **HIPK3** | Homeodomain Interacting Protein Kinase 3 |
| **HIV** | Human Immunodeficiency Virus |
| **hnRNP** | Heterogeneous nuclear Ribonucleoprotein |
| **HPLC** | High-Performance Liquid Chromatography |
| **HVS-1** | Herpes Simplex Virus 1 |
| **HuR** | Human antigen R |
| **ICAM** | Intercellular Adhesion Molecule1 |
| **ID-1** | Inhibitor of DNA binding1 |
| **IFN-γ** | Interferon gamma |
| **ILF3** | Interleukin enhancer binding factor 3 |
| **IRES** | Internal Ribosome Entry Site |
| **ISG** | Interferon Stimulated Gene |
| **ITCH** | Itchy E3 ubiquitin protein ligase |
| **KSHV** | Kaposi Sarcoma Herpes Virus |
| **LNA** | Locked-Nucleic Acids |
| **LPS** | Lipopolyssacharide |
| **m6A** | N6-methyladenosine |
| **MBL** | Muscleblind |
| **mcircRasGEF** | Mouse circular Ras GEF domain family member |
| **MDA5** | Melanoma Differentiation-Associated protein 5 |
| **miRNA/miR** | MicroRNA |
| **mRNA** | Messenger RNA |
| **NF90/110** | Nuclear Factor 90 / Nuclear Factor 110 |

| | |
|---|---|
| **NSP** | Non-Structural protein |
| **OH** | Hydroxyl Radical |
| **ORF** | Open Reading Frame |
| **ORFV** | Orf Virus |
| **PABPN1** | Polyadenylate-Binding Nuclear Protein 1 |
| **PCR** | Polymerase Chain Recation |
| **PDK1** | Pyruvate Dehydrogenase Kinase |
| **PES1** | Pescadillo ribosomal biogenesis factor 1 |
| **PKR** | Protein kinase R |
| **POLR2A** | RNA Polymerase II subunit Adt |
| **prM** | Pre-Membrane protein |
| **QKI** | Quacking |
| **RBP** | RNA Binding Protein |
| **RCM** | Reverse Complementary Motifs |
| **RdRp** | RNA-dependent RNA-polymerase |
| **RIG-I** | Retinoic-Acid Inducible Gene I |
| **RNA** | Ribonucleic Acid |
| **RNA Pol II** | RNA Polymerase II |
| **RNAse L** | Ribonuclease L |
| **RNAse R** | Ribonuclease R |
| **RSV** | Respiratory Syncitial Virus |
| **sgRNA** | Single guide RNA |
| **SHAPE** | Selective 2′Hydroxyl Acylation analyzed by Primer Extension |
| **sHP** | Small Hairpin |
| **siRNA** | Small interfering RNA |
| **SL** | Stem-Loop |
| **SMA** | Spinal Muscular Atrophy |
| **snRNP** | Small nuclear RNP |
| **SP** | Structural Protein |

| | |
|---|---|
| **SR** | Serine and arginine-rich proteins |
| **ssRNA** | Single-stranded RNA |
| **STAT3** | Signal Transducer and Activator of Transcription 3 |
| **SV40** | Simian Virus 40 |
| **TGEV** | Transmissible Gastroenteritis Virus |
| **TNF-α** | Tumour Necrosis Factor alfa |
| **TTR** | Transthyretin |
| **uORF** | Upstream ORF |
| **UTR** | Untranslated Region |
| **VEGF** | Vascular Endothelial Growing Factor |
| **VR** | Variable Region |
| **VSV** | Vesicular Stomatitis Virus |
| **WNV** | West Nile Virus |
| **ZIKV** | Zika Virus |
| **ZNF609** | Zinc Finger protein 609 |

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*I ara que tot acaba i tot comença*
*fem del passat i del present el repte*
*per un futur esperançat i càlid*

*Miquel Martí i Pol*