

## **RNA interference : impact on RNA virus replication and use to study virus-host interaction**

Aurélie De Cock

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Supervisor : Thomas Michiels

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## **Committee**

### **Supervisor**

Pr. Thomas Michiels  
de Duve Institute  
Université Catholique de Louvain

### **Chair**

Pr. Etienne De Plaen  
de Duve Institute  
Université Catholique de Louvain

### **Internal members**

Pr. Guido Bommer (co-promoter)  
de Duve Institute  
Université Catholique de Louvain

Pr. Anabelle Decottignies  
de Duve Institute  
Université Catholique de Louvain

Pr. Patrick Goubau  
Institut de recherche expérimentale et clinique  
Université Catholique de Louvain

Pr. Frédéric Lemaigre  
de Duve Institute  
Université Catholique de Louvain

### **External members**

Dr. Maria Carla Saleh  
Department of Virology  
Institut Pasteur Paris

Pr. Laurent Gillet  
Department of infectious and parasitic diseases  
Université de Liège



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# Abbreviations

|                |                                                     |
|----------------|-----------------------------------------------------|
| Actb           | beta-actin                                          |
| Ago            | argonaute protein                                   |
| AMP            | adenosine monophosphate                             |
| Aub            | aubergine protein                                   |
| ca-siRNA       | cis-acting siRNA                                    |
| CARD           | caspase activation and recruitment domain           |
| cDNA           | complementary DNA                                   |
| cGAMP          | cyclic GMP-AMP                                      |
| cGAS           | cyclic GMP-AMP synthase                             |
| CRISPR         | clustered regularly interspaced palindromic repeats |
| CVB            | Coxsackievirus B                                    |
| DCL            | dicer-like                                          |
| DCR            | dicer                                               |
| DGCR8          | DiGeorge Critical Region 8                          |
| dsRNA          | double-stranded RNA                                 |
| EEEV           | Eastern equine encephalitis virus                   |
| eIF            | eukaryotic initiation factor                        |
| EMCV           | encephalomyocarditis virus                          |
| ESC            | embryonic stem cell                                 |
| EV71           | Enterovirus 71                                      |
| FCS            | fetal calf serum                                    |
| FHV            | Flock house virus                                   |
| FMDV           | Foot-and-mouth disease virus                        |
| GBP            | guanylate-binding protein                           |
| GDP            | guanosine diphosphate                               |
| GFP            | green fluorescent protein                           |
| GMP            | guanosine monophosphate                             |
| GTP            | guanosine triphosphate                              |
| Gvin1          | very large interferon-inducible GTPase 1            |
| HBV            | Hepatitis B virus                                   |
| HCV            | Hepatitis C virus                                   |
| hGBP1          | human guanylate-binding protein 1                   |
| HIV-1          | Human immunodeficiency virus 1                      |
| HSV-1          | Herpes simplex virus 1                              |
| IFN            | interferon                                          |
| IFNAR          | interferon-alpha receptor                           |
| IFNGR          | interferon-gamma receptor                           |
| IFNLR          | interferon-lambda receptor                          |
| IKK $\alpha$   | I kappaB kinase-alpha                               |
| IKK $\epsilon$ | I kappaB kinase-epsilon                             |
| IL             | interleukin                                         |
| IL-10R         | interleukin-10 receptor                             |

|              |                                                              |
|--------------|--------------------------------------------------------------|
| IRES         | internal ribosome entry site                                 |
| IRF          | interferon regulatory factor                                 |
| IRG          | immunity-related GTPase                                      |
| ISG          | interferon-stimulated gene                                   |
| ISGF3        | interferon-stimulated gene factor 3                          |
| ISRE         | interferon-stimulated regulatory element                     |
| JAK          | Janus kinase                                                 |
| KSHV         | Kaposi's sarcoma-associated herpesvirus                      |
| LDLR         | low density lipoprotein receptor                             |
| LGP2         | Laboratory of Genetics and Physiology 2, also known as DHX58 |
| Loqs         | loquacious                                                   |
| MAVS         | mitochondrial antiviral signaling protein                    |
| MDA5         | melanoma differentiation-associated protein 5                |
| MEF          | mouse embryonic fibroblast                                   |
| MICB         | major histocompatibility complex class I-related chain B     |
| miRNA        | microRNA                                                     |
| mRNA         | messenger RNA                                                |
| MuHV-4       | Murid herpesvirus 4                                          |
| Mx           | orthomyxovirus resistance gene                               |
| MyD88        | myeloid differentiation primary response 88                  |
| nat-siRNA    | natural antisense transcript siRNA                           |
| NGS          | next-generation sequencing                                   |
| NK           | natural killer                                               |
| NoV          | Nodamura virus                                               |
| nt           | nucleotide                                                   |
| OAS          | oligoadenylate synthetase                                    |
| ORF          | open reading frame                                           |
| PABPC        | poly(A)-binding protein                                      |
| PACT         | protein activator of PKR                                     |
| PBMC         | peripheral blood mononuclear cell                            |
| P-bodies     | processing bodies                                            |
| PFV-1        | Primate foamy virus type 1                                   |
| piRNA        | PIWI-interacting RNA                                         |
| Piwi         | P-element induced wimpy testis                               |
| PKR          | protein kinase R                                             |
| Pol          | polymerase                                                   |
| pre-miRNA    | precursor microRNA                                           |
| pri-miRNA    | primary microRNA                                             |
| pri-ta-siRNA | primary trans-acting siRNA                                   |
| RDR          | RNA-dependent RNA polymerase                                 |
| RIG-I        | retinoic acid-inducible gene 1                               |
| RISC         | RNA-Induced Silencing Complex                                |
| RLR          | RIG-I-like receptor                                          |
| RNAi         | RNA interference                                             |
| RSV          | Respiratory syncytial virus                                  |
| SGS3         | suppressor of gene silencing 3                               |
| shRNA        | short hairpin RNA                                            |
| siRNA        | small interfering RNA                                        |

|          |                                                                       |
|----------|-----------------------------------------------------------------------|
| SNARE    | soluble N-ethylmaleimide-sensitive-factor attachment protein receptor |
| SOCS     | suppressor of cytokine signaling                                      |
| ssRNA    | single-stranded RNA                                                   |
| STAT     | Signal Transducer and Activator of Transcription                      |
| STING    | stimulator of IFN genes                                               |
| ta-siRNA | trans-acting siRNA                                                    |
| TAR      | trans-activation response element                                     |
| Tat      | trans-activator protein                                               |
| TBK1     | TANK-binding kinase 1                                                 |
| TLR      | toll-like receptor                                                    |
| TMEV     | Theiler's murine encephalomyelitis virus (Theiler's virus)            |
| TNF      | tumor necrosis factor                                                 |
| TRBP     | HIV-1 TAR RNA-binding protein                                         |
| TRIF     | TIR domain-containing adaptor protein inducing interferon beta        |
| TYK      | tyrosine-protein kinase                                               |
| USP18    | ubiquitin specific peptidase 18                                       |
| UTR      | untranslated region                                                   |
| VLIG     | very large interferon-inducible GTPase, also known as GVIN            |
| vsiRNA   | virus-derived siRNA                                                   |
| VSR      | viral suppressor of RNAi                                              |
| VSV      | Vesicular stomatitis virus                                            |
| Zuc      | zucchini endonuclease                                                 |



## Summary

microRNAs are short double-stranded RNAs of about 21 base pairs, which inhibit protein synthesis by binding target mRNA displaying sequence complementarity. microRNA can also hybridize to mRNA or genomic RNA of viruses in order to restrict their replication. It is well documented that microRNAs are involved in DNA virus biology. Many DNA viruses produce microRNA that regulate either viral or host gene expression. Certain DNA viruses also harness host microRNAs to regulate their replication or to modulate cellular immune responses. However, the role of cellular microRNAs in RNA virus biology is still debated. It is proposed that microRNAs contribute to cellular innate immunity against RNA viruses.

In the first part of my thesis, we assessed the general influence of cellular microRNAs on Theiler's virus replication. To do so, we developed a system to inactivate the *Dicer* gene, which encodes a crucial enzyme that participates to microRNA synthesis. Then, we compared virus replication in *Dicer*-positive and *Dicer*-negative cells. Transient *Dicer* inactivation led to a significant decrease of microRNA levels and to a strong increase of Vesicular stomatitis virus replication. In contrast, microRNAs depletion did not increase Theiler's virus replication.

In the second part of my thesis, we evaluated the effect of a miR-142-3p target sequence present in human rotavirus genome. Our results indicate that this microRNA does not inhibit, nor enhance the replication of an attenuated human rotavirus, although this microRNA binds the target sequence located in the viral genome.

Lastly, we used the RNAi technology to analyse the antiviral activity of a murine interferon-inducible GTPase called Gvin1. This protein has a molecular mass of 280 kDa and is the largest known GTPase in any species. The GTP-binding domain of Gvin1 shares sequence similarity with those of other interferon-inducible GTPases, such as Mx and guanylate-binding proteins, which exhibit an antiviral activity against certain viruses.

To study the putative antiviral function of Gvin1, we inhibited its expression by using a short hairpin RNA expressed from a lentiviral construct. Next, we compared the replication of RNA and DNA viruses in cells where Gvin1 expression was restricted or not. We showed that the inhibition of Gvin1 expression in interferon-treated cells increases the infection of Theiler's virus, Vesicular stomatitis virus and, in particular, Murid herpesvirus-4. Moreover, Gvin1 impacts the nucleic acids uptake mediated by cellular transfection and rearranges the actin cytoskeleton. These results, however, need to be confirmed with other constructs, to rule out that they resulted from off-target effects.

All together, our results suggest that Gvin1 plays a role in cellular resistance to viral infection by interfering with virus entry into cells.



# Introduction

Viruses are intracellular pathogens that infect all living organisms. They use the cellular metabolism to replicate and finally, to spread in their host. During infection, many viruses produce replication intermediates that may be detected by host cell sensors and thereby trigger antiviral responses. The immediate cellular defences against viruses range from RNA interference (RNAi) in plants and invertebrates to the more complex interferon (IFN) response in mammals.

RNAi was first reported in 1990 by C. Napoli and colleagues. In an attempt to alter the colour of petunia flowers, they overexpressed the gene encoding the chalcone synthase, a key enzyme responsible of the deep violet coloration of petunia [1]. This experiment resulted in the generation of white flowers whereas dark flowers were expected. They observed that the level of the endogenous enzyme was 50-fold lower than in wild type petunias, suggesting that the introduced transgene was suppressing the expression of the endogenous gene. Further investigations in plants indicated that this gene silencing phenomenon was due to a post-transcriptional inhibition of gene expression.

In 1998, Craig Mello and Andrew Fire reported a potent gene silencing after injection of double-stranded RNA (dsRNA) into *Caenorhabditis elegans* [2]. They were the first to identify the causative agent of RNAi and were awarded the 2006 Nobel Prize in Physiology or Medicine. Since then, numerous studies have contributed to the current understanding of the RNAi pathway in plants, insects and vertebrates.

RNAi is a general phenomenon of gene expression regulation that includes a range of mediators : microRNAs (miRNAs) are imperfect RNA duplexes, which are the physiological inhibitors of mRNA translation. Small interfering RNAs (siRNAs) are derived from endogenous transcripts or exogenous sequences. They exhibit full complementarity to their target sequence and mediate transposons silencing, repression of gene expression and cellular protection against viral infections. PIWI-interacting RNAs (piRNAs) help to maintain genome integrity in gonads.

These RNAi mediators reviewed in the following sections are listed in Table 1.

| Organism | RNAi mediators                     | Functions & features                                     |
|----------|------------------------------------|----------------------------------------------------------|
| Plants   | microRNA                           | mRNA cleavage / inhibition of mRNA translation           |
|          | trans-acting siRNA                 | mRNA degradation                                         |
|          |                                    | precursor undergoes a miRNA-mediated processing          |
|          | cis-acting siRNA                   | DNA or histone methylation / transposons silencing       |
|          | natural antisense transcript siRNA | mRNA degradation / cellular stress response              |
|          | virus-derived siRNA                | cellular defense against viruses                         |
| Insects  | microRNA                           | inhibition of mRNA translation / mRNA cleavage           |
|          | endo-siRNA                         | mRNA degradation / transposons and genes silencing       |
|          | PIWI-interacting RNA               | genome integrity in germ line cells (ovaries and testes) |
|          | virus-derived siRNA                | cellular defense against viruses                         |
| Mammals  | microRNA                           | inhibition of mRNA translation / mRNA cleavage           |
|          | PIWI-interacting RNA               | genome integrity in germ line cells (testes)             |

**Table 1.** RNAi mediators and their functions in plants, insects and mammals.

## 1. RNA interference in plants

In plants, distinct RNAi pathways exist that lead to the production of short dsRNAs [3]. They include the microRNA pathway, the trans-acting siRNA pathway, the cis-acting siRNA pathway and the natural antisense transcript siRNA pathway. These pathways however share essential processing enzymes :

- dsRNAs are processed into short RNA duplexes by a family of RNase III called Dicer.
- The passenger strand of the RNA duplex is degraded and the guide strand is integrated in an effector complex called RNA-induced silencing complex (RISC).
- The RISC complex contains a member of the Argonaute (Ago) family of proteins.

Base-pairing between the guide RNA and its nucleic acid target can lead to mRNA cleavage, translation arrest or DNA methylation. RNAi outcome in plants is determined by the usage of processing enzymes that have different substrate specificities. For example, *Arabidopsis thaliana* encodes four Dicer and ten Ago enzymes that are involved in the different forms of RNAi.

### 1.1. The microRNA pathway in plants

microRNAs (miRNAs) are dsRNA molecules of 21 to 22 nucleotides (nt) in length that are encoded in non-coding intergenic regions or within introns (figure 1A). miRNA coding genes are transcribed by RNA polymerase II (Pol II), which produces capped and poly(A)-tailed primary miRNAs (pri-miRNAs) carrying a stem-loop [4]. Following transcription, Dicer-like 1 (DCL1) cleaves the pri-miRNA and generates a RNA duplex containing the guide strand and the passenger strand (miRNA\*) of the mature microRNA [5]. The processing activity of DCL1 occurs in association with a double-stranded RNA binding protein named HYL1 [6]. Each strand of the duplex is then methylated by the methyltransferase HEN1 at the 2' hydroxyl group of their 3' terminal nucleotide. This methylation prevents degradation of miRNAs from the 3' end of both strands [7]. Next, mature microRNAs are led out of the nucleus by the exportin protein HASTY but some observations suggest the existence of a HASTY-independent miRNA export mechanism [8]. Once in the cytoplasm, miRNAs incorporate the RISC complex containing Ago1 protein and the passenger strand is degraded.

The majority of plant miRNAs have a perfect sequence complementarity with their target, leading to the catalytic cleavage of the target mRNA by Ago1 protein. This cleavage generates two mRNA fragments, 5' and 3', which are then degraded from the newly exposed ends : the 5' fragment is polyuridylated at its 3' end and is degraded by the exosome; the 3' mRNA fragment is degraded by 5'-3' exonuclease XRN4 [9]. Nevertheless, some evidences support that plant microRNAs can also act as transcriptional repressors. For example, the miR-172 has near perfect complementarity to its mRNA target AP2, but the consequence of their interaction is mainly an inhibition of mRNA transcription [10]. Moreover, plant miRNAs can accumulate in both nucleus and cytoplasm of the cell, suggesting that miRNAs could have nuclear function such as modification of homologous DNA [11].

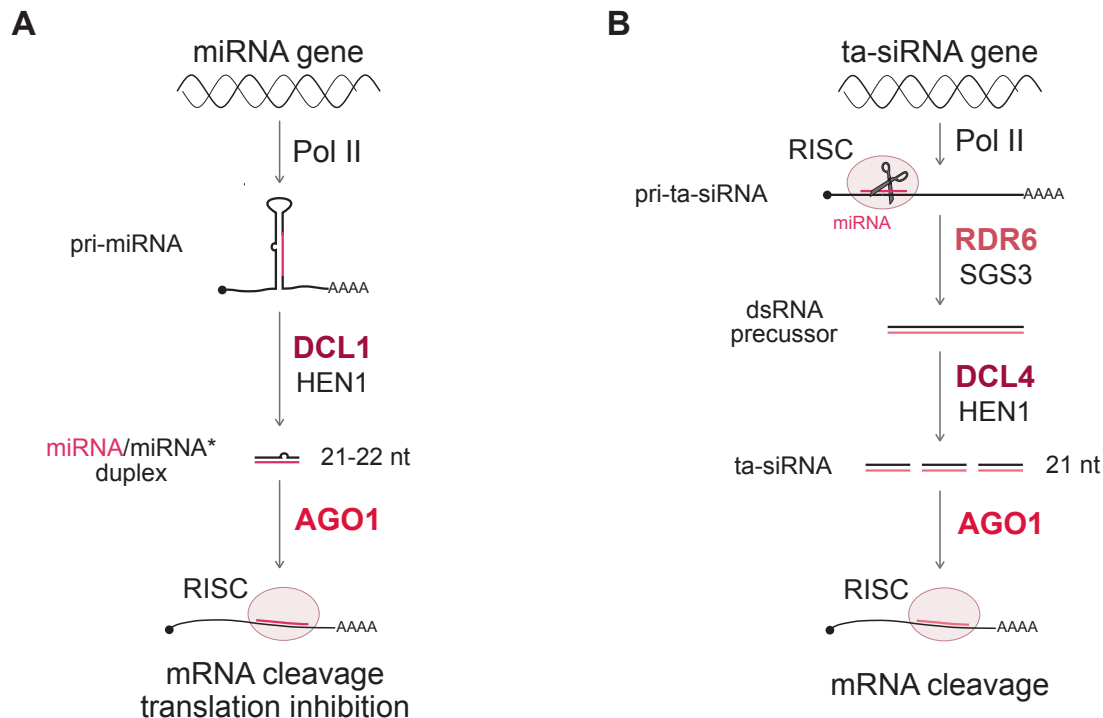


**Figure 1. microRNA and trans-acting siRNA pathways in *Arabidopsis thaliana*.**

(A) miRNAs derive from pri-miRNA precursors that are generated by the transcription of miRNA genes. This precursor is processed by DCL1 that produces a 21-nt miRNA/miRNA\* duplex. Both strands of the duplex are protected from degradation through the methylation of their 3' termini by HEN1. After nuclear export, the miRNA guide strand is incorporated into the Ago1-containing RISC complex. The guide strand binds to a complementary target sequence leading to mRNA cleavage or translational inhibition. (B) Pri-ta-siRNA precursors are transcribed by RNA Pol II and undergo a miRNA-mediated cleavage. One fragment of the precursor serves as a template for the synthesis of a long dsRNA by RDR6 and SGS3. This dsRNA is processed by DCL4 that produces 21-nt ta-siRNA duplexes. Following HEN1-mediated methylation, one strand of the ta-siRNA is loaded into RISC complex containing Ago1 and induces the cleavage of a target mRNA. This figure was adapted from S-W. Ding, 2010, [12].

## 1.2. The trans-acting siRNA pathway in plants

Trans-acting siRNAs (ta-siRNAs) are 21-nt long dsRNA molecules whose biogenesis requires components of both miRNA and siRNA pathways (figure 1B). ta-siRNA coding genes are transcribed by RNA Pol II that produces a long RNA precursor displaying a 5' cap and a poly(A) tail. This primary ta-siRNA (pri-ta-siRNA) precursor undergoes a miRNA-mediated cleavage that leads to the production of two RNA fragments [13]. The cut caused by the miRNA recruits the RNA-dependent RNA polymerase 6 (RDR6) and the suppressor of gene silencing 3 (SGS3), which start to synthesize a long dsRNA from one of the two pri-ta-siRNA fragments. Dicer-like 4 (DCL4) then cleaves this long dsRNA and generates several mature ta-siRNAs, which are methylated by HEN1. Mature ta-siRNAs associate with Ago1, integrate the RISC complex and cause degradation of their targeted mRNA. Four families of ta-siRNAs have been identified in *Arabidopsis thaliana* that play distinct roles in plant development.

### 1.3. The cis-acting siRNA pathway in plants

Heterochromatin-associated siRNAs are the most abundant endogenously produced siRNAs in plants (figure 2A). They derive from transposons or genomic repeated regions and are produced by a plant-specific DNA-dependent RNA polymerase named Pol IV that generates a long RNA molecule [14]. A dsRNA precursor is synthesized from this long RNA by the RNA-dependent RNA polymerase 2 (RDR2). Dicer-like 3 (DCL3) then cleaves this dsRNA precursor, giving rise to 24-nucleotide cis-acting siRNAs (ca-siRNAs). Following methylation by HEN1, ca-siRNAs are incorporated into RISC complexes, which contain Ago4 or Ago6 proteins and promote DNA methylation or histone modification [15]. Therefore, this siRNA pathway participates in the maintenance of genome integrity and in the control of transposons.

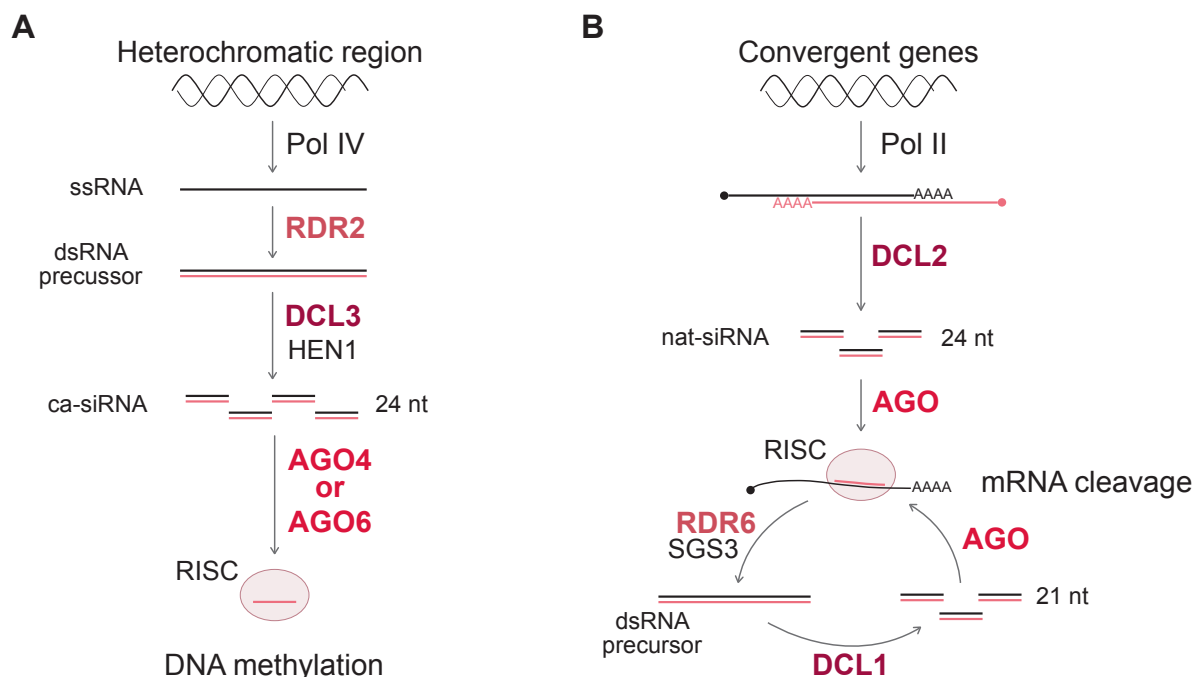


**Figure 2. cis-acting siRNA and natural antisense transcript siRNA pathways in *Arabidopsis thaliana*.** (A) Genomic regions rich in transposable elements or repetitive DNA are transcribed by RNA Pol IV that produces a single-stranded RNA (ssRNA). This ssRNA is then converted in dsRNA by RDR2. DCL3 processes this dsRNA precursor in 24-nt ca-siRNA duplexes. Following methylation by HEN1, ca-siRNA associate with Ago4 or Ago6 to mediate methylation of a target DNA locus. (B) Natural antisense transcripts produced by RNA Pol II form dsRNA within their overlapping regions. The dsRNA is processed by DCL2 into nat-siRNAs of 24-nt in length. This nat-siRNA incorporates the RISC complex containing an unidentified Ago and targets one of the parental transcripts. In association with SGS3, RDR6 forms an amplification loop to generate more nat-siRNAs, which reinforce the cleavage of the target transcript. This figure was adapted from S-W. Ding, 2010, [12].

### 1.4. The natural antisense transcript siRNA pathway in plants

A second groupe of long siRNAs are the natural antisense transcript siRNAs (nat-siRNAs) that derive from two overlapping transcripts from opposite DNA strands (figure 2B) [16]. In a first step, the nat-siRNA pathway requires Dicer-like 2 (DCL2), which produces 24-nucleotide long siRNA species that promote the cleavage of one of the two parental transcripts. In a second step, the cleaved transcript is converted into dsRNA by RDR6 and SGS3.

The newly synthesized dsRNA is then cleaved by DCL1 that generates secondary 21-nucleotide nat-siRNAs, thus amplifying the cleavage of the parental transcript. Few data are available on the activity of nat-siRNAs but they appear to be involved in the cellular stress response.

### **1.5. RNA interference-associated antiviral immunity in plants**

Besides playing an essential role in the regulation of plant gene expression, RNAi also constitute an important mechanism of defence against viruses [12,17]. Several evidences emphasize their antiviral function : (1) siRNAs derived from viral RNA templates are rapidly detected upon viral infection [18]; (2) most plant viruses have acquired viral suppressors of RNAi to interfere with RNAi-mediated antiviral immunity [19,20]; and (3) mutations in components of the RNAi pathway can increase plants susceptibility to viral infection [20].

#### **1.5.1. The biogenesis of virus-derived siRNAs in plants**

The majority of plant viruses have a RNA genome and produce RNA intermediates during their replication cycle. These viral RNAs are recognized by Dicer-like enzymes that initiate the production of virus-derived siRNAs (vsiRNAs). Sequencing data indicate that these vsiRNAs can be processed both from viral dsRNA replication intermediates and from viral genomic RNA that form local stem-loop structures [21-23]. The vsiRNAs take part to specific antiviral immunity through the targeting of their corresponding viral RNAs.

*Arabidopsis thaliana* genome carries four *dicer*-like genes. Only DCL2, DCL3 and DCL4 are able to recognize viral RNAs and produce vsiRNAs of 22, 24 and 21 nucleotides, respectively [24]. DCL4 is the main antagonist of RNA virus infection (figure 3). However, RNA virus infection of DCL4 mutants still generates vsiRNAs of 22-nt in length, showing that other DCL can substitute for DCL4 activity [25]. DCL2 is the main enzyme that can compensate for the absence of DCL4. Inactivation of both DCL4 and DCL2 is necessary and sufficient to restore systemic infection of suppressor-deficient RNA viruses [25,26].

DNA virus infection is mostly affected by DCL3, which produces 24-nt vsiRNAs. These small RNA molecules have also been detected during RNA virus infection in DCL4/DCL2 double mutant plants. DCL1 is responsible of microRNA production and its role in antiviral silencing is very limited. Some data suggest that certain hairpin structures formed in DNA virus transcripts are recognized by DCL1 as miRNA-like precursors. Likely, the cleavage of these viral RNA molecules by DCL1 improves their accessibility to DCL3 and DCL4 that generate vsiRNAs.

Following the detection and the cleavage of viral RNA molecules by Dicer-like enzymes, the primary vsiRNAs are methylated by HEN1 to protect them from degradation [27]. Ago proteins then interact with vsiRNAs and load them in specific RISC effector complexes to promote the cleavage of their viral RNA targets (figure 3) [28]. Immunoprecipitation experiments have shown that Ago1 associates with vsiRNAs during *in vivo* RNA virus infection [29]. Antiviral activity of Ago1 results in viral RNA slicing or, less frequently, in translational inhibition [30,31]. Several plant viruses express viral suppressors that block Ago1 cleavage activity, suggesting that Ago1 is a major factor in the defence against certain RNA viruses [32].

Ago2 is also an important antiviral factor. Ago2-deficient plants have shown hypersusceptibility to different RNA viruses [33,34]. In absence of Ago1, the levels of Ago2 are increased suggesting that Ago2 can take over the role of Ago1 if it is needed.

Ago7 also contributes to viral RNA clearance but works as a surrogate of Ago1 activity [35]. Finally, it has been suggested that Ago4 can interact with vsiRNAs and induce viral DNA methylation during Geminiviruses replication [36].

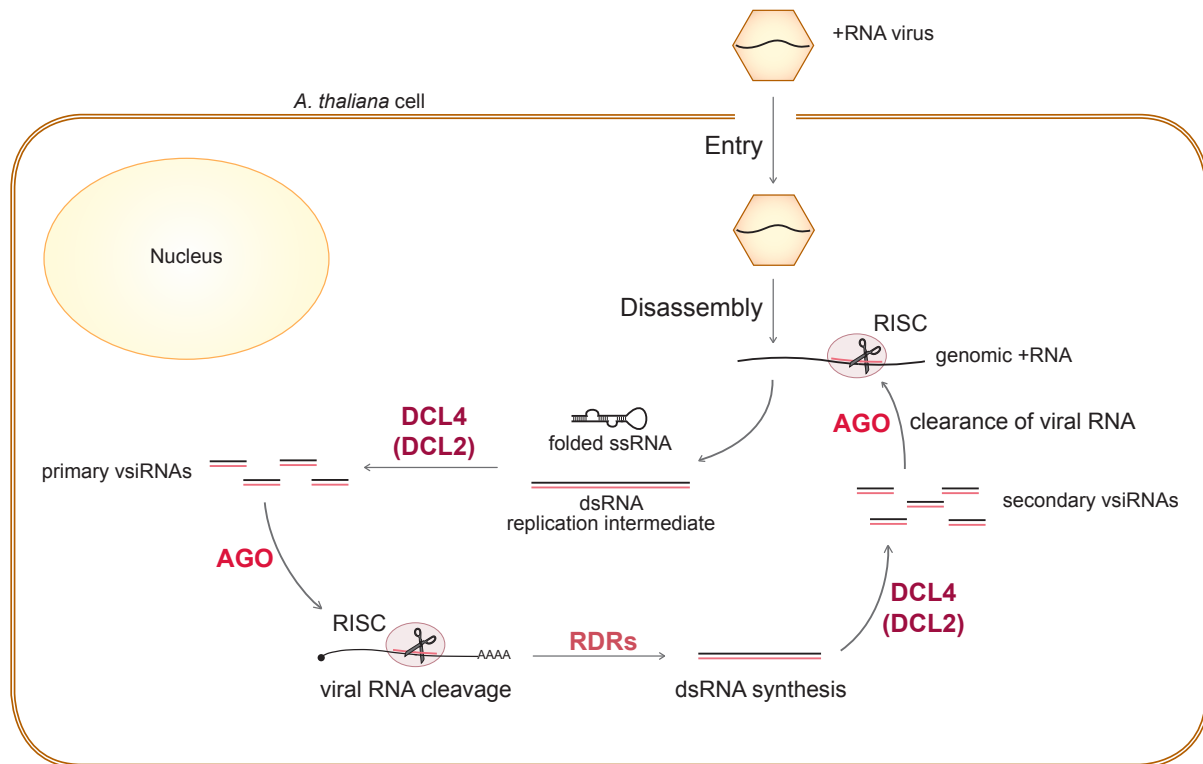


**Figure 3. RNAi-mediated antiviral immunity induced in *Arabidopsis thaliana* by infection of a positive-stranded RNA virus.** After entry and disassembly into plant cells, a RNA virus starts to replicate and produces RNA intermediates such as folded ssRNA or dsRNA molecules. These replication intermediates can be processed by DCL4, DCL2 or DCL3, which produce primary vsiRNAs. The primary vsiRNAs are then loaded into RISC complexes containing Ago proteins to guide the initial cleavage of viral RNAs. This cleavage triggers dsRNA synthesis by cellular RDRs. Secondary vsiRNAs processed from the new dsRNA associate with Ago proteins to establish a more potent antiviral response against the RNA virus. This figure was adapted from S-W. Ding, 2010, [12].

Once primary vsiRNAs are loaded into RISC complexes, they are guided toward their viral RNA targets. In addition, vsiRNAs can recruit specific RNA-dependent RNA polymerases (RDRs) that synthesize a complementary RNA. Aberrant viral RNAs lacking features like cap or poly(A) tail can also serve as substrates for host RDRs [37]. The newly formed dsRNAs are then cut by Dicer-like enzymes that generate secondary vsiRNAs (figure 3). Evidences have shown that RDR1, RDR2 and RDR6 contribute to the biogenesis of secondary vsiRNAs during RNA virus infection [38,39]. The activity of RDR6 is also required for systemic spreading of antiviral RNA silencing [40]. Data suggest that siRNAs are able to travel within the plant through plasmodesmata and phloem to immunize distant tissues [41]. Nevertheless, short and long distance movements of specific vsiRNAs need to be experimentally confirmed.

### **1.5.2. Viral suppression of antiviral RNAi in plants**

Most plant viruses have acquired viral suppressors of RNAi (VSRs), thereby highlighting the efficiency of RNAi-mediated antiviral defence [32]. VSRs are extremely diverse in sequence, structure and mode of action [19]. Indeed, VSRs can block all the steps of the antiviral RNAi pathway : viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification.

The most usual suppression strategy is the sequestration of double-stranded RNAs [42]. This mechanism is used by VSRs encoded by several virus genera and was best characterized for the P19 protein of tombusviruses (positive-stranded RNA viruses). Crystallographic studies have shown that P19 forms a tail-to-tail homodimer that acts as a ruler measuring the length of siRNA duplexes and binding them in a sequence-independent way [43,44]. P19 thus binds 21 to 25 nucleotides dsRNAs and prevents their incorporation into RISC complexes.

The suppression of antiviral RNAi also occurs through interactions between VSRs and the protein components of RISC complex. Protein P0 of the poleroviruses (positive-stranded RNA viruses) does not possess RNAi-binding activity but targets Ago1 for degradation before RISC assembly [45].

A single VSR can act at multiple steps of the antiviral silencing pathway. The suppressor of Cucumber mosaic virus (positive-stranded RNA virus), the 2b protein, has a dual mode of silencing inhibition. First, this viral suppressor binds short and long dsRNAs [46]. Secondly, 2b interacts with Ago1 protein to inhibit its slicing activity [29].

Other viruses have developed completely different suppression strategies. For example, Cauliflower mosaic virus (double-stranded DNA virus) produces a transcript containing a highly structured leader sequence. This leader region is the source of a massive production of viral-derived siRNAs [47]. These vsiRNAs do not restrict viral infection but serve as a trap to overload the RNAi silencing machinery [48]. Another virus, the Sweet potato chlorotic stunt virus (positive-stranded RNA virus), encodes a viral RNase III endonuclease that cleaves the 21-, 22- and 24-nt vsiRNAs into inactive 14-nt dsRNAs [49].

## 2. RNA interference in insects

RNAi in insects has mostly been studied in *Drosophila melanogaster*. In this organism, there are three well characterized RNAi pathways [50,51] : (1) the miRNA pathway in which miRNAs regulate the expression of host genes; (2) the siRNA pathway where siRNAs are generated from endogenous transcripts or exogenous sources such as viruses; and (3) the PIWI-interacting RNA pathway in which piRNAs control the epigenetic state of the genome and the activity of transposable elements in germ line cells.

### 2.1. The microRNA pathway in insects

The insect microRNA pathway is similar to that found in mammals (figure 4A) [52]. miRNA loci are located in intergenic or intronic regions. Several miRNA coding sequences also exist as clusters and their transcription gives rise to bicistronic or polycistronic transcripts.

miRNA loci are transcribed by RNA Pol II, which generates hairpin-structured pri-miRNA bearing a 5' cap and a 3' poly(A) tail. In the nucleus, the RNase III enzyme Drosha, with the help of the dsRNA-binding protein Pasha, cleaves the pri-miRNA to release a 70-nt precursor miRNA (pre-miRNA) containing a 2-nt 3' overhang [53]. This precursor is then exported to the cytoplasm by the RanGTP/Exportin5 complexes. There, Dicer1 (DCR1) and its dsRNA-binding partner Loquacious (Loqs) cleave the terminal loop structure of the pre-miRNA, liberating a 22-nt miRNA duplex [54,55]. The passenger strand (miRNA\*) of the duplex is degraded and the guide strand is loaded into the RISC complex, with contain the Ago1 protein. In *Drosophila melanogaster*, more than one quarter of miRNAs require additional processing after their association with Ago1. This processing is performed by a 3'-5' exonuclease called Nibbler that trims the 3' end of the loaded miRNA strand [56].

The miRNA/Ago1 complex generally targets the 3'-untranslated region (UTR) of transcripts having sequence complementarity with the miRNA. This interaction leads to translational inhibition and/or mRNA degradation. miRNA/mRNA interaction and its outcome will be further discussed in the chapter concerning the miRNA pathway in mammals. *Drosophila* miRNAs play important roles in regulating developmental processes, apoptosis, cell proliferation, metabolism and signalling pathways.

### 2.2. The endogenous siRNA pathway in insects

Endogenous siRNAs derive from transposable elements, from complementary annealed transcripts, and from long hairpin-structured transcripts [57] (figure 4B). In the cytoplasm, Dicer2 (DCR2), with the help of Loqs, cleaves these dsRNA structures to generate 21-nt siRNAs duplexes. Once associated with a RNA-binding protein named R2D2, DCR2 facilitates the loading of siRNAs duplexes in Ago2 protein-containing RISC complexes [58]. One strand of the siRNA duplex is then excised by Ago2 assisted by the endoribonuclease C3PO. The remaining guide strand is methylated by HEN1 to increase its stability. The guide strand binds targets having fully complementary base-pairing to induce RNA cleavage by Ago2. In flies, endogenous siRNAs are involved in transposon silencing and repression of genes implicated in stress resistance and aging [59,60].

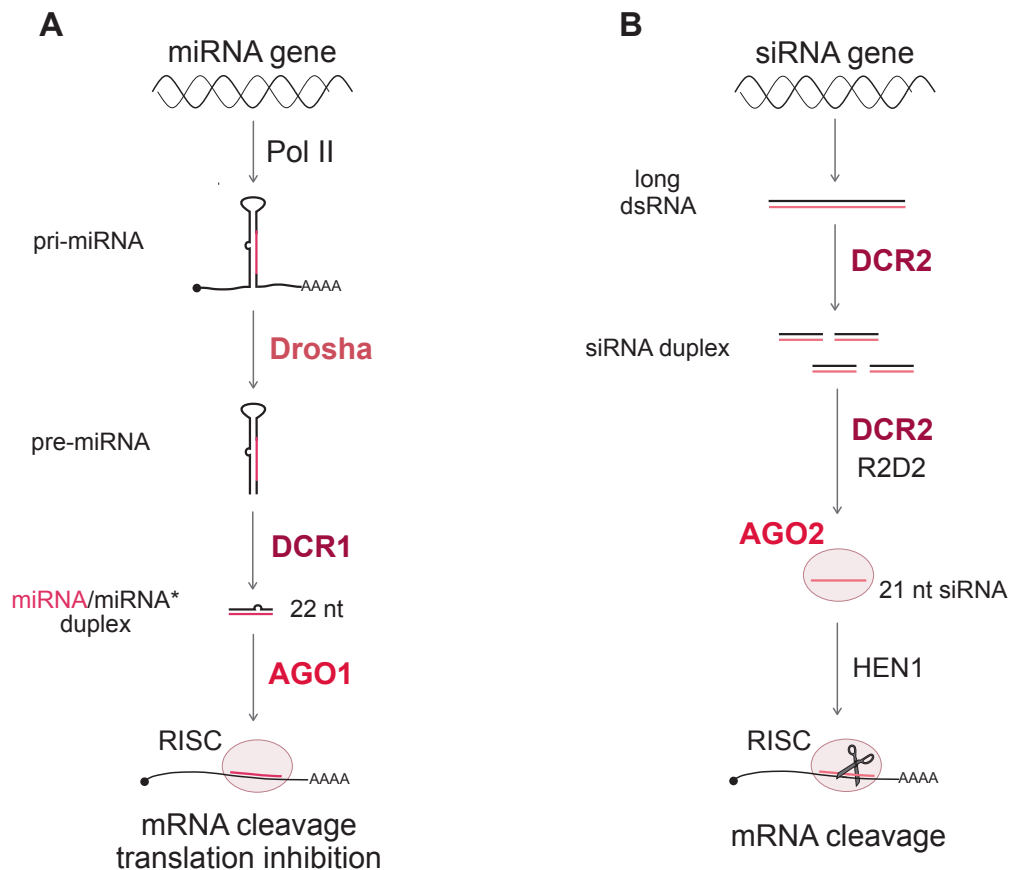


**Figure 4. microRNA and endogenous siRNA pathways in *Drosophila melanogaster*.** (A) Following transcription by RNA Pol II, pri-miRNA transcripts are sequentially processed by RNases Drosha, in the nucleus, and DCR1, in the cytoplasm. This processing leads to the production of 22-nt miRNA duplexes. The guide strand of the duplex associates with Ago1 in RISC complexes to mediate translational arrest or degradation of target mRNAs. (B) dsRNAs derived from the transcription of overlapping genes or structured loci are cleaved by DCR2, which generates siRNA duplexes. The guide strand of the duplex incorporates RISC complexes and, in association with Ago2, targets complementary RNAs resulting in their degradation. This figure was adapted from S-W. Ding, 2010, [12].

### 2.3. The PIWI-interacting RNA pathway in insects

PIWI-interacting RNAs (piRNAs) are small RNA molecules of 24 to 35-nt in length and are known as guardians of the genome integrity and stability in *Drosophila* germ line cells (figure 5) [61]. These small RNAs protect germ line cells from transposable elements by targeting the transcribed RNA of active transposons. The biogenesis of piRNAs is Dicer-independent, but involves three members of the Ago protein family : P-element induced wimpy testis (Piwi), Ago3 and Aubergine (Aub). The piRNAs are encoded by clusters of genes that are transcribed by RNA Pol II, giving rise to single-stranded RNA precursors. Following nuclear export, these precursors are recognized by the RNA-binding Yb protein that recruits them in the piRNA pathway. Through an unknown mechanism, RNA precursors are processed into piRNA intermediates bearing a 2'-3' cyclic phosphate at their 3' termini. In cytoplasmic foci, piRNA intermediates are cleaved by the nuclease Zucchini (Zuc) to produce a pool of primary piRNAs. These piRNAs are then loaded into effector complexes containing a Piwi protein.

These complexes are imported into the nucleus where they perform transposon silencing at the transcriptional level. In the cytoplasm, an amplification loop is initiated by Aub and Ago3 from the targeted RNA to produce secondary piRNAs.

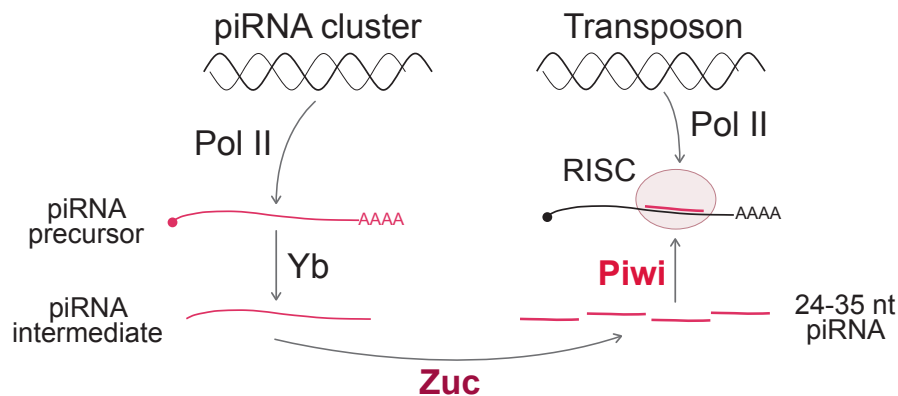


**Figure 5. PIWI-interacting RNA pathway in *Drosophila melanogaster*.** piRNA precursors are transcribed from genomic clusters by RNA Pol II. They are then recognized by Yb proteins and are processed into piRNA intermediates bearing a cyclic phosphate at their 3' termini. piRNA intermediates are cleaved by the nuclease Zuc that generates mature piRNAs. These mature piRNAs associate with Piwi protein and integrate a RISC complex that enters in the nucleus to repress the expression of transposon transcripts. This figure was adapted from S. Hirakata et al., 2015, [61].

## 2.4. RNA interference-associated antiviral immunity in insects

RNAi-mediated immunity is the main antiviral defence in insects [12,62]. RNAi is initiated after detection of viruses through their production of dsRNA replication intermediates, highly structured ssRNA or convergent transcripts. This yields virus-derived siRNAs that correspond to sequences derived from the viral RNA and trigger an antiviral RNAi response.

### 2.4.1. The biogenesis of virus-derived siRNAs in insects

Long viral dsRNAs initiate antiviral RNAi defences. This process involves the protein components of the endogenous siRNA pathway (DCR2, R2D2 and Ago2) [63] (figure 6). 21-nt vsiRNAs are generated by DCR2, which in association with R2D2, loads them in effector complexes containing Ago2. There, the passenger strand of the vsiRNA duplex is degraded and the guide strand is methylated by HEN1. The loaded vsiRNA targets viral RNA by sequence complementarity. Perfect base-pairing between the vsiRNA and its target leads to the cleavage of the viral RNA mediated by Ago2.

The importance of this antiviral mechanism is revealed by the fact that DCR2 or Ago2-deficient flies are unable to restrict viral replication [64]. Flies with a loss-of-function mutation in the gene encoding DCR2 show higher susceptibility to infection by different RNA viruses, including Flock house virus, Drosophila C virus and Sindbis virus [65]. More recently, piRNAs were also suggested to be involved in the antiviral response.

In mosquitoes, deep-sequencing data revealed that piRNAs of viral origin occur during infection with Chikungunya virus, Sindbis virus and Dengue virus [66-68]. The knockdown of Piwi and Ago3 proteins in mosquito cells decreases the amount of these virus-derived piRNAs. Moreover, silencing of Piwi in these cells increases the replication of Semliki Forest virus [69]. Therefore, arboviruses can trigger the piRNA pathway that contributes to antiviral defences in insects.

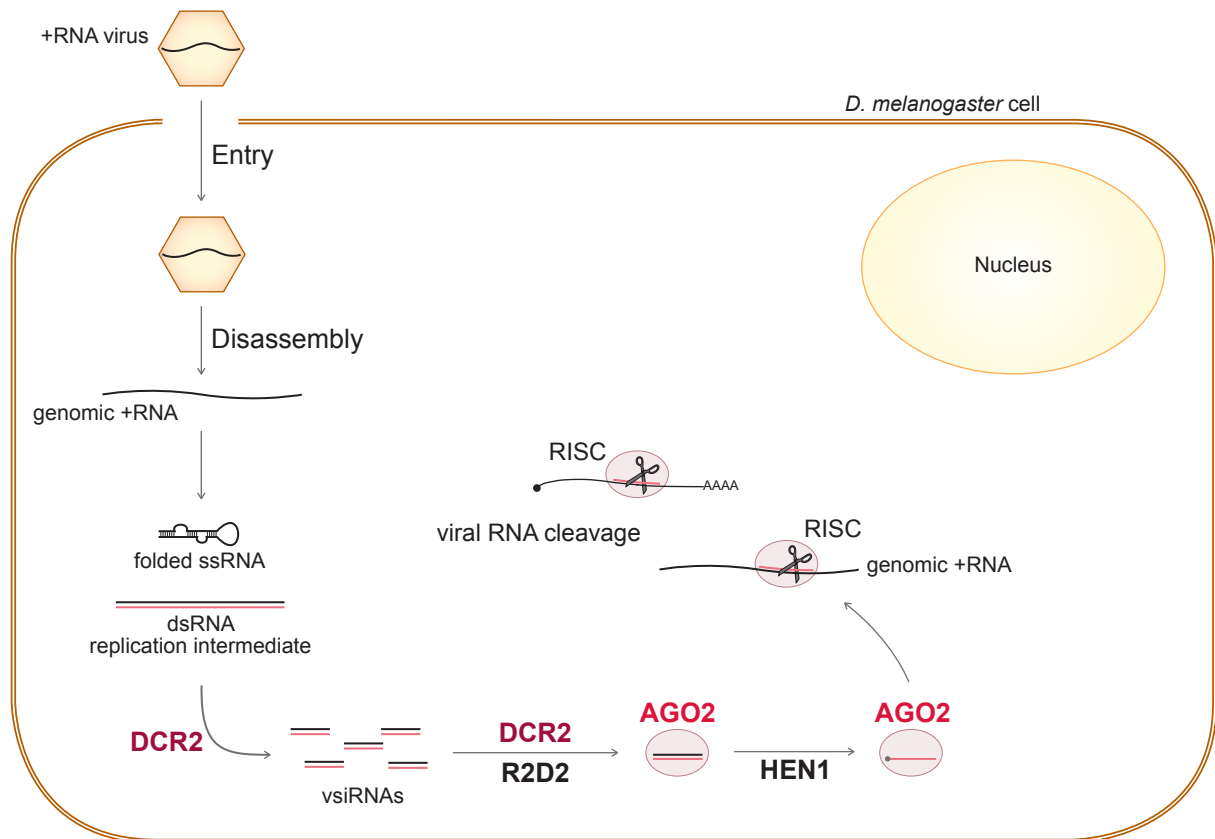


**Figure 6. RNAi-mediated antiviral immunity induced in *Drosophila melanogaster* by infection of a positive-stranded RNA virus.** During replication, positive-stranded RNA viruses produce replication intermediates such as highly structured RNAs or dsRNA molecules. Both RNA species are recognized by DCR2 that cleaves them into virus-derived siRNAs duplexes. These duplexes assemble with Ago2 into the RISC complex where the passenger strand is degraded. The remaining guide strand is methylated by HEN1 and induces virus clearance through RNA interference. This figure was adapted from S-W. Ding, 2010, [12].

Several data support the existence of systemic RNAi during an antiviral response in insects [63]. First, uninfected flies were inoculated with dsRNA molecules corresponding to a specific region of the Sindbis virus genome. Two days after injection, flies were infected and displayed protection against the virus. Secondly, flies infected with Sindbis virus expressing the green fluorescent protein (GFP) show a reduced expression of host-encoded GFP at distant sites from infection [70]. Finally, the study of mosquito cells infected with Semliki Forest virus has shown that dsRNAs can spread from cell-to-cell and inhibit the replication of incoming viruses in neighbouring cells [71]. Therefore, a systemic antiviral RNAi pathway seems to exist in arthropods. To establish an efficient systemic immunity, molecules that signal the viral infection need to be amplified and dispersed through the organism.

Currently, the nature of the signal, the signal amplification mechanism and the signal spreading mechanism are unknown in *Drosophila melanogaster*.

#### **2.4.2. Viral suppression of antiviral RNAi in insects**

To interfere with RNAi-based immunity, many insect viruses encode suppressor proteins. Given their mechanism of action, viral suppressors are generally classified into two groups : DCR2-targeting viral suppressors and Ago2-targeting viral suppressors.

One of the best known RNAi suppressor is the B2 protein of the Flock house virus (FHV). This protein binds dsRNAs in a size-independent way and interacts with DCR2 [72,73]. Moreover, B2 associates with the viral RNA-dependent RNA polymerase during FHV replication cycle to protect viral dsRNA intermediates from DCR2-mediated cleavage [74]. Therefore, the B2 protein inhibits multiple steps of the antiviral RNAi pathway as the biogenesis of vsiRNAs and their incorporation into the Ago2-containing RISC complex.

Unlike B2 protein, the 1A suppressor of the Cricket Paralysis virus does not interfere with the biogenesis of siRNAs. This protein interacts with Ago2 and inhibits the slicing activity of RISC [75].

Arboviruses are transmitted by arthropod vectors to a wide range of hosts, including humans. These viruses also replicate in mosquitoes where they establish persistent infection. Surprisingly, no arboviruses have been shown to encode RNAi suppressor proteins. However, West Nile virus and Dengue virus generate a small non-coding RNA from the 3' UTR of their genomic RNA. This non-coding RNA suppresses siRNA and miRNA pathways by interacting with Dicer and Ago2 proteins [76,77].

### 3. RNA interference in mammals

In mammals, two RNA silencing pathways are described : the microRNA pathway and the PIWI-interacting RNA pathway, which, as in insects, regulates transposable elements activity in germ cells. In the following section, I will focus on the microRNA pathway.

In human, more than thousand miRNAs have been discovered and prediction analyses suggest that more than 60% of human coding genes could be regulated by miRNAs [78]. Sequences encoding miRNAs are generally located in intergenic regions and transcribed from their own promoter. miRNA coding sequences can be organised in polycistronic clusters. The different miRNAs encoded by such clusters often target the same genes or different genes of the same pathways [79]. One third of miRNA coding sequences are however embedded in the introns of protein coding genes and are thus transcribed from their host gene promoter [80].

#### 3.1. The microRNA pathway in mammals

The biogenesis of microRNAs begins with the transcription of a long primary transcript (pri-miRNA) by the RNA polymerase II (figure 7) [81]. This RNA molecule is 5'-capped, 3'-polyadenylated and forms a local stem-loop structure [82]. In the nucleus, the microprocessor complex binds to and cleaves the pri-miRNA to release a 60-70-nt RNA precursor called pre-miRNA. The ribonuclease Drosha and the dsRNA-binding protein DiGeorge critical region 8 (DGCR8) are the main components of the microprocessor complex [83,84]. The cleavage occurs at the base of the stem-loop structure and releases a 2 nucleotide-long 3' overhang, characteristic of RNase III cleavage products.

The pre-miRNA is then exported to the cytoplasm by Exportin-5 [85]. The 2-nt 3' overhang and the length of the stem are crucial for recognition of the pre-miRNA [86]. Exportin-5 is a dsRNA karyopherin that, in association with RanGTPase, shuttles between the nucleus and the cytoplasm of the cell.

Once in the cytoplasm, the pre-miRNA is recognized and cleaved by the RNase III Dicer [87,88]. In mice, loss of Dicer leads to lethality early in the embryonic development, pointing out the crucial role of microRNAs in cellular differentiation and development [89]. Dicer associates with two dsRNA-binding proteins, the PKR-activating protein (PACT) and the HIV-1 TAR RNA-binding protein (TRBP), which are both necessary for Dicer substrate specificity and stability [90,91]. The cleavage takes place at both strands of the pre-miRNA near the loop structure and gives rise to a 21-nt microRNA duplex with 2-nt 3' overhangs. The mature microRNA consists in a guide strand that is incorporated in the RISC complex. Its complementary strand, called passenger strand (miRNA\*), is degraded. Recent publications have revealed that some miRNAs\* can also integrate RISC complexes [92,93].

The core component of the effector complex is the Ago protein. In human cells, four Ago are expressed but only Ago2 possesses a slicer activity [94]. Ago1, Ago3 and Ago4 can carry out non-cleavage-mediated inhibition although their precise function is unknown [95].

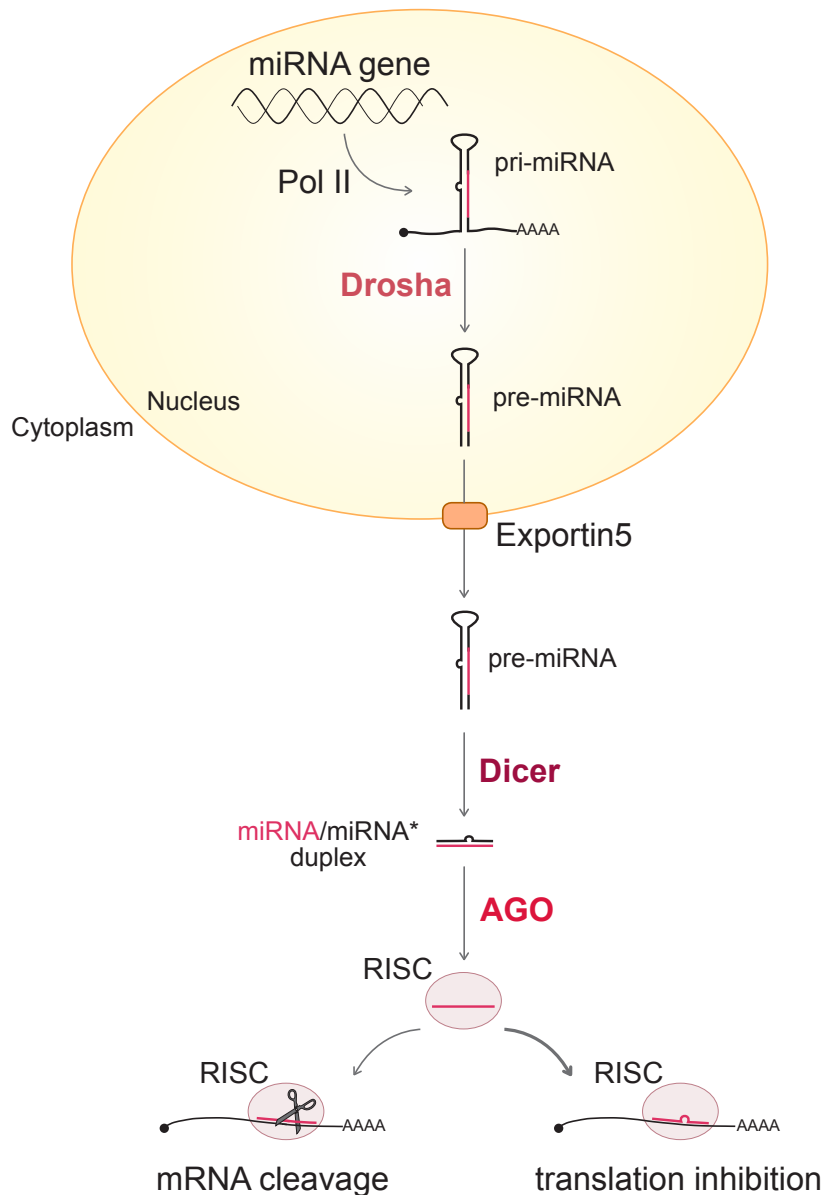


**Figure 7. The miRNA pathway in mammals.**

In the nucleus of the cells, pri-miRNAs are transcribed by RNA Pol II and are processed into 60-nt pre-miRNAs by the RNase III Drosha. These pre-miRNAs are then actively transported to the cytoplasm by Exportin-5. Once in the cytoplasm, they are processed into 21-nt miRNA duplexes by Dicer. The functional strand of the duplex is incorporated into RISC complex where it guides the Ago protein to target mRNAs. This complex mediates inhibition of gene expression through mRNA degradation or translational arrest.

Once it is loaded into the RISC complex, the miRNA guide strand binds a complementary sequence, preferentially in the 3'UTR of the targeted mRNA. A primary feature that dictates this binding is the base-pairing between the targeted transcript and the 5' end of the miRNA called "seed sequence" (residues 2-7) [96]. Given the limited length of the seed sequence, one microRNA can bind and repress several mRNA targets [97,98]. The fate of the mRNA is determined by the degree of complementarity between the microRNA and its target : a perfect base-pairing will lead to mRNA degradation and a partial base-pairing will induce inhibition of mRNA translation [94,99].

Only one complementary site is generally sufficient for efficient mRNA cleavage [100]. After Ago2 slicing, the poly(A) tail of the 3' mRNA fragment is removed and 5'-3' exonucleases are recruited for subsequent degradation [101]. The 5' fragment of the cleaved mRNA seems to be a substrate for the exosome.

Nevertheless, the sequence complementarity between the miRNA and its target is often imperfect. RNAi then results in a translational inhibition that can lead to mRNA destabilization [102-104]. Currently, this mechanism is only partially understood. Translational repression mediated by miRNAs can occur by interfering with the initiation step of mRNA translation. Human Ago2 can bind the cap of target mRNAs to prevent the recruitment of eIF4E, a factor involved in translation initiation [105]. Moreover, human RISC complex can associate with eIF6 to interfere with ribosome formation [106]. Some data also show that miRNAs inhibit the translation of target mRNAs during the elongation step [107].

Ago proteins, microRNAs and mRNA targets of miRNA have been shown to accumulate in processing bodies (P-bodies), which are the functional sites for mRNA turnover [108,109]. P-bodies contain proteins involved in mRNA remodeling, decapping, and deadenylation, as well as exonucleases [110]. Accordingly, depletion of a core component of P-bodies was shown to impair miRNA-based silencing [111].

### **3.2. RNAi-mediated antiviral immunity in mammals : state of the art**

Unlike in plants and invertebrates, virus-derived siRNAs (vsiRNAs) are not usually detected in mammalian cells. Recent high throughput sequencing data have, however, shown that vsiRNAs can accumulate in infected mammalian cells but at very low levels [112,113]. Moreover, transfection of long dsRNAs in mammalian somatic cells does not result in the production of functional siRNAs [114]. Introduction of long dsRNA leads, instead, to the activation of the interferon pathway that triggers the expression of hundreds antiviral genes. Thus, evidences argue against an antiviral activity of RNAi in somatic cells. In contrast, some data suggest that embryonic stem cells (ESCs), that lack a potent IFN response, could mount an RNAi response upon viral infection [115].

The interferon system stands for the primary line of defense against viruses. It is triggered by innate recognition of various viral products, such as 5'-triphosphorylated viral RNA, viral DNA or dsRNA. As for RNAi in plants and invertebrates, numerous mammalian viruses have evolved mechanisms to evade the IFN response.

It is still debated whether microRNAs encoded in the mammalian genome have a direct role in innate antiviral responses. Connections however exist between the interferon and miRNA pathways [116]. Several miRNAs target transcripts encoding proteins of the IFN system, including pattern-recognition receptors, IFN receptors, signal transduction proteins and interferon-stimulated genes. On the other hand, type I IFNs up-regulate the expression of at least 21 cellular miRNAs. Some of these miRNAs have been shown to target viral transcripts. RNAi suppressors have been described in mammalian viruses and are considered as a strong argument for the importance of miRNA-based antiviral immunity. Therefore, numerous studies have examined the potential antiviral function of host microRNAs.

### 3.2.1. Virus-derived siRNAs in embryonic stem cells

Introduction of long dsRNAs in mouse ESCs triggers a decrease of related mRNA levels [114,117,118]. Additionally, endogenous siRNAs derived from pseudogenes have been detected in mouse oocytes, which regulate the expression of mobile genetic elements [119]. Thus, mammalian ESCs are able to process long dsRNAs into functional siRNAs. This observation has led Maillard et al. to examine the potential antiviral role of RNAi in ESCs [120]. Undifferentiated mouse cells infected with Encephalomyocarditis virus generate vsiRNAs of about 22-nt that derive from viral dsRNA replication intermediates. Furthermore, these vsiRNAs are not detected in *Dicer* knockout cells and decrease in abundance upon cell differentiation. Maillard et al. also infected the same cells with a Nodamura virus (NoV) encoding protein B2, which binds short dsRNAs and suppresses RNAi. Upon infection with wild-type NoV, small amounts of vsiRNAs are observed in murine ESCs. In contrast, infection with B2-deficient NoV triggers the accumulation of vsiRNAs that reduce viral replication. Notably, ESCs lacking all mammalian Ago proteins partially rescued B2-deficient NoV infection.

In a recent study, Flemr et al. provided new insights into the processing of long dsRNA in mouse oocytes [121]. They identified a shorter *Dicer* isoform lacking the N-terminal domain whose expression is restricted to oocytes. This short *Dicer* isoform exerts an increased dsRNA cleavage activity. ESCs from *Dicer*-deficient mice rescued with this shorter *Dicer* isoform exhibit higher levels of endogenous siRNAs but similar levels of miRNAs compared to full-length *Dicer* expressing cells.

Unlike oocytes, ESCs do not express the short *Dicer* isoform that is more efficient at cleaving long dsRNA than pre-miRNA *in vitro*. They however express high levels of the long isoform, which therefore allows some levels of siRNA processing [122,123]. The *Dicer* cofactor, TRBP, has been shown to facilitate the processing of long dsRNAs, likely by interacting with the N-terminal domain of *Dicer*. On the contrary, the PACT cofactor was reported to inhibit the processing of long dsRNAs by *Dicer* [124,125]. Interestingly, TRBP and PACT expression are respectively higher and lower in ESCs than in differentiated cells [126].

Overall, these data suggest that embryonic stem cells and potentially other undifferentiated cell types can generate virus-derived siRNAs after viral infection. It should be noted that Maillard and others did not assess the direct binding of these vsiRNAs to viral mRNA and their ability to silence viral gene expression. As ESCs are unable to raise an efficient IFN response, RNAi might compensate for the lack of IFN-mediated antiviral activity in undifferentiated cells.

### 3.2.2. Virus-derived siRNAs in differentiated cells

Several studies have examined the ability of RNA viruses to generate small interfering RNAs in mammalian somatic cell lines. The insertion of an artificial stem-loop sequence element in the viral genome leads to the production of a functional siRNA during viral infection [127,128]. Further investigations have detected natural vsiRNAs in mammalian cells infected with various RNA viruses [129-133]. However, these vsiRNAs occur at such low levels that they appear inconsistent with an effective antiviral strategy. Data suggest, instead, that vsiRNAs are controllers of viral replication and viral protein production.

Virus-derived small RNAs are produced in adenovirus-infected cells and are involved in efficient adenovirus production [129]. Influenza A virus synthesizes small siRNAs that interact with the viral RNA polymerase and promote genome replication [131]. Along the same line, Enterovirus 71 produces vsiRNAs that target the IRES of the viral RNA genome to regulate viral proteins translation [134].

Recently, interesting results presented by Li and co-workers supported the role of RNAi during viral infection [135]. This group has found significant amounts of vsiRNAs in baby hamster kidney cells infected with a Nodamura virus lacking the B2 suppressor protein. Importantly, suckling mice infected with this B2-deficient Nodamura virus produced abundant viral siRNAs and became completely resistant to lethal infection. Nevertheless, identifying the cell type responsible for vsiRNAs production *in vivo* is crucial. This may imply that a high number of cells with pluripotent characteristics generates vsiRNAs, which is particularly relevant in newborn animals.

The use of different cell lines and viruses as experimental models has highlighted the complexity of vsiRNAs function in infected mammalian hosts. Results suggest that certain cell types can support vsiRNAs generation and function, whereas others have lost this capacity.

### **3.2.3. Cellular microRNAs targeting viral sequences**

Numerous studies have reported the targeting of viral RNAs by cellular microRNAs, leading to the attenuation of viral replication *in vitro*. The physiological importance of the phenomenon is, however, still a matter of debate. Repression of viral replication by cellular microRNAs has been documented for several viruses. These include primate Foamy virus type 1 (PFV-1), Vesicular stomatitis virus (VSV), Human immunodeficiency virus type 1 (HIV-1) and Hepatitis C virus (HCV).

The first reported example of a cellular miRNA that targets a viral RNA genome is miR-32. This microRNA has target sequences located in all transcripts deriving from the retrovirus PFV-1. Transfection of antagomir directed against miR-32 increases the accumulation of PFV-1 in HeLa cells, implying that this microRNA serves as an antiviral defense against PFV-1 [136].

Another example of a virus that is restricted by cellular miRNAs is VSV. This virus is an arbovirus that establishes persistent infection in insects and also infects animals because of the broad tropism of its envelope protein. The team of Otsuka demonstrated *in vivo* that two cellular microRNAs target the RNA genome of the virus [137]. In this study, they have generated a mutant mouse with a partial loss of Dicer expression (Dicer d/d mice). In some mouse tissues, Dicer can be detected at very low levels but in others, as in peritoneal macrophages, Dicer expression seems to be completely abolished. Infection of these Dicer d/d peritoneal macrophages with VSV produces greater titers of the virus. *In vivo*, the lack of Dicer expression results in increased lethality for mice that underwent intranasal inoculation of the virus. Increased viral replication can be attributed to the absence of miR-24 and miR-93 that target the VSV genome. miR-24 represses the expression of the viral polymerase whereas miR-93 reduces the amount of the P gene, a cofactor for the polymerase. Both genes are essential for viral replication.

The first studies on the interaction between cellular miRNAs and HIV-1 have shown that Drosha and Dicer restrict virus replication in peripheral blood mononuclear cells (PBMCs) from infected donors [138]. Moreover, knockdown of Drosha and of other components of the microRNA pathway results in virus reactivation in PBMCs isolated from HIV-infected patients treated with suppressive Highly active antiretroviral therapy [139]. In a step further, it has been demonstrated that miR-28, miR-125b, miR-150, miR-223 and miR-382 can target sequences near the 3' end of all HIV-1 transcripts and suppress the expression of viral mRNAs in resting CD4<sup>+</sup> T cells [140]. A pool of antagomirs directed against these miRNAs greatly counteracted their effect on the targeted mRNAs. Therefore, these findings argue for a role of cellular miRNAs in the establishment of viral latency in resting CD4<sup>+</sup> T cells. The same microRNAs are expressed in peripheral blood monocytes, which possess HIV-1 receptors but rarely become infected. The suppression of these miRNAs in monocytes facilitates HIV-1 infectivity, suggesting that microRNAs regulate the tropism of HIV-1 [141].

Target prediction analysis in HIV-1 genome has revealed that miR-29a and miR-29b could bind a sequence localised in the transcript of the accessory protein Nef. This viral protein is essential for efficient viral replication and pathogenesis *in vivo* [142]. Both microRNAs inhibit Nef expression and interfere with HIV-1 replication *in vitro* [143]. Another study has shown that the interaction between miR-29a and HIV-1 mRNA enhances the association of the viral transcript with RISC and P-body proteins [144]. Interestingly, a recent report has proved that IL-21, which is produced by activated CD4<sup>+</sup> T cells, suppresses initial HIV-1 infection by inducing the expression of miR-29 [145].

HCV is a unique case in the field of virus/microRNAs interaction. Indeed, viral replication of HCV can be both induced and repressed by cellular microRNAs. miR-122 is highly expressed in human liver. This microRNA binds two conserved sites in the 5' UTR of HCV and stimulates viral replication [146-148]. Some data suggest that increased viral replication could result from a stabilizing effect of miR-122 on the HCV genome [149]. This microRNA recruits Ago2 protein to the 5' end of the viral RNA and protects the genome from degradation mediated by cytoplasmic exonuclease Xrn1 [150,151]. A recent study of miR-122/HCV interaction has shown that this miRNA enhances HCV RNA levels by reducing the fraction of viral genome engaged in translation while increasing the fraction available for RNA synthesis [152].

Besides the positive role of miR-122, a specific set of cellular miRNAs is involved in the restriction of HCV gene expression. Eight microRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448) were found to be up-regulated in response to IFN signaling that is triggered during HCV infection [153]. Transfection of cells with corresponding miRNA-mimics reproduces the antiviral effects of IFN-beta (IFN- $\beta$ ) on HCV replication, suggesting that IFN could act on HCV replication through the up-regulation of these miRNAs. Moreover, IFN- $\beta$  treatment leads to a significant reduction in the expression of miR-122.

Overall, cellular microRNAs have the ability to repress or enhance viral replication and are able to modulate the tropism of a given virus.

### 3.2.4. Cellular microRNAs targeting picornaviruses

*Picornaviridae* is a family of single-stranded, positive-sense RNA viruses that contain a large open reading frame (ORF) flanked by 5' and 3' UTRs. The 5' UTR contains an IRES driving the translation of a single polyprotein, which is subsequently processed into mature viral proteins.

Some studies have examined the targeting of picornaviruses by host microRNAs [154,155]. One of the first studies was achieved by the team of R. Andino. This team investigated the effect of the insertion of microRNA target sites in the Poliovirus genome on viral replication [156]. The insertion of miRNA target sequences that exhibit perfect complementarity to cellular miRNAs strongly restricted viral replication and affected viral tissue tropism. Poliovirus engineered to be targeted by the neuronal-specific miR-124 lost their ability to replicate in the central nervous system, which lead to a significant attenuation of neurovirulence in infected mice.

Several works demonstrated that cellular microRNAs can directly bind to Picornavirus RNA genomes and inhibit viral infection *in vitro* [157-159]. Enterovirus 71 (EV71) possesses two target sites for miR-296-5p, located in the structural protein coding region. This microRNA restricts viral replication by targeting these two sites. However, the introduction of point mutations in miR-296-5p target sequences of EV71 abolishes the inhibitory effects of this miRNA [160].

In the same way, the ORF of Coxsackievirus B type 3 (CVB3) contains a target site for the cellular miR-342-5p, which decreases viral RNA and protein synthesis [159]. This target sequence is conserved among 5 different types of Coxsackievirus B, meaning that miR-342-5p may exert some beneficial influence to the virus. CVB3 also contains a target site for miR-10\* in the viral polymerase coding region. Surprisingly, miR-10\* promotes virus replication [161].

Cellular miRNAs also affect the replication of picornaviruses by targeting their IRES. Binding sites for miR-153, miR-220, miR-242 and miR-276 have been reported in the IRES of Foot-and-mouth disease virus (FMDV). The overexpression of these four miRNAs leads to the restriction of FMDV infection [157].

Theiler's murine encephalomyelitis virus (Theiler's virus or TMEV) belongs to the *Cardiovirus* genus within the picornavirus family. The DA strain of TMEV is responsible for persistent infection of the central nervous system of the mouse, whereas the GDVII strain produces a fatal encephalomyelitis. In this work, TMEV serves as a model to study the impact of cellular miRNAs on RNA virus replication. The replication cycle of Theiler's virus is illustrated on figure 8.

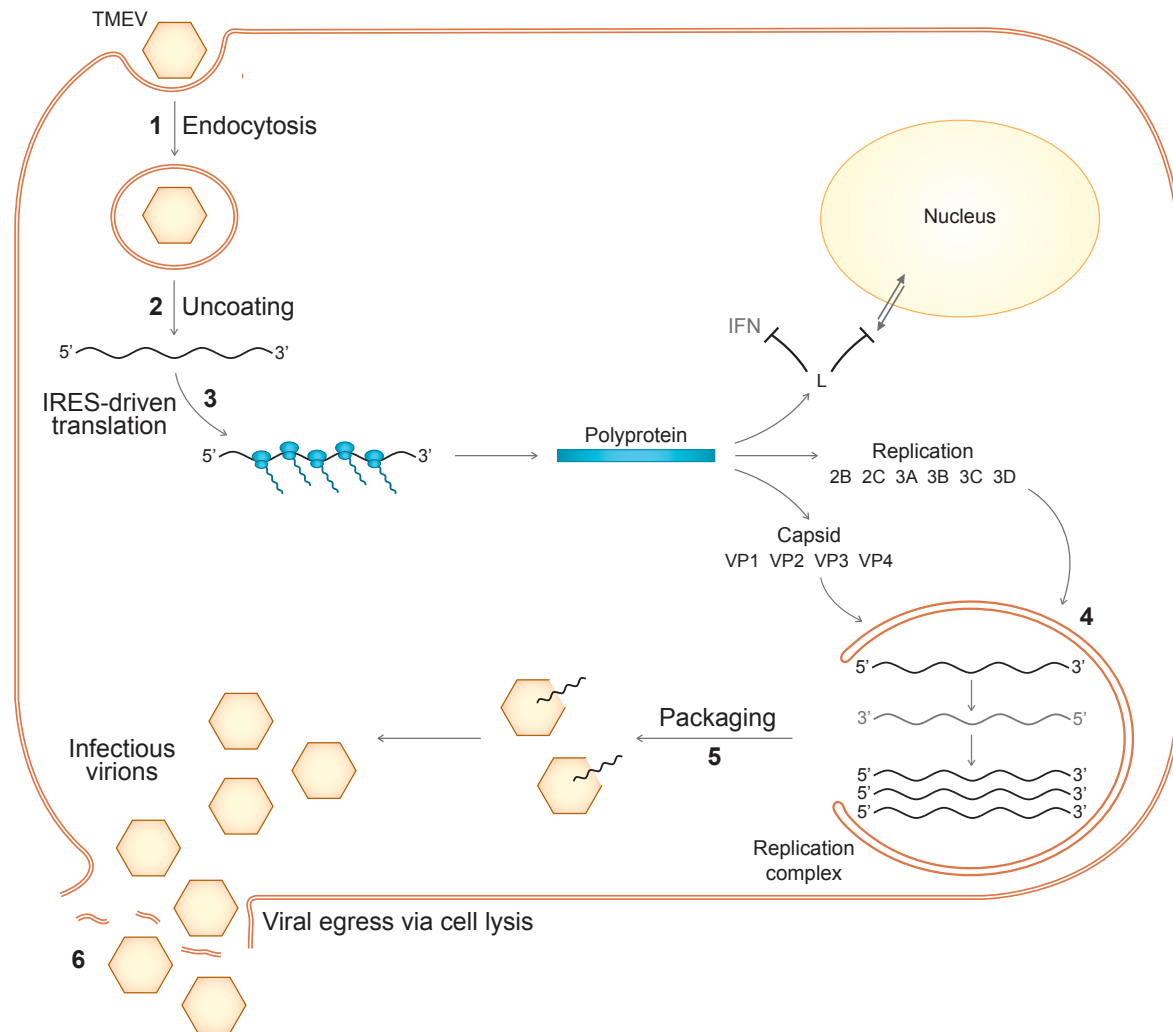


**Figure 8. Theiler's virus replication cycle.**

(1) Even if the attachment receptor is unknown, it is established that the persistent Theiler's virus strain (DA) binds sialic acid, whereas the neurovirulent strain (GDVII) binds heparan sulphate or a yet uncharacterized co-receptor. Following receptor binding, TMEV enters cells via endocytosis. (2) Then, the virus capsid undergoes a conformational change that induces the release of the viral genome into the cytoplasm. (3) The viral genome is 8100 nucleotides long and contains a single ORF. It is translated into a polyprotein that is subsequently cleaved into 12 mature proteins. VP1, VP2, VP3 and VP4 assemble to form the capsid. Proteins 2B, 2C, 3A, 3B, 3C and 3D participate in genome replication. The protein L inhibits the production of IFNs and the nucleocytoplasmic trafficking. Specific to Theiler's virus, a protein called L\* is generated by an ORF that overlaps the main ORF (not shown) (4) RNA replication occurs in complexes made of membrane vesicles derived from the endoplasmic reticulum. The viral RNA-dependent RNA polymerase (3D) synthesizes a negative-stranded RNA from the viral genome, which serves as a template for the production of new genomic RNAs. (5) Viral genomes are then packaged into preassembled procapsids. (6) Infectious virions accumulate in the cytoplasm leading to cell lysis and virus release.

### 3.2.5. Viral microRNAs interacting with host genes

It is well established that DNA viruses influence cellular miRNA expression to control key cell signaling pathways, but they also encode their own miRNAs. To date, more than 200 viral miRNAs have been identified, mostly in herpesviruses [162]. These viral miRNAs have been shown to target both viral and cellular transcripts. They can regulate the switch between latent and lytic replication, support viral replication or modulate immune responses.

During latency, Herpes simplex virus 1 (HSV-1) produces viral miR-H2 and miR-H6, which down-regulate the expression of immediate early genes products [163]. These viral genes encode ICP0 and ICP4 proteins that regulate the induction of lytic replication. Thus, the targeting of immediate early transcripts by viral miRNAs allows HSV-1 to be maintained at the latent stage.

The MICB (major histocompatibility complex class I-related chain B) transcript is targeted at three distinct sites by viral miRNAs generated by Human cytomegalovirus, Kaposi's sarcoma-associated herpesvirus and Epstein-Barr virus [164]. MICB protein is a ligand for NK and CD8<sup>+</sup> T cells that mediates their activation. MICB transcript targeting by multiple herpesvirus miRNAs leads to a reduction of infected cell killing by NK cells.

### **3.2.6. Mammalian virus-encoded suppressors of RNAi**

Viral suppressors of RNAi have been well documented for the majority of plant viruses. Such proteins have also been identified in some mammalian viruses, arguing for an antiviral role of RNAi in mammals. The first reported mammalian suppressor factors were the Vaccinia virus E3L protein and the Influenza A virus NS1 proteins. Further studies identified a dozen mammalian virus-encoded RNAi suppressors [165,166].

Human Influenza A virus NS1 protein is a non-structural protein that possesses a RNA binding domain. NS1 is a multifunctional protein that affects a variety of host cell functions [167]. NS1 inhibits host mRNA processing and blocks nuclear export of polyadenylated cellular transcripts [168]. This protein also complexes with the host translation initiation factor eIF4G1 to enhance the translation of viral mRNAs [169]. NS1 blocks the type I IFN system by binding dsRNA, which inhibits the activation of the RNA sensors TLR3 and RIG-I as well as the Protein kinase R (PKR) and the 2'-5' Oligoadenylate synthetase (OAS)/RNaseL effector systems [170-172]. The RNAi suppressor activity of NS1 was first reported in plant and insect organisms in which NS1 can bind siRNA duplexes and enhances viral pathogenicity [173-175]. The nuclear and cytoplasmic localization of NS1 suggests that it could trap precursors as well as mature forms of siRNAs. In mammals, the suppressor activity of NS1 needs to be confirmed because conflicting results were obtained when this viral protein was expressed with different reporter gene expression systems [176,177].

Vaccinia virus is a DNA virus that expresses a virulence factor called E3L. As NS1, this protein possesses a dsRNA-binding domain and interferes with the production of IFN- $\beta$  and the activation of PKR [178,179]. In fruit flies, this viral protein displays RNAi suppression activity through its dsRNA-binding domain [175]. In mammalian HEK293T cells, E3L was shown to affect shRNA-mediated repression of luciferase expression [176]. However, the group of Lantermann has contested the suppressor activity of E3L in mammalian cells [180]. They generated a recombinant Vaccinia virus that expresses the *lacZ* gene as a marker of gene silencing. Infection of HeLa cells expressing *lacZ*-specific siRNAs showed efficient inhibition of *lacZ* expression, independently of the presence of E3L.

HIV-1 expresses a transcription trans-activator (Tat) protein, which is crucial for virus replication in host cells. Tat regulates viral gene expression by binding a trans-activation response element (TAR) located in the HIV-1 long terminal repeat sequence.

TAR acts as a RNA docking site for Tat, which enhances transcription via the recruitment of a kinase that activates RNA Pol II [181,182]. Besides its role in viral gene expression, Tat also promotes HIV-1 replication by thwarting the interferon response [183,184]. Further, it has been shown that HIV-1 suppresses the expression of the polycistronic miRNA cluster miR-17/92 in infected PBMCs [138]. This RNAi suppressor activity is due to Tat, which blocks the miRNA processing step performed by Dicer [185]. In addition, HIV-1 TAR RNA can sequester an essential co-factor of Dicer, TRBP, which makes it unavailable for cellular miRNAs processing [186].

The non-structural VP35 protein of Ebola virus is a multifunctional protein. VP35 is an essential cofactor of the viral RNA polymerase and is required for virus assembly [187,188]. VP35 contains a dsRNA-binding domain that inhibits type I IFN production by interfering with the detection of viral RNA by the cytoplasmic sensor RIG-I [189-191]. VP35 also acts as a suppressor of RNAi in mammalian cells and its dsRNA-binding domain is essential for this activity [176].

Human Adenoviruses possess a dsDNA genome coding for two non-coding RNA molecules called VA I and VA II, which are transcribed by RNA polymerase III [192]. These VA RNAs form dsRNA structures that interact with host proteins to disrupt multiple cellular processes. VA I binds and inhibits PKR activity, thus allowing Adenovirus to escape a key antagonist of viral infection [193,194]. In addition, VA I interacts with other dsRNA sensing proteins such as RIG-I and OAS1, as well as the host RNAi machinery [195,196]. Viral VA I RNA interferes with cellular miRNAs biogenesis at three levels. First, VA I directly interacts with Exportin-5, thereby disrupting the transport of pre-miRNA from the nucleus to the cytoplasm [197]. Secondly, both VA I and VA II are processed by Dicer and saturate this enzyme, rendering it unavailable to cleave cellular pre-miRNAs [198]. Finally, after Dicer processing, VA RNAs can be incorporated into RISC where they interact with Ago2 protein [129,199].

All these mammalian RNAi suppressor proteins act by binding dsRNA molecules. It is likely that their primary function is the inhibition of IFN pathway, which also relies on dsRNA recognition and is crucial to control viral infections.

## 4. RNA interference as an experimental tool

Over the past fifteen years, the biochemical understanding of the RNAi pathway has led to the use of small RNAs as a genetic tool to control gene expression in mammalian cells and animals. In 2001, T. Tuschl and colleagues were the first to report that transfection of mammalian cell lines with 21-nucleotide RNA duplexes suppresses the expression of endogenous genes [200]. Following this initial report, small RNAs have become a standard tool for molecular biology owing to the development of several methods mediating RNAi.

### 4.1. Design of synthetic small dsRNA molecules mediating RNA interference

RNAi application can be mediated by two types of molecules : the synthetic short interfering RNAs (siRNAs) and the short hairpin RNAs (shRNAs).

siRNAs are RNA duplexes of 19 to 25 pb in length with 3' dinucleotide overhangs. These RNA duplexes are transfected into cells where the guide strand is loaded into RISC. Perfect complementarity between the siRNA guide strand and a single target induces mRNA cleavage and degradation. Transfection of siRNAs is a fast and simple method to elicit gene silencing but their effects are transient, due to cellular division.

shRNAs are RNA molecules transcribed from a DNA vector, which is introduced into cells through plasmid transfection or viral transduction. In contrast to siRNAs, shRNAs allow long-term inactivation of target mRNAs. Based on their design, two kinds of shRNA are used to mediate gene silencing. The first ones are transcribed in the cell nucleus under the control of a RNA polymerase III promoter. The transcript is forming a stem-loop structure and closely resembles pre-miRNA molecules. The second ones, which are also referred to as artificial pri-miRNA, are transcribed in the nucleus by a RNA polymerase II. The 5' capped and 3' polyadenylated transcripts then carry the stem-loop structure that is processed by Drosha before being exported from the nucleus toward Dicer.

### 4.2. Experimental usage of synthetic siRNA and shRNA

Both siRNAs and shRNAs induce a specific knockdown of gene expression and have been used to study gene function *in vitro*. RNAi technologies are widely used to assess the involvement of a given gene in a specific cellular pathway. Furthermore, RNAi screening allows performing functional analyses at whole genome scale to identify unknown gene products associated with a particular cell phenotype. In this way, arrayed or pooled screens are carried out using RNAi libraries containing one or more RNAi species directed against each target gene [201,202].

In an arrayed screening, a set of RNAi molecules targeting a single gene are individually transfected in separate wells of a micro-well plate. Analysis is achieved for each transfected well by cell morphological characterization, biochemical or reporter assays. Alternatively, microarrays can be used to perform reverse transfection : RNAi reagents are linked to microarray slides followed by the deposit of the cells. Recent applications of this approach have identified novel genes required for signal transduction, cell differentiation and host-pathogen interactions [203-205].

In a pooled screening, a shRNA library is introduced into a population of cells by viral transduction. A selective pressure is then applied to induce a given phenotype. Genomic DNA is isolated from selected cells to identify the shRNA of interest by sequencing or by microarray analysis. Pooled screens have contributed to identify genes involved in a variety of processes such as cancer [206]. In addition, pooled RNAi screening has been extended to examine gene function in mouse models. Murine cells are transduced *ex vivo* with a pool of shRNAs and are then transplanted into specific tissues and organs. This approach allows functional study in a more physiological context.

Numerous studies have exploited RNAi technologies to investigate gene function *in vivo*. In mouse, various methods have been used to deliver siRNAs and shRNAs to the desired cell type, tissue or organ [207]. For a systemic delivery, siRNA duplexes can be introduced naked, complexed with cationic liposomes or conjugated to cholesterol. Moreover, chemical modifications of the RNA backbone can increase siRNA stability in mouse serum. Nanoparticles-encapsulated siRNAs, antibody-protamin bound siRNAs or aptamer-siRNA chimeras allow tissue or cell type specific delivery of these RNAi reagents.

shRNA coding plasmids can be delivered by direct injection in the tail vein of the mouse although the use of viral vectors is usually preferred. Adenoviral vectors provide efficient delivery systems for a transient shRNA expression whereas retroviral vectors allow to stably silence target genes. Notably, these vectors enable the use of inducible transcription systems and tissue-specific promoters to induce temporal and spatial expression of shRNAs in mouse organism.

## 5. RNA interference as a therapeutic tool

RNAi technologies have a high potential for the development of novel therapies to inhibit genes involved in disease. Recent breakthroughs in therapeutic applications of RNAi have been made and are owed largely to progresses in *in vivo* delivery methods. Several diseases including viral infections, cancer, amyloidosis, metabolic and neurological disorders, are possible targets for RNAi-mediated treatment in future years [208]. Currently, pre-clinical and clinical trials are pending to assess efficacy and toxicity of RNAi therapeutics.

Several studies have successfully demonstrated that delivery of siRNAs or shRNAs into animal models inhibits infection of viruses [209]. Effective suppression of viral replication was observed in animals pre-treated with siRNAs targeting viral transcripts. Antiviral effects of RNAi were also observed when siRNAs or shRNAs were delivered after infection. This was the case for Influenza A virus, Respiratory syncytial virus (RSV) and Hepatitis B virus (HBV) [210-212]. These studies provide proof-of-principle for the use of RNAi reagents as prophylactic and therapeutic strategies against viral infections. Early phase clinical trials are actually testing RNAi based-therapy for potential applications to HBV and RSV treatment [213,214].

A major issue in antiviral RNAi therapy is the potential rapid evolution of viruses to escape RNAi suppression by acquiring mutations in the target sites. To overcome this hurdle, three main solutions have been proposed to delay or prevent the emergence of virus escape mutants : first, treatment with a combination of different siRNAs; secondly, targeting highly conserved regions in the viral genome, such as UTRs of RNA viruses; thirdly, targeting of host factors required for viral replication, such as virus receptors.

To prevent the emergence of HIV escape mutant in a clinical trial, a shRNA targeting a HIV exon has been used in combination with RNA-based inhibitors of the HIV TAR element and of the viral receptor CCR5. Hematopoietic progenitor cells were transduced with lentiviral vector *ex vivo* and then reinjected into infected patients [215]. Transfected cells were successfully engrafted in infused patients without any toxicity. Moreover, RNA expression from the lentiviral vector was detected in various cell lineages for up to 24 months. These results support the development of RNAi-based therapy for HIV treatment.

In another example, an antisense oligonucleotide has been developed to inhibit the liver-specific miR-122, which is essential for HCV replication. This oligonucleotide is complementary to miR-122 and exhibits high affinity and specificity for this microRNA, thereby blocking miR-122 biogenesis and function in hepatocytes [216]. A recent clinical trial has assessed long-term safety and efficacy in patients treated with this miR-122 inhibitor [217]. A significant decrease of viral RNA levels was observed with no detectable evidence of viral resistance. These data indicate that the targeting of a host factor required for efficient viral replication is a powerful and safe treatment strategy against HCV infection.

The development of RNAi for therapy is now focusing on avoiding off-target and undesired effects. Indeed, the base-pairing of the RNAi therapeutic with complementary sequence of an unrelated protein-coding mRNA can lead to inappropriate gene silencing.

siRNAs can also activate the type I interferon response through dsRNA recognition by RIG-I, PKR and Toll-like receptors [218,219].

Particular siRNA sequence motifs seem to be recognized by TLR7 in the endosomal compartment of dendritic cells, leading to secretion of inflammatory cytokines [220].

While induction of the immune system could be beneficial in antiviral therapy, it could also trigger undesired cellular toxicity in some clinical applications.

Lastly, large quantities of shRNA may inhibit the biogenesis of endogenous miRNAs, as shRNA needs to be processed by the RNAi machinery. Side effects of RNAi therapy have been observed in mice that received high doses of liver-directed shRNA coding vectors. Within 2 months, damages occurred in the liver of treated mice, leading to lethality [221]. Evidences showed that synthetic shRNA may block the normal export of endogenous pre-miRNAs from the nucleus and increased levels of Exportin-5 can reduce hepatotoxicity observed in mice treated with shRNA-containing vectors.

## 6. Innate immunity in mammals : The interferon system

In the early 1950s, it has been described that pre-treatment of cells with inactivated influenza virus confers resistance to subsequent infection with a live influenza virus [222]. However the factor responsible for this resistance was unknown. In 1957, the term « interferon » was proposed to describe the substance produced by cells that interferes with influenza infection [223]. Almost 20 years later, IFN was shown to be a family of small proteins produced and secreted by cells. IFN are classified into three distinct classes according to their receptor complex usage [224].

Type I interferons include IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\omega$ , IFN- $\delta$ , IFN- $\tau$  and IFN- $\zeta$  [225]. All of these IFNs bind a heterodimeric receptor complex formed by IFN- $\alpha$  receptor 1 (IFNAR1) and IFN- $\alpha$  receptor 2 (IFNAR2) subunits (figure 9) [226,227]. Type I IFN genes are clustered on chromosome 9 in human and exhibit different patterns of tissue expression [225]. Nearly every cell is capable of producing and responding to type I IFN.

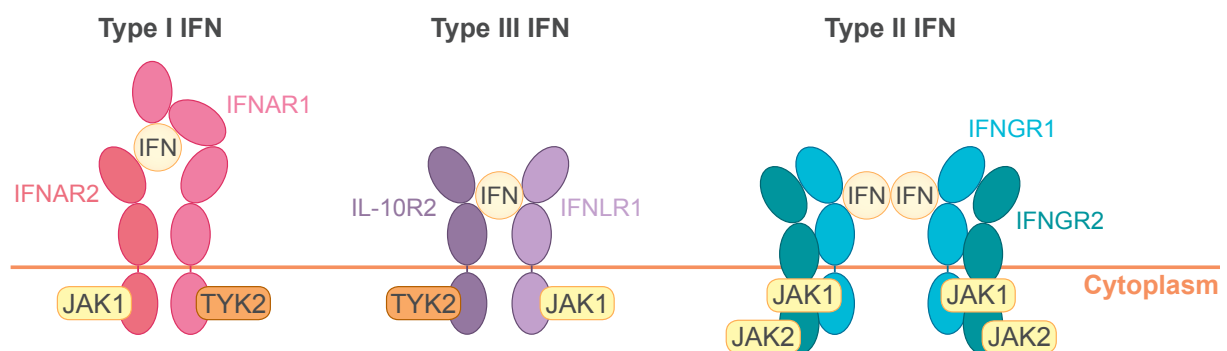


**Figure 9. Interferons and their cognate receptors.**

A heterodimer formed by IFNAR1 and IFNAR2 binds type I IFNs. IL-10R2 associates with IFNLR1 to bind type III IFNs. A tetramer consisting of two IFNGR1 chains and two IFNGR2 chains binds dimers of the type II IFN. This figure was adapted from W.M. Schneider et al., 2014, [228].

Type II interferons only comprise IFN- $\gamma$  that forms a homodimer. IFN- $\gamma$  signaling begins by the interaction of an IFN- $\gamma$  dimer with two IFN- $\gamma$  receptor 1 (IFNGR1) subunits, which then recruit two IFN- $\gamma$  receptor 2 (IFNGR2) subunits (figure 9) [229]. Type II interferon is produced by activated T lymphocytes and natural killer cells. IFNGR is widely expressed so that nearly all cell types can respond to IFN- $\gamma$  [230].

IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3 and IFN- $\lambda$ 4 form the type III interferons family and are the most recently discovered IFNs [231,232]. Their receptor complex is made of two distinct subunits : the interleukin-10 receptor 2 (IL-10R2) that is broadly distributed and the IFN- $\lambda$  receptor 1 (IFNLR1), which is mainly expressed in epithelial cells (figure 9) [233]. This receptor complex signals through the same JAK-STAT pathway as the type I IFN receptor complex [231,234].

## 6.1. The type I interferon pathway

Upon recognition of foreign nucleic acids, sensors activate a signaling cascade that ends up with type I interferon production. Innate immune sensors for nucleic acids can be divided into two groups depending on their expression pattern and their subcellular localization. The first group of sensors is composed of helicases that are expressed in almost all cell types and detect nucleic acids in the cytoplasm. The second group of sensors includes members of the Toll-like receptor (TLR) family that act predominantly in immune cells, such as dendritic cells, macrophages and B cells. These TLRs are localized in the cytoplasmic or in the endosomal membrane and inspect the extracellular space and the endosomal lumen for foreign nucleic acids detection.

### 6.1.1. Production of type I interferon

Numerous RNA viruses fulfill their entire replication cycle in the cytoplasm. Others cross the cytoplasm on their way in and out of the cell. Cells possess nucleic acid sensors to detect ongoing viral infection and lead to the production of IFN- $\alpha/\beta$ . The RIG-I-like receptors (RLRs) are a family of cytosolic RNA sensors composed of three members : RIG-I, MDA5 and LGP2 [235]. RIG-I and MDA5, but not LGP2, contain two caspase activation and recruitment domains (CARD) that mediate signaling to downstream adaptor proteins [236,237]. Given its lack of CARD, LGP2 is thought to be a positive or a negative regulator of the two others RLRs [238-240].

The range of viruses recognized by RIG-I and MDA5 differs in line with the predilection of these sensors for distinct RNA ligands (figure 10). RIG-I mostly associates with single-stranded RNA molecules containing a triphosphate group at their 5' end, which is absent in host mRNAs [241,242]. The ligand for MDA5 is less well characterized. This helicase is activated by long dsRNAs, such as the replicative forms of picornaviruses or the synthetic dsRNA analogue poly(I:C) [243,244]. Thus, MDA5 appears to target viruses that produce high amounts of dsRNA during their replication cycle, including positive-stranded ssRNA, dsRNA and even DNA viruses [245-247]. In the case of the latter viruses, dsRNA is believed to result from the annealing of convergent overlapping transcripts. Some viruses, including Dengue virus and West Nile virus, are recognized by both RIG-I and MDA5 [248,249].

Viral DNA is also known to activate the innate immune response but the activation mechanism was not understood until lately. The cyclic GMP-AMP synthase (cGAS) has been identified as the sensor for cytosolic DNA (figure 10) [250]. This protein belongs to the nucleotidyltransferase family and catalyses the production of cyclic GMP-AMP (cGAMP) [251]. cGAMP acts as a second messenger and activates the stimulator of IFN genes (STING) [252,253]. STING is a crucial adaptor protein that is located to the endoplasmic reticulum [254]. Once activated, this adaptator relocates to the Golgi complex and assembles into punctate structures that contain the TBK1 kinase [255,256]. By this way, STING induces the activation of TBK1, resulting in the phosphorylation of IRF3 and the subsequent production of type I interferon [257].

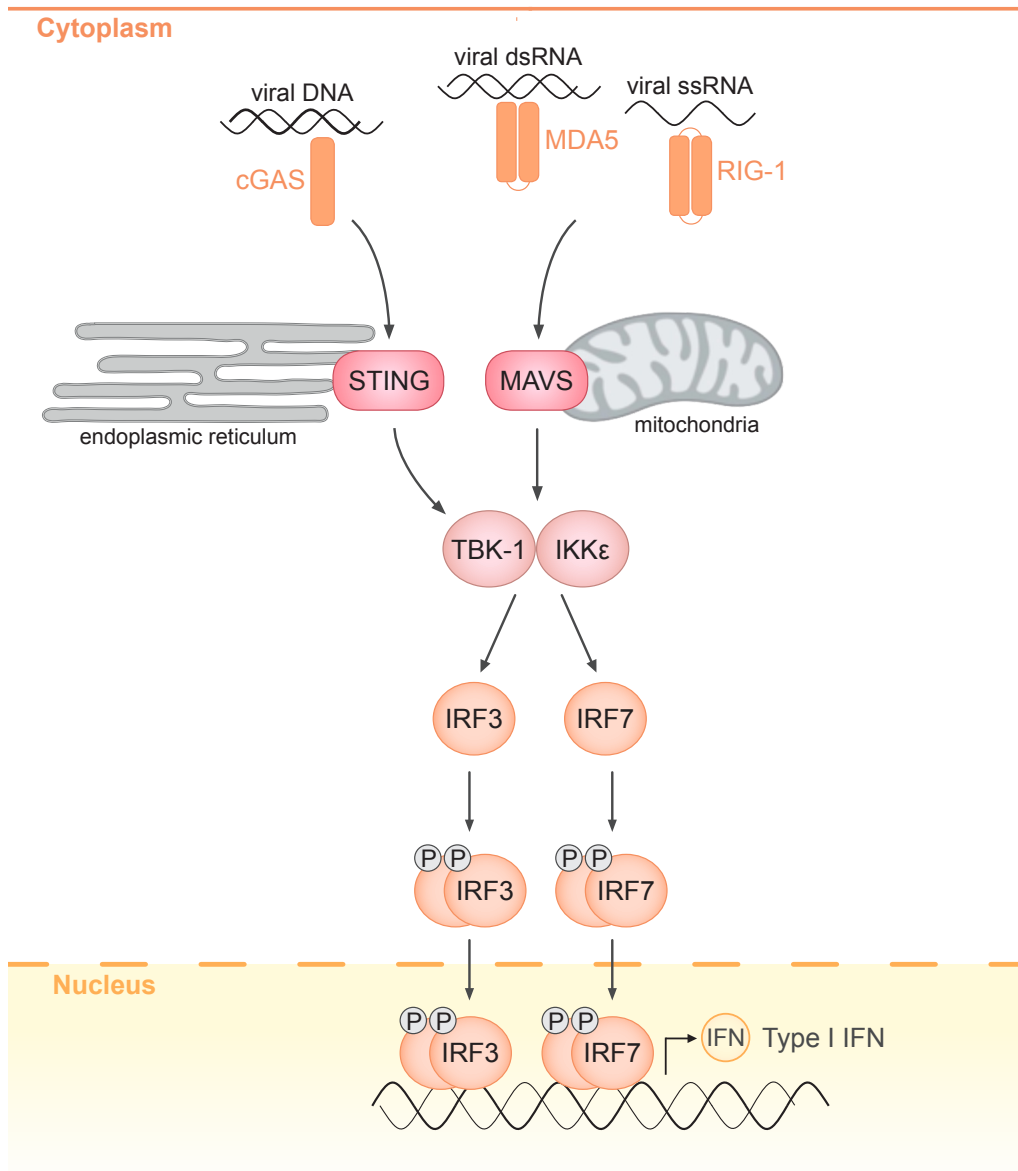


**Figure 10. Cytosolic nucleic acid pattern recognition.**

Cytosolic pattern-recognition receptors recognize foreign nucleic acids : RIG-I and MDA5 detect ssRNA and dsRNA respectively; cGAS recognizes DNA. Activation of RIG-I and MDA5 induces the polymerization of the adaptor MAVS located at the mitochondrial outer membrane [258,259]. This leads to the recruitment of kinases IKKε and TBK1 that phosphorylate the transcription factors IRF3 and IRF7, leading to the production of type I IFNs [260,261]. Activated cGAS transduces signals to the endoplasmic reticulum-associated adaptor STING, which recruits and activates the kinase TBK1. This kinase then activates transcription factors to induce type I IFNs transcription.

Several TLRs are sensors of viral products and are localized at the cytoplasmic and the endosomal membrane. The endosomal TLR3, TLR7, TLR8 and TLR9 sense distinct types of foreign nucleic acids. TLR2 and TLR4 recognize viral proteins in the extracellular space.

TLR3 is activated by viral dsRNA, which leads to the recruitment of the adaptor TRIF protein. In turn, TRIF transduces the signal to the two kinases TBK1 and IKKε, which phosphorylate and activate both IRF3 and IRF7 leading to the production of IFNs [260,262].

TLR7 and TLR8 detect ssRNA molecules of RNA viruses and recruit the adaptor MyD88 [263,264]. MyD88 forms a multiprotein signaling complex containing the kinase IKK $\alpha$  and the IRF7 transcriptional factor [265]. In this complex, IRF7 becomes phosphorylated by the kinase and translocates into the nucleus to induce the transcription of interferon mRNAs [266].

TLR9 recognizes unmethylated CpG DNA motifs that are present in viruses but are rare in mammalian cells [267]. Ligation of viral DNA by TLR9 triggers the MyD88-dependent pathway [268].

TLR2 and TLR4 are present on the cell surface and mainly recognize viral envelope proteins or viral proteins released into the extracellular space. TLR2 activation leads to the induction of proinflammatory cytokines and chemokines rather than the type I IFN production. TLR4 signals both through the MyD88 and the TRIF-dependent pathways.

### **6.1.2. Type I interferon signaling : the JAK-STAT pathway**

Upon interferon binding to their cell surface receptors, a signal is transmitted leading to the production of a series of proteins that are devoted to the control of pathogen infection [269].

All interferons signal through the JAK-STAT signaling pathway. Three of the four known Janus Kinases (JAKs) function in IFN signalling and are ubiquitously expressed [270]. These three kinases, called JAK1, JAK2 and TYK2 (tyrosine kinase 2), bind to one receptor chain on the inner side of the cellular membrane. The type I and type III heterodimeric IFN receptors associate with JAK1 and TYK2, whereas IFNGR associates with JAK1 and JAK2 (figure 9) [271-273]. Type I IFN signaling is illustrated on figure 11. It culminates in the transcriptional upregulation of more than 300 genes referred to as interferon-stimulated genes (ISGs).

### **6.1.3. The interferon-stimulated genes**

The binding of type I IFNs to their receptor leads to the induction of more than 300 ISGs, which establish a cellular antiviral state [228,274]. Some ISGs code for proteins that control the IFN production and response pathways, thereby providing either positive or negative feed-back loops. Most ISGs, however, encode effector proteins that impact an antiviral state to the cell.

#### *Amplification of IFN signaling*

Several ISGs are sensors of viral infection and signal transducing proteins, such as STAT1/2, IRF3/7/9, RLRs and some TLRs. Thus, Interferon enhances the expression of these proteins to strengthen IFN signaling and prime cells for improved pathogen detection.

Oligoadenylate synthetase (OAS) is induced by the IFN signaling cascade. Viral dsRNA detection by OAS leads to the synthesis of 2'-5'-oligoadenylates, which act as intracellular second messengers. These molecules bind inactive RNaseL to convert it in an active homodimer that cleaves host and viral RNAs. Small RNA cleavage products generated by RNaseL can in turn be detected by the RLRs to amplify and perpetuate IFN signaling [275].

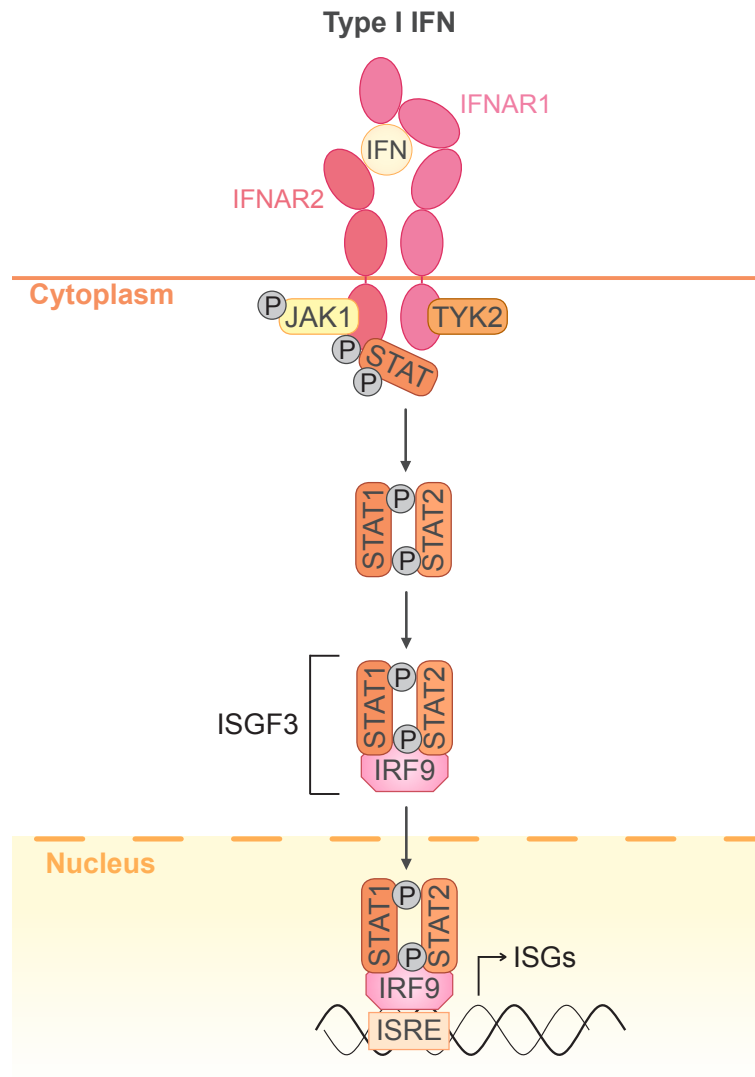


**Figure 11. Type I interferons receptor signaling.**

Following binding by type I IFNs, signal transduction is initiated by tyrosine kinases (JAK1 and TYK2), which phosphorylate their associated receptor chain. This leads to the recruitment and the phosphorylation of STATs [276-278]. Type I IFN signaling leads to the heterodimerization of STAT1 and STAT2 [279], which further associate with IRF9 to form the ISGF3 trimeric complex [280]. This complex translocates to the nucleus where it binds ISRE present in the promoters of interferon-stimulated genes [281,282]. This figure was adapted from W.M. Schneider et al., 2014, [228].

### *Interferon desensitization*

Certain ISGs negatively regulate IFN signaling to facilitate the return to cellular homeostasis. For example, SOCS proteins bind phosphorylated tyrosine residues on either IFN receptors or JAK proteins, resulting in the inhibition of JAK activity and STAT binding [283,284]. Moreover, SOCS mediate IFN receptors degradation through the proteasome.

USP18 also plays an important role in the long-term establishment of type I IFNs desensitization. This protein binds the intracellular domain of IFNAR2, which prevents the binding of JAK1 [285,286].

### *Antiviral effectors*

Numerous ISGs are antiviral effectors that target almost every stages of the viral life cycle [287]. For example, during infection, Mx proteins are highly induced by IFN. These proteins oligomerize to trap viral nucleocapsids and prevent replication of incoming viruses.

The kinase PKR is constitutively expressed but it is also an ISG. Upon detection of viral dsRNA, PKR monomers are phosphorylated and dimerize to form the active enzyme. Activated PKR phosphorylates the initiation factor eIF2- $\alpha$  to inhibit host and viral mRNA translation [288].

Other ISGs can inhibit entry, replication and assembly of viruses. They can also trigger apoptosis of infected cells and induce cytoskeletal remodelling.

In the following chapter, I will focus on one class of ISGs, the interferon-inducible GTPases, because my work included the characterization of one of these proteins.

## 7. The interferon-inducible GTPases

The interferon-inducible GTPase family belongs to the dynamin superfamily of high molecular weight GTPases and acts against several microbial classes [289,290]. This ISG family comprises at least 47 members in humans and mice, that can be classified into 4 subfamilies according to their paralogy and their molecular mass :

- The Mx proteins were among the first studied ISGs. They confer resistance almost exclusively to viruses.
- The immunity-related GTPases (IRGs) subfamily is composed of 21-47 kDa proteins that target pathogens in intracellular compartments.
- The guanylate-binding proteins (GBPs) are found in the cytosol and in intracellular membranes. They control the infection by both vacuolar and cytosolic pathogens.
- The last subfamily includes a group of very large inducible GTPases (VLIGs).

With the exception of Mx proteins that are only induced by type I and type III interferons, all of these IFN-inducible GTPases are transcribed in response to type I, type II and type III interferons [291-294].

### 7.1. The Mx proteins

In 1963, the team of Lindenmann studied the genetic factor establishing the resistance of a particular inbred mouse strain to Influenza A virus. The resistance phenotype was conferred by a single gene named Mx1 for orthomyxovirus resistance gene 1 [295,296]. Mx genes were subsequently identified in all vertebrates. Murine cells express two Mx proteins called Mx1 and Mx2, which are located in two different cellular compartments. Indeed, Mx1 is localized in the nucleus, whereas Mx2 is found in the cytoplasm. Human cells also express two Mx proteins, MxA and MxB, which are both localized in the cytoplasm [297]. The subcellular localisation of the Mx proteins influences their antiviral properties.

Mx proteins possess a molecular weight of about 80 kDa and display characteristic dynamin domains. Mx proteins bear a N-terminal GTPase domain that contains the highly conserved GTP-binding motif, a middle domain, and an effector domain with leucine zipper motifs at the C-terminal end (figure 12) [298]. The effector domain is implicated in viral target recognition [299]. The effector domain of mouse Mx1 also contains a nuclear localization signal that mediates its nuclear accumulation [300]. Another feature shared by the dynamin-like large GTPases is their capacity to assemble into oligomers. Purified Mx proteins form homo-oligomers resembling ring-like or helical structures [301,302]. Oligomerization likely arises from the attachment of the leucine zipper region of one protein to the middle domain of a second protein [303]. This self-assembly of Mx proteins seems to be crucial for their stability and their GTPase activity [304].

Mx family members show an antiviral activity against a wide range of viruses. The mouse Mx1 inhibits the multiplication of orthomyxoviruses known to replicate in the cell nucleus. This GTPase interacts with the ribonucleoprotein complex of Influenza A virus, thereby blocking viral mRNA synthesis of incoming viruses [305,306]. The mouse Mx2 confers resistance to viruses that replicate in the cytoplasm, such as Vesicular stomatitis virus and La Crosse virus [307]. Thus, rodents express two distinct Mx proteins whose antiviral activity relies on intracellular replication sites of viruses [308,309].

In contrast to rodent Mx GTPases, the human MxA protein targets diverse types of viruses regardless of their replication site. Indeed, this GTPase sequesters the nucleocapsids of nuclear viruses, such as Thogoto virus and Influenza A virus, to prevent their import into the nucleus [310,311]. In the same way, MxA traps the Hepatitis B virus capsid protein into perinuclear compartments [312]. Human MxA also inhibits the replication of cytoplasmic viruses including Vesicular stomatitis virus and Human parainfluenza virus, by targeting the viral nucleocapsids and blocking viral mRNA synthesis in the cytoplasm [313,314].

Until recently, human MxB was thought to be devoid of antiviral activity and was suggested to regulate nuclear import and cell cycle progression [315]. However, MxB was shown to bind HIV-1 capsid after entry and to affect virus uncoating, nuclear uptake and integration of viral DNA in the host genome [316-318].

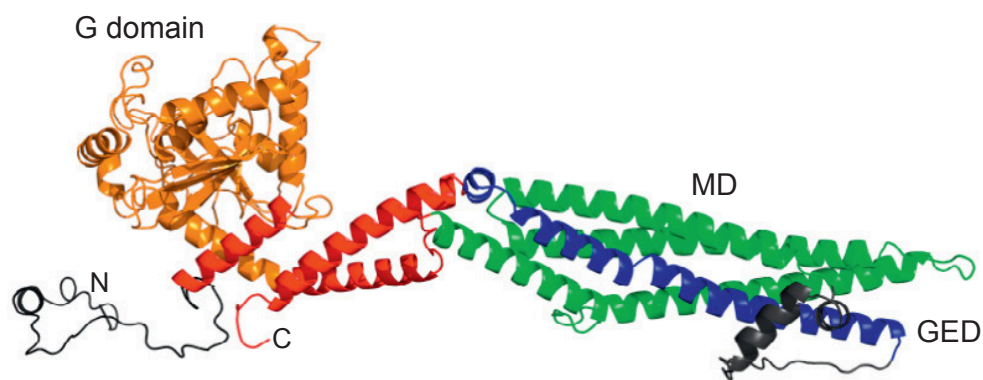


**Figure 12. Crystal structure of human MxA.**

The GTPase domain (G domain) is colored in orange, the middle domain (MD) in green and the GTPase effector domain (GED) in blue. The connection between the G domain and the MD consists of a bundle signaling element (red  $\alpha$ -helices), which is thought to transmit a signal from the GED to the G domain. This figure was adapted from O. Haller et al., 2015, [297].

Human and mouse Mx proteins seem to recognize viral nucleocapsid proteins of specific Mx-sensitive viruses. A possible model for Mx action proposes that nucleocapsid recognition by Mx dimers or tetramers leads to the recruitment of additional Mx molecules and self-assembly into ordered oligomers. This oligomerization results in the formation of ring-like structures that activate the GTPase activity of Mx proteins. This event induces mislocalization of the targeted viral proteins and the blockade of nuclear import [297,319].

## 7.2. The human guanylate-binding protein 1

Members of guanylate-binding proteins (GBPs) are induced by type I IFNs but are also and mostly induced by IFN- $\gamma$  [320]. These 65-73 kDa GTPases are well conserved in the vertebrates. The human genome contains 7 GBP genes that form a cluster on chromosome 1 [321]. The human guanylate-binding protein 1 (hGBP1) is the best-studied GBP. Expression of this protein is induced by IFNs as well as by cytokines such as IL-1 $\beta$ , IL-1 $\alpha$  and TNF- $\alpha$  [322]. hGBP1 is mainly localized in the cytoplasm but can be redirected to the Golgi apparatus upon IFN- $\gamma$  treatment. This redirection of hGBP1 requires GTP binding and farnesylation, which facilitates membrane interaction [323].

The three-dimensional structure of hGBP1 reveals a globular domain at the N-terminus of the protein, and a C-terminal helical domain consisting in a series of  $\alpha$ -helices (figure 13) [324]. The first five helices extend from the globular domain. They are followed by a loop allowing the folding of the next helix and its interaction with the globular domain. The last residues of the C-terminal domain also contain the farnesylation recognition motif. The globular domain of hGBP1 carries the consensus GTPase sequences for guanine nucleotide binding. As a member of the dynamin superfamily, hGBP1 possesses the classical G1, G2 and G3 motifs that are involved in phosphate groups recognition,  $Mg^{2+}$  binding, and GTP hydrolysis respectively. However, hGBP1 lacks the last canonical G4 motif that binds the guanine base [325]. Consistent with this feature, the GTPase has the ability to bind GMP and hydrolyses GTP in both GDP and GMP, GMP being the most prominent product [326]. Further analyses have shown that the hGBP1 can oligomerize and form dimers or tetramers in solution. Oligomerization is necessary for efficient GTP hydrolysis [327].

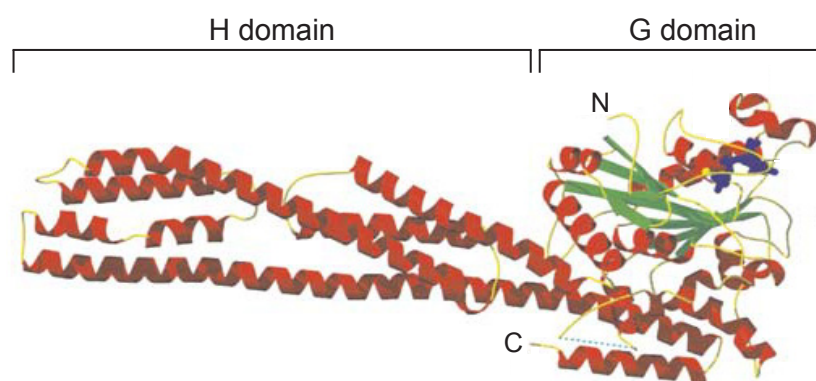


**Figure 13. Crystal structure of human GBP1.**

The structure shows the globular domain (G domain) and the extended  $\alpha$ -helical C terminal domain (H domain). The  $\alpha$ -helices are colored in red and the  $\beta$ -sheets in green. This figure was adapted from S. Martens et al., 2006, [290].

Despite its exact mechanism of action has not yet been resolved, hGBP1 was found to confer resistance to several pathogens *in vitro*. Infected HeLa cells expressing hGBP1 show reduced production of Vesicular stomatitis virus and Encephalomyocarditis virus (EMCV) [328]. Likewise, antisense RNAs targeting hGBP1 increase VSV and EMCV replication in IFN- $\gamma$  treated cells, suggesting that this GTPase mediates antiviral activity against both viruses. More recently, a study reported that hGBP1 confers resistance to Hepatitis C virus [329]. Indeed, HCV replication is suppressed by overexpression of hGBP1 in Huh7 cells. However, the viral protein NS5B interacts with hGBP1 to inhibit its GTPase activity, which is essential for its antiviral function.

hGBP1 also exhibits antibacterial activity. *Chlamydia trachomatis* are Gram-negative bacteria that replicate into intracellular compartments called inclusions. In HeLa cells infected with these bacteria, hGBP1 localizes to the inclusion membrane [330]. This localization requires the C-terminal helical domain containing the site for lipid addition. Membrane anchored hGBP1 can delay the growth of *Chlamydia trachomatis*.

In endothelial cells in culture, hGBP1 inhibits proliferation induced by inflammatory cytokines [331]. In addition, the GTPase represses the migration of endothelial cells by suppressing the expression of matrix metalloproteinase 1 [332].

In accordance with these biological activities, hGBP1 acts as a tumor suppressor in colorectal carcinoma and in mammary tumors [333,334]. Using an immunoprecipitation/mass spectrometry approach, Ostler and colleagues have identified actin as a binding partner of hGBP1 [335]. hGBP1 colocalizes with actin and is responsible for the actin remodelling observed in IFN- $\gamma$  treated cells. Oligomerization and GTPase activity are required for actin cytoskeleton rearrangement by hGBP1.

### 7.3. The very large inducible GTPase-1

In 2003, a fourth family of interferon-inducible GTPases has been discovered from cDNA libraries of mouse embryonic fibroblasts (MEFs) and macrophages stimulated with type II IFN [293]. This family was named very large inducible GTPases (VLIGs or GVINs), owing to the huge size of their proteic members. Gvin1 is the first specimen of this family and is found in 68 different species, ranging from zebrafish to chimpanzee. In the mouse genome, six Gvin1-related genes are clustered on chromosome 7. Four of them are non-coding genes. Two genes, GM4070 and GM1966, are predicted to produce proteins and show respectively 99% and 87% identity with Gvin1 at the nucleotide level. In the human genome, the VLIG family is represented by a single gene, homologue to Gvin1, that maps on chromosome 11. However, this gene is a pseudogene because it carries a frameshift in the coding sequence.

The mouse Gvin1 gene contains four exons and the entire open reading frame of 7284 bp is located in the last one. This gene encodes two different transcripts due to an alternative splicing in the 5' UTR, thus producing the same protein product. The Gvin1 protein has a molecular mass of 280 kDa and is the largest known GTPase in any species. The amino acid sequence of Gvin1 shows canonical motifs conserved in most GTPases. Furthermore, the GTP-binding motifs and their immediate sequence environment have the closest similarity to other interferon-inducible GTPases, in particular to the hGBP1 [293]. Amongst others, Gvin1 protein sequence contains the G1, G2 and G3 motifs but lacks the canonical G4 motif. The features of the GTP-binding domain of Gvin1 suggest that this GTPase has catalytic properties similar to those of interferon-inducible GTPases characterized by self-activation and oligomerization.

Up to now, only one study has examined the biological characteristics of Gvin1 [293]. The team of T. Klamp has shown that Gvin1 expression is induced by IFN- $\gamma$ , and to a somewhat lesser extent by IFN- $\beta$ , in MEFs and murine macrophages. In mice infected by *Listeria monocytogenes*, a strong increase of Gvin1 expression is observed in the liver, which appears to be IFN $\gamma$ -dependent. This GTPase strongly binds GDP but very weakly GTP and GMP. The study of subcellular localization of endogenous Gvin1 has revealed that this protein is mainly located in the nucleus of untreated MEFs. After stimulation with IFN- $\gamma$ , Gvin1 has either a nuclear or a cytoplasmic localization. The sequence similarity of Gvin1 GTP-binding domain with those of interferon-inducible GTPase families and its up-regulation by IFN suggest that Gvin1 could be implicated in the resistance against several pathogens. Yet, no cellular function has been assigned to Gvin1.



## Aims of the study

My research project focuses on two main topics : on the one hand, the study of the physiological role of cellular miRNAs on RNA viruses replication and, on the other hand, the use of RNAi to assess the antiviral activity of Gvin1.

### **1. Physiological role of cellular microRNAs on RNA viruses replication**

At the time I started to analyze the influence of miRNAs on viral infection, little was known about the physiological influence of miRNAs on RNA virus replication and this question is still largely unsolved today. It was well established that the presence of an artificial miRNA target sequence in the genome of poliovirus led to a strong inhibition of virus replication but it was also clear that escape mutants quickly emerged, that were resistant to miRNA activity. It was also reported that miRNAs could either repress or activate the replication of specific RNA viruses.

If miRNA could indeed inhibit the replication of RNA viruses, we hypothesized that evolution would have quickly eliminated miRNA target sites from viral genomes. On the other hand, conservation of miRNA target sites in viral genomes might indicate that viruses evolved to avoid infecting cells that express high amounts of the cognate miRNAs and therefore that miRNAs might shape the tropism of some viruses.

We addressed these questions with two subprojects. In the first one, we aimed to analyze the global influence of miRNA on RNA virus infection, using Theiler's virus as a model. In the second one, we specifically asked whether a miRNA target site that is conserved in rotavirus genomes could act to positively or negatively regulate replication.

#### **1.1. Global influence of cellular microRNAs on Theiler's virus replication**

This project was initiated during my Master thesis. We attempted to investigate the effect of miRNAs on Theiler's virus replication by inhibiting their biogenesis. Therefore, we planned to compare viral replication in cells expressing either the RNAi suppressor P19, or an inactive form of this protein. P19 is expressed by plant tombusviruses and sequesters 21 to 25 nucleotide dsRNAs. The group of O. Voinnet showed that P19 inhibits miRNA activity in human cells [136]. Thus, expressing P19 in mammalian cells should prevent miRNA biogenesis and thus allow to compare TMEV infection in miRNA-positive and miRNA-negative cells. To this end, we tested the activity of P19 in mouse cells. However, we were unable to observe any inhibitory activity of P19, neither on the activity of cellular miRNA, nor on TMEV replication.

In the first part of my thesis, we aimed to continue the assessment of the global influence of miRNAs on Theiler's virus replication.

First, we analysed the frequency of miRNA target sequences in the virus genome to test if selection against specific miRNA targets occurred during evolution. Then, we analysed the influence of such miRNA target sites on virus replication.

Secondly, with the same idea in mind as that of the P19 experiments, we developed a strategy to compare TMEV replication in *Dicer*-positive and *Dicer*-negative mouse embryonic fibroblasts (MEFs). Results obtained from this study are in press in *Viruses* and are presented in results section 1.

## **1.2. Impact of miR-142-3p on rotaviruses replication**

Preliminary works of Guido Bommer showed that Human rotavirus A genome contains an almost perfect target sequence for miR-142-3p. This miRNA is mainly expressed in the lymphoid system while rotaviruses replicate in epithelial cells of the intestine. We hypothesized that miR-142-3p could inhibit virus replication in lymphoid cells. Indeed, Human rotavirus A could have evolved to avoid replicating in these cells in order to prevent an early immune response.

Therefore, we compared the replication of an attenuated human rotavirus in simian epithelial cells expressing or not miR-142-3p. Data obtained from this work are presented in results section 2.

## **2. Antiviral activity of Gvin1**

In the second part of my thesis, we were interested to assess the antiviral activity of Gvin1. This project was initiated in our laboratory by Marguerite Kreit. She examined why IFN-treated neurons were sensitive to TMEV or VSV infection. A microarray analysis identified 15 genes whose expression was up-regulated in IFN- $\beta$ -treated MEFs, but that were little or not expressed in neurons [336]. I became involved in this project because it would much benefit from RNAi technology to test the involvement of these 15 genes in the antiviral response. I helped to set up the conditions for shRNA use and thereby technically contributed to the work of Marguerite Kreit on the antiviral role of ApoL9 [336]. Next, I continued a project related to another IFN-stimulated gene that is almost not expressed in neurons : the gene encoding the large GTPase Gvin1.

Gvin1 expression is inducible by type I and type II interferons. Gvin1 protein shares sequence similarities with others IFN-inducible GTPase families. These GTPase families, such as the Mx proteins family and the guanylate-binding proteins family, have shown antiviral activity against several RNA viruses. Therefore, it was likely that Gvin1 possesses a mechanism of action similar to those of these GTPase families and confers cellular resistance to viral infection.

To test this hypothesis, we used RNA interference to down-regulate the expression of Gvin1. Then, we compared viral replication of RNA and DNA viruses in mouse cells where Gvin1 expression was decreased or not. Data obtained for this second part are presented in results section 3.

## Results



## Section 1

### Cellular microRNAs repress Vesicular stomatitis virus but not Theiler's virus replication

Aurélie De Cock, Thomas Michiels<sup>1</sup>

In press in Viruses (MDPI)

<sup>1</sup>Laboratory of Virology, de Duve Institute,  
Université Catholique de Louvain, Brussels, Belgium.

In this work, we assessed the global influence of cellular microRNA on Theiler's virus replication (TMEV). First, we examined the genome of TMEV for the presence of under- or over-represented miRNA target sequences and tested whether these sequences would affect TMEV replication in cells overexpressing the corresponding microRNAs. Neither the most under-represented miR-770-3p nor the most over-represented miR-709 target sequence significantly affected TMEV replication, suggesting that miRNAs have little influence on TMEV. Next, we designed a strategy to compare virus replication in *Dicer*-positive and *Dicer*-negative mouse embryonic fibroblast populations. Short-term *Dicer* inactivation led to a > 10-fold decrease in miRNA abundance and strongly increased replication of Vesicular stomatitis virus. In contrast, miRNA depletion did not increase TMEV replication. In conclusion, RNA interference appears to have surprisingly little influence on TMEV replication and on TMEV genome evolution.



# Cellular microRNAs repress Vesicular stomatitis virus but not Theiler's virus replication

Aurélie De Cock <sup>1</sup>, Thomas Michiels <sup>2,\*</sup>

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<sup>1</sup> Université catholique de Louvain, de Duve Institute, VIRO B1.74.07, 74 avenue Hippocrate, B-1200 Brussels, Belgium; aurelie.decock@uclouvain.be

<sup>2</sup> Université catholique de Louvain, de Duve Institute, VIRO B1.74.07, 74 avenue Hippocrate, B-1200 Brussels, Belgium; thomas.michiels@uclouvain.be

\* Author to whom correspondence should be addressed; E-Mail: thomas.michiels@uclouvain.be; Tel.: +32-2-764-7429; Fax: +32-2-764-7495.

**Abstract:** Picornavirus' genomic RNA is a positive-stranded RNA sequence that also serves as a template for translation and replication. Cellular microRNAs were reported to interfere to different extents with the replication of specific picornaviruses, mostly acting as inhibitors. However, owing to the high error rate of their RNA-dependent RNA-polymerases, picornavirus quasi-species are expected to evolve rapidly in order to lose any detrimental microRNA target sequence. We examined the genome of Theiler's murine encephalomyelitis virus (TMEV) for the presence of under-represented microRNA target sequences that could have been selected against during virus evolution. However, little evidence for such sequences was found in the genome of TMEV and introduction of the most under-represented microRNA target (miR-770-3p) in TMEV did not significantly affect viral replication in cells expressing this microRNA. To test the global impact of cellular microRNAs on viral replication, we designed a strategy based on short-term *Dicer* inactivation in mouse embryonic fibroblasts. Short-term *Dicer* inactivation led to a > 10-fold decrease in microRNA abundance and strongly increased replication of Vesicular stomatitis virus (VSV), which was used as a microRNA-sensitive control virus. In contrast, *Dicer* inactivation did not increase TMEV replication. In conclusion, cellular microRNAs appear to exert little influence on Theiler's virus fitness.

**Keywords:** Theiler's virus, picornaviruses, *Dicer*, microRNA, evolution, interferon.

**PACS:** J0101

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## 1. Introduction

RNA interference (RNAi) is a key mechanism of post-transcriptional gene expression regulation in eukaryotic cells [337]. MicroRNAs (miRNAs) are short double stranded RNA molecules, formed from precursor transcripts carrying a hairpin structure. In the nucleus, this hairpin structure is cleaved off the precursor RNA by the Drosha endonuclease. The hairpin structure is then exported to the cytoplasm where it is processed by *Dicer* to generate mature miRNAs which are RNA duplexes of about 21 bp [338]. miRNAs are incorporated into RISC complex where one strand is degraded and the other strand acts as a guide to trigger translation arrest and/or degradation of target mRNAs. Targets are mostly determined by sequence complementarity of the seed sequence corresponding to the first eight nucleotides of the miRNA. According to the extent of base-pairing, target mRNA will undergo translation arrest or degradation [100]. Typically, miRNA can bind the 3' UTR of dozens of cellular mRNAs and thereby fine-tune the expression of corresponding proteins.

In mammals, miRNAs regulate various physiological processes such as cell proliferation, cell differentiation, cell signalling and apoptosis. Their dysfunction can lead to development abnormalities or to cancer [339].

In plants and insects, small interfering RNAs (siRNAs) play a crucial role in antiviral immunity [20,340]. Double stranded viral RNAs are recognized and processed by a Dicer-like enzyme into virus-derived siRNAs. These siRNAs are then loaded into the RISC complex and bind their viral RNA target with perfect base pairing to induce viral RNA degradation [74]. Plants also express RNA-dependent RNA polymerases that amplify virus-derived siRNAs to establish a systemic RNA silencing defence against viral infection [39]. In return, the majority of plant viruses and some insect viruses express proteins that interfere with the RNA silencing pathway [42,72,341]. Mutations in the RNAi machinery can increase the susceptibility of these organisms to viral infection [65,342]. Thus, in these eukaryotes, RNAi is a key antiviral mechanism.

As plants and insects viruses, some mammalian viruses have been shown to express proteins that inhibit the miRNA synthesis machinery (e.g. influenza NS1, Ebola virus VP35, HIV-1 Tat) [138,175,176,343]. These viral proteins act by binding double-stranded (ds) RNAs and thereby also inhibit other antiviral pathways that depend on dsRNA recognition, notably the interferon (IFN) pathway, which is critical to control viral infections. Thus, the biological significance of the RNAi suppressor activity of these proteins remains unclear.

It is still debated how much miRNAs can directly target viral genomes and have antiviral activity in mammals. *In vivo*, Dicer-deficient mice display increased susceptibility to Vesicular stomatitis virus (VSV) infection [137] due to impaired expression of miR-93 and miR-24 which directly target viral RNA. Similarly, miR-29 was reported to bind the 3'UTR of HIV mRNA, thereby inhibiting its translation and inducing its relocation into processing bodies (P bodies). These data support a direct role for host miRNAs in antiviral immunity.

In mammals, a link does exist between the miRNA and the IFN pathway. Interferon beta (IFN- $\beta$ ) treatment of hepatocytes raises the expression of several cellular miRNAs whose seed sequence is complementary to hepatitis C virus (HCV) RNA [153]. These miRNAs can directly target viral RNA to attenuate HCV replication. Mahajan et al. suggest that HCV could, however, take advantage of this replication reduction to establish a persistent infection [344]. Recently, it has been described that miR-142-3p inhibits the replication of Eastern equine encephalitis virus in murine macrophages and dendritic cells [345]. By limiting infection of hematopoietic cells, this RNA virus might limit the induction of innate immunity and thereby promote infection.

Infection of Dicer-deficient mouse embryonic fibroblasts (MEFs) with a large variety of viruses (e.g. Influenza virus, VSV, HIV, Human herpes simplex virus 1) suggested that absence of cellular miRNAs had little if any impact on the replication of these viruses [346]. Bogerd et al. concluded that human viruses evolved to escape targeting by cellular miRNAs. A recent study compared the antiviral function of miRNAs and IFN by using recombinant VSV viruses expressing either a viral suppressor of the RNAi machinery or a viral inhibitor of the IFN pathway [347]. Inhibition of RISC-associated small RNAs resulted in a slight decrease of viral infection, likely due to a reduced repression of interferon-stimulated genes by miRNAs. In contrast, IFN inhibition strongly enhanced viral replication [347].

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) belongs to the *Picornaviridae* family that includes, among others, the prototypic poliovirus. Typical picornavirus genomes are about 8000 nucleotide-long positive-stranded RNA molecules encoding a unique polyprotein. In the picornavirus cycle, replication and translation are inter-dependent as the full-length genome is used as a template for both processes. As replication occurs in semi-confined compartments known as replication complexes, it is unclear how much the viral genome would be accessible to miRNAs activity. Several studies have examined the interaction between host miRNAs and picornaviruses. Introduction of artificial miRNA target sequences in the genome of poliovirus or Cocksackie virus A21 led to a dramatic reduction of viral load *in vitro* and *in vivo*, in cells that expressed the corresponding miRNA [154,156].

*In vivo*, however, virus escape mutants rapidly emerged that lost their miRNA target sequence, suggesting that picornavirus genomes can easily adapt to minimize the impact of RNAi. On the other hand, certain picornaviruses like Foot-and-mouth disease virus (FMDV), Encephalomyocarditis virus (EMCV), Enterovirus 71 (EV71) and Coxsackie virus B3 (CVB3), naturally carry cellular miRNA binding sites in their genome [157-160]. Targeting of these sequences by cellular miRNAs generally decreases viral replication, suggesting that viruses carrying such target sequences evolved to avoid replicating in specific cell types that express high amounts of these miRNAs. In the case of CVB3, however, a miRNA targeting the viral RNA-dependent RNA polymerase coding sequence was reported to enhance virus biogenesis [161].

The aim of this study was to assess the global influence of miRNAs on Theiler's virus replication. We first analysed the frequency of miRNA sequences in the virus genome to test if selection against specific miRNA targets occurred during evolution. Next, we developed a transient *Dicer* inhibition system to compare viral replication levels in *Dicer*-positive and *Dicer*-negative cells.

## 2. Materials and Methods

### 2.1. Cell culture

L929 cells (ATCC) were maintained in Dulbecco modified Eagle medium (Lonza ref 12-604F) supplemented with 10% foetal calf serum (Sigma) and 100 U/ml of penicillin-streptomycin (Lonza).

Mouse embryonic fibroblast (MEFs) were isolated from a *Dicer<sup>fllox/null</sup>* embryo derived from a *Dicer<sup>fllox/fllox</sup>* female mouse that had been crossed with a *Dicer<sup>wt/null</sup>* male (kind gift of Frédéric Lemaigre, de Duve Inst., Brussels) [348]. MEFs were isolated by standard procedures, as described previously [349] and grown in Dulbecco Modified Eagle medium (DMEM, Lonza ref 12-604F) containing ultraglutamine and 4,5 gr/L glucose, supplemented with 10% of foetal calf serum (Sigma) and 50 units/ml of penicillin/streptomycin (Lonza). Genomic DNA was then extracted from MEFs to genotype the *Dicer* gene by PCR. Briefly, cells were centrifuged for 5 min at 4°C and then washed twice with PBS. After resuspension in 500 µl PBS, MEFs were warmed at 99 °C during 20 min followed by 10 minutes centrifugation. Supernatant was used for PCR amplification. The following primers were used for PCR screening : 23F-460R amplify the floxed allele of *Dicer* gene and 458F-460R amplify the excised or the null allele of *Dicer* gene (23F ATT GTT ACC AGC GCT TAG AAT TCC ; 458F TCG GAA TAG GAA CTT CGT TTA AAC ; 460R GTA CGT CTA CAA TTG TCT ATG). After screening, one *Dicer<sup>fllox/null</sup>* MEF population was immortalized by stable transfection of a pBABE-neo vector expressing the Simian virus 40 large T antigen.

### 2.2. Viruses

The VSV derivative expressing GFP was a gift from Martin Schwemmle (University of Freiburg, Germany).

KJ6 and KJ7 are derivatives of the persistent DA1 strain of Theiler's murine encephalomyelitis virus (TMEV) [350]. Both viruses carry capsid mutations that enhance L929 cell infection [351]. KJ7 additionally possesses the GFP coding sequence inserted between codons 5 and 67 of the L-coding region [349].

A KJ6 derivative (ADC43) was constructed in which 3 miR-770-3p target sequences were introduced by site-directed mutagenesis. The target site sequences thus introduced in the viral genome were those predicted for one of the randomly generated virus sequence (see below) that contained 3 miR-770-3p target sites (5'-CCG GCA GUG UCG UCC ACG-3', 5'-UUG CGC CGA ACA GUC GCC CAU G-3', 5'-CCG UGU CAC GGC ACG CUU ACG-3').

Viruses were propagated and titrated on BHK-21 cells [352]. Recombinant viruses used in this study are listed in Table 1.

**Table 1.** Recombinant viruses, lentiviral vectors and reporter vectors used in this study

| Vector/virus <sup>1</sup> | Parental <sup>1</sup> | Characteristics                                                                        |
|---------------------------|-----------------------|----------------------------------------------------------------------------------------|
| (p)KJ6                    | TMEV (DA1)            | Capsid adapted to infect L929 cells                                                    |
| (p)KJ7                    | (p)KJ6                | GFP-coding sequence replacing codons 5-67 of L protein                                 |
| (p)ADC43                  | (p)KJ6                | TMEV carrying 3 extra target sequences for miR-770-3p                                  |
| VSV-GFP                   | VSV                   | VSV carrying a GFP-coding sequence                                                     |
| (p)TM945                  | pCCLsin <sup>2</sup>  | Lentiviral vector carrying P <sub>CMV</sub> -MCS-IRES-mCherry <sup>2</sup>             |
| (p)ADC82                  | (p)TM945              | Lentiviral vector carrying P <sub>CMV</sub> -Cre recombinase-IRES-mCherry <sup>2</sup> |
| (p)ADC48                  | pLKO.1                | Lentiviral vector expressing shRNA corresponding to miR-770-3p                         |
| (p)ADC38                  | pGIPZ                 | Lentiviral vector expressing genomic miR-142-3p                                        |
| (p)ADC1                   | (p)TM897              | Lentiviral vector carrying P <sub>PGK</sub> - <i>Renilla</i> luciferase <sup>2</sup>   |
| (p)ADC30                  | (p)ADC1               | Luciferase reporter with 4 tandem miR-770-3p target sequences                          |
| (p)ADC39                  | (p)ADC1               | Luciferase reporter with 4 tandem miR-142-3p target sequences                          |
| (p)ADC62                  | (p)ADC1               | Luciferase reporter with 4 tandem mutated miR-770-3p target sequences                  |
| (p)ADC64                  | (p)ADC1               | Luciferase reporter with 4 tandem copies of mutated miR-142-3p target sequences        |
| pADC13                    | pcDNA.3               | <i>Photinus</i> luciferase (firefly) from pGL-2 basic (Promega)                        |

<sup>1</sup> (p): names of plasmids that carry a viral genome start with a “p” while corresponding viruses lack the “p”. For example, plasmid pKJ6 carries the genome of virus KJ6.

<sup>2</sup> pCCLsin = pCCLsin.PPT.hPGK.GFP.pre; PCMV = cytomegalovirus promoter; PPGK = human phosphoglycerate kinase promoter; MCS = multi-cloning site.

### 2.3. miRNA target sequence predictions

We compared the wild type (WT) sequence of the viral RNA with corresponding randomly generated sequences to identify target sites of miRNAs that were “under-represented” in the WT viral genome. To do this, we used the original amino acid sequence of the TMEV polyprotein that we back-translated to generate 100 different nucleotide sequences that encode the same polyprotein sequence. To avoid codon usage bias, we excluded rare codons from the computational generation of viral nucleotides sequences. Then, we used the RNA22 server to identify potential binding sites of murine miRNAs in the WT viral RNA and in the hundred back-translated nucleotide sequences [353].

### 2.4. Construction of a CRE expressing lentiviral vector and cell transduction

Lentiviral vectors were derived from pCCLsin. PPT. hPGK. GFP.pre (kindly provided by Luigi Naldini, Ospedale San Raffaele - Milano, Italy) [354]. pTM945 was generated by inserting, in the backbone of this vector, a cytomegalovirus promoter, a multi-cloning site, an IRES from TMEV and the mCherry coding sequence. The ORF of bacteriophage P1 Cre recombinase was subcloned in this plasmid using *Bsi*WI and *Xba*I restriction sites (pADC82). Lentivirus particles were generated by transient transfection of 293T cells grown in 75-cm<sup>2</sup> Petri dishes as previously described [336]. Cells were transduced with lentiviral particles 4 or 8 days before infection. Lentiviral vectors used in this study are presented in Table 1.

### 2.5. Generation of L929 cells overexpressing miR-770-3p and construction of a miR-142-3p expressing vector

A lentiviral vector was derived from pLKO.1, that drives the expression of a sequence corresponding to miR770-3p from a RNA polymerase III promoter [355].

To this end, complementary forward and reverse primers containing the short hairpin RNA (shRNA) sequence of miR-770-3p were annealed and cloned in pLKO.1, using *AgeI/EcoRI* restriction sites. Lentiviral particles were generated, as described previously [336] and used to transduce L929 cells. RT-qPCR was used to assess the expression of miR-770-3p in transduced and mock-transduced L929 cells, using Taqman® MiRNA Reverse Transcription Kit and Taqman® Gene Expression Master Mix (Applied Biosystems). Expression of miR-770-3p was not detected in L929 cells and was upregulated > 271±68 times in the cell populations transduced with ADC48 as compared to background.

miR-142-3p was overexpressed in cells by transient transfection of pADC38, a pGIPZ (GE Dharmacon) derivative that drives the expression of artificial miRNAs based on the backbone of miR30, from a RNA polymerase II promoter. To construct this plasmid, a 430 bp genomic sequence encompassing the miR-142-3p short hairpin sequence was amplified by PCR from C57BL/6 mouse genomic DNA and subcloned in pGIPZ using *XhoI/BamHI* restriction sites.

## 2.6. Reporter vectors and Luciferase assays

As reporter vector, we used a lentiviral vector (pADC1) that expresses the *Renilla* luciferase gene derived from pGL4.78 (Promega). In the 3' UTR region of the *Renilla* luciferase gene, we subcloned a *BsiWI/XbaI* fragment carrying 4 tandem target sequences for miR-770-3p and miR-142-3p, yielding plasmids pADC30 and pADC39, respectively. Control plasmids pADC62 and pADC64 were obtained similarly by cloning 4 tandem miR-770-3p or miR-142-3p target sequences that carry point mutations preventing their recognition by their cognate miRNA (Table 2).

As a control of transfection efficiency, we used a pcDNA3 derivative, pADC13 carrying the *Photinus pyralis* (firefly) luciferase gene from pGL2 (Promega). Transfection was performed with TransIT-LT1 reagent (Mirus) according to the manufacturer's protocol. Luciferase assays were carried out with the Dual-Luciferase Reporter Assay System (Promega) and results are reported as relative luciferase units (Luciferase of the reporter/Luciferase of the control vector). Reporter vectors used in this study are listed in Table 1. Corresponding nucleotides sequences of miRNA binding sites are given in Table 2.

**Table 2.** Nucleotide sequence of miRNA binding sites incorporated in reporter vectors

| Vector   | miRNA binding site                     | Nucleotides sequence of miRNA binding sites |
|----------|----------------------------------------|---------------------------------------------|
| (p)ADC30 | Target sequences for miR-770-3p        | CCA GCT CCA CGT CAG GCC CAC G               |
| (p)ADC39 | Target sequence for miR-142-3p         | TCC ATA AAG TAG GAA ACA CTA CA              |
| (p)ADC62 | Mutated target sequence for miR-770-3p | CCC GCA CCC CGA CAC GCT CAT G               |
| (p)ADC64 | Mutated target sequence for miR-142-3p | TCT ATT AAA TAA GAG ACT CTT CA              |

## 2.7. RNA extraction and quantitative RT-PCR

Total RNA preparations, reverse transcription and quantitative PCR (RT-qPCR) were performed as previously described [356]. Primers used for amplification are presented in Table 3. qPCR standards consisted of 10-fold dilutions of quantified plasmid DNA carrying the sequence to be amplified. Plasmids pTM793 (*Actb*), pCS40 (*Oasl2*), pMIP01 (*Usp18*), pSPA31 (*Stat1*) and pSPA33 (*Pkr*) correspond to pCR4-Topo (Invitrogen) derivatives carrying the corresponding amplicons. Plasmid pTM410 carries nucleotides 1-1730 of TMEV (DA1 strain), plasmid pMD2-VSV-G carries the sequence encoding VSV glycoprotein and plasmid pCDNA3-*Irfnb* carries the mouse gene encoding IFN-β. Reference plasmids are given in Table 1.

Quantitative RT-PCR for miR-let-7b was performed as previously described [357,358]. Forward (ACA CTC CAG CTG GGT GAG GTA GTA GGT TGT) and reverse primers (CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG AAC CAC AC) were kindly provided by Frédéric Lemaigre (de Duve Institute, Brussels, Belgium).

Expression of miR-770-3p was quantified by RT- qPCR in different tissues of C57BL/6 mice, using 10 ng of total RNA prepared previously [349]. RT was performed using TaqMan® MiRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. 1 µl of cDNA was used to perform qPCR using the TaqMan® Gene Expression Master Mix (Applied Biosystems) in an ABI 7300 thermal cycler, according to the manufacturer's protocol. PCR programs were 95 °C for 10 min, 40 cycles at 95 °C for 10 sec and 60 °C for 1 min.

**Table 3.** Primers used for gene expression analysis by RT-qPCR and reference plasmids

| Gene         | Name*        | Sequence 5' – 3'              | Plasmid             |
|--------------|--------------|-------------------------------|---------------------|
| <i>Actb</i>  | TM421 (Fw)   | AGA GGG AAA TCG TGC GTG AC    | pTM793              |
|              | TM422 (Rev)  | CAA TAG TGA TGA CCT GGC CGT   |                     |
| TMEV         | TM348 (Fw)   | GAA CGT CAG CAT TTT CCG GC    | pTM410              |
|              | TM349 (Rev)  | GGT GTG AAG AGC GGC AAG TG    |                     |
| VSV-G        | TM846 (Fw)   | TTG CTG CTC CAA TCC TCT CA    | pVSV-G              |
|              | TM847 (Rev)  | TCG AAC ACC TGA GCC TTT GA    |                     |
| <i>Ifnb</i>  | TM642 (Fw)   | ATG AAC AAC AGG TGG ATC CTC C | pcDNA3- <i>Ifnb</i> |
|              | TM643 (Rev)  | AGG AGC TCC TGA CAT TTC CGA A |                     |
| <i>Oasl2</i> | TM638 (Fw)   | GGA TGC CTG GGA GAG AAT CG    | pCS40               |
|              | TM639 (Rev)  | TCG CCT GCT CTT CGA AAC TG    |                     |
| <i>Usp18</i> | TM1116 (Fw)  | TGC AGG GTC TGT TCA CCA TC    | pMIP01              |
|              | TM1117 (Rev) | GCA CAT GTC GGA GCT TGC TA    |                     |
| <i>Stat1</i> | TM881 (Fw)   | GAG GGG CCT CTC ATT GTC AC    | pSPA31              |
|              | TM882 (Rev)  | GAT TCC TGG GCT CTG TCA CC    |                     |
| <i>Pkr</i>   | TM887 (Fw)   | CGC TGG CAG AAC TCA ATC AC    | pSPA33              |
|              | TM888 (Rev)  | CTC CGG TCA CGA TTT GTT CA    |                     |

\* (Fw) Forward and (Rev) Reverse

## 2.8. Flow cytometry

Cells were trypsinized and resuspended in phosphate-buffered saline containing 5% of filtered foetal calf serum and 0.5% of paraformaldehyde. Data acquisition was performed on a LSR Fortessa cell analyser (BD biosciences) using the FACSDiva software. Data were analysed using FlowJo 9.6.4. In cells transduced with mCherry-expressing vectors, viral replication (GFP fluorescence) was measured in the mCherry-positive-gated population. The rate of infection was defined as the percentage of GFP-positive cells. Viral replication was estimated by the median of GFP fluorescence intensity in the GFP-positive population. Infection efficiency was calculated by combining the rate of infection and viral replication (percentage of GFP-positive cells multiplied by median of GFP fluorescence of these cells in the mCherry-positive population).

## 2.9. Statistical analysis

Statistical significance was calculated for at least 3 replicates, using the Student t test. (\*,  $P < 0.005$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

### 3. Results

#### 3.1. Under-represented miRNA target sequences in TMEV genome

It was proposed that viral genomes might have evolved to suppress miRNA target sequences in order to escape miRNA-mediated replication inhibition [359]. We analysed whether TMEV evolved to suppress the presence of specific miRNA target sequences in its genome. Therefore, we compared the frequency of predicted miRNA target sequences that are present in the coding sequence of the DA1 strain of TMEV and in randomly generated nucleotide sequences that encode an identical polyprotein sequence.

The miR-770-3p target sequence was the most under-represented in TMEV genome (no occurrence in TMEV and a mean of 1.39 occurrences in the 100 randomly generated sequences) (Figure S1A). As this miRNA is mainly expressed in the brain and spinal cord (Figure S1B), we hypothesized that the virus could have evolved to suppress these target sequences, thus avoiding the restriction imposed by miR-770-3p on viral replication in the central nervous system.

To investigate this hypothesis, we generated L929 cells that over-express miR-770-3p and constructed a TMEV mutant virus, ADC43, where 3 predicted miR-770-3p target sites were introduced by site-directed mutagenesis. Then we compared the replication of the WT and mutant virus in L929 cells that overexpressed or not the miRNA construct (Figure 1).

shRNA expressed in these cells efficiently decreased *Renilla* luciferase expression from transfected reporter plasmids carrying the corresponding miRNA target sequences in the 3'UTR of the luciferase gene (Figure 1B).

Replication of the WT and mutant viruses were assessed, by RT-qPCR, 8 hours after infection. As shown in Figure 1C, the presence of miR-770-3p target sequences in the viral genome did not restrict viral replication in cells expressing this miRNA (Figure 1C).

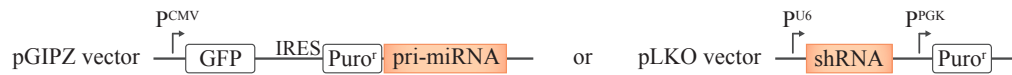
#### 3.2. Cre-mediated Dicer inactivation reduces expression and activity of cellular miRNAs

In order to investigate the global impact of cellular miRNAs on viral replication, we attempted to derive cells that would differ only by the expression of *Dicer*. To do so, we immortalized and cloned *Dicer<sup>flox/null</sup>* MEFs, which carry one *Dicer* allele with lox sequences framing exons 22 and 23, and one *Dicer* "null" allele (i.e. with a deletion of the RNaseIII domain) [348] (Figure 2A). All attempts to derive a *Dicer<sup>-null</sup>* cell line from these *Dicer<sup>flox/null</sup>* cells, by transiently expressing the Cre recombinase from either adenovirus or plasmid vectors, led to gradual growth arrest of the cells (not shown). These cells were thus not suitable to compare viral replication in *Dicer*-positive and *Dicer*-negative cells. We therefore designed a short-term *Dicer* inactivation strategy based on Cre-expressing lentiviral vectors.

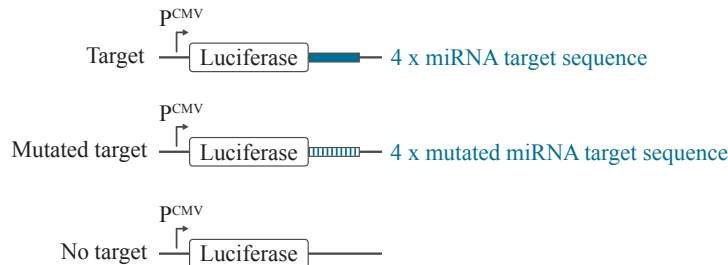
*Dicer<sup>flox/null</sup>* MEFs were transduced with a lentiviral vector (ADC82) co-expressing mCherry and Cre, in order to inactivate the floxed *Dicer* allele (Figure 2A). *Dicer* expression and miRNA abundance were analysed 4 and 8 days after transduction of the Cre vector. WT *Dicer* expression was reduced by 30-fold at both time points (Figure 2B). Abundance of the cellular miRNA miR-let-7b decreased by 7-fold at day 4 and by 11-fold at day 8 post-transduction (Figure 2C).

**A**

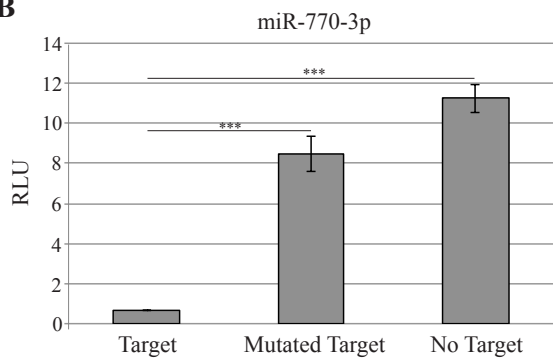
miRNA expression vector



Reporter vector



**B**



**C**

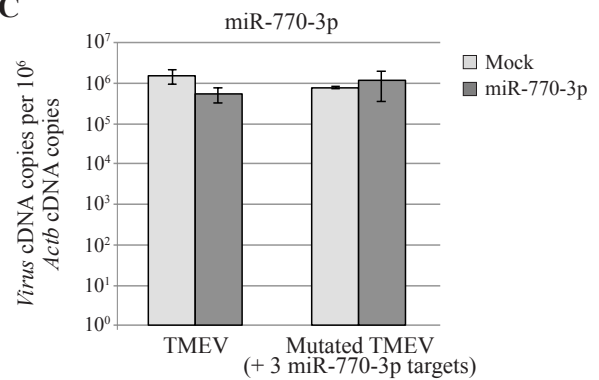


**Figure 1.** Lack of influence of miR-770-3p on TMEV replication. (A) Illustration of miR expression and reporter vectors. miR expression was achieved by expressing either an artificial primary miRNAs using the pGIPZ vector or a short hairpin RNA, using the pLKO.1 vector. Reporter vectors carry a *Renilla* luciferase gene with 0 (No target) or 4 wild type (Target) or mutated (Mutated target) miR target sequences. (B) L929 cells expressing miR-770-3p from the pLKO vector were transduced with the indicated reporter constructs and luciferase activity (Relative Luciferase Units : RLU) was assessed 24h post transduction. (C) L929 cells expressing miR-770-3p were infected for 8 hours with wild type and mutant TMEV derivatives before replication analysis by RT-qPCR (C). Viruses were KJ6 (TMEV) and ADC43, a KJ6 derivative carrying 3 extra binding sites for miR-770-3p. Histograms show the mean and SD of a triplicate experiment (out of 2).

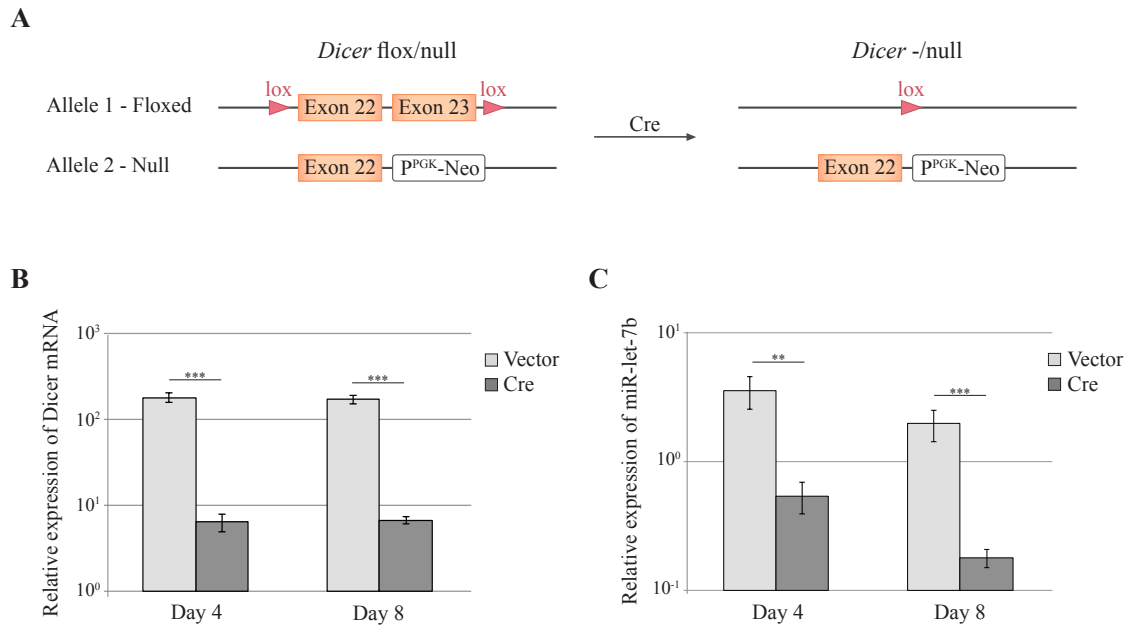


**Figure 2.** Short-term *Dicer* inactivation influence on cellular miRNA expression. (A) Illustration of *Dicer* alleles present in MEFs before and after Cre-mediated recombination. (B, C) Four and 8 days after transduction of *Dicer*<sup>flox/null</sup> MEFs with either TM945 expressing mCherry alone (Vector) or ADC82 co-expressing mCherry and Cre (Cre), WT *Dicer* mRNA (B) and mature miR-let-7b (C) were quantified by qRT-PCR. Histograms show the mean and SD of a typical experiment (out of 2) performed in triplicate.

We further tested the influence of *Dicer* inactivation on a reporter system where miR-142-3p expressed from a pGIPZ derivative inhibits the expression of a reporter luciferase construct carrying miR-142-3p target sequences. In cells transduced with the control lentiviral vector that does not express Cre, miR-142-3p triggered an important decrease of *Renilla* luciferase activity (Figure 3A–3B). In cells transduced with the Cre-expressing vector, the decrease of luciferase activity was reduced, as expected from *Dicer* inactivation (Figure 3A–3B).

Thus, Cre expression in *Dicer*<sup>flox/null</sup> MEFs induces a significant decrease of miRNAs expression and of miRNAs function that is detectable 4 days and more pronounced 8 days after transduction.

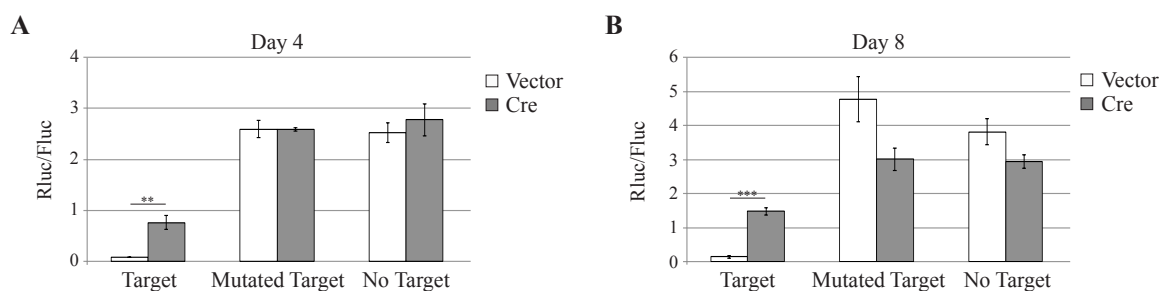


**Figure 3.** Effect of *Dicer* inactivation on miR activity. *Dicer*<sup>flox/null</sup> MEFs were transduced for 4 (A) or 8 (B) days with TM945 expressing mCherry alone (Vector) or ADC82 co-expressing mCherry and Cre (Cre). Cells were then co-transfected with pADC38 (pGIPZ-miR-142-3p), with indicated *Renilla* luciferase (Rluc) reporter constructs carrying miR-142-3p targets and with pADC13 encoding firefly luciferase (Fluc) from the CMV promoter. Histograms show the mean and SD of the Rluc/Fluc ratio, which reflects the absence of miR function.

### 3.3. Knockdown of Cellular miRNAs increases VSV but not TMEV replication

Next, we analysed the influence of *Dicer* inactivation on TMEV replication. VSV was used as a control since replication of this virus was reported to be susceptible to host miRNA activity. Thus, *Dicer*<sup>flox/null</sup> MEFs were transduced with the lentiviral vectors co-expressing Cre and mCherry (ADC82) or expressing mCherry alone (TM945). Four and eight days after transduction, cells were infected with VSV or TMEV derivatives expressing GFP and viral replication was monitored by flow cytometry. This strategy allows the specific analysis of virus replication (GFP fluorescence) in Cre-expressing (i.e. *Dicer*<sup>-</sup>/null, mCherry fluorescence) cells. As shown in Figure 4A, expression of Cre in *Dicer*<sup>flox/null</sup> MEFs led to a significant increase of VSV infection efficiency at 8 days post transduction (Figure 4A). In contrast, Cre triggered a slight decrease of TMEV infection efficiency (Figure 4B). Analysis of viral replication by RT-qPCR and plaque assay confirmed that the inactivation of the floxed *Dicer* allele by CRE increased VSV but not TMEV replication (Figure 4C–4F).

### 3.4. *Dicer* inactivation increases *Oasl2* and *Usp18* expression

To verify that the increase of VSV replication was not due to an impairment of the interferon response in *Dicer*-deficient cells, we used RT-qPCR to measure the expression of the gene coding interferon beta (*Ifnb*) and of 4 interferon-stimulated genes (ISG) (Figure 5). In non-infected cells (left histograms), *Dicer* inactivation did not influence *Ifnb*, *Stat1* or *Pkr* expression but clearly induced *Oasl2* expression. This suggests that basal *Oasl2* levels are maintained low by miRNAs, as reported by Seo *et al.* who suggested that miRNAs limit ISG expression to avoid their detrimental effects on cell proliferation [360]. As expected, infection of MEFs with either VSV or TMEV increased the expression of *Ifnb* (Figure 5A) and of *Oasl2*, *Usp18* and *Pkr* (Figure 5B – 5C, 5E). Upregulation of *Ifnb* and of ISG expression correlated with the extent of virus replication, in agreement with the fact that viral RNA is the main trigger of IFN- $\beta$  expression. ISG expression was thus higher in Cre-expressing MEFs infected with VSV (Figure 5A). This shows that *Dicer* inactivation did not increase VSV replication through inhibition of the IFN response.

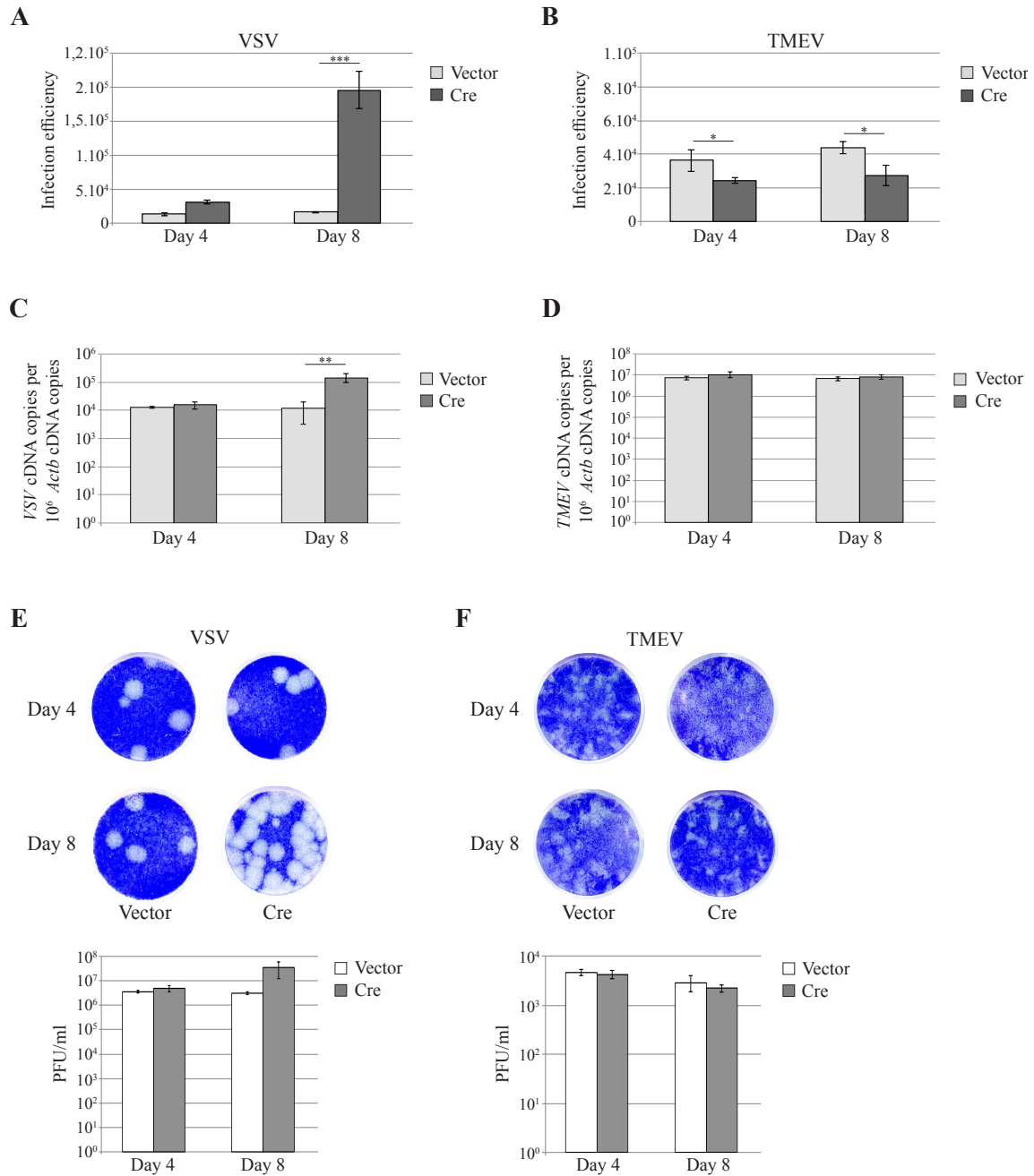


**Figure 4.** Impact of *Dicer* inactivation on VSV and TMEV infection. Infection by GFP expressing derivatives of VSV and TMEV was compared in *Dicer*<sup>flox/null</sup> MEFs that were transduced for 4 or 8 days with TM945 expressing mCherry alone (Vector) or with ADC82 co-expressing mCherry and Cre (Cre). Cells were then infected in triplicate with 10 PFU per cell of VSV during 7h or with 1 PFU per cell of TMEV during 8h. Histograms show the mean and SD of VSV and TMEV infection efficiency as determined by flow cytometry (A, B)(1 representative experiment out of 2), RT-qPCR (C, D) or plaque assay (E, F).

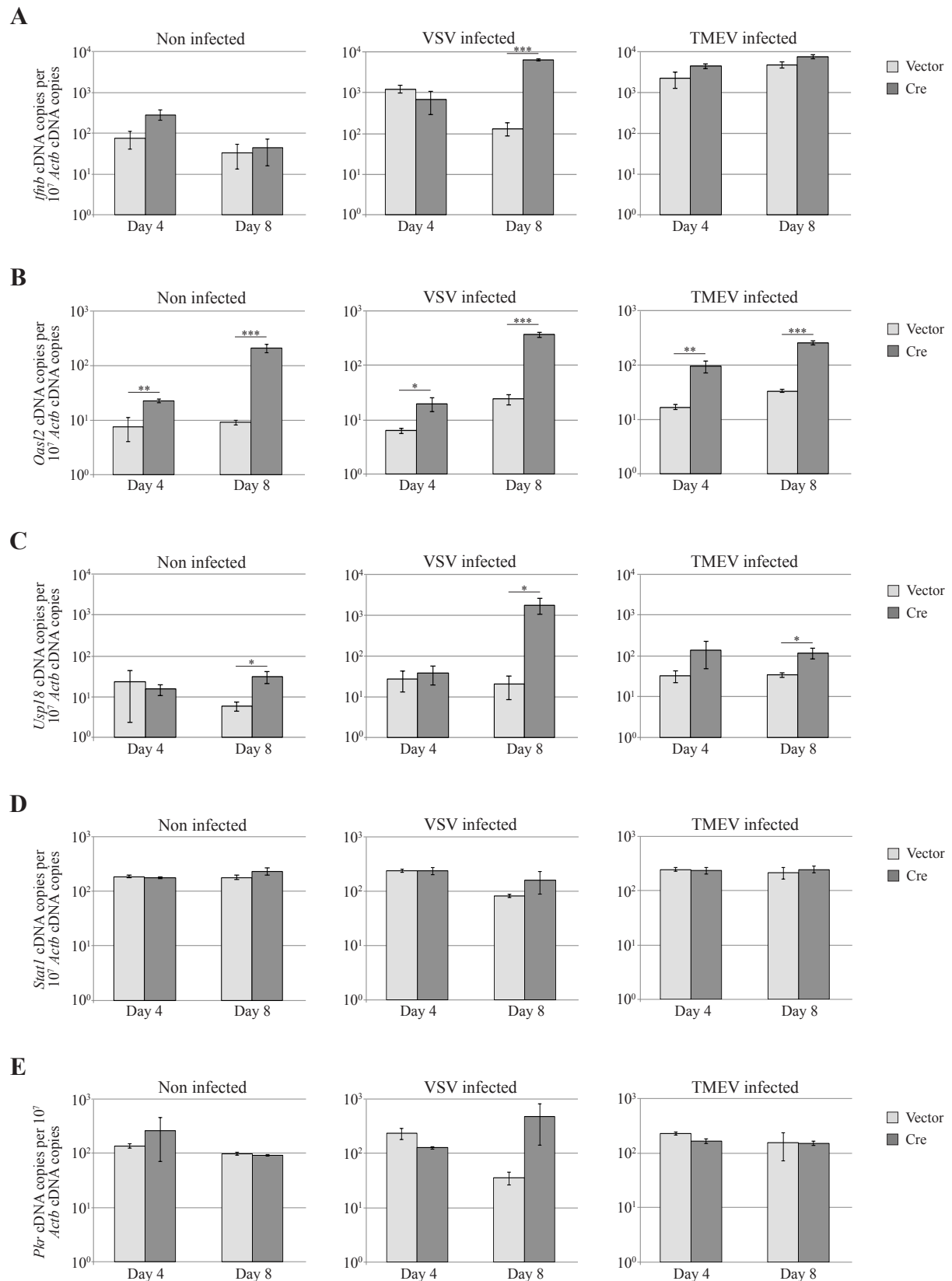


**Figure 5.** IFN response in *Dicer*-positive and *Dicer*-negative MEFs. (A-E) *Ifnb* (A), *Oasl2* (B), *Usp18* (C), *Stat1* (D) and *Pkr* (E) expression was quantified by RT-qPCR in non-infected, VSV-infected or TMEV-infected *Dicer*<sup>flox/null</sup> MEFs transduced for 4 or 8 days with lentiviral vectors expressing mCherry alone (Vector) or co-expressing mCherry and Cre (Cre). Infection conditions were as in Figure 4.

#### 4. Discussion

In this work, we wanted to evaluate the global influence of miRNAs on TMEV replication. We first analysed whether miRNAs shaped the viral genome sequence by selection against miRNA target sequences. The most under-represented miRNA target sequence in the TMEV genome (miR-770-3p) was absent from the viral genome and occurred at a mean frequency of 1.39 in randomly generated sequences coding the same protein, the probability to find 0 miR-770-3p target sequence in random sequences being 0.24. Also, overexpression of a shRNA corresponding to miR-770-3p in L929 cells did not significantly affect replication of TMEV mutants where predicted miR-770-3p target sequences were introduced. The latter results must, however, be taken with caution because this shRNA was expressed from a RNA polymerase III promoter, which reportedly generates RNA that might be off one or few nucleotides [361]. The integrity of the 3' end of the shRNA was attested by the RT-PCR procedure but alterations of the 5' end could not be excluded. Taken together, these results fail to suggest a strong selection pressure exerted against the presence of miRNA target sequences in the genome of TMEV.

We next engineered a *Dicer* inactivation system that enables the comparison of virus replication in otherwise identical *Dicer*-positive and *Dicer*-negative cells. Complete inactivation of *Dicer* consistently led to cell growth inhibition. Therefore, short-term *Dicer* inactivation was used to analyse the impact of *Dicer* inactivation on the replication of TMEV and VSV, before the occurrence of cell growth arrest.

Eight days after Cre-mediated deletion of *Dicer*, the amount and activity of cellular miRNAs were significantly decreased. Replication of VSV was strongly enhanced while replication of TMEV was not affected. Enhancement of VSV replication in *Dicer*-negative cells was not a consequence of miRNA-mediated inhibition of the interferon antiviral response since the loss of *Dicer* did not prevent the expression of IFN-stimulated genes. Inhibition of VSV replication was thus likely a direct effect of cellular miRNAs, in line with a previous report showing that VSV can be targeted by miR93 and miR24 [137]. In contrast, TMEV replication was not increased after *Dicer* inactivation. On the contrary, a very small but reproducible decrease of TMEV replication was observed. This increased TMEV replication may result from the upregulation of some ISGs, as observed for *Oasl2*, that may contribute to restrict viral replication.

Altogether, our results suggest that TMEV exhibits little susceptibility to the action of cellular miRNAs. A recent study showed that the IFN system was much more active than RNAi to restrict the replication of VSV [347]. In the case of TMEV, the balance between RNAi and IFN antiviral effects tips even more toward IFN, even if TMEV evolved to produce proteins that act to antagonize both IFN production and IFN effector activity [362,363].

**Supplementary Materials:** Figure S1 is available online at [www.mdpi.com/link](http://www.mdpi.com/link), Figure S1: Distribution of the number of predicted miR-770-3p targets in viral genomes and expression of miR-770-3p and miR-709 in mouse tissues.

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**Author Contributions:** ADC and TM conceived and analyzed the experiments; ADC performed the experiments; ADC and TM wrote the text.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Supplementary figure

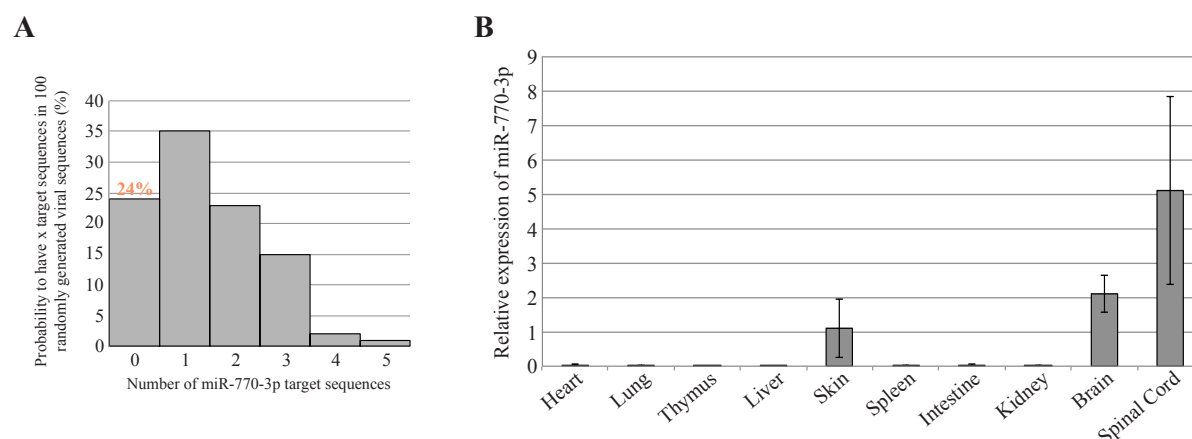


**Figure S1.** Distribution of the number of predicted miR-770-3p targets in viral genomes and expression of miR-770-3p in mouse tissues. (A) Distribution of the number of predicted miR-770-3p target sequences in 100 randomly generated sequences coding the same polyprotein. The sequence of virus DA1 contains 0 target sequence which occurs with a probability of 0.24 in randomly generated sequences. (B) Distribution of miR-770-3p in mouse tissues, as assessed by RT-qPCR.

## Section 2

### “miR-142-3p does not restrict the replication of an attenuated human rotavirus”

**Aurélie De Cock**, Guido Bommer<sup>2</sup>, Thomas Michiels<sup>1</sup>

<sup>1</sup>Laboratory of Virology, de Duve Institute,  
Université Catholique de Louvain, Brussels, Belgium.

<sup>2</sup>Laboratory of Biochemistry and Metabolic Research, de Duve Institute,  
Université Catholique de Louvain, Brussels, Belgium.

In the course of my thesis, we were interested to assess the impact of a miR-142-3p target sequence present in Human rotavirus A genomes on virus replication. We thus engineered MA-104 cell clones that overexpress this microRNA in order to examine the replication of an attenuated rotavirus in these cells. Despite miR-142-3p were active, our data showed that this microRNA does not restrict viral replication.



## 1. Introduction

Rotaviruses cause severe diarrhea in infants and young children worldwide. Approximately 95% of children have encountered rotavirus gastroenteritis at the age of five [364,365]. Each year, about 500 000 children die from rotavirus infection, most of them living in developing countries. These viruses have been classified in eight species (A-H), the group A being the most common infectious agent. Rotaviruses belong to the *Reoviridae* family and their genome is composed of segmented double-stranded RNA (dsRNA) [366]. The eleven genomic segments are enclosed in a capsid formed by three concentric layers of proteins [367]. These viruses replicate in enterocytes of the small intestine, leading to the destruction of the epithelium. Once attached to their surface receptor, rotaviruses enter the cell by endocytosis and reach the early endosome [368-370]. In this cellular compartment, the outer capsid protein VP7 is removed to release a double-layered particle into the cytosol. The RNA-dependent RNA polymerase VP1 is then activated and transcribes capped positive-sense RNA from each dsRNA genome segment. These RNA molecules serve either as mRNAs for viral protein synthesis or as templates for genome replication. Non-structural proteins NSP2 and NSP5 interact to form large cytoplasmic inclusions called viroplasms, in which viral components accumulate and contribute to genome replication and assembly of viral progeny [371-373]. The new virions bud into the endoplasmic reticulum where the outer capsid protein VP7 resides and allows the formation of triple-layered particles. Virions are finally released from infected cells and are exposed to gastrointestinal proteases that cleave the cell-attachment VP4 protein to produce fully infectious viruses [374,375]. Two attenuated live vaccines have been developed to prevent rotavirus infection [376].

microRNAs (miRNAs) are short dsRNA molecules that regulate protein synthesis and thereby regulate various cellular physiological processes. Besides their role in the regulation of gene expression, cellular miRNAs can restrict the replication of RNA viruses by directly targeting viral RNAs [344,377]. Recently, the hematopoietic specific miR-142-3p has been shown to bind target sites located in the 3' UTR of Eastern equine encephalitis virus (EEEV) genome [345]. This interaction results in a significant reduction of viral replication and thereby limits viral tropism for macrophages and for dendritic cells. *In vivo*, this inhibition leads to decreased viral replication in the lymph nodes and to the enhancement of disease progression.

The majority of rotavirus A genomes contains an almost perfect binding site for miR-142-3p (figure 1). This target site is located in segment 11 of the viral genome that encodes the non-structural proteins NSP5 and NSP6 from two overlapping reading frames. Guido Bommer has shown that the overexpression of miR-142-3p can restrict HA-tagged NSP5 expression from a transfected expression vector. As rotavirus is able to infect certain lymphocyte subpopulations in the intestine [378], we hypothesized that this virus had maintained or acquired a miR-142-3p target site to limit its replication in particular cell-types. As in the case of EEEV, this may result in a delay of the immune response and a spreading of the virus from the intestinal tract without being prematurely attacked by the immune system.

5' GATAAAAGTAGGAAACACTACC 3' Human rotavirus Strain A sequence type 1  
 3' AGGUA-UUUCAUCCUUUGUGAUGU 5' miR-142-3p

5' CCATAGAAGTAGGAAACACTACC 3' Human rotavirus Strain A sequence type 2  
 3' AGGUAU-UUCAUCCUUUGUGAUGU 5' miR-142-3p

**Figure 1. Predicted binding sites of miR-142-3p to the NSP5/NSP6 transcript of Human rotavirus A.** Two types of miR-142-3p target sequences have been reported in Human rotavirus A genomes. The sequence complementarity between miR-142-3p and its viral target sequences of type 1 (upper alignment) or type 2 (lower alignment) is predicted to be almost perfect.

## 2. Materials and Methods

### 2.1. Cell culture

MA-104 cells (ATCC) were cultured in Minimum Essential Medium (EMEM – Lonza 12-611F) supplemented with 10% fetal calf serum (Sigma), 100 U/ml of penicillin-streptomycin (Lonza), non-essential amino acids, and sodium pyruvate.

### 2.2. Virus and cell infection

As rotavirus model, we used the attenuated live vaccine Rotarix (GlaxoSmithKline AROLA547BA, strain G1P[8]) to perform infection experiments. The genomic fragment coding for the NSP5/NSP6 proteins contains the type 1 sequence of miR-142-3p binding site (figure 1). The sequence of this region was confirmed in our virus stock.

For infection experiments, MA-104 cells were first washed and incubated during 1h with EMEM without FCS at 37°C. In the same time, Rotarix was incubated with trypsin (Sigma type IX-S, 10 µg/ml final concentration) at 37°C during 30 min, followed by resuspension in serum free EMEM. Cells were then infected with 0.01 MOI of the viral suspension and incubated during 1h at 37°C. Finally, MA-104 cells were washed and resuspended in serum free EMEM supplemented with 0.5 µg/ml of trypsin.

### 2.3. miRNA expression vector and lentiviral vector production

The pGIPZ plasmid (Dharmacon) was used to express miR-142-3p. To this end, we PCR-amplified the sequence of miR-142-3p from C57BL/6 genomic DNA. Sense and antisense primers contained, at their 5' end, *Xho*I and *Bam*HI restriction site respectively. The PCR product was then subcloned in pGIPZ using *Xho*I/*Bam*HI restriction sites, to yield plasmid pADC38. Lentivirus particles were produced by transient transfection of HEK293T cells grown in 75-cm<sup>2</sup> Petri dishes, by the calcium phosphate method [336]. We used 7.5 µg of pGIPZ, 2.25 µg of pMD2.G (Addgene) and 5.625 µg of psPAX2 (Addgene). Cell supernatants were harvested at 24h and 48h post-transfection.

### 2.4. Reporter vectors and Luciferase assays

As reporter vector, we used a lentiviral vector (pADC1) that expresses the *Renilla* luciferase gene derived from pGL4.78 plasmid (Promega). In the 3' UTR region of *Renilla* luciferase, we subcloned a *Bs*WI/*Xba*I fragment carrying 4 tandem copies of target sequences complementary to miR-142-3p, yielding plasmid pADC39. A control plasmid, pADC64, contains 4 tandem copies of the corresponding mutated target sequences.

Plasmid pADC147 was obtained similarly by cloning 4 tandem copies of the target site corresponding to that present in the Rotarix genome. As a control of transfection efficiency, we used a pcDNA3 derivative, pADC13, carrying the *Photinus pyralis* (firefly) luciferase gene from pGL2 (Promega). Transfection was performed with TransIT-LT1 reagent (Mirus) according to the manufacturer's protocol. Luciferase assays were carried out with the Dual-Luciferase Reporter Assay System (Promega).

## 2.5. RNA extraction and quantitative RT-PCR

Total RNA preparations, RT and quantitative PCR were performed as previously described [356]. To quantify miR-142-3p in different tissues of C57BL/6 mice, we used 20 ng of total RNA prepared previously [349]. RT was performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. cDNAs were diluted at 1:20 and 5 µl of this dilution were used to perform qPCR using TaqMan® Gene Expression Master Mix (Applied Biosystems – 4369016) in an ABI 7300 thermal cycler, according to the manufacturer's protocol. PCR programs were 95 °C for 5 min, 40 cycles at 95 °C for 10 sec and 60 °C for 1 min.

Primer sequences were the following : TM421 (forward) AGA GGG AAA TCG TGC GTG AC and TM422 (reverse) CAA TAG TGA TGA CCT GGC CGT for *Actb*, TM1597 (forward) CAA CGC TGT AGA ACG AAA GCT A and TM1598 (reverse) CGA AGT TAA TAT AGC AGG ATC CAT T for the viral polymerase *VP1*. *Actb* was used as a reference gene to normalize *VP1* and miR-142-3p quantification.

## 3. Results and Discussion

If miR-142-3p prevents rotavirus replication in particular cell-types, this miRNA should be expressed in cells where rotavirus does not replicate. As rotaviruses replicate in the intestine, we looked at miR-142-3p expression in this tissue as well as in nine other murine organs. This miRNA was mainly detected in the spleen and, to a lesser extent, in the lung, the skin, the thymus and the intestine (figure 2).

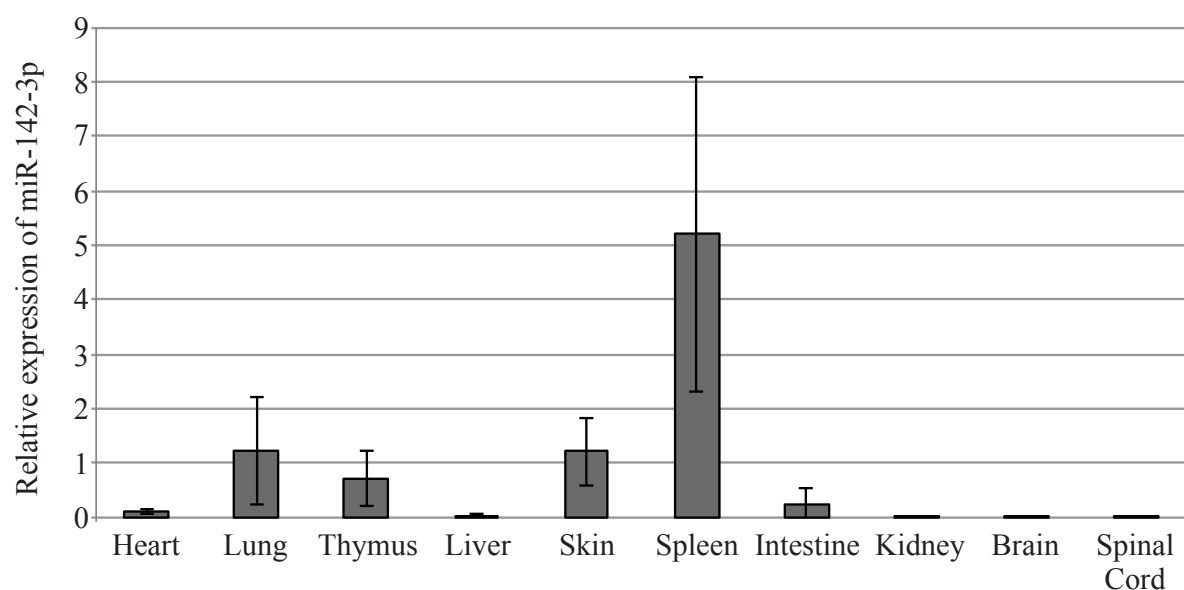


**Figure 2. Tissue distribution of miR-142-3p.** Expression of miR-142-3p was quantified by RT-qPCR in different tissues of naïve C57BL/6 mice. Histograms show mean and SD for a triplicate experiment.

The high expression of miR-142-3p in the spleen suggests that this miRNA is mostly expressed in hematopoietic cells.

To investigate the effect of miR-142-3p on rotavirus infection, we compared the replication of rotavirus in cells that over-expressed or not miR-142-3p. As rotavirus model, we used the Rotarix attenuated live vaccine.

Therefore, MA-104 cell clones that over-expressed miR-142-3p were obtained by transduction of a pGIPZ vector expressing miR-142-3p. To examine the biological activity of miR-142-3p, we generated a reporter plasmid carrying corresponding miRNA target sequences in the 3' UTR of the luciferase gene. These target sites correspond to either sequences fully complementary to miR-142-3p or target sequences identical to that present in the Rotarix genome. MA-104 cells expressing miR-142-3p were transfected with the reporter plasmids and luciferase assay was carried out 24h after transfection.

miR-142-3p expressed in these cells was active since it efficiently decreased *Renilla* luciferase expression from transfected reporter plasmid carrying the perfectly complementary target sequences (figure 3A). miR-142-3p also strongly reduced luciferase expression in cells expressing a reporter plasmid carrying the rotavirus target sequences. This suggests that miR-142-3p can bind the Rotarix target sequence.

Next, replication of Rotarix was assessed, by RT-qPCR, at different time points after infection of control cells expressing an empty pGIPZ vector and of cells expressing miR-142-3p. Expression of miR-142-3p did not restrict the replication of Rotarix (figure 3B).

In Conclusion, these data argue against a deleterious effect of miR-142-3p on the replication of a rotavirus having a target sequence in the NSP5/NSP6 coding region.

The viral protein NSP5 was reported to play an essential role in the viral replication cycle. siRNA-mediated knockdown of NSP5 expression affected viral protein synthesis, viral genome replication and infectious particles production [379].

Recently, the team of D. Trobaugh demonstrated that miR-142-3p limits Eastern equine encephalitis virus infection in myeloid cells by direct binding to viral RNA [345]. This virus replicates in the cytoplasm of the cell and the viral genome consists in a positive single stranded RNA. As EEEV, rotavirus performs its entire replication cycle in the cytoplasm but viral RNA synthesis and genome assembly take place in specific viral structures that segregate viral components from the surrounding environment. Viral mRNA production occurs inside double-layered particles following the release of the virus from the endosomes to the cytoplasm. Viral protein synthesis and genome replication are sequestered in viroplasms created by NSP5/NSP2 interaction, thus, protecting the assembly of newly formed viral particles. It is thus possible that viral mRNAs are not accessible to microRNA targeting. This hypothesis is however not supported by the successful inhibition of rotavirus replication observed by Campagna et al. after transfection of siRNAs targeting segment 11 [379].

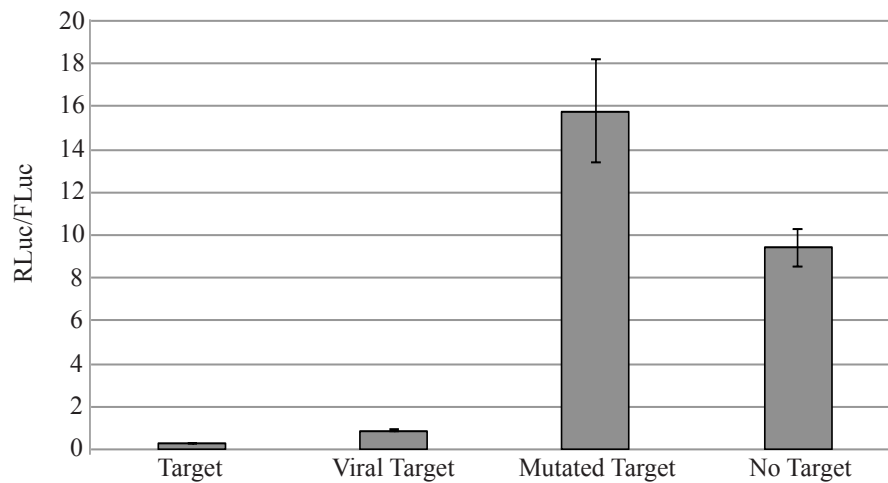
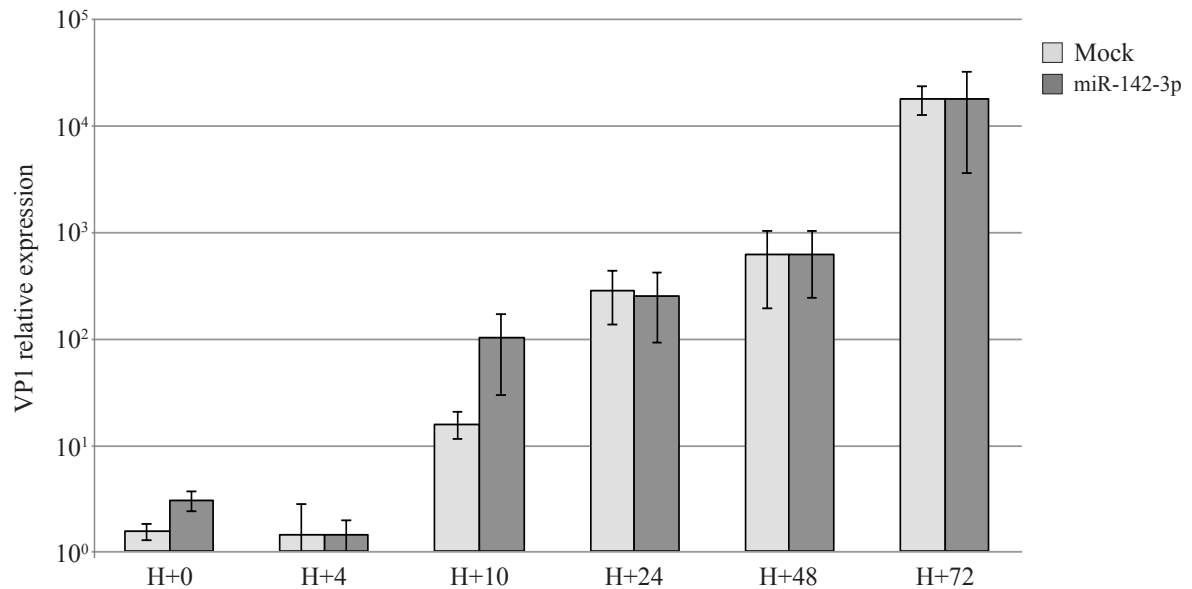
**A****B**

**Figure 3. Lack of influence of miR-142-3p on Rotarix replication.** (A) miR-142-3p expression was achieved by expressing an artificial primary microRNA (pri-miR) using the pGIPZ vector. Reporter vectors carry a *Renilla* luciferase gene with 0 target sequences (No target), WT target sequences (Target), mutated target sequences (Mutated Target) or target sequence corresponding to that of the Rotarix genome (Viral Target). MA-104 cell clones expressing miR-142-3p were transduced with the indicated reporter constructs (RLuc) and with pADC13 encoding firefly luciferase (FLuc). Luciferase activity was assessed 24h post transduction. (B) Replication of Rotarix was assessed through the quantification of the viral polymerase VP1 by RT-qPCR. Total RNA extraction was performed at 0h, 4h, 10h, 24h, 48h and 72h post-infection. Histograms show the mean and SD for a triplicate experiment.



### Section 3

#### **“Gvin1 contributes to cellular antiviral activity against Theiler’s virus, Vesicular stomatitis virus and Murid herpesvirus-4”**

**Aurélie De Cock**, Marguerite Kreit<sup>1</sup>, Laurent Gillet<sup>2</sup>, Thomas Michiels<sup>1</sup>

To be submitted

<sup>1</sup>Laboratory of Virology, de Duve Institute,  
Université Catholique de Louvain, Brussels, Belgium.

<sup>2</sup>Laboratory of Immunology – Vaccinology, Department of infectious and parasitic diseases  
Université de Liège, Belgium.

In our laboratory, Marguerite Kreit initiated this project by analysing why primary neurons treated with IFN- $\alpha/\beta$  remained very susceptible to viral infection. The hypothesis was that some interferon-stimulated genes that are protective against viral infection in fibroblasts might be less expressed in neurons. She observed that the *Gvin1* gene was very weakly expressed in IFN-treated primary neurons compared to IFN-treated primary fibroblasts. She also observed that Gvin1 knockdown increased TMEV replication.

This work showed that Gvin1 participates to cellular antiviral defenses against Theiler’s virus, vesicular stomatitis virus and Murid herpesvirus 4. Gvin1 also enhances transfection-mediated nucleic acids uptake and induces actin cytoskeleton remodelling. Our data strongly suggest that Gvin1 influences the endocytosis pathway.



## 1. Introduction

Over the past few years, interferon-inducible GTPases have emerged as potent anti-microbial proteins [289,290]. This protein family comprises at least 47 members in human and mouse, that can be classified into 4 subfamilies, Mx, IRGs, GBPs and VLIGs.

The Mx proteins were discovered in 1963 and were among the first studied interferon-stimulated genes (ISGs) [295]. These GTPases almost exclusively target viruses and depend on type I and type III interferon (IFN) stimulation. Mice express two Mx proteins, the nuclear Mx1 and the cytoplasmic Mx2, which target different viruses depending on their intracellular replication sites. Mx1 inhibits the infection by orthomyxoviruses, such as Influenza A virus, known to replicate in the cell nucleus, whereas Mx2 confers resistance to viruses that replicate in the cytoplasm [305-307]. In contrast, the two human Mx proteins are localized in the cytoplasm and target diverse types of viruses regardless to their replication site [310,311,313,317,318]. Nevertheless, human and mouse Mx proteins seem to share the same mechanism of action that consists in the sequestration and/or disruption of nucleocapsids [298,319].

The immunity-related GTPases (IRGs) are typically 47 kDa proteins that act against bacteria and parasites located in intracellular compartments [294]. After IFN stimulation, IRGs are abundantly expressed and accumulate around intracellular membranes of infected cells. The first evidence about the immune role of IRGs came from the observation that *Mycobacterium Tuberculosis*-containing phagosomes recruit Irgm1 to promote the fusion of this compartment with lysosomes in infected macrophages [380]. Thereafter, several IRGs were shown to target certain bacterial phagosomes and Chlamydial inclusion bodies in monocytes, macrophages and epithelial cells [381-384]. Numerous IRGs also disrupt vacuoles containing the *Toxoplasma gondii* parasite in IFN- $\gamma$  activated macrophages, fibroblasts and astrocytes [385,386]. Following IRGs recruitment, pathogen-containing vacuoles are targeted to lysosomal degradation through the interaction of IRGs with various partners, such as autophagic and SNARE adaptors [381,387,388].

The guanylate-binding proteins (GBPs) are split between cytoplasm and intracellular membranes to target compartment-enclosed pathogens as well as bacteria that have escaped their vacuole to replicate in the cytosol. These 65-73 kDa GTPases are induced by type I IFN but IFN- $\gamma$  is their most potent inducer [320]. Besides bacteria and protozoa, GBPs promote defences against viruses. Human GBP1 (hGBP1) and GBP3 inhibit Influenza A replication in IFN- $\gamma$ -stimulated lung epithelia [389]. Moreover, human GBP1 confers resistance to Vesicular stomatitis virus, Encephalomyocarditis virus and Hepatitis C virus in infected cells [328,329]. As other interferon-inducible GTPases, the hGBP1 carries the consensus sequences for GTP binding and hydrolysis named G1, G2, G3 motifs. However, this protein lacks the last canonical G4 motif that binds the guanine base [325]. This protein is also able to form oligomers required for efficient GTP hydrolysis [327]. Recently, hGBP1 has been shown to interact with filamentous actin, which induces actin remodelling in IFN- $\gamma$ -treated cells [335].

The last family of interferon-induced GTPases, named very large inducible GTPases (VLIGs), has been detected more recently in cDNA libraries of mouse embryonic fibroblasts and macrophages stimulated with type II IFN [293].

Gvin1 (also called VLIG1) is the prototypic protein of this family and is found in 68 different species, ranging from zebrafish to chimpanzee. The Mouse genome encodes six similar genes in addition to *Gvin1*, but only two of them are predicted to produce a protein : *GM4070* and *GM1966*. They show respectively 99% and 87% identity with *Gvin1* at the nucleotide level. In this paper, *Gvin1* and *GM4070*, which encode identical proteins, were collectively referred to as Gvin1. In human, the single gene homologous to *Gvin1* contains a frameshift in the open reading frame and is therefore considered as a pseudogene. *Gvin1* generates a 9 kb transcript encoding a 280 kDa protein that is the largest known GTPase in any species. The GTP-binding domain of this protein shares sequence similarities with other interferon-inducible GTPases, in particular with the hGBP1. Indeed, Gvin1 protein contains G1, G2 and G3 motifs but lacks the canonical G4 motif. In unstimulated mouse embryonic fibroblasts (MEFs), Gvin1 protein was reported to be located in the nucleus. Upon IFN- $\gamma$  treatment, Gvin1 is detected in the nucleus or in the cytoplasm of distinct mouse embryonic fibroblasts. Yet, no cellular function has been assigned to Gvin1.

We showed previously that IFN- $\beta$  treatment triggers a surprisingly low resistance of primary mouse neurons to viral infection (M. Kreit) [336]. A transcriptomic analysis revealed that 15 ISGs are very weakly induced or expressed in neurons as compared to fibroblasts, after IFN- $\beta$  stimulation. *Gvin1*, encoding the very large IFN-inducible GTPase 1, is among these genes. Its expression was not detected in primary neurons. The aim of this study was to analyse the putative antiviral function of Gvin1. We showed that Gvin1 knockdown increases susceptibility to diverse types of viruses, including Theiler's virus (TMEV), Vesicular stomatitis virus (VSV) and Murid herpesvirus 4 (MuHV-4). Gvin1 knockdown enhanced cellular uptake of nucleic acids and induced actin cytoskeleton remodelling.

## 2. Materials and Methods

### 2.1. Cell culture

L929 cells (ATCC), BALB/3T3, Neuro-2A (ECACC), mouse embryonic fibroblasts and J774.1 macrophages were maintained in Dulbecco's modified Eagle medium (Lonza) supplemented with 10% fetal calf serum (Sigma) and 100 U/ml of penicillin/streptomycin (Lonza).

### 2.2. Interferon

IFN-beta (IFN- $\beta$ ) was produced by transfection of pcDNA3-IFN- $\beta$  in 293T cells [390]. Cell supernatants containing up to 400 000 U/ml of IFN- $\beta$  were diluted in culture medium and used at 0.5 or 1 U/ml to treat L929 cells before infection.

### 2.3. Viruses

Theiler's murine encephalomyelitis viruses used in this study, KJ7 and KJ26, were GFP-expressing derivatives of the persistent DA1 and of the neurovirulent GDVII strain, respectively [336,349]. Both viruses contain capsids adapted to infect L929 cells efficiently [351]. These viruses were produced as described [352], by reverse genetics, from plasmids pKJ7 and pKJ26, carrying the full length viral cDNA.

The Vesicular stomatitis virus (Indiana strain) derivative expressing GFP was a gift from Martin Schwemmle (University of Freiburg, Germany).

The MuHV-4 derivative expressing the GFP gene obtained as a bacterial artificial chromosome was a gift from Laurent Gillet (University of Liège, Belgium) [391,392]. This virus was generated and purified as described previously [393]. All viruses were propagated and titrated on BHK-21 cells. Recombinant viruses used in this study are listed in Table 1.

#### 2.4. RNA interference and lentiviral vector production

Scramble shRNA was a gift of David Sabatini (Addgene plasmid #1864) [394]. The shRNA targeting the 3'UTR of Gvin1 transcript (TRCN0000246462) were selected from the RNAi library collection (Sigma) and cloned in pLKO.1 derivatives [355] where the puromycin resistance gene was replaced either by the mCherry coding sequence (pMK84) or by the geneticin resistance gene (pTM988), using *Bam*HI and *Kpn*I restriction sites [336]. shRNA sequences were cloned in both pMK84 and pTM988 by using *Age*I and *Eco*RI restriction sites introduced in the hybridized primers. Lentiviral particles were produced, as described [336], by transient transfection of 293T cells grown in 75 cm<sup>2</sup> Petri dishes by the calcium phosphate method. After L929 transduction, the efficiency of RNA silencing was assayed by measuring Gvin1 gene expression by RT-qPCR, in IFN- $\beta$ -stimulated cells (figure S1). Lentiviral vectors used in this study are presented in Table 1.

**Table 1.** Recombinant viruses, lentiviral vectors and reporter vectors used in this study

| Vector/virus <sup>1</sup> | Parental <sup>1</sup> | Characteristics                                                                      |
|---------------------------|-----------------------|--------------------------------------------------------------------------------------|
| (p)KJ7                    | TMEV (DA1)            | GFP-coding sequence replacing codons 5-67 of L protein                               |
| (p)KJ26                   | TMEV (GDVII)          | GFP-coding sequence replacing codons 1-67 of L protein                               |
| VSV-GFP                   | VSV                   | VSV carrying a GFP-coding sequence                                                   |
| MuHV-4-GFP                | MuHV-4                | MuHV-4 carrying a GFP-coding sequence                                                |
| (p)MK84                   | pLKO.1                | mCherry coding region replacing puro <sup>R</sup>                                    |
| (p)TM988                  | pLKO.1                | Geneticin resistance gene replacing puro <sup>R</sup>                                |
| (p)MK77                   | (p)MK84               | shRNA targeting Gvin1 3' UTR                                                         |
| (p)ADC131                 | (p)MK84               | Scramble shRNA targeting none of the known cellular mRNAs                            |
| (p)ADC118                 | (p)TM988              | shRNA targeting Gvin1 3' UTR                                                         |
| (p)ADC148                 | (p)TM988              | Scramble shRNA targeting none of the known cellular mRNAs                            |
| (p)ADC1                   | (p)TM897              | Lentiviral vector carrying P <sub>PGK</sub> - <i>Renilla</i> luciferase <sup>2</sup> |

<sup>1</sup> (p): names of plasmids that carry a viral genome start with a "p" while corresponding viruses lack the "p". For example, plasmid pKJ7 carries the genome of virus KJ7.

<sup>2</sup> P<sub>PGK</sub> = human phosphoglycerate kinase promoter.

#### 2.5. Reporter vectors and luciferase assays

As reporter vector, we used a lentiviral vector (pADC1) that expresses the *Renilla* luciferase gene derived from pGL4.78 plasmid (Promega), from the PGK promoter. Transfection experiments were performed in 24-well culture plates using TransIT-LT1 reagent (Mirus), according to manufacturer's protocol. For cell electroporation, confluent cells cultured in a 25 cm<sup>2</sup> Petri dish were detached with trypsin-EDTA, centrifuged at 1000 rpm during 5 min and washed with cold PBS. Centrifugation and washing steps were repeated twice.

The cells and 5 µg of plasmid DNA were mixed in a final volume of 750 µl cold PBS. The cells were then electroporated at 1500 V, 25 µF with a maximal resistance using a Gene Pulser Xcell electroporation system (BIORAD). Electroporated cells were then suspended in 10 ml of culture medium and equally seeded in 24 wells of a culture plate. Luciferase assays were carried out with the *Renilla* luciferase Reporter Assay System (Promega) and luminescence was measured with a GloMax 20/20 luminometer (Promega). Reporter vectors used in this study are listed in Table 1.

## 2.6. RNA extraction and quantitative RT-qPCR

To minimize the use of laboratory animals, RT-qPCR were performed on samples of mouse RNA prepared for other experiments. RNAs from organs of naïve C57BL/6 mice were obtained from Hermant et al. [349]. Samples from FVB/N mice electro-injected with IFN-expressing and control plasmids were obtained from Sommereyns et al. [233].

Total RNA preparations, reverse transcription (RT) and quantitative PCR (qPCR) reactions were performed as previously described [356]. qPCR standards consisted of 10-fold dilutions of quantified plasmid DNA carrying the sequence to be amplified : pTM793 (*Actb*), pMK73 (*Gvin1*) and pCS40 (*Oasl2*). These plasmids are derived from pCR4-TOPO (Invitrogen) in which corresponding PCR fragments were cloned. Real-time PCR primer sequences used are the following : TM421 (forward) AGA GGG AAA TCG TGC GTG AC and TM422 (reverse) CAA TAG TGA TGA CCT GGC CGT for *Actb*, TM638 (forward) GGA TGC CTG GGA GAG AAT CG and TM639 (reverse) TCG CCT GCT CTT CGA AAC TG for *Oasl2*, TM1530 (forward) CAC CAA TCC CAT TCA GAG CA and TM1531 (reverse) CTC GGT GGT CTG TTG TCT GC for *Gvin1*. TM1530 and TM1531 primers also detect *GM4070* expression.

## 2.7. Cell infection and flow cytometry

L929 cells were infected with 0.5 PFU/cell of KJ7 during 9 hours, 1 PFU/cell of KJ26 during 12 hours, 4 PFU/cell of VSV during 7 hours or 4 PFU/cell of MuHV-4 during 18 hours. After dissociation with trypsin-EDTA, cells were suspended in PBS containing 5% FCS and 0.5% of paraformaldehyde. Data were acquired with a LSR Fortessa flow cytometer (BD Bioscience) using the FACSDiva software and analysed using FlowJo 9.6.4. In cells transduced with mCherry-expressing vectors, viral replication (GFP fluorescence) was measured in the mCherry-positive-gated population. The rate of infection was defined as the percentage of GFP-positive cells. Viral replication was estimated by the median of GFP fluorescence intensity in the GFP-positive population. Infection efficiency was calculated by combining the rate of infection and viral replication (percentage of GFP-positive cells multiplied by median of GFP fluorescence of these cells in the mCherry-positive population).

## 2.8. Immunostaining and microscopy

L929 cells were fixed for 10 min with PBS containing 4% paraformaldehyde and then permeabilized for 5 min with PBS containing 0.1% of Triton X-100. For actin staining, cells were incubated with phalloidin-FITC (Sigma) for 1h, at a 1/100 dilution, in PBS. Finally, cells were washed thrice with PBS containing 0.01% of Tween and mounted with Mowiol for fluorescence microscopy. Spinning disk confocal microscope or DMIRB inverted microscope (Leica) equipped with an AxioCam MRm camera (Zeiss) were used to perform fluorescence microscopy.

## 2.9. Statistical analyses

Results are expressed as mean  $\pm$  standard deviations. Statistical significance was analysed using the two-tailed Mann-Whitney *U* test : \* p-value < 0.05; \*\* p-value < 0.01; \*\*\* p-value < 0.001.

## 3. Results

### 3.1. Tissue distribution of *Gvin1* expression and type I IFN induction

We analysed *Gvin1* mRNA expression in different mouse organs. In untreated mice, *Gvin1* was readily expressed in all the tested organs, but to a lesser extent in brain, spinal cord, ovary and thymus (figure 1A). Circulating IFN- $\alpha$  induced *Gvin1* expression between 2 and 4.5 fold (figure 1B).

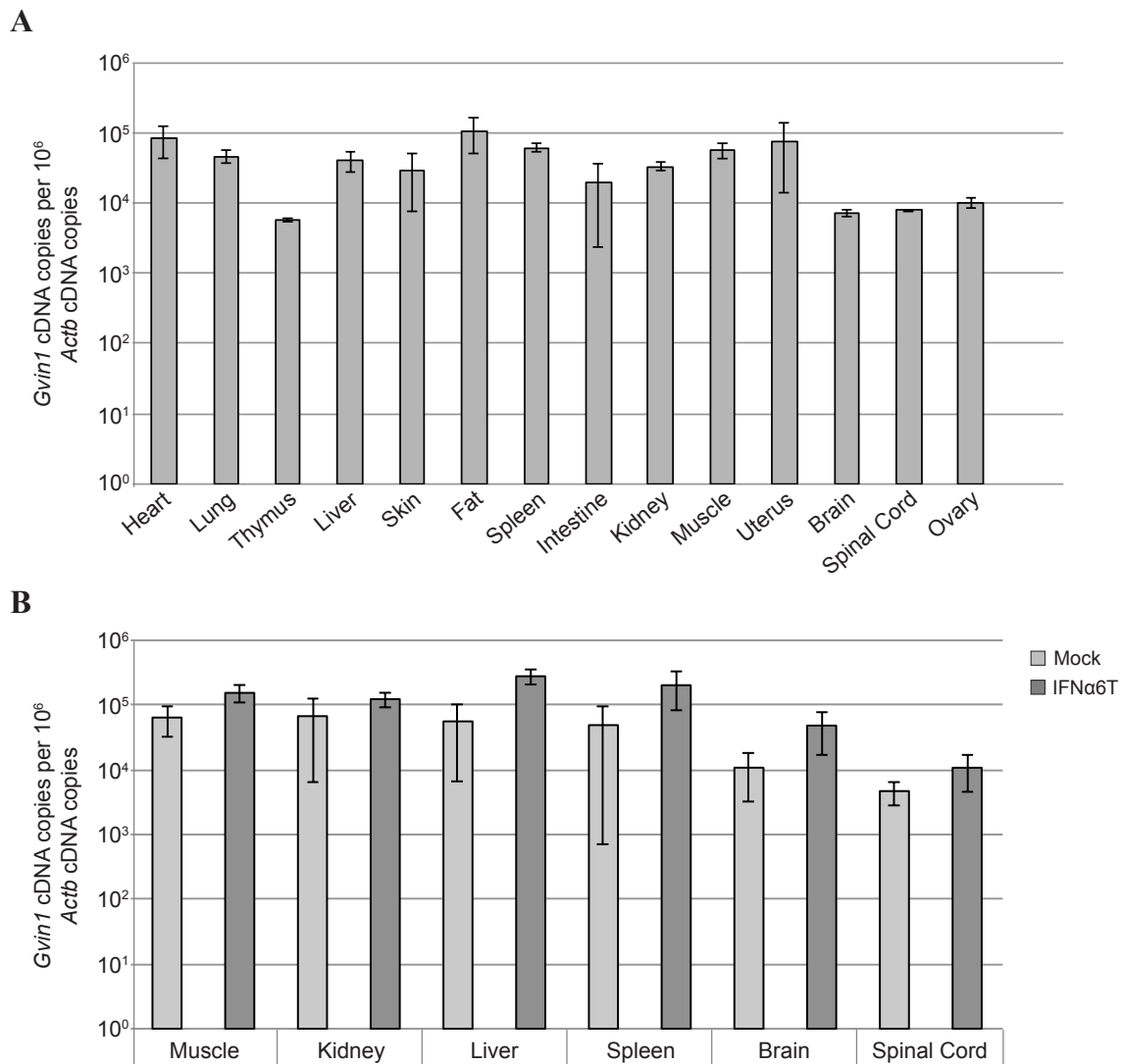


**Figure 1. *Gvin1* expression in mouse tissues.** *Gvin1* transcripts were quantified by RT-qPCR in tissues of naïve C57BL/6 mice (**A**, n=3) and in tissues of FVB/N mice electro-injected in the tibialis muscle with plasmid pcDNA3-IFN- $\alpha$ 6T (**B**, n=6). Control mice were electro-injected with an empty pcDNA3 plasmid. Histograms show mean and SD.

Basal *Gvin1* expression was detected in five murine cell lines and was more prominent in J774.1 macrophages (figure 2). After IFN- $\beta$  stimulation, *Gvin1* mRNA expression was highly enhanced in L929, BALB/3T3, mouse embryonic fibroblasts (MEFs) and J774.1 macrophages. In contrast, IFN- $\beta$  treatment did not induce *Gvin1* expression in Neuro2A although this cell line strongly responded to interferon stimulation, as shown by *Oas/2* mRNA up-regulation (figure 2). *GM1966* expression was detected in none of the five tested cell lines (not shown).

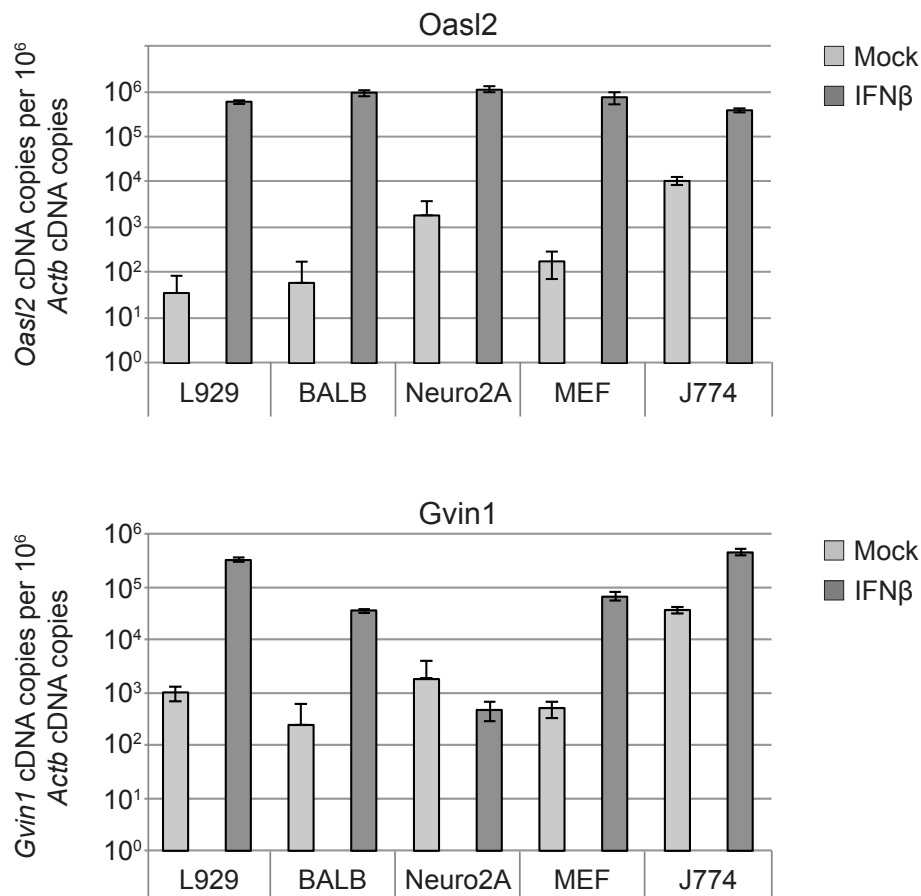


**Figure 2. *Gvin1* expression in murine cell lines.** *Oas/2* and *Gvin1* cDNAs were quantified by RT-qPCR in L929, BALB/3T3, Neuro-2A, MEFs and J774.1 macrophages. 24h before RNA extraction, cells were mock treated or treated with 100 unit/ml of IFN- $\beta$ . Histograms show mean and SD for a triplicate experiment.

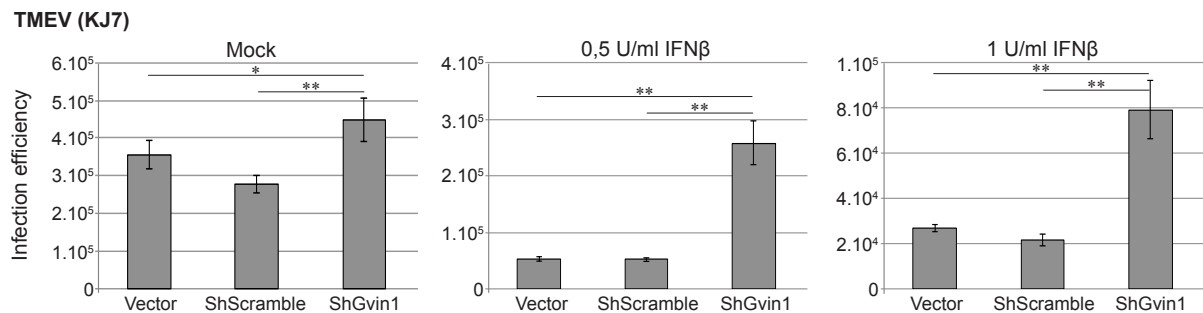
### 3.2. *Gvin1* knockdown promotes TMEV, VSV and MuHV-4 infection

To assess the antiviral activity of *Gvin1*, we employed a shRNA-based knockdown approach. Indeed, its high basal level in murine cell lines and the large size of its open reading frame rendered *Gvin1* overexpression difficult to achieve.

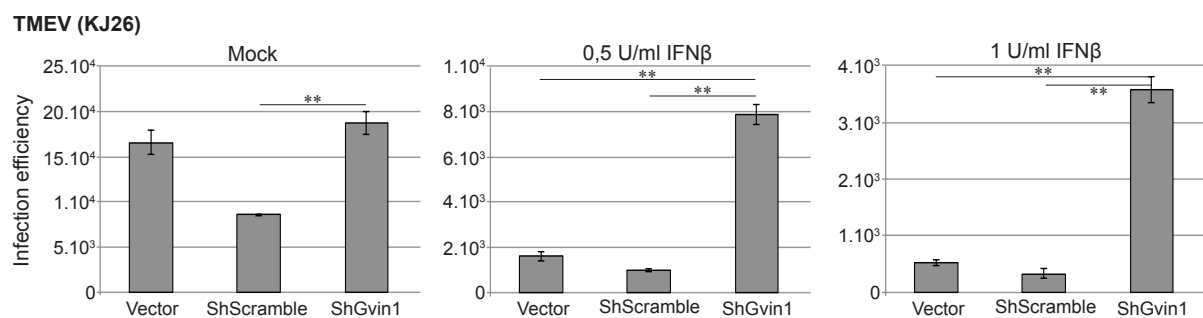
Thus, we constructed lentiviral vectors, derived from pLKO.1, co-expressing mCherry and either a shRNA targeting the 3' UTR of *Gvin1* (pMK77) or a scramble shRNA targeting none of the known cellular mRNAs (pADC131). Following the transduction of L929 with these shRNA constructs, cells were mock-treated or treated with 0.5 or 1 U/ml of IFN- $\beta$  during 24h and then infected with GFP-expressing viruses. The effect of *Gvin1* knockdown on viral replication was examined by flow cytometry, by following viral replication (GFP fluorescence)

in transduced cells expressing the shRNA (mCherry fluorescence). Viruses were KJ7 and KJ26 derivatives of persistent and neurovirulent Theiler's virus (TMEV) strains respectively, Vesicular stomatitis virus (VSV) and Murid herpesvirus 4 (MuHV-4) (figure 3).

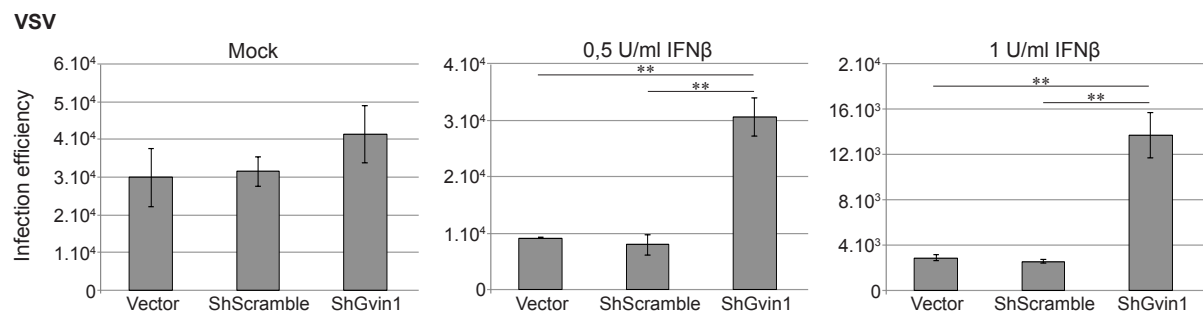
**A**



**B**



**C**



**D**

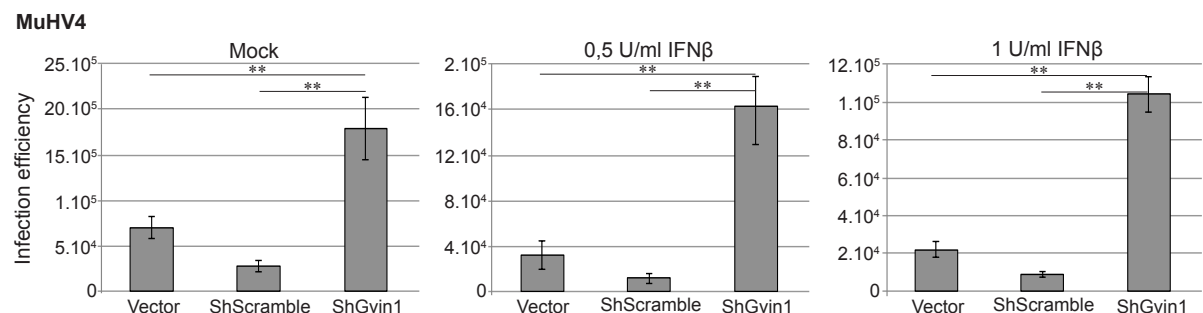


**Figure 3. Down-regulation of Gvin1 expression increases TMEV, VSV and MuHV-4 infection efficiency.** Infection efficiency was determined by FACS in L929 cells that were transduced with the empty pMK84 (Vector) or with the vector expressing either the scramble shRNA (shScramble) or the shRNA targeting *Gvin1* (shGvin1). Cells were treated with low doses of IFN-β (mock, 0.5 or 1 unit/ml) 24h prior to infection with KJ7 (**A**), KJ26 (**B**), VSV (**C**) and MuHV-4 (**D**). Histograms show mean and SD for a typical experiment (out of 3) performed in triplicate.

Gvin1 knockdown in L929 cells that were not treated with IFN had some impact on KJ7 and KJ26 infection efficiency (figure 3A & 3B), and led to a clearer increase in MuHV-4 infection efficiency (figure 3D). In cells stimulated with 0.5 or 1 U/ml of IFN- $\beta$ , knockdown of Gvin1 expression significantly increased the percentage of infected cells (figure S2) and the infection efficiency for all the tested viruses (figure 3A, 3B, 3C & 3D).

Thus, Gvin1 exerts antiviral activity against representatives of positive- and negative-stranded RNA viruses and DNA viruses. Interestingly, basal levels of Gvin1 expression are sufficient to promote some extents of antiviral activity.

### 3.3. *Gvin1* knockdown enhances the cellular uptake of nucleic acids.

Next, we attempted to examine a potential effect of Gvin1 on the transduction of a lentiviral vector. To this end, we transduced ADC1, a lentivirus expressing the firefly luciferase, into L929 cells that expressed the Gvin1 shRNA or an empty vector. As a control of transduction, we transfected these cells with pADC1 plasmid DNA. Unexpectedly, the results emphasized an improvement of transfection efficiency in cells expressing the shRNA targeting Gvin1, rather than an enhancement of virus transduction (figure 4). Indeed, Gvin1 knockdown slightly decreased lentiviral transduction efficiency but clearly increased plasmid transfection.

Therefore, we postulated that Gvin1 might act on nucleic acids uptake, and thus likely on endocytosis.

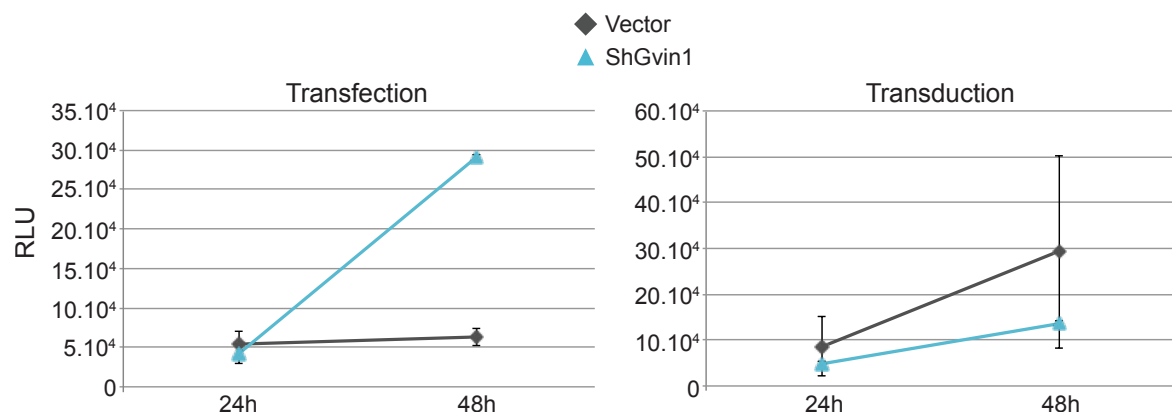


**Figure 4. Down-regulation of Gvin1 expression enhanced transfection efficiency.** L929 cells expressing the empty pMK84 (Vector) or the vector expressing the shRNA targeting *Gvin1* (shGvin1) were transfected with pADC1 or transduced with the corresponding lentiviral vector ADC1. Luciferase activity (Relative Luciferase Units : RLU) was assessed 24h and 48h after transfection or transduction. Graphics show mean and SD for a representative triplicate experiment.

Next, we compared DNA transfection and DNA electroporation (which is expected to bypass the endocytosis step) of a non-replicative reporter vector encoding *Renilla* luciferase. L929 cells expressing shGvin1 (pADC118), shScramble (pADC148) or an empty vector (pTM988) were transfected or electroporated with this reporter vector and luciferase activity was measured at five different time points. As shown in figure 5A, the luciferase signal raised identically until 24h in all three transfected cell types. 48h after transfection, luciferase signal still increased in cells expressing the shRNA targeting Gvin1.

On the contrary, the luciferase signal slightly dropped in cells expressing a scramble shRNA or an empty vector (figure 5A). Thus, knockdown of *Gvin1* increased the luciferase signal from 48h post-transfection. In electroporated cells, the kinetics of luciferase activity was comparable between cells expressing the shRNA targeting *Gvin1*, a scramble shRNA or an empty vector (figure 5B).

Therefore, knockdown of *Gvin1* extended the uptake of nucleic acids after transfection, but not after electroporation.

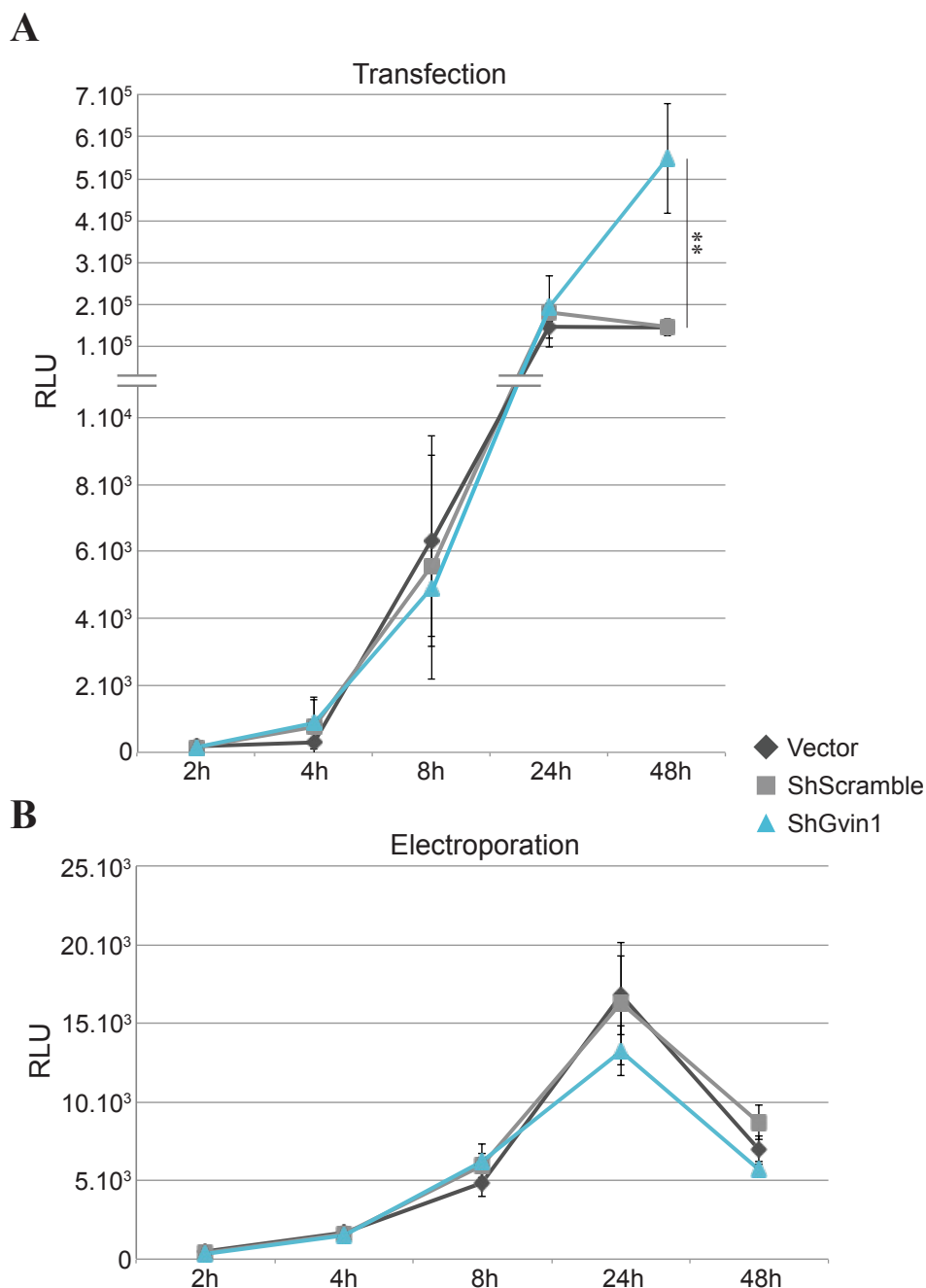


**Figure 5. Down-regulation of *Gvin1* expression increases cellular uptake of transfected nucleic acids.** L929 cells were transduced with the empty pTM988 or with the vector expressing either the shRNA scramble (shScramble) or the shRNA targeting *Gvin1* (shGvin1). These cells were then transfected (**A**) or electroporated (**B**) with pADC1 plasmid expressing *Renilla* luciferase. Luciferase activity (Relative Luciferase Units : RLU) was assessed 2h, 4h, 8h, 24 and 48h after transfection or electroporation. Graphics show mean and SD for a typical experiment (out of 3) performed in triplicate.

### 3.4. *Gvin1* knockdown disrupts actin cytoskeleton

Given the potential involvement of *Gvin1* in endocytosis, we examined the effect of *Gvin1* knockdown on the actin cytoskeleton. To this end, cells expressing sh*Gvin1*, shScramble or an empty vector were fixed with paraformaldehyde and stained with fluorescein-conjugated phalloidin. As shown in figure 6A, knockdown of *Gvin1* induced actin aggregation. The percentage of cells containing actin granules was significantly higher in sh*Gvin1* expressing cells than in cells expressing a scramble shRNA or an empty vector (figure 6B).

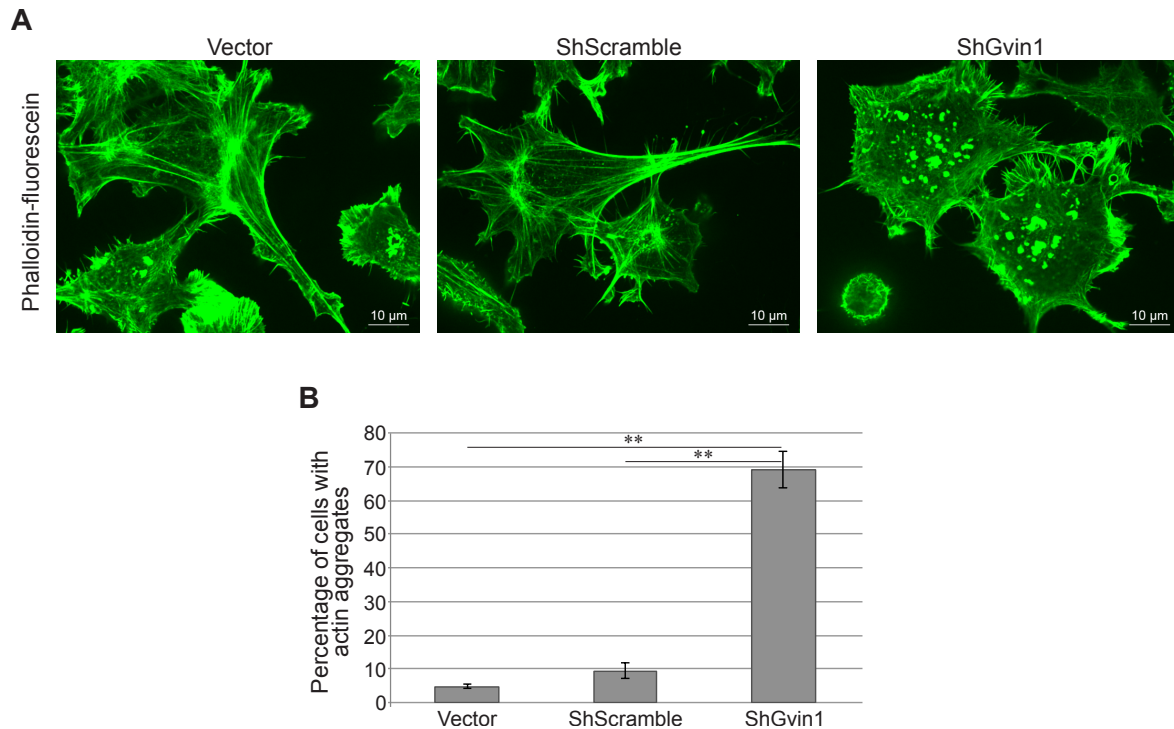


**Figure 6. Down-regulation of *Gvin1* expression disrupts actin cytoskeleton.** L929 cells were transduced with the empty pTM988 or with the vector expressing either the shRNA scramble or the shRNA targeting *Gvin1*, prior to immunolabeling and analysis by confocal microscopy. **(A)** Illustration of immunolabeling with phalloidin coupled to fluorescein. **(B)** Graphs show the percentage of L929 cells displaying actin aggregates. Histograms show mean and SD for a typical experiment (out of 2) performed in triplicate.

## 4. Discussion

*Gvin1* possesses the characteristics of other proteins of the interferon-inducible GTPase family [293]. It is mainly induced by type II interferon and preferentially binds GDP. All IFN-inducible proteins family have been implicated in anti-microbial immunity. However, no cellular function has been assigned to *Gvin1* until now.

In this work, we identified *Gvin1* as an antiviral ISG that is expressed in many mouse tissues. In the central nervous system, the basal level of *Gvin1* was lower, probably because *Gvin1* expression was very low in neurons. Expression of this gene was up-regulated by type I interferon in various cell lines, but not in the Neuro2A neuroblastoma cell line.

Down-regulation of Gvin1 expression increased cellular susceptibility to Theiler's virus, Vesicular stomatitis virus and Murid herpesvirus 4, suggesting that this protein displays a broad spectrum antiviral activity. Indeed, viruses used in this study are representative of different viral families that differ in their way to replicate into their cellular host. TMEV is a positive-stranded RNA virus whereas VSV possesses a negative-stranded RNA genome. Both replicate in the cytoplasm of the cell. MuHV-4 has a DNA genome and replicates in the nucleus. Thus, Gvin1 should act on a common step of the replication cycle of these diverse viruses.

Our data showed that Gvin1 also influences the uptake of nucleic acids and triggers a rearrangement of the actin cytoskeleton. While we performed this work, an article revealed that in human, who lack a functional *Gvin1* gene, hGBP1 induces actin remodelling by interacting with actin fibers and regulatory cytoskeletal proteins [335]. Since viruses used in this study enter cells via endocytosis (figure 7), we hypothesized that Gvin1 might impact a step of the endocytic pathway.

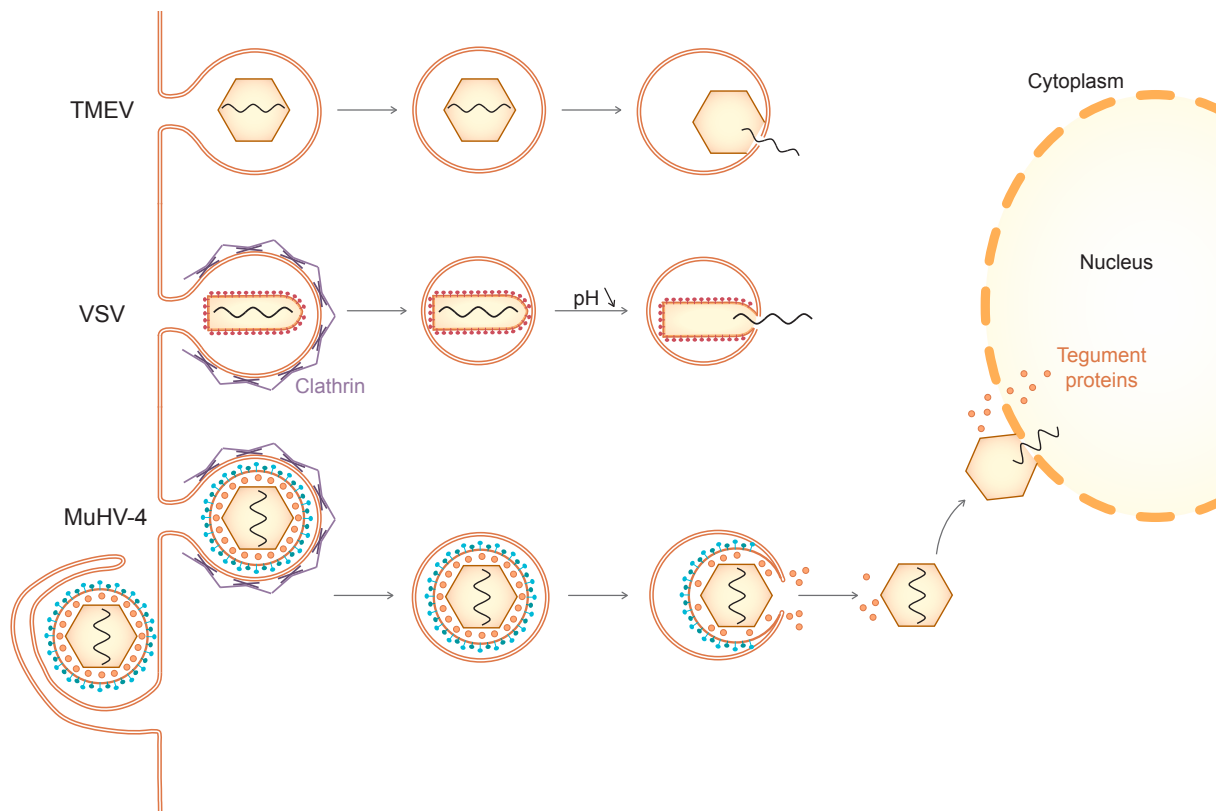


**Figure 7. Cell entry of TMEV, VSV and MuHV-4.**

Persistent and neurovirulent Theiler's virus strains bind sialic acid and heparan sulfate respectively, which are present at the cell surface. Following endocytosis, TMEV capsid undergoes a conformational change that induces the release of the viral genome into the cytoplasm. LDLR serves as the cellular receptor for Vesicular stomatitis virus [395]. After receptor attachment, VSV enters cells through clathrin-coated vesicles that fuse with the early endosomes [396]. The acidic environment of the endosomes triggers a conformational change of the viral glycoprotein, which induces the fusion of the viral envelope with the endosomal membrane and the subsequent release of the genome into the cytoplasm. MuHV-4 entry is likely similar to that of KSHV, which enters cell via clathrin-mediated endocytosis or via macropinocytosis, depending on the cell type [397]. Following fusion of the viral envelope with the endocytic vesicles, viral capsid and tegument proteins are released into the cytoplasm and migrate toward the nucleus. The viral DNA is delivered into the nucleus through the nuclear pores.

Currently, we are testing the influence of Gvin1 knockdown on the kinetics of MuHV-4 entry. Therefore, MuHV-4 virions were bound to L929 cells expressing either the scramble shRNA or the shRNA targeting Gvin1 at 4°C during 2h. Cells were then washed with ice-cold PBS to remove the unbound virions and either fixed or incubated at 37°C to allow virion endocytosis. After 1 or 2 hours of incubation, cells were fixed and virion entry was visualised by immunofluorescence. First, cells were stained for the ORF75c tegument protein, allowing us to follow the course of MuHV-4 endocytosis. After incubation at 4°C, ORF75c localizes around the plasma membrane; after 1h at 37°C, it is both in the cytoplasm and in the nucleus; after 2h at 37°C, ORF75c locates almost entirely in the nucleus [398]. We observed that the intensity of ORF75c staining increased in the nucleus of shRNA Gvin1 expressing cells (figure 7). This suggested that Gvin1 knockdown enhances the endocytosis of MuHV-4. However, we were unable to reproduce the obtained results.

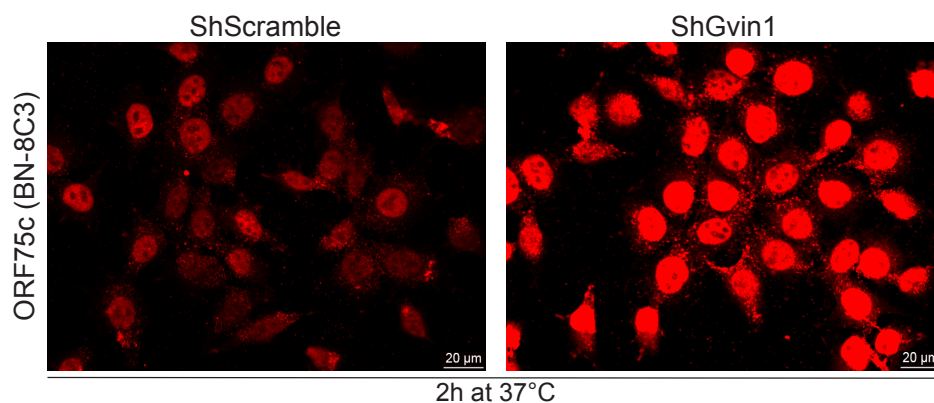


**Figure 8. Gvin1 knockdown increases the intensity of ORF75c staining.** MuHV-4 virions (3 PFU per cell) were bound to L929 cells expressing either the scramble shRNA or the Gvin1 shRNA (2h at 4°C). Cells were then washed and further incubated to allow virion endocytosis (2h at 37°C). Cells were fixed for 10 min with PBS containing 2% paraformaldehyde. Cells were then permeabilized for 5 min with PBS containing 0.1% of Triton X-100. Blocking occurred for 1h at room temperature in PBS containing 10% of filtrated FCS. Cells were stained for the ORF75c tegument protein with mAb BN-8C3 diluted at 1/3. AlexaFluor-568 conjugated secondary antibody was incubated for 1h at a 1/400 dilution. Finally, cells were mounted with Mowiol for fluorescence microscopy. Antibody BN-8C3 was a kind gift of Laurent Gillet.

Secondly, cells were stained for the ORF25 capsid protein that migrates to the nuclear periphery after incubation for 2h at 37°C [398]. We observed an increased accumulation of ORF25-stained dots in cells expressing the shRNA targeting Gvin1 as compared with cells expressing the scramble shRNA (figure 8). These data also suggested that Gvin1 knockdown improves MuHV-4 endocytosis. To pursue this work, we plan to stain the nuclear envelope by using an anti-lamin antibody and to perform z-stack images in order to visualize virions located in the nuclear periphery. Next, we plan to compare, through a computational analysis, the number of ORF25-stained dots in the cytoplasm of cells expressing either the Gvin1 shRNA or the scramble shRNA.

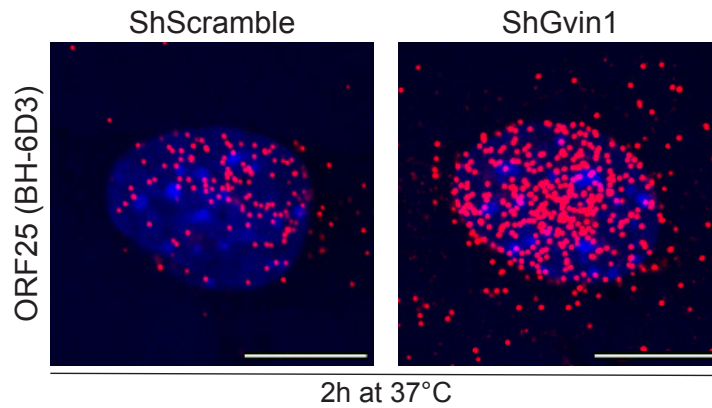


**Figure 9. Gvin1 knockdown increases the accumulation of ORF25-stained dot.** MuHV-4 virions (3 PFU per cell) were bound to L929 cells expressing either the scramble shRNA or the Gvin1 shRNA (2h at 4°C). Cells were then washed and further incubated to allow virion endocytosis (2h at 37°C). Immunolabeling protocol was as in figure 7. Cells were stained for the ORF25 capsid protein with mAb BH-6D3 diluted at 1/30 (red). AlexaFluor-568 conjugated secondary antibody was incubated for 1h at a 1/400 dilution (scale bar : 10  $\mu$ m). For nuclear staining, cells were incubated in Hoechst 33258 (blue). Finally, cells were mounted with Mowiol for fluorescence microscopy. Antibody BH-6D3 was a kind gift of Laurent Gillet.

Importantly, our results are based on a single shRNA construct and, up to now, we cannot rule out some off-target effects. We tried to validate our conclusions by using an alternative method to confirm the antiviral activity of Gvin1. To this end, we transfected a plasmid encoding the open reading frame of Gvin1 in our cells expressing the shRNA targeting Gvin1 3' untranslated region. Preliminary results showed that Gvin1 overexpression restricted KJ26 infection. Unfortunately, we were unable to reproduce this experiment. In a second trial, we attempted to inactivate Gvin1 expression by using CRISPR/Cas9 system. However, no cell clone could be obtained that had 4 mutated alleles (2 for *Gvin1* and 2 for *GM4070*), suggesting that the complete lack of Gvin1 compromises cell growth. Currently, we are testing shRNAs targeting different regions of the Gvin1 transcript to confirm our data.

In conclusion, our work suggests that Gvin1 confers resistance to various viruses by interfering with viral endocytosis.

## Supplementary figures

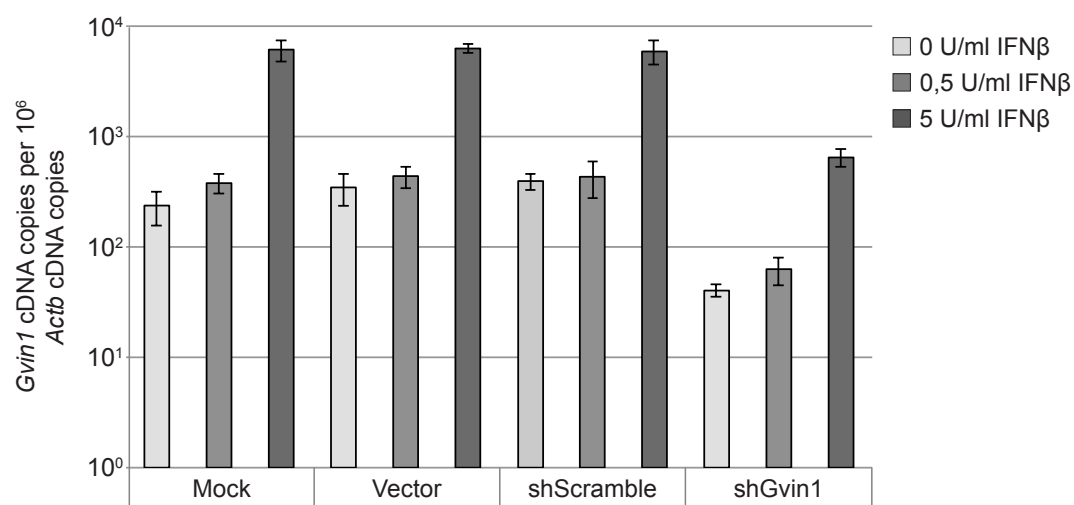
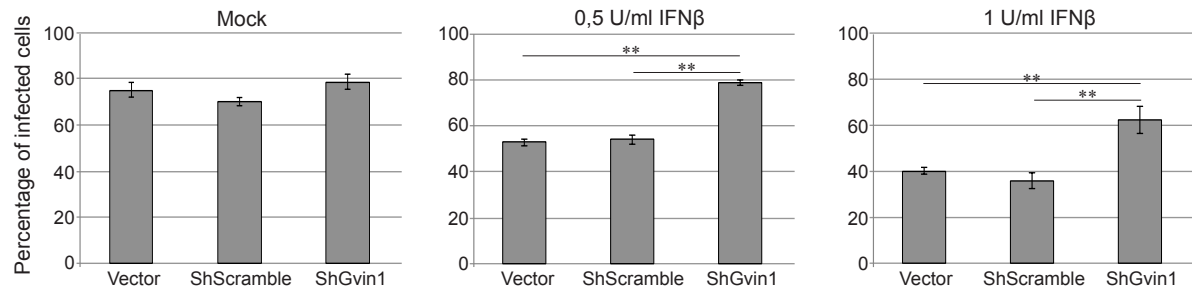


**Figure S1. *Gvin1* expression in IFN- $\beta$  treated L929 cells.** *Gvin1* cDNAs were quantified by RT-qPCR in L929 transduced with the empty pTM988 or with the vector expressing either the scramble shRNA (shScramble) or the shRNA targeting *Gvin1* (shGvin1). 24h before RNA extraction, cells were mock treated or treated with 0,5 unit/ml or 5 unit/ml of IFN- $\beta$ . Histograms show mean and SD for a triplicate experiment.

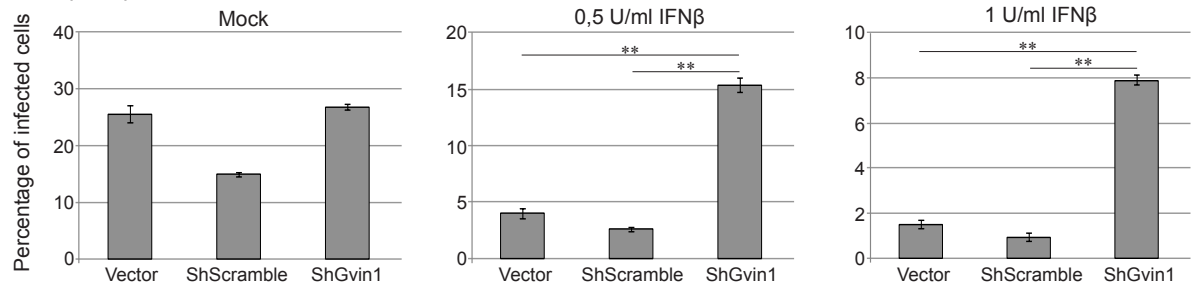
**A**

**TMEV (KJ7)**



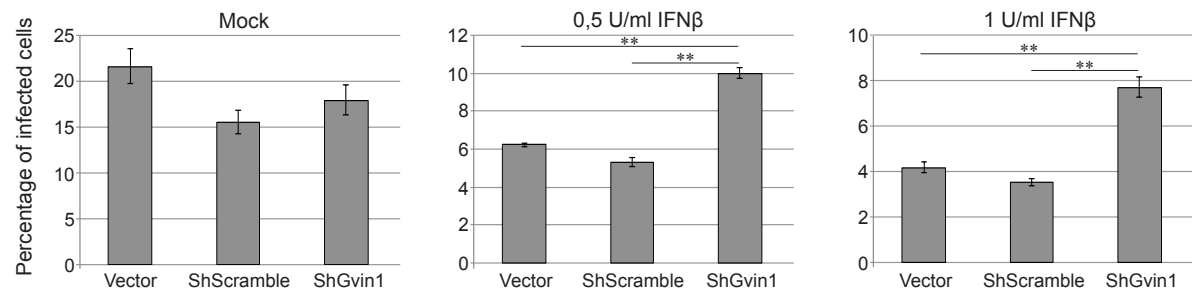
**B**

**TMEV (KJ26)**



**C**

**VSV**



**D**

**MuHV4**

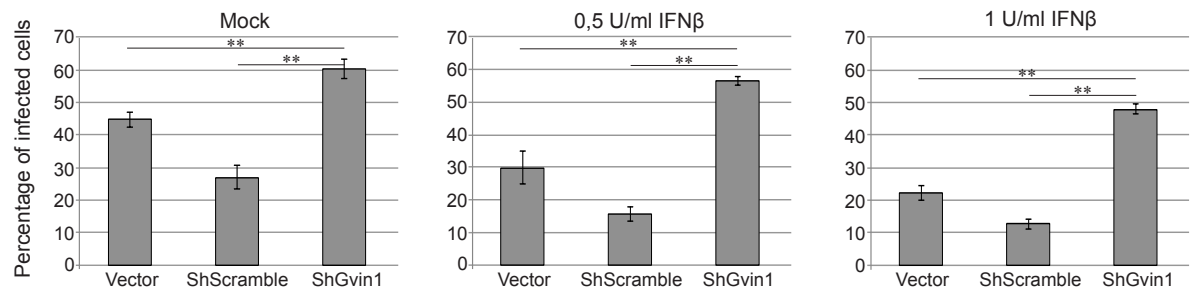


**Figure S2. Down-regulation of *Gvin1* expression increases the percentage of cells infected with TMEV, VSV and MuHV-4.** Percentage of infected cells was determined by FACS in L929 cells that were transduced with the empty pMK84 (Vector) or with the vector expressing either the scramble shRNA (shScramble) or the shRNA targeting *Gvin1* (shGvin1). Cells were treated with low doses of IFN- $\beta$  (mock, 0.5 or 1 unit/ml) 24h prior to infection with KJ7 (A), KJ26 (B), VSV (C) and MuHV-4 (D). Histograms show mean and SD for a typical experiment (out of 3) performed in triplicate.



## Discussion

During their replication cycle, viruses generate perfect dsRNAs, stem-loop structured RNAs or convergent overlapping transcripts that can serve as Dicer substrates. In plants, nematodes and arthropods, this leads to the production of virus-derived siRNAs, which directly bind viral RNA species and suppress virus accumulation. Therefore, RNAi is an essential antiviral immune response in these organisms. In support of an antiviral role of RNAi, the majority of viruses that infect these organisms have acquired viral suppressors of RNAi that are required for an effective viral spreading through the target host. Whether a similar antiviral function for RNAi exists in mammals remains a debated issue.

In this work, we addressed the question of how much cellular microRNAs could influence the replication of two mammalian RNA viruses, Theiler's virus and Human rotavirus. In parallel, we analysed the antiviral activity of an interferon-inducible GTPase, called Gvin1, by using RNA interference as an experimental tool.

In the following chapters, I will discuss our findings in the context of the current literature.

## 1. Sensitivity of TMEV to cellular microRNAs

In this study, we assessed the influence of cellular microRNAs on Theiler's virus (TMEV) replication. First, we examined the genome of TMEV for the presence of under-represented microRNA target sequences and tested whether these sequences would affect TMEV replication in cells overexpressing the corresponding microRNAs. However, introduction of 3 copies of the most under-represented miR-770-3p target sequence in TMEV did not affect replication of the recombinant virus in cells expressing the corresponding shRNA.

Secondly, we developed a strategy to compare virus replication in *Dicer*-positive and *Dicer*-negative mouse embryonic fibroblast populations. Short-term *Dicer* inactivation led to a significant decrease in microRNA abundance and strongly increased replication of Vesicular stomatitis virus (VSV), which was used as a miRNA-sensitive control virus. In contrast, microRNAs depletion did not increase TMEV replication. In conclusion, RNA interference appears to have little influence on TMEV replication.

### 1.1. Is TMEV able to evolve in order to suppress microRNA target sequences ?

In the first part of this project, we assessed the possibility that selection against specific miRNA target sites occurred during evolution of TMEV genome in to escape miRNA-mediated replication inhibition. Our analysis does not allow to exclude this hypothesis due to the small number of under-represented target sequences examined. Among 591 murine miRNA target sequences examined, only 7 were under-represented in the coding sequence of TMEV and as compared to randomly generated nucleotide sequences encoding an identical polyprotein. It would be worth to extend the functional analysis performed for miR-770-3p to the six additional under-represented miRNA target sequences.

As other RNA viruses, TMEV possesses a high mutation rates because its RNA polymerase lacks proofreading activity. As a consequence, spontaneous mutations appear frequently during viral replication, allowing a rapid evolution of the virus. Therefore, TMEV could easily avoid the binding of host miRNAs. This assumption is supported by several *in vitro* studies showing that RNA viruses readily mutate and escape inhibition by exogenous siRNAs [359,399,400]. It is thus surprising that our above results failed to show any influence of miR-770-3p.

Despite the expression of an error-prone RNA polymerase, certain viral genomic RNAs contain conserved binding sites for cellular miRNAs. Thus, these viral genomes could have evolved to acquire miRNA target sequences to modulate their replication or their cellular tropism [344]. The best known example is the binding of the liver-specific miR-122 to the 5' UTR of hepatitis C virus (HCV) genomic RNA that increases viral RNA stability and promotes translation and replication [401]. The interaction between miR-122 and HCV is critical for the viral life cycle. Another example of virus that harnesses host miRNAs to modulate its life cycle was reported by D. Trobaugh and co-workers [345]. They have reported that the hematopoietic cell-specific miR-142-3p binds three highly conserved sites in the 3' UTR of eastern equine encephalitis virus (EEEV) genomic RNA. This interaction leads to the restriction of viral replication in myeloid-lineage cells, which increases disease severity because it limits myeloid cells infection and the consequent innate immunity induction.

Predictions made in the case of TMEV identified the miR-709 target sequence as a sequence that was more abundant in the genome of TMEV (3 sites) than in random sequences coding the same polyprotein (not shown). Nevertheless, mutation of these target sequences did not significantly affect TMEV replication in cells expressing miR-709. Thus, it is unclear whether the genome of TMEV evolved to eliminate or acquire specific miRNA target sequences.

A possible way to pursue this part of the project and to determine whether miRNA impact on the evolution of the TMEV genome could be to passage the virus repeatedly in *Dicer*-negative and *Dicer*-positive cells and to sequence (NGS) the viral genome for the possible (dis)appearance of miRNA binding sites.

## 1.2. Why is TMEV insensitive to cellular microRNAs ?

In the second part of this project, we inactivated *Dicer* to test the global impact of cellular miRNAs on TMEV replication. *Dicer* inactivation led to a significant decrease of miRNA amount and activity. It is worth noting that miRNA levels continued to decrease up to 8 days after CRE-mediated *Dicer* gene inactivation, suggesting that miRNAs are very stable in the cells. Replication of VSV was strongly enhanced while TMEV replication was not affected by *Dicer* inactivation. Four hypotheses could explain the lack of sensitivity of TMEV to cellular miRNAs : (1) the influence of miRNAs differs depending on the cell types; (2) TMEV rapidly adapts by acquiring escape mutations in its genome; (3) viral RNAs are inaccessible to cellular miRNAs during virus replication; (4) TMEV expresses a suppressor protein that inhibits miRNA activity.

(1) Our experiments showing the absence of miRNA influence on TMEV replication were conducted in mouse embryonic fibroblasts. In infected mouse, TMEV replicates first in neurons and then persists in cells of the central nervous system. Thus, TMEV could be targeted by certain miRNAs that are expressed in these specific cells but not in mouse embryonic fibroblasts. To investigate this hypothesis, *Dicer* inactivation experiments could be repeated in other primary cells derived from mouse *Dicer*<sup>flax/null</sup>, especially in neurons, macrophages and astrocytes.

(2) TMEV genome could mutate very quickly under the selective pressure exerted by cellular miRNAs. To test this, we could use the plaque assay technique to compare viral replication in *Dicer*-positive and *Dicer*-negative cells. In this experiment, an increase of plaques number in *Dicer*-negative cells would indicate a limitation of the initial infection by miRNAs. An increase of plaques size in *Dicer*-negative cells would indicate a restriction of viral replication by cellular miRNAs.

(3) We could also examine the availability of viral RNAs to cellular miRNAs during TMEV replication. Indeed, virus RNAs could be inaccessible either because they are protected by certain viral proteins, or because their cellular localization prevents the RISC complex to access viral target sequences. Therefore, we could assess the accessibility of viral RNAs by expressing perfectly complementary siRNAs to different regions of the viral genome in infected cells.

(4) Finally, the TMEV genome could encode a protein that inhibits the activity of cellular miRNAs. To test this hypothesis, we could express a reporter gene carrying miRNA target sequences in infected cells. The expression of a suppressor protein by TMEV should prevent the binding of the corresponding miRNA on its target sequences and the subsequent degradation of the reporter transcript.

## 2. Sensitivity of human rotavirus to miR-142-3p

In this work, we assessed the effect of a miR-142-3p target sequence present in the eleventh segment of the human rotavirus genome. This segment encodes the non-structural protein NSP5. Our data indicated that this microRNA does not inhibit, nor enhance the replication of an attenuated human rotavirus, although this microRNA binds the target sequence located in the viral genome.

### 2.1. Why does miR-142-3p not inhibit human rotavirus replication in vitro ?

We initially thought that viral mRNA might not be accessible to the targeting of cellular miRNAs. Indeed, viral RNA synthesis and virion assembly take place in specific viral structures, called viroplasms, which segregate viral components from the surrounding environment. However, this hypothesis is not supported by the successful inhibition of rotavirus production observed by Campagna et al. after transfection of siRNAs targeting segment 11 of this virus [379].

During the replication cycle, the rotavirus RNA exists under different forms. The viral genome is composed of 11 dsRNA segments, each encoding a single protein. Following uncoating and cytoplasm penetration of the double-layered particles, dsRNA segments are transcribed by the viral polymerase, as positive-sense RNAs, which serve either as mRNAs for the synthesis of viral proteins or as templates for synthesis of negative-sense RNAs during genome replication. Silvestri and co-workers showed that viral mRNAs are susceptible to siRNAs targeting, but that positive-sense RNAs serving as templates for dsRNA synthesis are unlikely to be targeted [373]. The work of Campagna et al. revealed that siRNA targeting NSP5 mRNA strongly decreased NSP5 protein levels. The impairment of NSP5 expression inhibited viroplasms formation, genome replication, virion assembly and the synthesis of others viral proteins such as the polymerase VP1 [379]. These data indicate that NSP5 plays an essential function during virus replication. In our work, we examined the effect of miR-142-3p on rotavirus replication by quantifying viral RNA levels. The influence of NSP5 on replication described above may too weak to observe its impact by RT-qPCR. It would be interesting to look at NSP5 protein levels by western blotting. However, no anti-NSP5 antibodies were available when we performed the experiments. As NSP5 associates with NSP2 to form viroplasms, one could also visualize viroplasm formation by NSP2 immunolabeling in miR-142-3p expressing MA-104 cells.

Rotavirus mRNAs are 5'-capped but are not poly(A) tailed. Instead, they possess a consensus sequence (GACC) at their 3' end, which is bound by the viral non-structural protein NSP3 [402]. This protein also binds to eIF4G leading to the impairment of cellular mRNAs translation while enhancing rotavirus mRNAs translation [403,404]. NSP3 evicts the cytoplasmic poly(A)-binding protein (PABPC) that usually interacts with eIF4G and the 3' poly(A) tail of cellular mRNAs to promote their circularization and to stimulate their translation. During rotavirus infection, PABPC accumulates in the cell nucleus where it inhibits the nucleocytoplasmic transport of poly(A)-containing mRNAs [405,406]. As pri-miRNAs carry a poly(A) tail, they may be trapped by PABPC in the nucleus during rotavirus infection, thus preventing their processing into mature miRNAs. Such a mechanism could explain why we failed to observe any influence of ectopically produced miR-142-3p on viral replication.

### 3. Antiviral activity of Gvin1

Gvin1 is a very large interferon-inducible GTPase that shares sequence similarity with the antiviral proteins Mx and hGBP1 belonging to the same GTPase family. Knockdown of Gvin1 expression in interferon-treated cells increased the infection of Theiler's virus, Vesicular stomatitis virus and, in particular, Murid herpesvirus-4. Moreover, it impacted nucleic acids uptake after cellular transfection and promoted a rearrangement of the actin cytoskeleton. Experiments are in progress to confirm that the observed effects do not result from off-target effects of the shRNA used. If the data are confirmed, our results collectively suggest that the antiviral effect of Gvin1 may occur through inhibition of virus entry into cells.

#### 3.1. What is the mechanism of action of Gvin1 ?

As Gvin1, the immunity-related GTPases (IRGs) and the guanylate-binding proteins (GBPs) belong to the interferon-inducible GTPase family. Both IRGs and GBPs have been shown to target pathogens that either replicate in intracellular vesicles or enter cells via endocytosis.

Following bacterial or parasitic infection, IRGs accumulate around intracellular compartments containing these pathogens. During *Mycobacteria* or *Salmonella* infection, human IRGM and its murine ortholog, Irgm1, recruit autophagic and SNARE adaptor proteins to deliver phagocytosed bacteria to lysosomes [381,407]. The mouse Irgm3 interacts with others IRGs and acts as a regulator of their activity. Upon *Chlamydia serovars* infection, Irgm3 releases Irga6 and Irgb10 that disrupt the chlamydial inclusion body [384,408]. IRGs also target vacuoles containing *Toxoplasma gondii*. The accumulation of Irgb6, Irgb10, Irga6 and Irgd around the parasite vacuole induces membrane disruption, which leads to either necrosis or autophagic elimination of the damaged vacuole, depending on the host cell type [386,409,410].

In addition to IRGs, several GBPs, including hGBP1, have been reported to localize at the surface of pathogens containing compartments. In cells infected with *Chlamydia trachomatis* and transfected with either hGBP1 or hGBP2, GBPs accumulate to the chlamydial inclusion membrane leading to the delay of bacteria growth [330]. Mouse GBP5 is induced in IFN-treated macrophages and localizes on the membrane of the phagosomes containing *Salmonella enterica*. This GBP promotes programmed cell death associated with inflammation, a process called pyroptosis [411,412]. In *Toxoplasma gondii* infected cells, several murine GBPs accumulate around the parasitic vacuole and are thought to be involved in the immune response against *Toxoplasma gondii* [413].

Given the mechanism of action of IRGs and GBPs, it is tempting to speculate that Gvin1 acts in a similar way and accumulates at the membrane of endosomes containing incoming viruses (figure 14). Thus, Gvin1 accumulation could lead either to endocytosed virus delivery to lysosomes, or to virus sequestration in endosomes, or to the early disruption of the endosomal membrane. In the latter case, the virus would be unable to properly release its genome or its nucleocapsid into the cell cytoplasm.

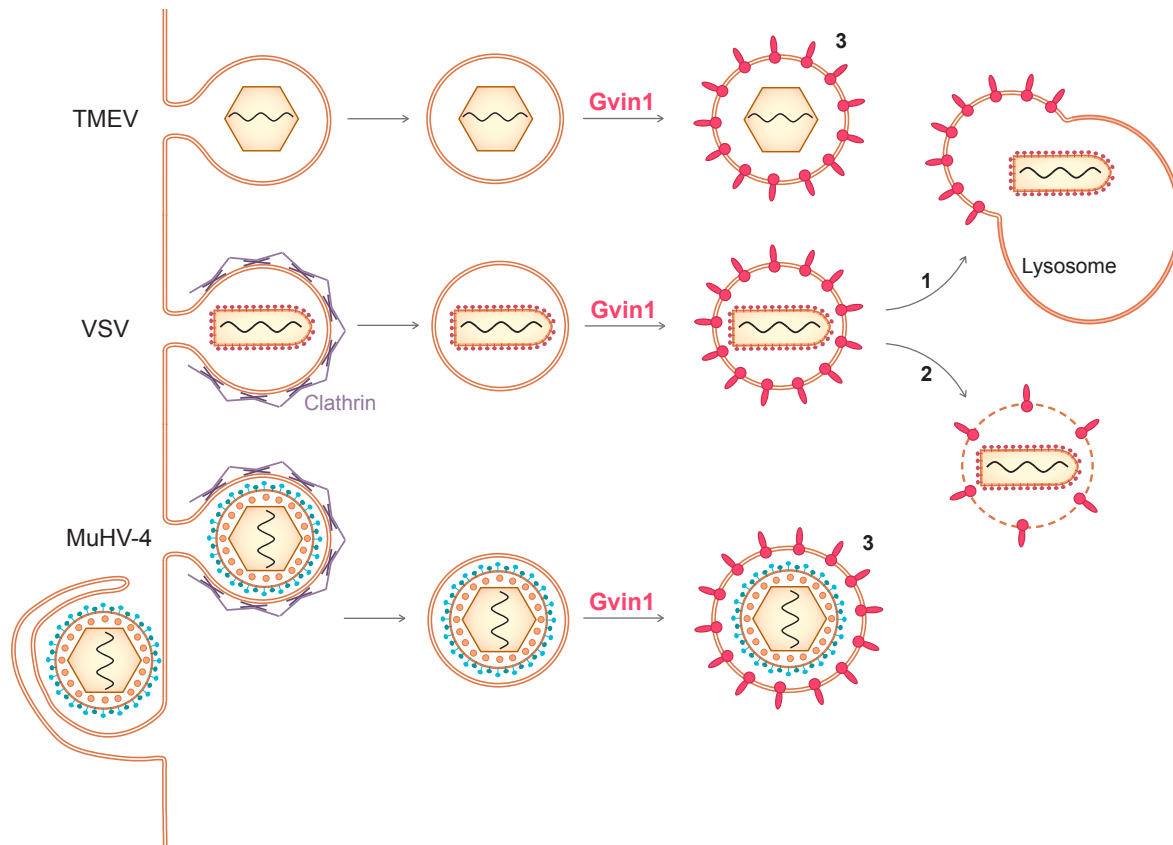


**Figure 14. Potential Gvin1 mechanism of action.**

Gvin1 could accumulate around endosomes containing an incoming virus. This could lead to three possible consequences : (1) the endocytic vesicle is delivered to lysosomes, leading to virus elimination. (2) Gvin1 disrupts the endosomal membrane, thereby preventing proper conformational change of the viral capsid or fusion of the viral envelope with the endosomal membrane necessary for the release of the virus genome into the cytoplasm. (3) Gvin1 traps incoming viruses into endocytic vesicles.

### 3.2. Perspectives

Our results suggest that Gvin1 confers cellular resistance against viral infection, likely by blocking virus endocytosis. To continue this work, we should first confirm the inhibitory activity of Gvin1 on endocytosis. In this aim, we examined the effect of Gvin1 down-regulation on transferrin uptake into cells. Fluorochrome-conjugated transferrin was bound at 4°C during 1h to L929 cells expressing either an empty vector or a scramble shRNA or a shRNA targeting *Gvin1*. Cells were then washed with ice-cold PBS to remove unbound transferrin and incubated at 37°C to allow endocytosis. The uptake of transferrin was examined by flow cytometry after different incubation times. We observed no effect of Gvin1 knockdown on transferrin uptake (figure 15). However, it is possible that Gvin1 does not interfere with transferrin endocytosis, which follows an unusual endocytic pathway. Indeed, following the release of iron inside early endosomes, transferrin is recycled to the plasma membrane. Thus, it would be interesting to examine the effect of Gvin1 knockdown on the endocytosis of others compound, such as LDL or dextran that, unlike transferrin, reach the late endosomes.

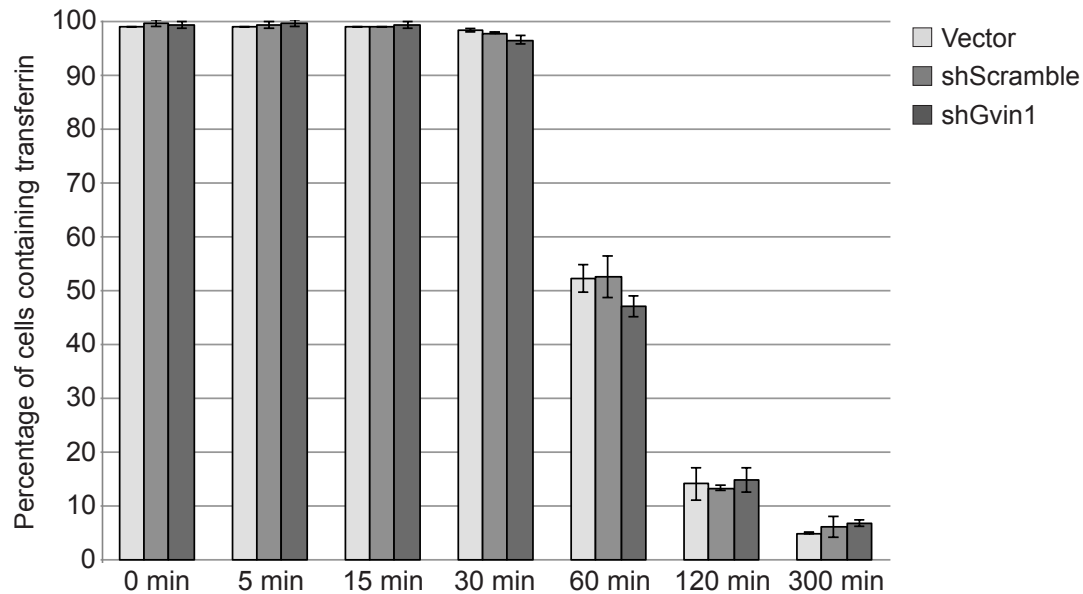


**Figure 15. Gvin1 knockdown does not impact transferrin uptake.**

L929 cells expressing either the empty pTM988 or the scramble shRNA (shScramble) or the shRNA targeting *Gvin1* (shGvin1) were starved during 1h and then incubated at 4°C during 20 min. Alexa Fluor 488 conjugated transferrin (20 µg/ml) was added to cells on ice, which were next incubated at 4°C during 1h. Cells were washed with cold PBS and suspended in complete medium on ice. Cells were then incubated at 37°C during indicated times. Following incubation, cells were first washed with PBS and secondly with an acidic buffer to remove transferrin from the cell surface. Finally, cells were dissociated with trypsin-EDTA and suspended in PBS containing 5% FCS and 0.5% of paraformaldehyde. Data were acquired with a LSR Fortessa flow cytometer (BD Bioscience) using the FACSDiva software. Histograms show mean and SD for a typical experiment (out of 3) performed in triplicate.

Secondly, we could determine which type of endocytosis is impaired by Gvin1. Therefore, it would be interesting to investigate the effect of the inhibition of a specific endocytic pathway on Gvin1 activity. Endocytosis occurs through 4 main pathways : clathrin-mediated endocytosis, cholesterol-dependent endocytosis, macropinocytosis and phagocytosis. Some chemical inhibitors can be used to specifically block one of these endocytic pathways without impairing the others. For example, monodansylcadaverine inhibits clathrin-mediated endocytosis by stabilizing clathrin-coated vesicles. Filipin binds to cholesterol in the plasma membrane and blocks cholesterol-dependent endocytosis. Macropinocytosis is impaired by amiloride, which acts by inhibiting  $\text{Na}^+/\text{H}^+$  exchange at the cell surface. Thus, we could test whether the treatment of L929 expressing the shRNA targeting *Gvin1* with these inhibitors can thwart the increase of viral infection observed in these cells.

Our data also showed that the down-regulation of Gvin1 expression induces a rearrangement of the actin cytoskeleton. Thereby, we could assess the direct interaction of Gvin1 with actin filaments by immunoprecipitation.

Finally, it would be interesting to evaluate the resistance conferred by Gvin1 during bacterial infection. Indeed, Gvin1 expression was shown to be induced in the liver of mice infected with *Listeria monocytogenes* [293].

#### 4. RNA interference and interferon system interplay

RNA-mediated silencing is an evolutionarily conserved mechanism generally triggering repression activities such as post-transcriptional gene down-regulation, RNA degradation, heterochromatin formation or DNA methylation. The key components of the RNAi machinery are found in members of the four eukaryotes kingdoms : protists, plants, fungi and animals [414]. Phylogenetic analyses suggest that a relatively complex RNAi system was already present in the last common ancestor of eukaryotes and consisted of at least one Argonaute-like protein, one Piwi-like protein, one Dicer enzyme and one RNA-dependant RNA polymerase. The primary function of these proteins was likely the defense against viruses and transposable elements. In contrast to lower eukaryotes, mammals possess a protein-based innate antiviral response, the IFN system, which is a key player in antiviral defense. Type I IFNs have appeared much later during evolution, since these proteins are found only in reptiles, birds and mammals (GeneTree ENSGT00760000119150). Thus, in these organisms, the RNAi antiviral response has probably been replaced by the more complex IFN system. In this way, cells avoid potential off-target effects that virus-derived siRNAs may exert on the expression of partially complementary host mRNAs.

Yet, a link exists between RNAi and the IFN pathway (figure 16) [116]. Several miRNAs have been shown to target transcripts encoding components of the type I IFN response, such as pattern-recognition receptors, IFN receptors, signal transduction proteins and interferon-stimulated genes (ISGs). Also, 21 cellular miRNAs are up-regulated and 15 are down-regulated by type I IFN. These IFN-regulated miRNAs are involved in the control of type I IFN production and action, but also target viral transcripts. Hepatitis C virus, Hepatitis B virus and HIV mRNAs are targeted by cellular miRNAs induced by type I IFN [144,153,415]. Notably, the liver-specific miR-122, essential for HCV replication, is down-regulated upon IFN- $\beta$  stimulation. In addition to cellular miRNAs, IFNs down-regulate the expression of proteins, such as Dicer and Argonaute, which are involved in miRNAs processing.

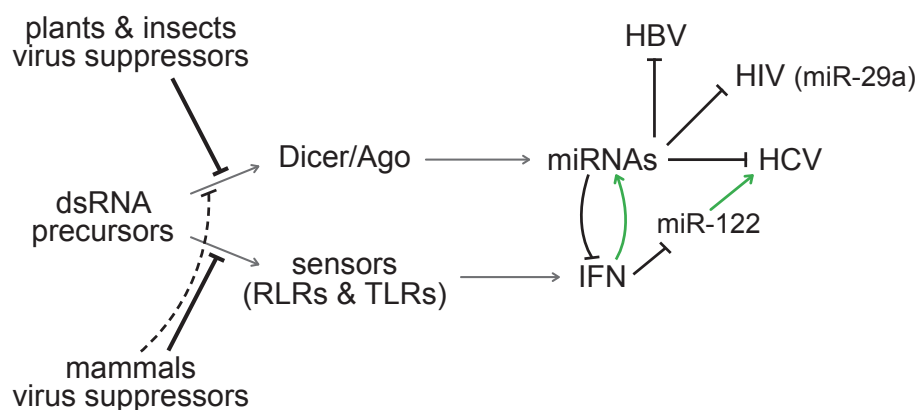


**Figure 16. RNAi and interferon pathways interplay.**

21 cellular miRNAs are reported to be up-regulated by IFN. Several of these miRNAs target viral RNA, including HIV and HCV transcripts. Some miRNAs, such as miR-122, as well as Dicer and Ago proteins are down-regulated upon IFN stimulation. In turn, numerous miRNAs target transcripts encoding proteins involved in the type I IFN pathway. IFN-mediated immunity is crucial during virus infection of a mammalian host. Numerous mammalian viruses thus express suppressor proteins of sensors that trigger the IFN response. In contrast, RNAi is the major defense against virus infection of plants and insects. Therefore, viruses infecting these organisms evolved suppressor proteins that antagonize the RNAi pathway.

## 5. Final conclusion

The discovery and the understanding of the RNAi pathway have revolutionized the field of gene expression control and brought new insights into the interplay between viruses and host cells. RNAi serves an essential antiviral innate response against viral infection in plants and invertebrates. In mammals, many studies documented direct interactions between microRNAs and mammalian viruses. Certain DNA viruses harness host miRNAs or produce their own miRNAs in order to ease viral infection. Interactions between cellular microRNAs and RNA viruses are less well understood and accumulating data argue against a major role of miRNAs in antiviral immunity. RNA viruses may instead exploit the miRNA machinery to modulate their replication or their tropism. Others, such as Theiler's virus, seem to be insensitive to the miRNA-mediated inhibition.

RNAi has been used as an experimental tool *in vitro* and *in vivo* to elucidate gene function and to discover novel cellular pathway. RNAi technologies provide the capacity to silence virtually any protein-coding gene. Short-hairpin RNAs allowed us to highlight a potential antiviral activity of Gvin1. The recent development of CRISPR/Cas9 system has brought new possibilities to achieve functional genomic studies through gene knockout. Both technologies, RNAi and CRISPR/Cas9, can complement each other to elucidate gene function.



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