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Régulation de l'accumulation de microARN viraux et cellulaires au cours de l'infection par le cytomegalovirus murin
/
Regulation of cellular and viral microRNAs accumulation upon mouse cytomegalovirus infection

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INTRODUCTION

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INTRODUCTION

1. FOREWORD

RNA silencing covers several processes taking part in a powerful mechanism that ultimately leads to the control of gene expression both at the transcriptional and post-transcriptional level. One of the key features of RNA silencing conserved in most eukaryotes such as plants, insects, animals, worms, and fungi, is the involvement of small RNA species of 19 to 30 nucleotides (nt) in length that act as specificity determinants.

The first glimpse of what would become a challenging, fast growing research field came with the experimental observation that the expression of a transgene homologous to an endogenous locus led to the suppression of expression of both genes. When Napoli and collaborators tried to overexpress a transgene encoding the chalcone synthase (CHS) protein in petunia, they observed an unexpected color pattern. The CHS gene encodes for a key enzyme involved in flavonoid biosynthesis that is responsible for the production of anthocyanins, compounds responsible for the coloration of petals. In addition to the expected more intense purple coloration than in wild type petals, some transgenic plants and their progeny showed partially white petals. The authors further showed that the introduction of the CHS transgene led to the "extinction" or "silencing" of the expression of both the introduced, and the endogenous gene at the posttranscriptional level (Napoli, 1990). This report was the first to describe a phenomenon of co-suppression. Later, this transgene-induced gene silencing was linked to the fact that viruses are also initiators and targets of gene silencing (Ratcliff *et al*., 1997). These informative studies resulted in the description of gene silencing as a plant innate defense system naturally occurring upon viral infections.

Due to its action on target RNAs after their initial transcription, this silencing mechanism has been referred to as post-transcriptional gene silencing (PTGS). It quickly became evident that double-stranded (ds)RNA was a key molecule in the mechanism (Baulcombe, 1996; Dougherty and Parks, 1995; Metzlaff *et al*., 1997), a notion that was definitively adopted with the discovery of Andrew Fire and Craig Mello in 1998 that long double-stranded RNA could lead to the degradation of a homologous transcript in *C. elegans*, a process they coined RNA

interference (RNAi) (Fire *et al*., 1998). It then took little time before the specificity determinant allowing the targeting in *trans* of homologous transcripts was identified. This feat was accomplished in 1999 by A. Hamilton and D. Baulcombe who showed that in both transgene-induced and virus-induced silencing, small RNAs of about 25 nt in length accumulated to high levels in silenced plants (Hamilton and Baulcombe, 1999). These small RNAs derived from double-stranded RNAs, and were able to act as guide to silence sequence complementary RNAs. They were further characterized and named short-interfering RNAs (siRNAs) by S. Elbashir and T. Tuschl (Elbashir *et al*., 2001b). The understanding of the physico-chemical properties of siRNAs (21-22 nt long with a 2 nt 3' overhang on each side of the duplex) later enabled the Tuschl laboratory to successfully apply the potential of RNAi in mammalian cells by using synthetic siRNAs (Elbashir *et al*., 2001a).

Simultaneously to the research on PTGS, geneticists were looking for developmental timing mutants in the worm *C. elegans*. In 1993, both G. Ruvkun and V. Ambros laboratories identified by forward genetic screens a novel heterochronic gene termed *lin-4*. Interestingly, the product of *lin-4* is not a protein, but a small non-coding RNA, which acts by pairing to complementary sites in the 3' untranslated region (UTR) of another RNA, *lin-14*, to modulate its translation (Lee *et al*., 1993; Wightman *et al*., 1993). Later on, in 2000, Ruvkun and collaborators reinforced this concept by showing that the relative amount of another small RNA, *let-7*, was also responsible for the developmental fate of cell lineage (Reinhart *et al*., 2000). The mode of action of *let-7* is also mediated by complementary binding to the 3'UTR region of *lin-14, lin-28, lin-41, lin-42* and *daf-12*. These non-coding RNAs relating to the developmental timing of *C. elegans* were coined small temporal (st) RNAs and opened the view to a broader role of small regulatory RNAs in gene regulation. Surprisingly, stRNAs were for a long time thought to be an oddity of worms, until people realized that *let-7* was actually conserved in a variety of other organisms, including human (Pasquinelli *et al*., 2000). This observation resulted in a frenzied search for other RNAs similar to *let-7*, which eventually led to the definition of micro (mi)RNAs by the laboratories of T. Tuschl, D. Bartel and V. Ambros. Altogether, these three groups identified a total of 100 miRNAs in *Drosophila*, *C. elegans* and human (Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Lee and Ambros, 2001). To date, the number of miRNAs deposited in the miRNA registry miRBase (http://www.mirbase.org/) (Griffiths-Jones *et al*., 2008; Kozomara and Griffiths-Jones, 2011) is nearing 17,000 distributed in more than 150 species.

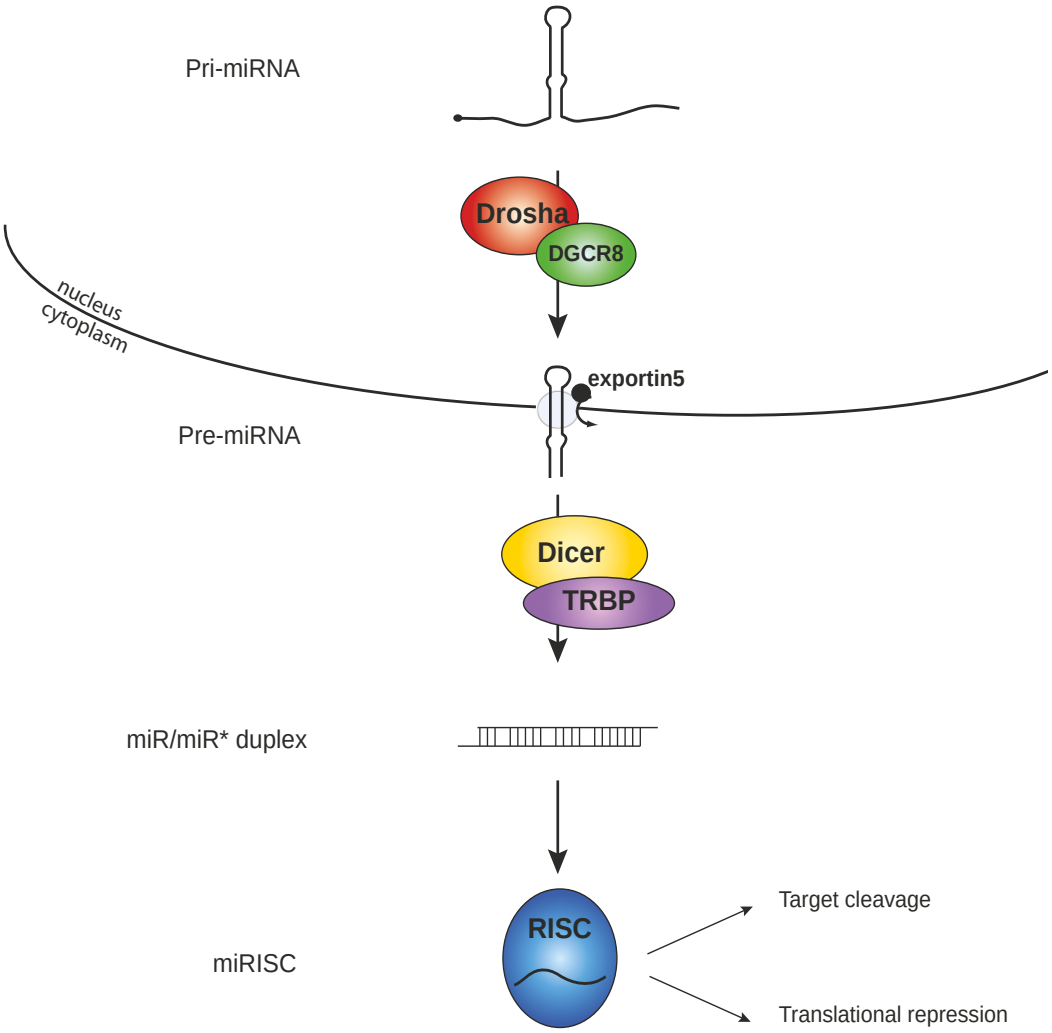


Figure 1: Biosynthesis and activities of miRNAs in mammals.

The primary transcript is mainly transcribed by RNA polymerase II, and presents stem-loop structures. In the nucleus, the combined action of Drosha and DGCR8 drives the production of a precursor of miRNA. Exportin 5 allows their export through the nuclear pore to the cytoplasm where they are cleaved by Dicer to generate a miRNA duplex. One strand is incorporated into the Argonaute containing RISC complex and acts either by cleavage of the target transcript or mostly by inhibition of its translation.

Finally, RNA silencing has been defined over years as a general mechanism involved in a broad range of biological processes: antiviral defense, transposon mobility control, gene expression regulation, histone methylation, chromatin establishment (see ref. (Baulcombe, 2004; Carrington and Ambros, 2003; Lippman and Martienssen, 2004; Voinnet, 2005) for review).

2. BIOGENESIS OF miRNAs

2.1. Biogenesis of miRNAs in mammals

2.1.1. Generation of the miRNA precursor

MiRNAs typically derive from large primary transcripts (pri-miRNA) that are transcribed by RNA polymerase II. In rare cases, they can also be processed from RNA polymerase III transcripts (see 4.2.3.1). The pri-miRNA transcript is capped and polyA tailed and can derive either from independent non-coding transcription units, introns, or sometimes from open-reading frames of protein-coding genes (Baskerville and Bartel, 2005; Rodriguez *et al*., 2004). The region of the pri-miRNA that folds into a typical hairpin structure is recognized and processed by the type III ribonuclease Drosha (Lee *et al*., 2003; Zeng *et al*., 2005). Drosha is the catalytic part of the microprocessor complex, in which the major co-factor identified is the DiGeorge Syndrome Critical Region Gene 8 (DGCR8) protein. DGCR8 recognizes and binds the stem-loop precursor within the pri-miRNA, thereby allowing the exact positioning and cleavage of Drosha to give rise to the pre-miRNA (Denli *et al*., 2004; Gregory *et al*., 2004; Han *et al*., 2004; Landthaler *et al*., 2004). The binding is facilitated by oligomerization of DGCR8 in a cooperative manner, probably involving the formation of a trimer of DGCR8 dimers (Faller *et al*., 2010). The pre-miRNA is folded into a hairpin of about 70 nt in length that possesses 2 nt 3' overhangs; a feature preferred by another type III ribonuclease, Dicer. It is then exported to the cytoplasm *via* a member of the importin-β family of proteins, exportin 5, through the nuclear pore in a Ran-GTP dependant manner (Figure 1). Exportin 5 was initially identified as the nuclear export factor for double-stranded RNA binding proteins (Brownawell and Macara, 2002) as well as for the adenovirus long RNA (VA1) (Gwizdek *et al*., 2003). Its role in pre-miRNA export was later assessed by three independent groups (Bohnsack *et al*., 2004; Lund *et al*., 2004; Yi *et al*., 2003).

In a few cases, pri-miRNA can be encoded within introns borders. The existence of such intron-derived small RNAs has been shown in *Drosophila* and *C. elegans* as well as in mammals. In this particular case, the pre-miRNAs are named mirtrons and are excised in a Drosha-independent manner by direct splicing. After splicing, the resulting branched intron can mimic the secondary structural features of a pre-miRNA. The 3' end of the « pre-miRNA » is defined by the 3' splice site. It is exported to the cytoplasm, and processed by Dicer to generate a miR/miR* duplex (Berezikov *et al*., 2007; Ruby *et al*., 2007).

2.1.2. Generation of a mature functional miRNA

Once in the cytoplasm, the pre-miRNA is processed by Dicer into an RNA duplex, very similar to an siRNA duplex (Hutvágner *et al*., 2001). Dicer has been shown to act in complex with the HIV-1 TAR RNA-binding protein (TRBP) (Kim, 2005) and sometimes the PACT protein (Lee *et al*., 2006). After this step, either the mature strand, or guide strand, or both strands are incorporated into the RNA-induced silencing complex (RISC), which invariably contains a member of the Piwi/Argonaute family (Carmell *et al*., 2002). Both Dicer and Argonaute proteins were initially identified as key proteins in the RNAi pathway (Bernstein *et al*., 2001; Hammond *et al*., 2001). Even if the process of how Dicer substrates are loaded into RISC is not yet fully understood, the incorporation of a single strand of the duplex, either siRNA or miRNA, seems to be a stepwise process. It was first described that the extremity of the duplex with the weakest pairing will define the 5' end of the guide strand (Khvorova *et al*., 2003). More recent data show that actually, for siRNAs at least, a first step is the physical association of the duplex with Argonaute2 followed by an activation step whereby the passenger strand is eliminated in the presence of accessory proteins (Ye *et al*., 2011) or depending on an intrinsic property of Argonaute proteins (Wang *et al*., 2009a). Several studies raised a difference between requirement for RISC loading of miRNAs and siRNAs, for example in the necessity of the presence of Dicer helicase activity (Sakurai *et al*., 2011; Welker *et al*., 2011). It has also been proposed that some pre-miRNAs could interact with Ago2 before their binding by Dicer, which would result in internal cleavage of the 3' arm of the pre-miRNA (Diederichs and Haber, 2007). Finally, it seems that in animals a 5'U tends to improve the loading of the mature strand over that of the passenger (or star) strand into RISC. This process is unrelated with the selection of one strand depending on weakness of base pairing (Seitz *et al*., 2011).

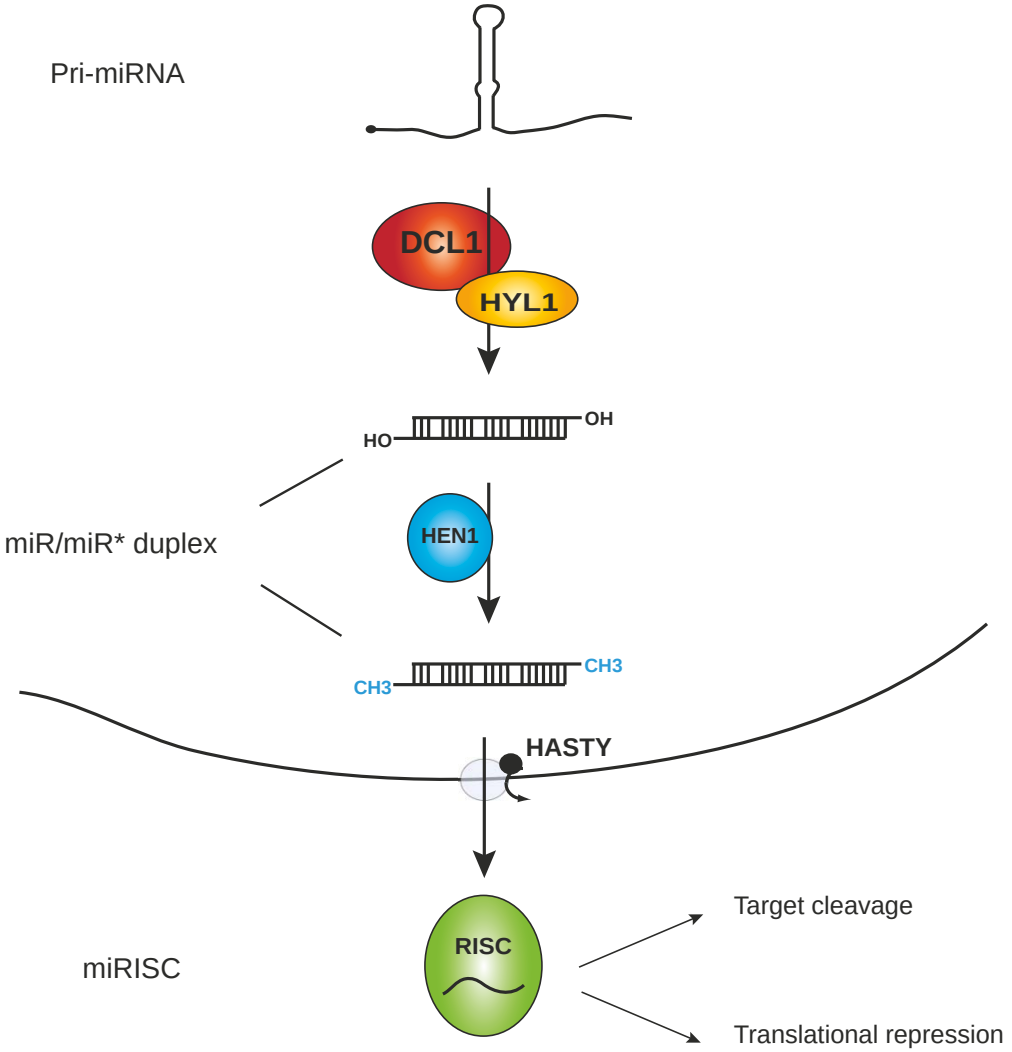


Figure 2: Biosynthesis and activities of miRNAs in *Arabidopsis*

The primary transcript presents stem-loop structures and is transcribed by RNA polymerase II. In the nucleus, the combined action of DCL1, HYL1 and HEN1 drives the production of methylated mature miRNAs. The exportin HASTY allows their export to the cytoplasm where they are incorporated into RISC. They act either by cleavage of the target trancript that will be later degraded or by inhibition of its translation.

RISC assembly has been described as an event coupled with Dicing (independent of ATP) but others argue that these processes are uncoupled (Yoda *et al*., 2010). The activation step requires a helicase activity that could be provided by Dicer or a specific helicase such as P68 (Salzman *et al*., 2007) and/or the slicer activity of Ago2 (Gu *et al*., 2011).

In rare cases, pre-miRNA processing can be Dicer independent. Thus, miR-451 has been shown to rely on Argonaute 2 for its maturation, which occurs *via* endogenous cleavage of the pre-miRNA within the 3' arm of the hairpin followed by exonucleolytic shortening of the small RNA (Cheloufi *et al*., 2010; Cifuentes *et al*., 2010).

2.2. Biogenesis of plant miRNAs

The biogenesis of plant miRNAs is for most part similar to the animal one (Figure 2). The maturation of the pri-miRNA into the single stranded miRNA is also a stepwise process. There are however some differences, the main one being that Drosha and DGRC8 homologs are absent in *Arabidopsis*. Instead, the Dicer Like 1 (DCL1) protein, together with the double-stranded RNA binding protein HYPONASTIC LEAVES 1 (HYL1) and C2H2-zinc finger protein SERRATE (SE), seems to be the only enzyme responsible for the processing of both the pri-miRNA and the pre-miRNA (Kurihara and Watanabe, 2004; Park *et al*., 2002; Vazquez *et al*., 2004a). In addition, the two processing events occur within the nucleus. HASTY (HST), the homolog of Exportin 5 in Arabidopsis, exports the miRNA/miRNA* duplex to the cytoplasm (Park *et al*., 2005). Another major difference with mammalian miRNA biogenesis is the methylation of plant miRNAs by the HUA ENHANCER 1 (HEN1) protein. HEN1 is a methyltransferase, which adds a methyl group on the hydroxyl at position C2 of the terminal ribose of both miRNA mature and star forms (Li *et al*., 2005; Yang *et al*., 2006b; Yu *et al*., 2005). Such a modification can also be found on animal small RNAs, but to date is not known to occur on miRNAs. Instead, it is a feature of Piwi-interacting RNAs in mammals and siRNAs in *Drosophila* (Hartig *et al*., 2007; Horwich *et al*., 2007). Independently of the small RNA considered, it seems that the mechanism of 2'O methylation is conserved, although some differences exist in the mode of action of HEN1 among the different organisms. As an example, the plant HEN1 recognizes the structure of the miRNA/miRNA* duplex and methylates both strands (Yang *et al*., 2006b), while the drosophila ortholog methylates single-stranded siRNAs (Horwich *et al*., 2007; Saito *et al*., 2007). While the exact role of the methyl group is still not fully understood, it has been proposed that one purpose of the 3' modification

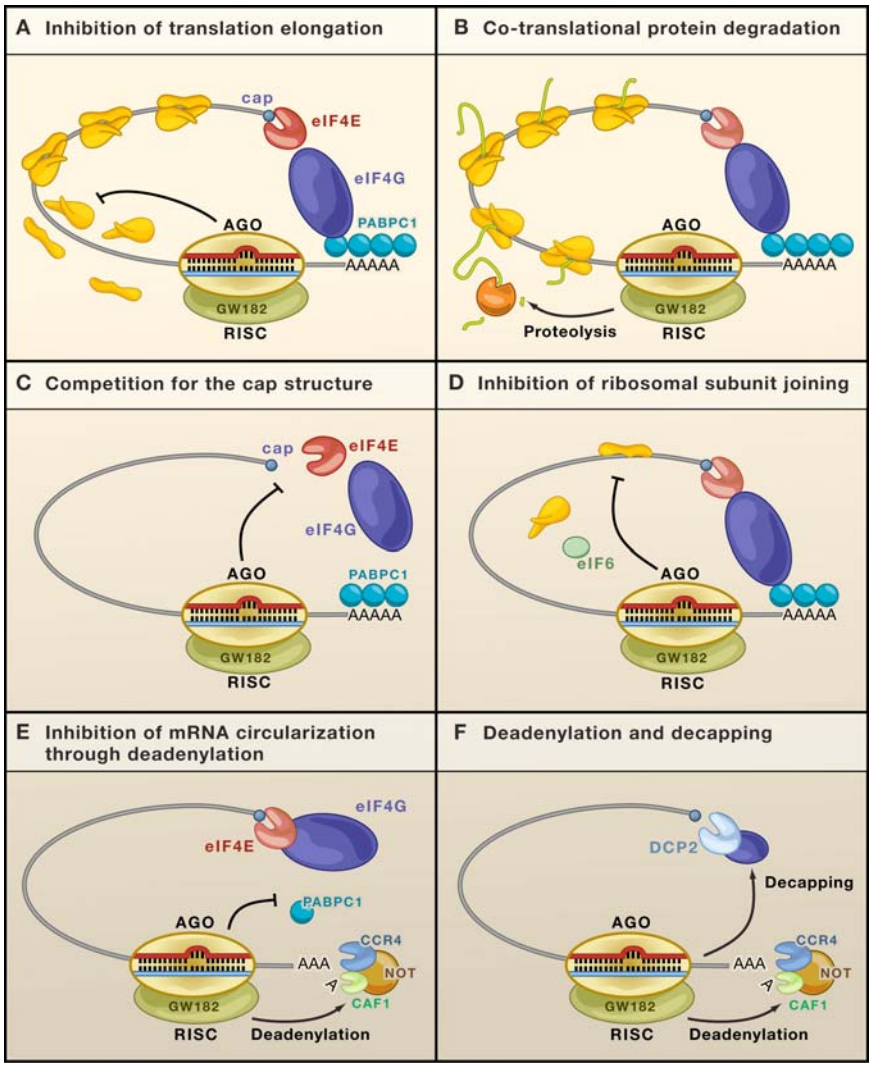
of small RNAs is to protect them against degradation. Indeed, in plants mutated in the HEN1 gene, the level of miRNA is dramatically reduced, which seems to be a consequence of their 3' modification *via* the addition of non-templated uridines (Li *et al*., 2005). Hence, the presence of the 2'O methyl group might impede upon activity of miRNA-modifying enzymes such as: ligases, terminal nucleotydyltransferases and polymerases, and therefore preventing their degradation.

2.3. Mode of action of miRNAs

In addition to some variations in the biogenesis of plant and animal miRNAs, there are also differences in their respective mode of action. It has indeed been shown that plant miRNAs, due to their almost perfect pairing to their target mRNAs, mostly trigger the cleavage of the RNA. This mode of regulation has been reported for a limited number of mammalian miRNAs (Yekta *et al*., 2004). The vast majority of animal miRNAs have been shown to engage translational repression of their targets *via* a pairing that involves several mismatches. This idea has been now re-evaluated and the extent of the differences between plant and animal miRNAs action tend to be lowered. Indeed, as in mammals, translational inhibition is probably widespread in plants, and involves the action of Ago1 and 10, as well as the decapping partner VARICOSE (Brodersen *et al*., 2008).

The active RISC is directed to its target RNA by the loaded mature miRNA that provides the sequence specificity. It is admitted that the most prominent sequence determinant to provide the specificity between the small RNA and its target is represented by the 5' proximal nucleotides 2 to 8, termed the "seed" (for review see (Bartel, 2009)). To date, most target prediction programs rely on this particular feature to define potential miRNA targets (for review see (Rajewsky, 2006)). The key component of RISC is a member of the Argonaute family. These proteins contain three conserved domains, PAZ, MID and PIWI. The PIWI domain has a catalytic activity in the form of an RNaseH-like fold (Liu *et al*., 2004). The junction of the MID and PIWI domains forms a binding pocket where the 5' monophosphate first nucleotide of the small RNA is anchored, whereas the PAZ domain binds reversibly to the 3' end of the miRNA (Jinek and Doudna, 2009; Parker *et al*., 2005; Wang *et al*., 2008; Wang *et al*., 2009c). There are four Argonautes in mammals, but only Ago2 has cleavage activity (Liu *et al*., 2004).

After RISC binding, the targeted transcript may undergo two possible fates, which are partially depending on the degree of complementarity between the transcript and the targeting small



From Eulalio et al., Cell, 2008

Figure 3: Different mechanisms triggering miRNA-mediated translational inhibition of mRNAs.

The possible levels of miRNA mediated action are: (A) ribosome drop-off or elongation blocking, (B) cotranslationnal degradation of the nascent protein, (C) initiation inhibition, competition Ago/ eIF4E for cap binding, (D) eIF6 mediated inhibition of large ribosomal subunit recruitment, (E) inhibition of the formation of the closed loop mRNA conformation.
mRNA decay is thought to be activated in all of these previous situations (F).

RNA. In case of a perfectly matched binding site and only when RISC contains Argonaute2 protein (Hutvagner *et al*., 2004; Meister *et al*., 2004), the targeted transcript will be cleaved in most of the cases and degraded by exonucleases. When the pairing is only incomplete, i.e. *via* the seed only or *via* the seed plus some binding of the 3' end of the miRNA, the translation of the target mRNA will be inhibited. This translation block can be achieved in different ways from the inhibition of translation initiation to the destabilization of the RNA by de-adenylation and decapping (reviewed in (Eulalio *et al*., 2008) & Figure 3). The fact that miRNA induced repression is mostly due to translational inhibition in mammals rather than mRNA destabilization has been re-evaluated when it was shown that translationally repressed mRNAs are also subjected to destabilization (Baek *et al*., 2008; Selbach *et al*., 2008).

Several steps can potentially be targeted during the course of miRNA-loaded RISC (miRISC) action. Pillai and coworkers first showed in 2005 that the translation inhibition occurs at the initiation step, because miRNAs and their associated targets are not found in polysome and the cap structure is required (Pillai *et al*., 2005). This process probably involves the displacement of the translation initiation factor 4E (eIF4E), the cap-interacting factor of the eIF4F complex.

The involvement of post-initiation mechanisms has also been described, such as repression of the 80S complex assembly or translation elongation. In one study, miRNAs and their targets were found to be associated with polysomes, leading to the idea that the nascent polypeptide could be degraded while its translation is already active, after either ribosome dissociation or ribosome drop off (Petersen *et al*., 2006). However, this study remains controversial (Ding and Grosshans, 2009).

Another option to prevent mRNA translation is to prevent its availability from the translation machinery and/or to induce its decay *via* the classical mRNA degradation pathway in the cell, *i.e*. deadenylation and decapping. These events usually take place in cytoplasmic processing or GW182 bodies (also referred to as p-bodies). The miRNA target can be translocated to these cytoplasmic foci, which contain the Ago interacting protein GW182, the CAF1-CCR4-NOT deadenylase complex, the decapping enzymes DCP1 and DCP2, some decapping activators, as well as RNA helicases (for review see (Eulalio *et al*., 2009b)). The deadenylation step can be mediated by the interaction with GW182 (Behm-Ansmant *et al*., 2006) but also requires the recruitment of the CCR4-NOT1 deadenylase complex (Eulalio *et al*., 2009a) and the polyA-binding protein (PABP) (Fabian *et al*., 2009). Interestingly, the sequestration of repressed mRNAs in GW/P-bodies is reversible in certain stress conditions such as serum starvation (Bhattacharyya *et al*., 2006). Another subcellular structure, the multivesicular body, a specialized late endosomal compartment, has been proposed to contribute to miRNA function

or miRISC turnover (Gibbings *et al*., 2009; Lee *et al*., 2009) as they have been shown to be enriched in GW182, Ago2, and some miRNAs, but not DCP1.

Although the paradigm of miRNA-mediated regulation is through downregulation of their targets, exceptions to these notions are emerging. Interestingly, it has been shown that in some conditions miRNAs can activate the translation of their targets. Thus, miR-122 can upregulate the hepatitis C virus (HCV) polyprotein translation (Henke *et al*., 2008) and miR-10a the translation of a ribosomal protein (Orom *et al*., 2008). The general impact of miRNA-mediated translation activation needs to be elucidated more carefully, even though some studies have pointed toward a role in cell cycle control (Mortensen *et al*., 2011; Vasudevan *et al*., 2007).

3. Non-canonical miRNAs and other small RNAs

3.1. Non-canonical miRNAs

3.1.1. Transfer RNA and snoRNA-derived miRNAs

Recent studies suggest that cleavage of transfer RNAs (tRNAs) could generate products with microRNA-like features (reviewed in (Pederson, 2010)). These tRNA-derived small RNAs (type II tsRNA) have been described in humans to be preferentially loaded onto Ago3 and Ago4 RISC complexes and restricted to cytoplasm (Haussecker *et al*., 2010). It is interesting to note that such tRNA-derived non-conventional small RNAs are also found in organisms where the RNAi machinery has been almost totally lost upon evolution like *Trypanosoma cruzi* (Garcia-Silva *et al*., 2010).

It was also shown that the human chromosome 19 miRNA cluster (C19MC) transcription requires RNA pol III *via* the presence of Alu elements. As the mature sequence of about 50 human miRNAs is predicted to lie within Alu elements, this is also probably the case for other cellular miRNAs (Borchert *et al*., 2006). However, in the precise case of C19MC, another study assessed that C19MC miRNAs are processed from introns of large RNA Pol II transcripts (Bortolin-Cavaille *et al*., 2009). Further experiments are then required to decipher the potential role of Alu-mediated miRNA transcription.

Human small nucleolar RNAs (snoRNAs) can also generate snoRNA-derived-miRNA-like. It has been shown that a subset of snoRNAs, instead of acting at the modification and processing of ribosomal RNAs (rRNA) and small nuclear RNAs (snRNA) in the nucleus, can be exported to the cytoplasm and then processed by Dicer to give rise to 20-22 nt long small RNAs. Such

snoRNA-derived-miRNAs are found associated with Ago1 and Ago2 in human. These RISC loaded small RNAs are efficiently able to regulate either a reporter RNA or an endogenous target. This has been shown for the snoRNA ACA45, but other snoRNA loci seem to present with similar features (Ender *et al*., 2008).

3.1.2. shRNAs

It has also been described that natural short hairpin RNAs (shRNAs) can mimic a pre-miRNA structure so that they are directly processed by Dicer and are DGCR8 independent. Such shRNAs are termed endogenous siRNAs (endosiRNAs) and were observed in mouse ES cells (Babiarz *et al*., 2008).

3.2. miRNA-offset RNAs (moRNAs)

By preparation of libraries of small RNAs in the simple chordate *Ciona intestinalis*, it has been found that small RNA species are generated from sequences immediately adjacent to pre-miRNA. These miRNA-offset RNAs are approximatively 20 nt long products from an RNase III processing activity (Shi *et al*., 2009), probably one end being generated by Drosha. Such small RNAs have been proposed to be present in *Drosophila melanogaster* (Ruby *et al*., 2007), mouse embryonic stem cells (Babiarz *et al*., 2008), and human (Langenberger *et al*., 2009). Whether or not these small RNAs are loaded in RISC complex or the understanding of their biogenesis is still unclear.

3.3. Small RNAs in plants

The diversity of small RNAs is much greater in plants than in mammals. One universal feature shared by every type of small RNAs is that they originate from long double-stranded RNA (dsRNA) molecules. Double-stranded RNAs are known to be triggers of RNA silencing, and can originate from various sources like inverted-repeat sequences, convergent transcription events, as well as transcripts with internal stem-loop structures (Brodersen and Voinnet, 2006; Chuang and Meyerowitz, 2000). In *Arabidopsis thaliana* genome there are 6 RNA-dependent-RNA-polymerases (RDRP), that in the presence of aberrant single-stranded RNA, can also

synthesize double stranded RNA (Dalmay *et al*., 2000; Gazzani *et al*., 2004; Wassenegger and Krczal, 2006). Long dsRNAs can also originate from exogenous sources such as viral replication intermediates, and inverse-repeat or sense and antisense insertion of transgenes (Chuang and Meyerowitz, 2000; Metzlaff *et al*., 1997; Voinnet, 2005).

3.3.1. Repeat-associated siRNAs (rasiRNAs)

Heterochromatin formation, *via* cytosine methylation on DNA and histone methylation, is mandatory to regulate the expression of transposons or repeat-associated regions in the genome and their associated siRNAs are required to maintain genome integrity (Henderson and Jacobsen, 2007; Matzke *et al*., 2001). The biogenesis of associated small RNAs requires RNA polymerases IV transcription (RNA pol IV), and RNApol IV and RDR2 activities generate dsRNA (Herr *et al*., 2005; Kanno *et al*., 2005; Onodera *et al*., 2005). After dicing by Dicer like 3 (DCL3) the 24 nt rasiRNA are incorporated into AGO4 complex and guide heterochromatin formation (Chan *et al*., 2004; Qi *et al*., 2006; Xie *et al*., 2004; Zilberman *et al*., 2004).

3.3.2. Secondary endo-siRNAs

Together with the classical RNA pol II miRNA loci, endo-trans-acting-siRNA genes (TAS genes) can lead to the synthesis of small RNAs that will be able to target and cleave complementary mRNAs (Vazquez *et al*., 2004b; Xie *et al*., 2005b). Unlike miRNA genes, TAS genes do not code for unique small RNA species, but for several unrelated siRNAs. Silencing mediated by tasiRNAs is mainly involved in developmental stage transitions and organ polarity definition (Peragine *et al*., 2004). After pol II transcription, a first miRNA-guided cleavage event of the primary ta-siRNA transcript occurs: TAS1/2 and TAS4 require a single miRNA binding site (respectively miR173 and miR828), whereas TAS3 requires 2 miR390 binding sites (Allen *et al*., 2005; Rajagopalan *et al*., 2006; Yoshikawa *et al*., 2005). The initial cleavage event involves either AGO1 or AGO7 (Allen *et al*., 2005; Marin *et al*., 2010; Montgomery *et al*., 2008) and it has been proposed that these ta-siRNA triggers are mostly 22nt long (Chen *et al*., 2010; Cuperus *et al*., 2010). In turn, RDR6, the RNA helicase SDE3 (silencing defective 3) and SGS3 (suppressor of gene silencing 3) activities can trigger the synthesis of a long double-stranded RNA that will be processed by DLC4 into phased ta-siRNA duplexes (Dunoyer *et al*., 2005; Gasciolli *et al*., 2005; Peragine *et al*., 2004; Vazquez *et al*., 2004b; Xie *et al*., 2005b).

The resulting 21 nt long ta-siRNA species can then cleave their target mRNA, like auxin response factor family members ARF1, 2, 3, or 4 in the case of TAS3 derived ta-siRNAs (Marin *et al*., 2010).

3.3.3. Natural-antisense transcript derived siRNAs (nat-siRNAs)

Small-interfering RNAs derived from overlapping transcript of adjacent genes located on opposite strands have been described. As an example, the transcripts of genes SRO5 and P5CDH can pair over approximately 750 bp, giving the possibility for a recognition and cleavage by DCL2 into nat-siRNAs of 24 nt (Borsani *et al*., 2005). These siRNAs accumulate in response to environmental abiotic or biotic stresses (Borsani *et al*., 2005; Katiyar-Agarwal *et al*., 2006). The biogenesis pathway of these nat-siRNAs requires the presence of DCL2, RDR6, SGS3 and NRPD1A, a subunit of the RNA polymerase IV (Borsani *et al*., 2005).

3.4. Piwi-interacting RNAs (piRNAs)

These recently discovered small RNAs play a major role in the regulation of transposon mobility in the germline. Indeed, transposable element silencing is required to maintain genomic stability. Piwi-RNAs have only been found up to now in the germline of animal species and also somatic support cell of the gonads (follicle cells), where the integrity of the genomic information has to be strongly protected in order to be transmitted to the next generation. These small RNAs are proposed to act at the transcriptional, transcript destruction, and translational inhibition levels (for review see (Aravin *et al*., 2007; Senti and Brennecke, 2010)).

4. SMALL RNAs IN VIRAL INFECTIONS

4.1. In plants and insects

A common feature of antiviral pathways across organisms is the recognition of pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs, such as dsRNA, but also bacterial lipopolysaccharides (LPS), hypomethylated DNA, and flagellin, requires the presence of a host receptor (pattern recognition receptor, PRR) (for review see (Kawai and Akira, 2011;

Kumar *et al*., 2011)). For example, recognition can involve Toll-like receptors (TLR), NOD-like receptors (NLR), and RIG-I-like receptors (RLR), which were initially discovered in *Drosophila melanogaster*, plant, and mammalian organisms respectively. These receptors can be either membrane-associated (e.g. TLR) or soluble in cytoplasm (RLR, NLR) (Medzhitov, 2007).

These PRRs play critical roles in triggering the antiviral immunity response in animals (especially so in mammals), however insects and plants rely on another powerful mechanism to fight viral diseases. Indeed, in these organisms, it has been well documented that the RNA silencing machinery can control viral infections (Ding and Voinnet, 2007; Galiana-Arnoux *et al*., 2006). A vast majority of viruses accumulate to some extent dsRNA products during the infection cycles. Such products can originate from replication intermediates, or fold-back structures within single-stranded viral transcripts or from the viral genome itself. The presence of dsRNA of viral origin serves as a substrate for Dicer proteins, which will process it into virus-derived small interfering RNAs (siRNAs), which in turn can be loaded into RISC to target degradation of viral transcripts and genomic RNA.

4.1.1. Generation of viral siRNAs in plant

Ruiz *et al*. showed in 1998 that viruses can induce PTGS of an endogenous transgene (VIGS) but that the presence of the virus is required for maintenance of silencing (Ruiz *et al*., 1998). Shortly after this discovery, the Baulcombe laboratory showed that the virus itself is a source of small RNAs in plants (Hamilton and Baulcombe, 1999), a finding that was later confirmed in insects (Li *et al*., 2002). In order to give rise to viral siRNAs (vsRNA), the presence of double stranded RNA that will trigger the antiviral silencing is necessary. The most likely source of dsRNA during a RNA virus infection is from the replication intermediate, when the genomic RNA is copied by the viral RNA dependent RNA polymerases (vRdRP) into opposite polarity RNA molecules. This minus strand will then be the template for both genomic RNA and full length mRNA or subgenomic mRNA synthesis, which will result again in the generation of double stranded RNA corresponding to the 5' part of the genome. However, in a number of cases, specific secondary structures in the single stranded RNA genome also play an important part in siRNA production, as was first observed with vsRNAs cloned from tombusvirus-infected plants (Szittya *et al*., 2002). Similar observations have been made with potyvirus, carmovirus as well as viroids (Ho *et al*., 2006; Itaya *et al*., 2007; Molnar *et al*., 2005).

2005). Secondary structures that can lead to the generation of vsRNA can also be found in DNA viruses as in the extensive fold-back structure located at the 5'end of the 35S transcript of the cauliflower mosaic virus (CaMV) (Moissiard and Voinnet, 2006). However a recent report argues that these vsRNAs would in fact derive from long sense and antisense transcripts originating from this region (Blevins *et al*., 2011).

Viral dsRNA is mainly processed by DCL4 and the resulting siRNAs are assembled into Ago1 to silence complementary target mRNA. Of note, even if the dicing itself could in theory have a powerful enough antiviral activity, the slicing mediated by Ago1 is also an essential component of the antiviral RNA silencing pathway. Although DCL4 is the main antiviral DCL protein, its function can be compensated by DCL2 (Deleris *et al*., 2006). For some viruses, DCL3 also produces vsRNAs, however they are not responsible for the degradation of viral transcripts (Deleris *et al*., 2006; Diaz-Pendon *et al*., 2007).

4.1.2. Generation of viral siRNAs in Drosophila

The first experimental evidence of the role of RNA silencing as an antiviral response in an animal organism came from the study of flock house virus (FHV) infection of *Drosophila* (Li *et al*., 2002). Small RNA pathways in *Drosophila* share common feature with that of the plant system, as they involve several Dicer and Argonaute proteins driving different aspects of the silencing mechanism. Thus, while Ago1, Dicer-1, and its cofactor R3D1 are involved in the miRNA pathway (Jiang *et al*., 2005; Lee *et al*., 2004; Okamura *et al*., 2004), the siRNA pathway that is activated upon detection of foreign dsRNA requires the presence of Ago2, Dicer-2, and its cofactor R2D2 (Lee *et al*., 2004; Liu *et al*., 2003; Okamura *et al*., 2004). These proteins have also been implicated in the antiviral response, and like in plants, mutants in these genes are hypersusceptible to viral infections (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006). Interestingly, it has been shown that RNAi does not only act directly as an antiviral immune response in *Drosophila*, but that some components of the silencing machinery could activate other types of innate immune response. Indeed, the DExD/H box helicase domain of Dicer-2 can induce the expression of the Vago gene to control viral load (Deddouche *et al*., 2008). The helicase domain of Dicer-2 shares homologies with the helicase domain of the RIG-I receptor in mammals the activation of which leads to the expression of interferon genes (Loo and Gale, 2011). Therefore, despite the lack of "RNAi" mechanism directed toward

viral infection in mammals, these proteins could potentially represent a conserved set of sensors for viral infections (Deddouche *et al*., 2008).

4.1.3. Suppressors of silencing

The silencing machinery has been implicated in the response to a broad range of viruses. This is well illustrated by the fact that, at least in plants, most if not all viruses, be their genome composed of RNA+, RNA-, dsRNA, ssDNA or dsDNA, seem to encode at least one suppressor of silencing (Li and Ding, 2006). The viral suppressors of silencing (VSR) are usually RNA binding proteins that can interact either with the initiator dsRNA molecules or with the effector siRNA, but can also directly target the RNA silencing components involved. Here are a few examples of the mode of action of VSR proteins. The tombusvirus P19 protein has a high affinity for 21nt long siRNA duplexes, thus inhibiting their loading into RISC and probably also interfering with the spreading of the silencing signal (Lakatos *et al*., 2004; Silhavy *et al*., 2002; Vargason *et al*., 2003). The 2b protein, encoded by the cucumber mosaic virus, is also able to bind dsRNA to drastically reduce the level of viral siRNA produced by DCL4, DCL2 and DCL3 (Diaz-Pendon *et al*., 2007). In addition this protein inhibits both the siRNA and miRNA pathways by interfering with the cleavage activity of Ago1 (Zhang *et al*., 2006). The polerovirus P0 protein also interact with Ago1 and promotes its degradation *via* the proteasome pathway (Baumberger *et al*., 2007; Bortolamiol *et al*., 2008). The helper component-proteinase (HC-Pro) is a multifunctional protein found among potyviruses that act as a VSR by interfering with the RDR6 dependent transitivity process and may inhibit HEN1 activity (Jamous *et al*., 2011). The TMV suppressor that resides in the viral 126-kDa small replicase subunit also interferes with the HEN1-mediated methylation of small RNAs (Vogler *et al*., 2007). The transitivity process can as well be inhibited by P19, but the effect of these two VSR may be indirect, and operate *via* the sequestration of siRNA duplex or by limiting DCL4 activity (Moissiard *et al*., 2007).

In insects, the proteins B2 of FHV and 1A of Drosophila C virus interact with dsRNA to inhibit Dicer-2 processing (Chao *et al*., 2005; Li *et al*., 2002; van Rij *et al*., 2006). Another viral suppressor of silencing encoded by the Cricket paralysis virus interacts with Ago2 to inhibit its activity (Nayak *et al*., 2010).

4.2. In mammals

4.2.1. RNAi and viruses

Introduction of dsRNA in mammalian cells activate the powerful but non-specific interferon response *via* the protein kinase RNA-activated (PKR). When PKR binds to dsRNA it is then dimerized and in turn activated by auto-phosphorylation. This active form phosporylates different substrates including eIF2, leading to a block in cellular translation, but also the inhibitor of NFkB (IkB), leading to the upregulation of the expression of antiviral interferon cytokines, and ultimately to apoptosis ((Chu *et al*., 1999; Meurs *et al*., 1993) and for review see (Garcia *et al*., 2007)). The existence of this pathway precludes the use of long dsRNA to trigger RNAi, but can be bypassed by the use of siRNA duplexes. Mammalian organisms are therefore in theory capable of taking advantage of the RNA silencing machinery to fight viral infections, but this has been masked by the development of the interferon response during evolution. In fact, early attempts failed at detecting the production of viral siRNAs upon infection of human cells with various RNA viruses (Cullen, 2006; Pfeffer *et al*., 2005). However, with the advent of next generation sequencing technology, this issue has been reexplored and recent data described that small RNAs deriving from RNA viruses can be detected during infection with viruses such as poliovirus, hepatitis C virus (HCV), and vesicular stomatitis virus (VSV) (Parameswaran *et al*., 2010). Although the exact nature of these viral small RNAs is not clear, their existence raises the need to further decipher the role of small RNAs derived from RNA viruses in mammals. This is especially true since it was recently shown that a pre-miRNA sequence introduced into the genome of the influenza A virus could be processed efficiently without interfering with the replicative capacity of the virus (Varble *et al*., 2010). Interestingly, a recent study also showed that another Drosha independent, but Dicer dependent, production of miRNA could occur after incorporation of a primary miRNA into the positive RNA genome of the cytoplasmic Sindbis virus (Shapiro *et al*., 2010).

4.2.2. Cellular small RNA action on viruses

There is no clear evidence to date that RNA silencing can act directly as an antiviral defense mechanism in mammals. However, mounting evidence demonstrates that there are interactions between small RNAs and viruses (Figure 4). However, these involve cellular miRNAs rather than the production of viral small RNAs. The Voinnet laboratory was the first to show that a cellular miRNA, miR-32, could perturb the replication of the primate foamy retrovirus (PFV-1)

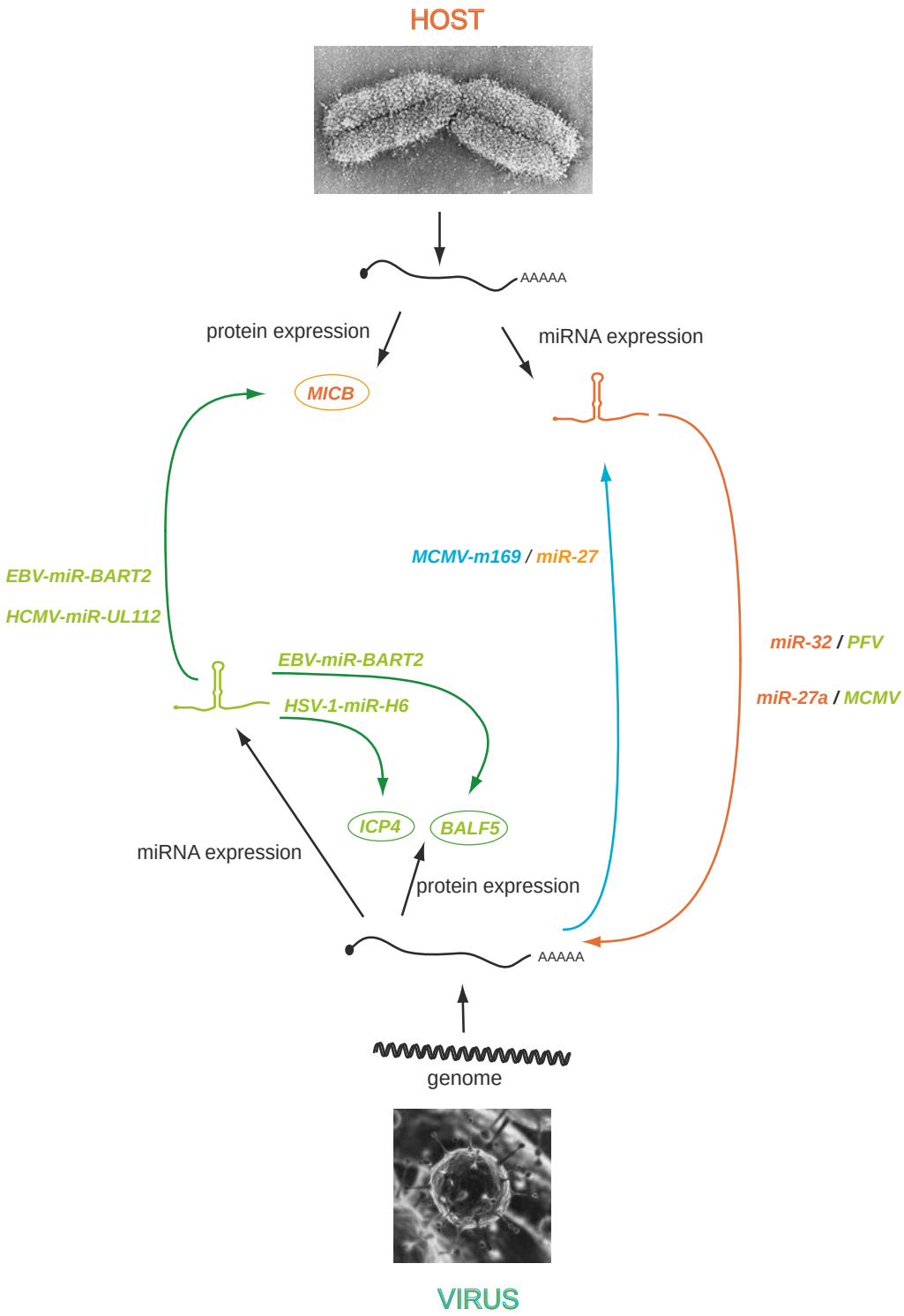


Figure 4: Host-virus interactions involving small RNAs in mammals.

The host genome encodes transcripts that can give raise to miRNAs. Some host miRNAs can affect virus replication (examples given in orange). Viral transcripts can also give raise to miRNAs that can in turn target either viral mRNAs or cellular mRNAs (examples given in green). Finally, viral transcript can itself affect the accumulation of a cellular miRNA (example given in blue).
See text for details.

in human cells by a direct targeting of the viral mRNA (Lecellier *et al*., 2005). Huang *et al*. later reported similar findings for HIV-1. They found that the 3' end of the HIV-1 messenger RNA is targeted by miRNAs, miR-28, miR-125b, miR-150, miR-223 and miR-382, and that the miRNA-mediated regulation of these viral transcripts played a role in the maintenance of viral latency (Huang *et al*., 2007). Another study showed that cellular miRNAs could interact directly with viral RNAs. The laboratory of J. Han generated a hypomorphic Dicer mutant mouse and tested its susceptibility to different viruses. They found that the animals were hypersusceptible to VSV infection, and that the effect was due to two miRNAs, miR-24 and miR-93, which could target the L and P transcripts (Otsuka *et al*., 2007). MiR-24 seems to be an important miRNA in virus-host interactions as its expression is also regulated upon EBV and ascovirus infections (Cameron *et al*., 2008; Hussain and Asgari, 2010).

Intriguingly, not all virus/miRNA interactions have a negative outcome for the virus. Indeed, the liver specific miRNA, miR-122, can bind to the 5' non-translated region of the hepatitis C virus genome, and this binding is mandatory to achieve an efficient viral replication (Jopling *et al*., 2005). The targeting of HCV genomic RNA by miR-122 apparently allows both efficient internal ribosome entry site (IRES) dependent translation, and replication (Henke *et al*., 2008; Jangra *et al*., 2010). Another hypothesis that emerged recently is that the binding of miR-122 protects the viral genome from degradation, or prevents the induction of innate immune responses that would be deleterious to the virus (Machlin *et al*., 2011).

The importance of miR-122 for HCV is such that it represents a promising therapeutic target. Several reports have shown that it is indeed possible to specifically and efficiently block this miRNA using antisense approaches, including in an *in vivo* setting, and that the inhibition of miR-122 correlates with a drop in HCV titer in chimpanzee (Lanford *et al*., 2010).

Other small RNAs are involved in the control of virus replication. In a polyomavirus infection (BK virus), the replication of DNA requires several partners. First of all, the large T antigen is required to initiate the replication by binding at the viral origin of replication. This allows the host DNA polymerase α-primase (Pol-primase) to synthetize DNA primers necessary to initiate the complete DNA replication (Mahon *et al*., 2009; Tikhanovich and Nasheuer, 2010). A recent report by Tikhanovich and colleagues showed that small RNAs of cellular origin, of unknown function, can interact with the origin of replication of the BK virus to inhibit the initiation of replication (Tikhanovich *et al*., 2011).

A

Family/subfamily	Genus/species	Mature miRNA
Polyomavirus	BK polyomavirus	1
	JC polyomavirus	1
	Simian virus 40	1
	Merkel cell polyomavirus	1
	Mouse polyomavirus	1
Papilloma/polyoma	Bandicoot papillomatosis carcinomatosis virus type 1	1
	Bandicoot papillomatosis carcinomatosis virus type 2	1
Ascovirus	Heliothis virescens ascovirus HvAV	1
Adenovirus	hAV	1
Herpesviridae	**Simplexvirus**	
Alphaherpesvirinae	Herpes Simplex Virus 1	16
	Herpes Simplex Virus 2	18
	Herpes B virus	3
	Mardivirus	
	Mareks disease virus	14
	Mareks disease virus type 2	18
	Herpesvirus of turkeys	17
	Varicellovirus	
	Bovine herpesvirus 1	10
	Iltovirus	
	Infectious laryngotracheitis virus	7
Betaherpesvirinae	**Cytomegalovirus**	
	Human cytomegalovirus	11
	Muromegalovirus	
	Mouse cytomegalovirus	18
	RCMV	24
Gammaherpesvirinae	**Lymphocryptovirus**	
	Epstein Barr virus	25
	Rhesus lymphocryptovirus	36
	Rhadinovirus	
	Rhesus monkey rhadinovirus	7
	Kaposi sarcoma-associated herpesvirus	13
	Mouse gammaherpesvirus 68	15

B

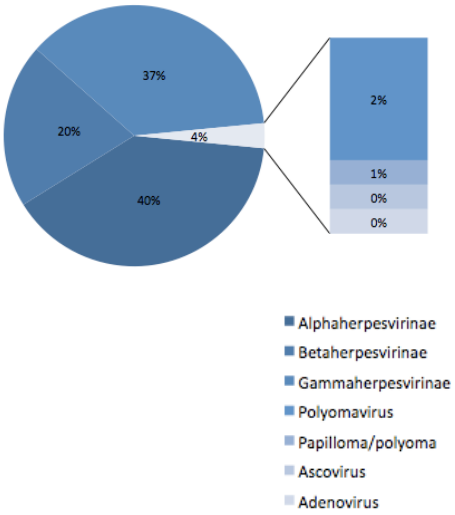
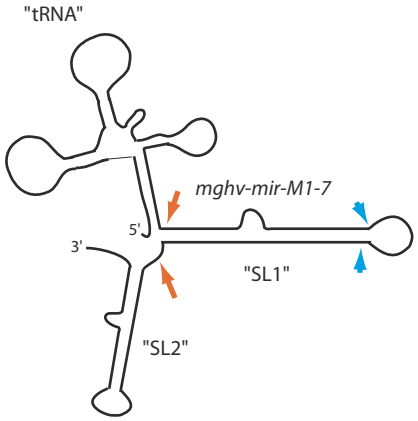


Table 1: (A) Virally encoded miRNAs according to the miRBase database, version 17, june 2011. (B) Repartition of viral miRNA by virus family.



Adapted from Bogerd et al., Mol. Cell, 2010

Figure 5: Cleavage events leading to the generation of a tRNA-derived microRNA.

Pri-miRNA transcript structure and unconventionnal processing of MHV68 miR-M1-7. The RNA structure presents a 5′ “tRNA” domain, a central stem-loop 1 (“SL1”) domain and a 3′ (“SL2”) domain, as indicated.

Orange arrows, RNaseZ cleavage sites, blue arrows, Dicer cleavage sites.

4.2.3. Virus encoded miRNAs

While it is still not clear whether siRNAs of viral origin can be produced in mammalian cells, it is now well established that certain mammalian viruses have hijacked the cellular machinery to produce their own miRNAs (Figure 4). The first virus-encoded miRNAs were identified in the Epstein-Barr virus (EBV) in 2004 in the Tuschl laboratory (Pfeffer *et al*., 2004). By cloning and sequencing small RNAs from cells latently infected with EBV, Pfeffer *et al*. identified five small RNAs fitting all the characteristics of miRNAs, paving the way for a whole new field of research.

4.2.3.1. The diversity and particularities of viral miRNAs

To date, viral miRNAs that have been described mostly belong to *Herpesviridae*, with some others: *Polyomaviridae,* and *Adenoviridae* (Table 1). Almost 240 miRNAs of viral origin are listed in the latest release of the miRBase registry (Griffiths-Jones *et al*., 2008; Kozomara and Griffiths-Jones, 2011). They all originate from DNA viruses that are replicating and are transcribed in the nucleus of the host cell, which means viral RNAs have technically access to the initial step of canonical miRNA biogenesis. Indeed, the vast majority of viral miRNAs use the exact same pathway as cellular ones for their biogenesis. One notable exception is the murine herpesvirus 68 (MHV68), which uses a non-conventional approach to express its miRNAs. Indeed, in the original description of MHV68 miRNAs, it was proposed that tRNA promoters drove the transcription of pri-miRNA by RNA polymerase III (Pfeffer *et al*., 2005). This was later confirmed by the Cullen laboratory, which showed that in addition to being Pol III transcripts, these miRNAs were processed by the RNase Z and not by Drosha (Bogerd *et al*., 2010). Dicer then performs the maturation of the pre-miRNA classically (Figure 5).

The vast majority of viral miRNAs are not evolutionary conserved between distant virus relatives or with their host. However, there are a few notable exceptions where some limited sequence homologies can be found between a viral and a cellular miRNA, but this rather seems to be due to a co-evolution in function. Thus, eight mature miRNAs encoded by the rhesus lymphocryptovirus (rLCV) are actually conserved in the close homolog *gammaherpesvirinae* EBV suggesting that an evolutionary pressure maintained these sequences (Cai *et al*., 2006). There also are a number of cases where the role of the miRNA has been conserved between distantly related viruses with no conservation at the primary sequence level. Similar to rLCV and EBV, miRNAs have been identified in the rhesus rhadinovirus (RRV), a monkey model of

Virus	miRNA	Target	Function	References
SV40	miR-S1	T antigen	Early protein	Sullivan *et al.* 2005
BKV	miR-B1	T antigen	Early protein	Seo et al., 2008b
JCV	miR-J1	T antigen	Early protein	Seo et al., 2008b
MCV	miR-M1	T antigen	Early protein	Seo et al., 2008a
EBV	miR-BART2 miR-BART1-5p, -16, 17	BALF5 LMP1	DNA polymerase Signaling molecule, TNF receptor mimic	Barth et al, 2008 Pfeffer et al. 2004 Lo et al. 2007
HCMV	miR-UL112-1	UL112/113, UL120/121 UL123 (IE72, IE1) UL114 UL117	IE protein IE protein, viral transactivator Uracil DNA glycosylase Maturation of replication compartments	Grey et al. 2007 Grey et al. 2007 Murphy et al. 2008 Stern-Ginossar, 2009 Huang et al. 2011
HSV-1	miR-H2-3p miR-H6	ICP0 ICP4	IE protein IE protein	Umbach et al., 2008 Umbach et al., 2008
HSV-2	miR-I miR-II, miR-III	ICP34.5 ICP34.5, ICP0	Pathogenicity factor	Tang et al., 2008a Tang et al., 2008b
HvAv	miR-1	ORF1	DNA polymerase	Hussain et al. 2008
KSHV	miR-K12-9*	ORF50/Rta	Viral transactivator	Bellare 2009
ILTV	miR-I5	ICP4	IE viral transactivator	Waidner, 2011

Table 2: Viral targets of viral miRNAs.

Virus	miRNA	Target	Function	References
KSHV	miR-K12-6-3p and others miR-K12-11 miR-K12-5 miR-K12-11, -6 miR-K12-7 miR-K12-10a	THBS1 BACH1, Fos BCLAF1 MAF MICB TWEAKR	Angiogenesis regulator Transcripiton factors Pro-apoptotic protein Transcription factor NK cell ligand Pro-apoptotic protein	Samols et al. 2007 Gottwein et al., 2007 Skalsky et al., 2007 Ziegelbauer et al., 2009 Hansen et al., 2010 Nachmani et al., 2009 Aben et al., 2010
EBV	miR-BHRF1-3 miR-BART5 miR-BART2	CXCL-11 PUMA MICB	Chemokine Pro-apoptotic protein NK cell ligand	Xia et al., 2008 Choy et al., 2008 Nachmani et al., 2009
HCMV	miR-UL112-1 miR-US25-1	MICB CCNE2 H3F3B TRIM28	NK cell ligand G1/S cyclin E2 H3 histone family 3B Transcriptionnal corepressor	Stern-Ginossar et al., 2007 Grey et al. 2010 Grey et al. 2010 Grey et al. 2010
MDV	miR-M4	PU.1	Transcription factor	Zhao et al., 2008
MCMV	miR-M23-2	CXCL16	Chemokine	Dölken et al., 2010

Table 3: Cellular targets of viral miRNAs

Kaposi's sarcoma associated HerpesVirus (KSHV). Although the miRNA sequences are not conserved between these evolutionary related viruses, their genomic location is in a locus similar to that found in KSHV (Schafer *et al*., 2007).

4.2.3.2. Cellular and viral targets of virus-encoded miRNAs

Numerous targets, both cellular and viral, have been described in the literature. Many of these miRNAs/mRNA targets relationships were the subject of a book chapter that I wrote together with Sébastien Pfeffer (see appendix). I provide in Tables 2 and 3 an update of the current knowledge of cellular and viral targets of miRNAs.

5. Human and mouse cytomegaloviruses

5.1. The herpesvirus family

The *Herpesviridae* family consists of three sub-families *Alphaherpesvirinae* (*Simplexvirus, Varicellovirus, Mardivirus* and *Iltovirus* genera), *Betaherpesvirinae* (*Cytomegalovirus, Muromegalovirus* and *Roseolovirus* genera) and *Gammaherpesvirinae* (*Lymphocryptovirus* and *Rhadinovirus* genera). These viruses infect predominantly mammals and birds, although a certain number of species also infect lower vertebrates such as reptilian, amphibian and some invertebrate hosts.

The viral cycle is classically divided into distinct stages: adsorption to the cell membrane, entry, release of the nucleocapsid, uncoating, replication, assembly of newly formed virion particles and release (for example, see HCMV viral cycle in Figure 6). The pattern of viral gene expression can be divided into three classes of genes, immediate-early genes (IE genes or alpha), early genes (E genes or delayed-early or beta) and late genes (or gamma). IE gene expression does not require any viral gene expression while the expression of early genes depends on functional IE gene products. Finally, the expression of late genes occurs after viral DNA replication (reviewed in (Knipe and Howley, 2007)).

All herpesviruses share the same virion morphology. The virion is spherical with a diameter of about 200 nm that is composed of the capsid, tegument, envelope, and the core. The core represents the packed double stranded DNA molecule. The capsid is icosahedric and composed of viral structural proteins. The tegument surrounds the capsid and is composed of several viral proteins, but its structure and function are not fully understood. Finally the envelope arises from the cellular membrane, decorated by viral proteins. Its composition is highly species-specific.

Herpesviruses are highly adapted to their hosts. This is especially true for the ß-herpesvirinae sub-family, one genus is usually restricted to one host. The co-evolution between these viruses and their host is also illustrated by the efficient immune response from the host, leading severe symptoms to be usually restricted to immunocompromised or very young individuals, in whom the ability to mount a cellular immune response is compromised or just not fully developed. At the same time, herpesviruses have developed multiple ways of counteracting or modulating the cellular immune response to establish lifelong latent infections. The best-characterized cell types involved in latency are neurons for the *Alphaherpesvirinae*, the monocyte lineage for the *Betaherpesvirinae*, and lymphocytes for the *Gammaherpesvirinae* (reviewed in (Cohrs and Gilden, 2001)).

5.2. HCMV pathogenesis

HCMV was first described as a major health concern as soon as it was identified as the causal agent of several pathologies, most notably cytomegalic inclusion disease (CID). The distribution of HCMV is worldwide and its prevalence has been estimated to range between 50 to 100% in human populations, mainly depending on geographical and socioeconomic factors (Stagno and Cloud, 1994). The major medical issues of HCMV relate to primary infection in immunocompromised patients or very young children, or with transplacental infection of the fetus. The pathogenesis of HCMV covers neurologic damages in newborns, like sensorineural hearing loss, and CMV retinitis in acquired immunodeficiency syndrome (AIDS) (Pass *et al*., 2006; Silva *et al*., 2010).

The main modes of transmission of HCMV are direct contact with infected body secretions and transplacental. Salivary glands represent an important site of shedding, and infectious particles are found in body secretions, such as saliva, but also urine, breast milk, representing a

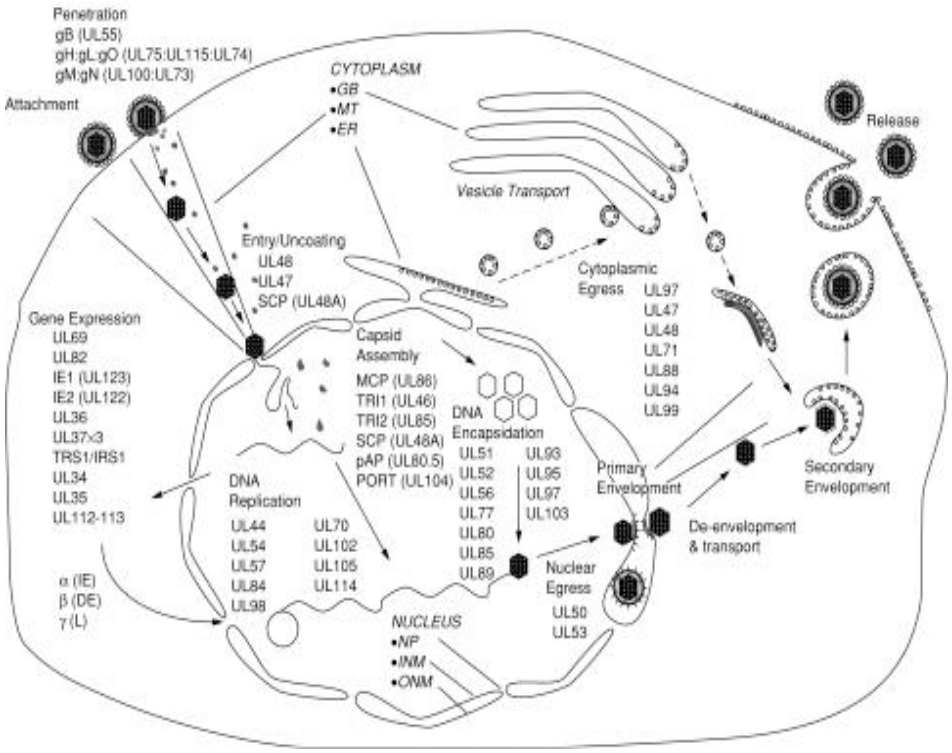


Figure 6: HCMV life cycle and conserved gene functions in betaherpesviruses.

After attachment, the entry involves direct fusion to the cell membrane or endocytosis. Once the nucleocapsid is in the cytoplasm, it migrates to the nucleus and the DNA is released into it, where viral gene expression and replication occur. Finally, viral particles are produced and released out of the cell (gene designations are the ones of HCMV).
Picture from: Arvin *et al.*, Cambridge University Press, 2007.

significant route for mother to child transmission and seminal or cervical secretions, route for sexual transmission.

5.3. CMV tropism and entry

While laboratory CMV strains efficiently replicate in fibroblasts in culture, their natural targets *in vivo* include multiple cell types. The cell type specificity that can occur in different viral strains is due to the high accumulation of mutations leading to adaptation to specific host cells. Interestingly, once established, these mutations are stable in adapted viral strains.
In human, permissive cell types range from epithelial and endothelial cells, to dendritic cells and macrophages. The attachment of betaherpesvirus particles to the cell surface involves an initial interaction with heparan sulfate followed by interaction with secondary receptors that allows fusing of the envelope to the cellular membrane (Compton *et al*., 1993). The exact mechanisms involving cell surface attachment and membranes fusion are not yet fully understood.

5.4. Viral cycle

After attachment to cell surface receptors and fusion between the cellular membrane and the envelope, the viral nucleocapsid is released into the cytoplasm and can then be translocated toward the nucleus *via* association with microtubules. The nucleocapsid then interacts with the nuclear pores followed by release of the genomic DNA into the nucleus. Viral DNA replication starts after IE and E gene expression (approximatively 16 hours post infection). CMV infection stimulates cellular mRNA and protein expression but not cellular DNA replication.
The assembly of the capsid, consisting of five viral core proteins possessing an icosahedral T=16 symmetry, and the encapsidation of genomic DNA start in the host cell nucleus. The mature capsid is thought to bud out of the nucleus in a two-step process, consisting of an envelopment and de-envelopment step. The tegument is acquired in the perinuclear compartment, and the lipid membrane with its associated viral glycoproteins originates from the Golgi-derived cytoplasmic compartments. The extensive reorganization of Golgi and endoplasmic reticulum (ER) during the infection, leads to the characteristic cytoplasmic inclusions of CMV infected cells. The ultimate source of the virion envelope is the ER or the

endoplasmic reticulum-Golgi intermediate compartment (ERGIC). Not only mature viral particles are formed, but also non-infectious enveloped particles and dense bodies are produced and contain similar envelope contents. Viral particles exit the cell by the cellular exocytosis pathway. ((Arvin *et al*., 2007) and Figure 6)

5.5. Genome organization and replication

HCMV possesses a linear double stranded DNA genome of about 235 kb in size, and thus represents the biggest genome of all the human herpesvirus, while MCMV genome is about 230 skb. CMV genomes potentially encode between 150 and 200 gene products based on open reading frames prediction. Although HCMV and MCMV share no extended DNA sequence homology, some of their predicted protein coding regions do show an evolutionary conservation. The genome is flanked by direct terminal repeats that enclose the cis-acting signals for cleavage and packaging of the progeny genomes (Arvin *et al*., 2007). There is another important cis-acting region in both HCMV and MCMV, the lytic origin of replication (*ori*Lyt), which plays essential roles in DNA replication, in association with virally-encoded origin-binding proteins, DNA replication enzymes and auxiliary factors. The initiation of replication at the *ori*Lyt requires the formation of an RNA-DNA hybrid, and the *ori*Lyt-associated RNA can be found bound to the viral genome in the mature capsid (Masse *et al*., 1992; Masse *et al*., 1997).

One of the first modifications to occur upon virus entry in the nucleus is the association to histone to try to silence the viral genome. The regulation of major intermediate-early promoter is mediated by several tegument, as well as some IE proteins. For example, one of the major components forming the tegument, the ppUL82 protein, is involved in the control of the acetylated state of histone to initiate viral IE gene expression. Increase acetylation allows the chromatin to be in a more relaxed structure that is accessible for gene transcription and replication. (Cantrell and Bresnahan, 2006; Saffert and Kalejta, 2006; Woodhall *et al*., 2006). Histone modifications have also been shown to be linked to the establishment of latency in MCMV infection (Murphy *et al*., 2002).

Viral gene expression is subjected to several regulations that are subject to intense studies. The timing of gene expression is not only of special importance for the viral lytic cycle but has also a pivotal significance in regard of latency and maintenance of the virus integrity.

5.6. Immunity and CMV infection

5.6.1. Cellular immune response

Natural killer (NK) T lymphocytes cells are the first immune cells to control viral infection. They are activated by IFN or by direct binding of cellular or viral proteins to NK cell receptors. These receptors includes Ly49H in mouse, which recognizes the MCMV glycoprotein m157, or NKG2D in human, which binds the HCMV glycoprotein UL16 (Sutherland *et al*., 2001). The class I major histocompatibility complex (MHC class I) is also an inhibitory ligand required to regulate NK cells activity. Its absence on the cell surface triggers the activation of the NK response (recognition of the « missing self ») (Karre *et al*., 1986). The action of NK cells is mediated *via* the action of cytoplasmic granules that release proteases, perforins and associated molecules, which will promote apoptosis or lysis of the targeted cell (Erlach *et al*., 2008)).
The life-long suppression of active viral replication is mainly due to a strong and broad T-cell response as demonstrated by the reactivation of HCMV upon immuno-suppressive treatment (Sylwester *et al*., 2005).
Humoral immunity also plays a role in protecting the host from CMV infection. Antibodies against many viral proteins are found in the serum of infected individuals. Recently, a large panel of neutralizing antibodies with high potency in neutralizing HCMV infection has been characterized (Macagno *et al*., 2010), which represents a potential powerful therapeutic target.

5.6.2. Viral modulation of the host cell immune pathway

Both HCVM and MCMV use similar mechanisms to modulate the immune response, although they might sometimes do so with evolutionarily distinct gene products. This is one of the reasons why MCMV is a good model to study HCMV pathology.

5.6.2.1. Inhibition of MHC class I presentation

Several viral proteins regulate the MHC class I complex to lower the targeting of cytotoxic T cells and natural killer cells. In HCMV, US3 is the earliest of several viral proteins that disrupt MHC class I antigen presentation, by inhibiting the processing and the transport steps (Jones *et*

al., 1996). The US6 gene, coding for a membrane glycoprotein, is also involved in binding and selective degradation of MHC class I, leading to a delayed trafficking, and it also inhibits antigen peptide transport (Ahn *et al*., 1997). US2 and US11 are also involved in the down-regulation of MHC class I presentation (Schempp *et al*., 2011). This strategy is fully conserved in MCMV and involves the genes m04, m06 and m152 (Hengel *et al*., 1999).

5.6.2.2. Inhibition of the interferon response

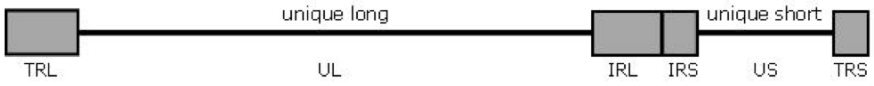
HCMV infection also alters the progression of the cell cycle by modulating cyclins and the expression pattern of other cell cycle regulatory proteins (Fortunato *et al*., 2000; Hertel and Mocarski, 2004). For example, HCMV encodes a homolog of interleukin-10, which inhibits cytokine production and lowers the cytotoxic T cell response (Chang *et al*., 2004; Kotenko *et al*., 2000). HCMV-IE1-p72 suppresses signal transducer and activator of transcription (STAT) signaling, a member of the JAK-STAT pathway, preventing IFN activation (Paulus *et al*., 2006; Zimmermann *et al*., 2005) The modulation of the interferon response is conserved during MCMV infection as illustrated by the targeting of STAT2 by pM27 as well as other strategies (Hengel *et al*., 2005).

5.6.2.3. Inhibition of apoptosis

Suppressors of apoptosis and necrosis have been identified in the genomes of both HCMV and MCMV (reviewed in (Brune, 2011)). For example, two of HCMV's IE genes are able to suppress apoptosis. UL37, in addition to its gene regulation activities, encodes a mitochondrial localized inhibitor of apoptosis vMIA, and UL 36, which encodes the conserved betaherpesvirus cell death suppressor vICA, inhibits caspase 8 activation (Goldmacher *et al*., 1999; Reboredo *et al*., 2004; Skaletskaya *et al*., 2001).

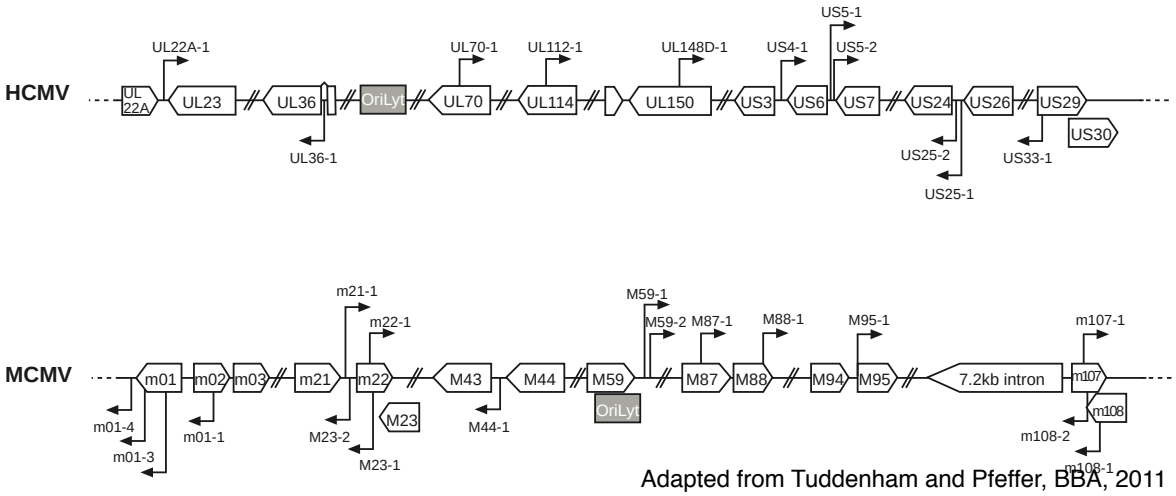
Interestingly, HCMV does not promote cellular replication, but rather blocks cellular DNA synthesis and cell division. Instead of inducing the S phase of the cell cycle, HCMV promotes the arrest into G2/M phase, thereby allowing the transcription of some HCMV genes into a pseudo G1 phase (Wiebusch *et al*., 2003; Wiebusch and Hagemeier, 2001).

A



From Kotenko et al., PNAS, 2000

B



Adapted from Tuddenham and Pfeffer, BBA, 2011

Figure 7:

(A) Schematic map of the CMV genome. The CMV genome is organized as two regions of unique sequences, unique long (UL) and unique short (US), each being flanked by a set of terminal (TR) and internal (IR) inverted repeats (TRL/IRL and IRS/TRS) (grey boxes).

(B) Genomic localization of HCMV and MCMV encoded miRNAs.

5.7. CMV and miRNAs

5.7.1. CMV-encoded miRNAs

5.7.1.1. HCMV miRNAs

HCMV miRNAs were first described by Pfeffer *et al*. who cloned and sequenced small RNAs from lytically infected primary fibroblasts and identified nine miRNAs (Pfeffer *et al*., 2005). Dunn *et al*. also described in 2005 two of these miRNAs, miR-UL22A and miR-US25, and respectively showed that they were expressed, at early and immediate-early stages of infection (Dunn *et al*., 2005). Grey *et al*. used a predictive approach to look for conserved stem–loop structures between HCMV and the closely related chimpanzee cytomegalovirus (CCMV). Out of 110 HCMV stem loop sequences that were conserved in CCMV, they predicted 13 potential miRNAs, of which they validated five. Two of those were not described before (miR-US4-1 and miR-UL70-1) (Grey *et al*., 2005). Again, these miRNAs are expressed with an immediate early (UL70-1) or early kinetics (US4-1, US5-1, US5-2, UL36-1). HCMV miRNAs are spread throughout the viral genome and are located either in non-coding regions, intronic regions or complementary to known viral ORFs (Figure 7). As an example, miR-UL-112–1 is located antisense to the viral uracil DNA glycosylase UL114. One of these miRNAs, miR-US36-1, is located in the intron of the UL36 gene, but does not seem to follow the immediate-early expression pattern of this gene. This indicates that there could be an alternate promoter driving its expression or that there is a stabilization of the intron after UL36 transcription.

5.7.1.2. MCMV miRNAs

The murine cytomegalovirus (MCMV) encodes 18 pre-miRNAs that are early and late gene products (Buck *et al*., 2007; Dolken *et al*., 2007). Similar to HCMV, these miRNAs are distributed throughout the genome (Figure 7), and expressed either individually or in clusters. Unlike the closely related HCMV and CCMV, MCMV miRNAs share no sequence homology with HCMV. In addition, their genomic localization differs between the two viruses, with the notable exception of miRNAs in the M23 region of MCMV which corresponds to the UL23 region of HCMV (Tuddenham and Pfeffer, 2011). Notably, it has been shown that MCMV miRNAs are expressed from both strands of the viral DNA genome. Thus, the star sequence of miR-M23-2 is complementary to 16 nt of the miR-m21-1 sequence. The same sequence

complementarity exists for miR-M23-1-5p and miR-m22-1, as well as for miR-m107-1 and miR-m108-2-3p. These miRNAs are all detectable by Northern blot, so it seems that these genomic localizations do not interfere with their accumulation.

5.7.2. Targets of CMV miRNAs

The precise functions of most individual viral miRNAs remain to be determined, but a number of studies have started to unveil some of the roles that these small RNAs might play. Intuitively, one could imagine that the involvement of viral miRNAs in the viral life cycle could occur at two main levels: (1) to modify the cellular environment so as to provide an optimized setting for the infection to take place, or (2) to act directly on the virus to fine-tune the expression of its genes. In the first scenario, viral miRNAs would for example be an additional tool for viruses to escape the immune response to remain unnoticed by the organism for extended periods of time. In the second hypothesis, the miRNAs would regulate the expression of viral genes at key steps of the virus like cycle (notably, the transition from the lytic to the latent stage of infection). Like cellular miRNAs, viral miRNAs can engage in different possible interactions with their targets, either *via* a fully complementary binding, or a more canonical binding involving the seed of the miRNA.

5.7.2.1. Viral targets

The first targets of viral miRNAs identified were of viral origin. In the original paper describing the identification of virus-encoded miRNAs, it was proposed that one of EBV's miRNAs, namely miR-BART2, could potentially target the viral DNA polymerase BALF5. This hypothesis was inferred from the fact that miR-BART2 gene is expressed from a position opposite to the genomic location of the BALF5 transcript, which was predicted to be cleaved in a position corresponding exactly to a miRNA-mediated slicing (Pfeffer *et al*., 2004). This hypothesis has since been confirmed (Barth *et al*., 2008). To identify viral targets of HCMV-miR-UL112-1, Grey *et al*. used the previously state computational approach to predict putative binding sites in regions that were conserved between the human and the chimpanzee cytomegaloviruses. They came up with a list of 14 candidate 3′ UTRs, of which they could validate three by luciferase assay (UL112/113, UL120/121 and UL123). Surprisingly, UL114, which is perfectly complementary to miR-UL112-1 because it lies antisense to it on the

genome, could not be validated as a target (Grey *et al*., 2007). Although this was only measured by a reporter assay, this finding could indicate that even when there is extensive complementarity between a miRNA and its target, this does not necessarily translate into a measurable regulation. It could be that in this particular case, the binding site is not accessible due to a strong local secondary structure, or to the interaction with an RNA-binding protein. Murphy *et al*. also used a bioinformatic approach to predict viral targets of HCMV miRNAs. They validated the regulation of HCMV UL123 (IE1) by the same miRNA, miR-UL112-1 using luciferase reporter assays and western blot analysis (Murphy *et al*., 2008).

5.7.2.2. Cellular targets of viral miRNAs

The identification of cellular targets remains a challenge due to the lack of amenable high-throughput validation method. What holds true for cellular miRNAs is also true for viral miRNAs. Additionally, due to the lack of cross-species conservation, finding targets for viral miRNAs can prove even more difficult than for host miRNAs. Indeed, a number of approaches have relied on bioinformatic prediction to pre-define candidates to be followed-up on. To increase the signal-to-noise ratio in these predictions, predicted miRNA binding sites are often restricted to regions that are conserved between species (Rajewsky, 2006). This approach cannot be directly transposed to viral miRNAs, which are, for a very large proportion, not conserved with the host miRNAs or between distantly related viral species. Nevertheless, there are notable exceptions to this observation like the cellular miR-155 and the KSHV-miR-K12-11 that display significant homology (including the seed sequence) and down regulate an extensive set of common mRNA targets (Gottwein *et al*., 2007; Skalsky *et al*., 2007). The same seed homology has also been observed for miR-155 and the miR-M4 from the herpesviridae Marek Disease Virus 1 (MDV1) (Zhao *et al*., 2008).

Stern-Ginossar *et al*. designed a novel algorithm for target prediction that did not rely on cross-species conservation. Rather, they looked for the occurrence of repeated motifs within 3′ UTRs and searched for their potential interactions with HCMV miRNAs. This approach enabled them to predict and validate the NK cell ligand MICB as a target of HCMV miR-UL112-1(Stern-Ginossar *et al*., 2007). Interestingly, the binding of miR-UL112-1 to MICB 3′-UTR occurs in a non-canonical manner with a two-nucleotide bulge within the seed, and a stronger binding of the 3′ end of the miRNA. Intriguingly, a closely related isoform of MICB, MICA, which differs only by one nucleotide in the putative miR-UL112-1 binding site, is not a target of this miRNA.

More recently, Grey and collaborators performed RISC pull-down experiment in infected cells as well as isolation of miRNPs by streptavidin beads pull-down in cells transfected with a synthetic biotinylated hcmv-miR-US25-1. They identified multiple cellular targets of HCMV involved in cell cycle control like cyclin E2. One unusual feature of the binding of US25-1 to its targets relies in the enrichment of seed sequence matches in the 5'UTR of the transcripts (Grey *et al*., 2010).

5.7.3. Roles of viral miRNAs during the viral life cycle

5.7.3.1. Viral replication

HCMV is a fascinating illustration of how critical miRNAs can be for viral replication. Two reports have shown that one of HCMV miRNAs, miR-UL112-1, can target immediate-early viral genes (Grey *et al*., 2007; Murphy *et al*., 2008). Grey *et al*. first identified UL112/113, UL120/121 and UL123 (encoding the HCMV IE72, functional homolog of MCMV IE1 protein) as targets of miR-UL112-1, and both UL112/113 and IE72 encode important products for viral replication. They also showed that the overexpression of miR-UL112-1 during infection could inhibit immediate-early gene expression and viral DNA replication. Since IE72/IE1 is necessary for efficient viral replication through trans-activation of early and late viral genes, one could imagine that its repression by a miRNA may play an important role in the establishment of a latent or persistent infection. These results were confirmed by Murphy *et al*. who independently predicted the same binding site for miR-UL112-1 in the 3′-UTR of IE72 (IE1). They also confirmed the involvement of this miRNA in IE1 down-regulation, and constructed a mutant virus in which miR-UL112-1 expression was impaired. Although replication of the mutant virus was not perturbed in fibroblasts, IE1 accumulated to a significantly higher level in cells infected by the mutant virus compared to wild type virus. However, the analysis of this type of miRNA mutant virus may not be optimal in cell culture condition.

5.7.3.2. Control of the immune response

We have already seen that the control of the immune response is a critical step during CMV infection. In this regard, the virus also takes advantage of its miRNAs. HCMV miR-UL112, shown to play important roles in the control of viral replication, is also involved in the

regulation of the cellular gene MICB, essential step for the survival of HCMV infected cells (Stern-Ginossar *et al*., 2007). The regulation of MICB by HCMV-miR-UL112-1 also takes place in the real context of infection; cells that express miR-UL112-1 ectopically are less sensitive to specific killing by NK cells. Moreover, a mutant virus not expressing miR-UL112-1 resulted in infected cells being more effectively killed by NK cells after infection, very much like what has been previously observed with SV40 virus and cytotoxic T lymphocyte response (Stern-Ginossar *et al*., 2007; Sullivan *et al*., 2005). It is worth noting that the viral UL16 protein has already been shown to lower the expression of MICB at the cell surface (Cosman *et al*., 2001). Interestingly, miR-UL112-1 acts synergistically with a cellular miRNA to suppress MICB expression (Nachmani *et al*., 2010). Thus there seems to be a cumulative effect due to viral mechanisms, including protein factors and miRNAs that helps the virus achieve the difficult task of evading the host immune response. The role of MICB regulation upon viral infection is also illustrated by its conserved targeting upon KSHV and EBV infections (Nachmani *et al*., 2009).

6. REGULATION OF miRNA EXPRESSION

Although the biogenesis of miRNAs has been extensively studied over the past 10 years, it is only recently that the research focus has shifted to the study of the regulation of their biogenesis. Recent work showed that this regulation could occur at every step of biogenesis, from transcription of the pri-miRNA, to the stability of the mature miRNA.

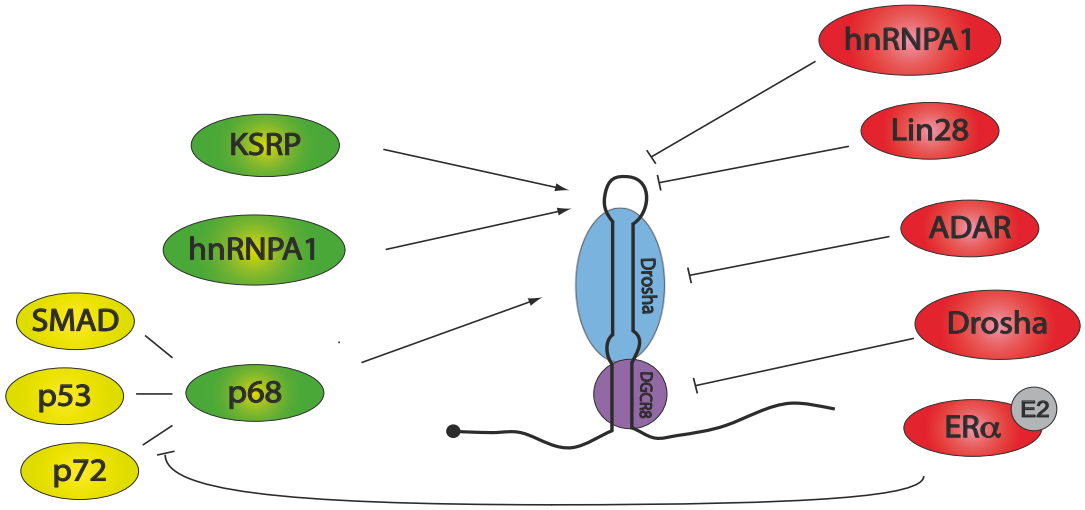
6.1. Transcriptional regulation

There is little to no difference between the transcriptional regulation of a miRNA gene, and an mRNA gene. As we saw earlier, miRNA primary transcripts are almost exclusively transcribed by RNA Pol II and are capped and polyadenylated as for protein-coding transcripts. This implies that every mechanism involved in the control of coding genes (transcription factors, enhancers, chromatin modifications) is also controlling the expression of miRNA genes.
The vast majority of studies on the transcriptional regulation of miRNAs has been associated with the study of the roles of miRNA in cancer. The c-MYC transcription factor, a well-known

proto-oncogene, controls cell proliferation, growth and apoptosis, and its dysregulation is involved in many human malignancies (Meyer and Penn, 2008). It has been shown that MYC mediates the decreased expression of several tumor suppressor miRNA genes such as miR-15a. This widespread down-regulation by MYC promotes tumorigenesis (Chang *et al*., 2008). Surprisingly, c-MYC is also able to regulate the expression of miRNAs gene in the opposite way. It can indeed enhance the transcription of the oncogenic miR-17-92 cluster besides other miRNAs in some tumors (Garzon *et al*., 2006; He *et al*., 2005). This cluster contains miR-17-5p and miR-20, two miRNAs repressing the expression of the cell cycle regulator E2F1 (O'Donnell *et al*., 2005). Interestingly, c-MYC can also directly promote E2F1 transcription (Coller *et al*., 2007). Another example of regulation at the transcriptional level is the up-regulation of miR-34 by P53. The induction of P53 by DNA damage and oncogenic stress leads to the stimulation of expression of miR-34 and other effectors ultimately leading to cell cycle arrest and apoptosis (He *et al*., 2007).

The transcriptional regulation of miRNA genes also occurs in plants. Nevertheless, plant miRNAs are mostly transcribed from specific intergenic loci unlike mammalian miRNA loci that can be found in introns of protein-coding genes (Baskerville and Bartel, 2005; Xie *et al*., 2005a). This implies that they might be under the control of independent promoters that respond classically to biotic and abiotic stresses. For example, it is known that the accumulation of miR399 primary transcripts is highly sensitive to phosphate availability (Bari *et al*., 2006). The regulation of miRNA transcription in *Arabidopsis thaliana* also takes place during plant development, and often involves feed-back loops between a transcription factor and a miRNA. Hence, miR156 regulates the expression of the Squamosa Promoter Binding Protein-Like (SPL) gene, and enables the switch between vegetative and reproductive phase (Wang *et al*., 2009b; Wu *et al*., 2009). The level of miR156 in flowering plants is controlled by the homeodomain proteins PENNYWISE (PNY) and POUND-FOOLISH (PNF) (Lal *et al*., 2011).

In *A. thaliana* lateral root (LR) development, the ARF proteins play a central role. Interestingly, ARF4 expression is controlled by the action of a tasiRNA arising from the TAS3 locus upon its cleavage by miR390. Repression of ARF allows the growth of lateral roots. In addition, ARF2, ARF3, and ARF4 affect auxin-induced miR390 accumulation. This feedback regulation permits the fine-regulation of the growth of lateral roots in response to variations in auxin concentration (Marin *et al*., 2010; Yoon *et al*., 2010).



Adapted from Krol et al. 2010

Figure 8: Regulation of biogenesis and stability of the pre-miRNA.

- KSRP promotes the processing of the pri-let-7a by Drosha.
- p72, p53 and SMAD in association with p68 enhance the processing by Drosha.
- hnRNPA1acts both as a positive regulator of pri-miR-18a processing in HeLa cells, as well as a negative regulator of let-7 processing by competition with KSRP.
- Editing of pri-miRNAs or pre-miRNAs by adenosine deaminases (ADAR1 and ADAR2) affects accumulation of mature miRNAs, and might also influence miRNA target specificity.
- ERα can inhibit the Drosha complex formation upon oestradiol activation.
- Drosha can inhibit miRNA processing via its inhibition of DGCR8 levels.

More data should appear in the literature about the transcriptional regulation of miRNA genes. Indeed, the tissue or cell specific expression of miRNA, as described for example during the development of the brain, as well as in shaping tissue identity, requests some further explanations (Krichevsky *et al*., 2003; Krichevsky *et al*., 2006; Nicolas *et al*., 2011; Tuddenham *et al*., 2006; Yang *et al*., 2011).

6.2. Regulation of miRNA processing

The first hint that miRNA processing was tightly controlled came from the observation that for some miRNAs, the accumulation of the mature sequence was highly tissue dependent, while the expression of their precursors was ubiquitous (Obernosterer *et al*., 2006). This is consistent with the fact that post-transcriptional regulation of miRNA processing allows harmonious development. Indeed, the primary transcripts of the let-7 family accumulate at high levels in the course of mouse development but are not processed (Thomson *et al*., 2006). The same kind of regulation has been proposed to play a role in oncogenesis process (Woods *et al*., 2007).
By acting on Drosha, Dicer, or their respective protein partners, the efficiency of the stepwise process leading to the generation of a mature miRNA can be regulated, and, in turn, represents an efficient way to control the accumulation of the active miRNA.

6.2.1. Activation of Drosha processing

Post-transcriptional cross-regulation has been described in the Drosha/DGCR8 complex. The Kim laboratory showed that the Drosha active complex is able to cleave the DGCR8 messenger RNA, while DGRC8 stabilizes Drosha by protein-protein interactions (Han *et al*., 2009). This particular regulation by proteins of the microprocessor complex itself, is not the only way to regulate the stepwise processing of miRNA, numerous proteins are involved in this phenomenon. For example, it has been described that Smad proteins, key transcription factors involved in TGFß signalling (for review see (Li and Flavell, 2008)), control Drosha mediated miRNA maturation (Davis *et al*., 2008). This study showed that the contractile phenotype in vascular smooth muscle cells, which is controlled by TGFß and bone morphogenic proteins (BMP) signaling, is regulated by miR-21. The TGFß signaling pathway induces an increase in pri-miR-21 processing due to the reruitment of Smad proteins to the Drosha microprocessor complex *via* the RNA helicase p68 (DDX5), inducing pri-miRNA processing.

Fukuda and collaborators first described the role of DEAD-box RNA helicases to promote Drosha processing in 2007 (Fukuda *et al*., 2007). The RNA helicase p68 is also able to associate with the central tumor suppressor P53 upon DNA damage to increase the Drosha processing of several miRNAs with growth-suppressive functions (Suzuki *et al*., 2009).

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is known to be an antagonist of the action of the SR family of protein involved in the regulation of splicing (for review, see (Shepard and Hertel, 2009)). It can also act as a positive regulator of Drosha processing by binding to the conserved terminal loop of pri-miR18a, reshaping its secondary structure and mediates its efficient processing (Michlewski *et al*., 2008).

Another RNA-binding protein, the KH-type splicing regulatory protein (KSRP), known to promote mRNA decay, acts also at the Drosha processing level by binding to the loop of the miRNA precursors and promoting their biogenesis (Ruggiero *et al*., 2009; Trabucchi *et al*., 2009; Gherzi *et al*., 2004) (Figure 8).

6.2.2. Inhibition of Drosha processing

Several studies report the role of proteins that inhibit Drosha function. As an example, the estrogen receptor alpha (ERalpha) is stimulated upon estradiol binding, and can inhibit the association between Drosha and some pri-miRNA (Yamagata *et al*., 2009). Another example is given with the role of hnRNP A1, which can also positively regulate Drosha (see above). Its direct binding to a conserved loop in the pri-miR-let-7a structure in somatic cells inhibits the processing by Drosha. This inhibition could be explained by the fact that the binding of hnRNP A1 competes with the binding of KSRP, which promotes the Drosha processing of pri-let-7a (Michlewski and Caceres, 2010). The accumulation of pri-let-7 due to the inhibition of its processing can also be due to the action of the RNA binding protein LIN28 (Newman *et al*., 2008; Viswanathan *et al*., 2008). The binding of LIN-28 to the endogenous primary let-7 transcript has been recently described to occur co-transcriptionally (Van Wynsberghe *et al*., 2011).

6.2.3. Regulation of Dicer processing

Examples of the regulation of Dicer processing have been extensively linked to the terminal modification of miRNAs precursors, but also to the role of the protein LIN28 that was discussed previously. LIN28 has first been proposed to induce the uridylation by

TUT4/Zcchc11 of the precursor of let-7, in turn preventing its processing by Dicer in embryonic stem cells (Heo *et al*., 2008; Heo *et al*., 2009). This mechanism has been shown to be conserved in *C. elegans,* where Lin-28 and PUP-2 trigger the uridylation and defect in processing of let-7 (Lehrbach *et al*., 2009). A recent publication showed that the default in Dicer processing of pre-miR-1 was due to the combined action of LIN28 and TUT4/Zcchc11, and that this may contribute to the cardiac dysfunctions observed in myotonic dystrophy (Rau *et al*., 2011).

6.3. Role of editing

Another mode of regulation of pri- or pre-miRNA processing involves editing. Editing is a classical cellular process that acts at the level of mRNA, tRNA and rRNA. In mammals, the modification of these RNAs involves cytidine to uridine (C to U) and adenosine to inosine (A to I) deamination editing, as well as non-templated nucleotide additions and insertions. These mechanisms produce transcriptome and proteome variability. Viruses use editing for example to express alternative protein variants. Nevertheless, it has been shown that most targeted RNA regions are non-coding regions, leaving a broad possible action for editing processes on the regulation of 3'UTR, splicing events, and regulation of small RNAs (for review see (Hundley and Bass, 2010; Zamyatnin *et al*., 2010)).

The first editing process described, which is specific to mammals, was the deamination by APOBEC (Apolipoprotein (apo)B mRNA editing complex) proteins of cytidine (C) to form uracil (U). The role of APOBEC proteins has been extensively described, especially because of their antiviral role during HIV infection (review in (Goila-Gaur and Strebel, 2008)). They also play a role in HSV-1 and EBV infections (Suspene *et al*., 2011). As such, editing represents an important process for therapeutic strategies in viral infections.

The other editing mechanism, prevalent in higher eukaryotes, is that of A to I editing, and is carried out by the adenosine desaminase acting on RNA (ADAR) proteins. Inosine preferentially pairs with cytosine instead of uridine, leading to mispairing within dsRNA structures and consequently to modification of pairing events (Levanon *et al*., 2004).

A direct consequence of pri-miRNA editing is the change implied on target regulation by modification of the miRNA binding site, especially the seed. Blow and collaborators showed that about 5% of the mature miRNAs analyzed in their study showed editing of their mature sequence, which can in theory expand their target set. This has been confirmed by the

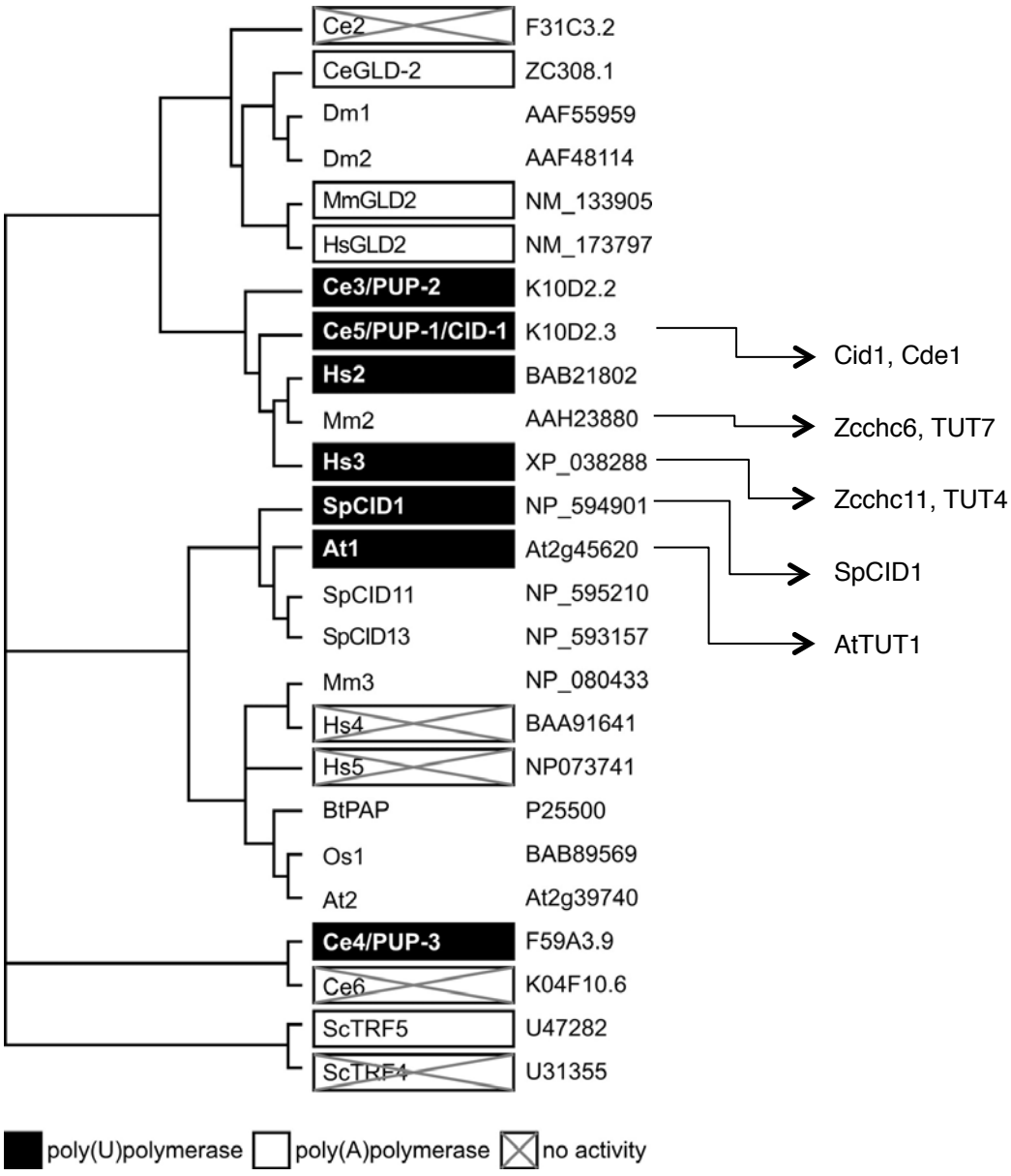
identification of targets that were specific of the edited miR-376, as well as the specific targeting of the tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) receptor (TWEAKR) by the non-edited kshv-miR-K12-10a (Abend *et al*., 2010; Kawahara *et al*., 2007b).

Editing has also been shown to perturb the maturation of miRNAs. This can happen at the level of Drosha processing, as in the case for pri-miR-142 (Yang *et al*., 2006a), or at the level of Dicer processing, which has been shown for pre-miR-151 (Kawahara *et al*., 2007a). As stated above, viral miRNAs are also subject to editing, and, as for cellular miRNAs, this can also affect their processing. Thus, EBV pri-miR-BART6 is edited in EBV latently infected cells, interfering with the processing of the mature miRNA in turn affecting the regulation of miR-BART6 targets, the viral EBNA2, and Dicer (Iizasa *et al*., 2010).

6.4. Regulation of miRNA stability/half life

The last step of miRNA regulation is obviously represented by the regulation of their mature form. It has been recently proposed that a broad range of mechanisms can affect the stability of these molecules. As we carefully examined this aspect of the regulation of small RNAs stability in the course of my thesis, I will introduce the state of the art of this topic in the next chapter.

The regulation of miRNA stability in both plant and mammalian systems, for the latter during the course of MCMV infection will be discussed in detail in the following chapter as the subject of this thesis.



From Kwak and Wickens, RNA, 2007.

Figure 9: Phylogenic tree of potential polyU polymerases.

Alignment of potential polyU polymerases, the black boxes represent enzymes with a polyU polymerase activity (as assessed *in vitro*), white boxes classical polyA polymerase, white crossed boxes enzyme without any proven activity *in vitro* and no box represents proteins for which the polymerase activity test had failed.

Chapter 1

Looking for the enzyme responsible for miRNA 3' modification

I previously outlined that several studies raised the fact that RNA tailing can be linked to the regulation of small RNA processing. As demonstrated already in plants in HEN1 backgrounds (Li *et al*., 2005), the addition of 3' non-template nucleotides is also a way to promote the degradation of small RNAs in the absence of a protective methyl group in 3'. Addition of nucleotides, especially by polyadenylation, was initially known to stabilize mRNA and enhance translation (Coller *et al*., 1998). However, it appears that transient poly(A) on RNA could also favor their rapid degradation by exonucleases in the cytoplasm (Slomovic *et al*., 2010), expanding the role of cytoplasmic tailing, not only for stabilization of RNA molecules, but rather to regulate their stability, ultimately leading to their eventual degradation.
The specific 3' elongation of small RNAs, especially by uridylation, has also been extensively observed for several years in *C. elegans* (Ruby *et al*., 2006), flies (Seitz *et al*., 2008), humans (Landgraf *et al*., 2007; Morin *et al*., 2008), as well as in plants (Li *et al*., 2005).

1. Enzymatic activities involved in RNA tailing and composition of the tail

The first glimpse of the importance of uridine addition on RNAs was given by Shen and Goodman who showed that mRNA fragments resulting from miRNA-mediated cleavage were also modified by 3' tailing. They proposed that uridine addition on cleaved mRNAs was a signature to trigger their degradation by exonucleases (Shen and Goodman, 2004). Indeed, the cleavage of the *A. thaliana* mRNAs ARF10 and MYB33 after perfect pairing of miR160 and miR159 respectively induced the appearance of several length isoforms of the 5' cleaved fragment. By circularization and reverse transcription of the targeted messenger RNA, they found that the miRNA cleavage site was extended by non-templated uridine stretches. In this particular case, the 3' tailing of the messenger RNA seems to induce a 5' to 3' decay of the transcript after recruitment of decapping enzymes rather than to activate 3' to 5' exoribonucleases. In the case of messenger RNA, this makes particular sense because it prevents the possible translation of the intact, capped, 5' end of the mRNA and therefore the synthesis of a non-functional protein.

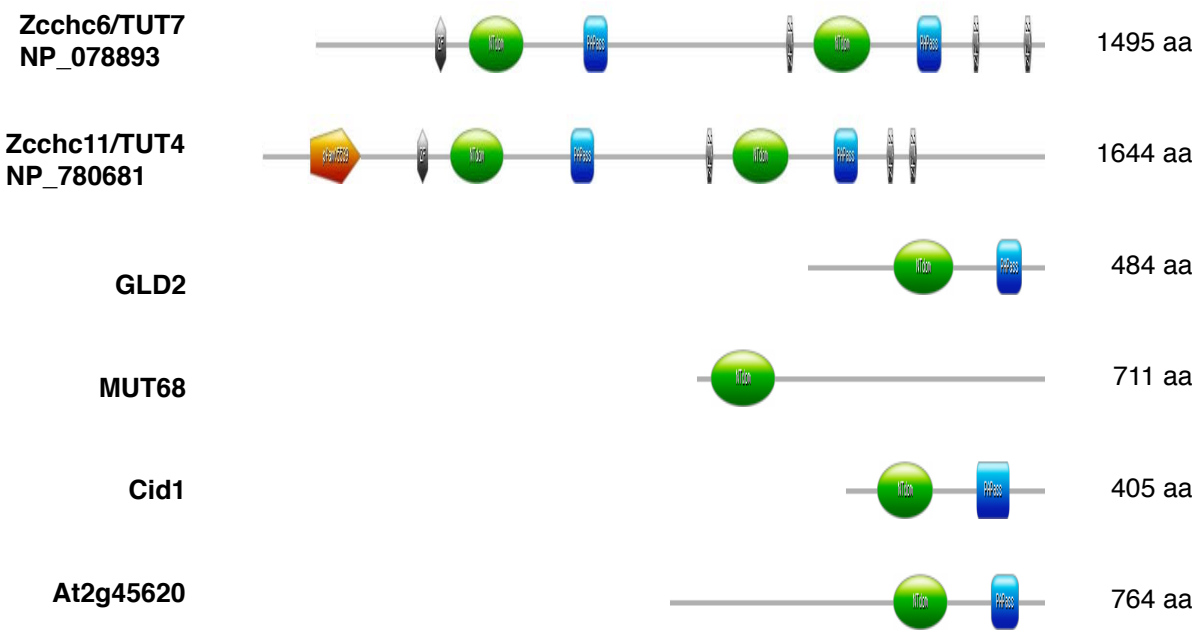


Figure 10: Alignment of potential terminal U transferases that could be involved in miRNA modification.

In green are represented nucleotidyltransferase (NT) domain of poly(A) polymerases and terminal uridylyl transferases (NT domain of DNA pol ß, cd05402). In blue are PAP associated domains (pfam03828). In grey are zinc fingers (PS00028 and PS50158). In orange is pneumovirinae attachment membrane glycoprotein G (pfam05539).

Conserved domains identification arised from NCBI conserved domain database as well as PROSITE database. Pictures have been realized with the PROSITE MyDomains - Image Creator.

Next, Rissland and collaborators discovered that the *Schizosaccharomyces pombe* Cid1 protein was able to uridylate polyadenylated mRNAs, more specifically the actin mRNA during S-phase arrest (Rissland *et al*., 2007). Moreover, they postulated that the human Zcchc6 protein was a Cid1 homolog capable of uridylating mRNA in human. At that time, Rissland and collaborators argued that the poly-U polymerase activity of SpCID1 and Zcchc6 were probably specific to these enzymes. However, Kwak and Wickens later reported that this characteristic was probably much more widespread than previously thought, when they showed that many enzymes characterized as polyA polymerases also possessed polyU polymerase activity (Kwak and Wickens, 2007). They tested different template-independent polymerases from worm, fly, human, mouse, yeast and plant, all sharing homologies with the *C. elegans* GLD-2 nucleotidyl transferase for their capacity to add As or Us to the 3' extremities of RNA substrates. They identified several enzymes that added preferentially Us to RNAs, and were therefore classified as polyU polymerases. Among these, the authors listed *S. pombe* and *C. elegans* CID-1, *C. elegans* PUP-2 and 3, two human genes they named Hs2 and 3, as well as a plant gene they named At1 (cf. Figure 9).

All these proteins share some common features (Marchler-Bauer *et al*., 2011): at least one catalytic domain related to the nucleotidyltransferase (NT) domain of DNA polymerase ß and similar proteins (Ntpolß), a polyA polymerase (PAP) associated domain, common in RNA modifying enzymes, as well as several Zinc finger motifs, which often are RNA-binding domains ((Laity *et al*., 2001) and Figure 10).

1.1. Direct regulation of NKkB pathway by Zcchc11/TUT4

A first report depicted Zcchc11 as a negative modulator of LPS-induced TLR signaling (Minoda *et al*., 2006). Upon TLR activation, IκB kinase (IKK) is phosphorylated and able to direct the degradation of an inhibitor of κB (IκB). The degradation of IκB unmasks the NLS (nuclear localization signal) of the transcription factor NFκB, thus leading to its nuclear translocation and activity. The release of NFκB requires the ubiquitin ligase activity of the oligomerized TRAF6 protein which requires TRAF6 oligomerization, promoted by a protein named TIFA. Minoda and collaborators were able to show by immunoprecipitation that the N-terminal part of Zcchc11 binds to TIFA upon LPS activation. This interaction is linked to a lowering of the activation of NFκB, leading to the classification of Zcchc11 as a cytoplasmic negative regulator of NfkB-mediated gene activation. Among the plethora of genes that are

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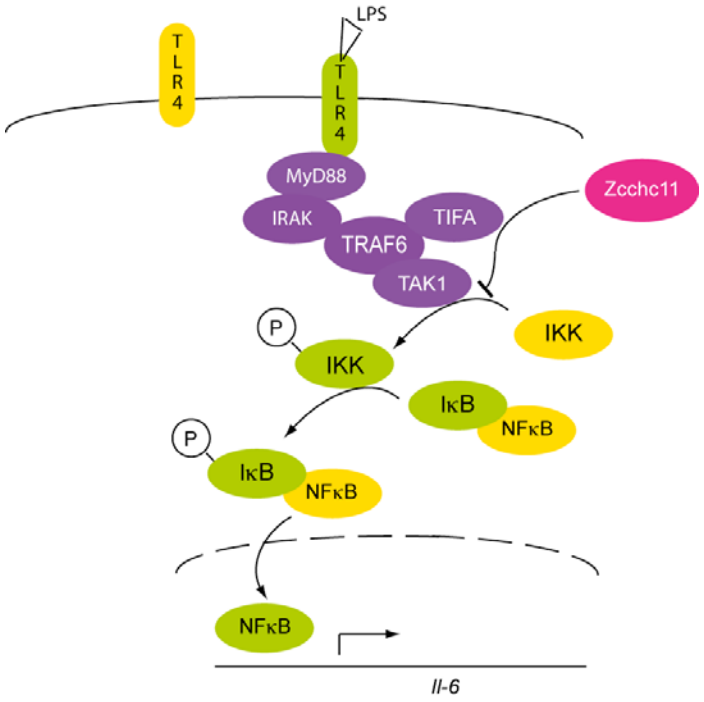
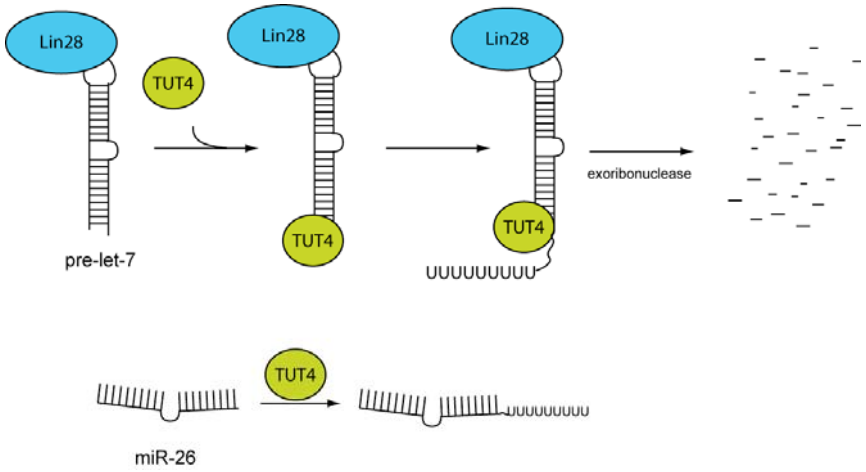


Adapted from Minoda *et al.*, Biochem. Biophys. Res. Commun., 2006.

B



Adapted from Krol *et al.*, Nat. Rev. Genet., 2010.

Figure 11:
(A) Toll-like receptor signaling modification via Zcchc11 regulation of TIFA (TRAF-interacting protein with a forkhead-associated (FHA) domain) / TRAF6 (tumor necrosis factor receptor-associated factor 6).
(B) Other roles of Zcchc11 on the degradation of both pre-let-7 and miR-26.

transcribed upon NFκB activation, there is the interleukin-6 (Il-6) gene ((Minoda *et al*., 2006) and Figure 11).

Zcchc11 is tissue specific (thymus, spleen, testis and lung) and contains several known motifs. It encodes a zinc finger (RNA or DNA interaction), three zinc knuckles (often RNA binding domains), two polyA polymerase-associated domains (common in RNA modifying enzymes) and a polß domain (mediates nucleotidyltransferase activity) (Jones *et al*., 2009). These motifs strongly suggested to Jones and collaborators that this protein was a ribonucleotidyl transferase with a preference for single stranded RNA. They showed that Zcchc11 was indeed able to uridylate a mature miRNA and interestingly, that the uridylation of miR-26 also abrogates interleukin-6 miRNA-related repression, reinforcing the NKκB-mediated negative regulation of Il-6 (Figure 11).

1.2. PAPD4/GLD2/TUTase2

Burrough *et al*. assessed that both uridylation and adenylation of miRNAs is common (present and conserved) in *Drosophila* and vertebrates. PAPD4 (also called GLD2 for germline defective 2) adenylates a wide range of miRNA loci. It localizes in the cytoplasm in *C. elegans*, *D. melanogaster*, *X. laevis*, and in mammals, it is also found in the nucleus (Burroughs *et al*., 2010).

Among the miRNAs that are modified by GLD2, several are implicated in cell cycle progression and tumorigenesis, consistent with a previously determined role for PAPD4 in controlling the cell cycle in *C. elegans* (Read *et al*., 2002; Saitoh *et al*., 2002; Wang *et al*., 2002). Strikingly, there is no obvious correlation between the stability of the concerned miRNAs and their adenylation, as shown with the analysis of small RNA libraries in PAPD4 knockdown cells. However, one possible effect could be related to the targeting effectiveness of the modified miRNA. This may be linked to a default in RISC incorporation, as adenylation seems to prevent Ago2 and Ago3 association, maybe in the same way as uridylation of a pre-miRNA by Lin28/TUT4 prevents its Dicer uptake (Burroughs *et al*., 2010).

A report of Katoh *et al*. showed that a single non-templated adenine addition on the mature miR-122 by the cytoplasmic poly(A) polymerase GLD-2 was able to selectively stabilize miR-122 in the liver, an important regulatory miRNA that has important roles in hepatic functions (Katoh *et al*., 2009). The adenylation of miR-122 is specifically carried out by GLD-2 on the

mature single stranded form of the miRNA to stabilize miR-122 after dicing, and the loss of adenylation leads to an extended degradation of the miRNAs by 3' exonucleases (Katoh *et al*., 2009). However, the depletion of this protein did not lead to severe developmental defects, giving a clue that another redundant enzyme could efficiently cover the functional role of GLD-2, a protein initially described to control germline progression through meiosis (Read *et al*., 2002). Nevertheless, the mono-adenylation of miR-122 and thus its stability leads to an efficient down regulation of the translation of cytoplasmic polyadenyation element binding protein (CPEB) in mouse fibroblasts (Burns *et al*., 2011). Reduced level of CPEB protein bound to p53 3'UTR lowers the anchoring of GLD4 (PAPD5), a non-canonical polyA polymerase responsible of the control of p53 polyA tail and translation, thus jeopardizing the fate of the cell between growth and senescence.

2. Degradation of small RNAs

Two different enzymes, Xrn2 and SDN1, have been already reported to contribute to the degradation of small RNAs in plants and in *C. elegans*.

2.1. Xrn2 and target-mediated miRNA protection

Xrn2/Rat1p, a 5' to 3' exoribonuclease was previously shown to be involved in tRNA quality control in yeast (Chernyakov *et al*., 2008) and, in the nucleus of *A. thaliana*, it was shown to degrade the excised loop of pri-miRNAs (Gy *et al*., 2007). It has been reported to efficiently degrade both mature and star miRNA molecules in the nematode *C. elegans* (Chatterjee *et al*., 2011; Chatterjee and Grosshans, 2009). In this particular case, the pairing of the miRNA to its natural target protects it from the degradation by Xrn2, which is the opposite from the observation of Ameres *et al*. who showed that in some cases pairing of a miRNA to a perfect or almost perfect target led to the degradation of the miRNA (Ameres *et al*., 2010). Moreover, the Xrn2-dependent degradation of miRNAs that do not have access to a target, could in part explain the difference in accumulation between the mature miRNA and its star sequence. This target-mediated miRNA protection (TMMP) could therefore be an additional means to ensure the correct loading of mature miRNAs.

2.2. SDN1 and degradation of small RNAs in *Arabidopsis thaliana*

SDN1 has been identified as a member of the small RNA degrading nucleases (SDN) in Arabidopsis thaliana after searching for Eri-1 homolog genes (Ramachandran and Chen, 2008). Eri-1, a conserved exonuclease in *C. elegans* and *S. pombe*, is responsible for the degradation of siRNA duplexes that possess two 3' nucleotides overhangs (Kennedy *et al*., 2004), therefore reducing the efficiency of RNA interference *in vivo*. The SDN family of enzymes, especially SDN1, is able to degrade single stranded small RNAs, in a 3' to the 5' direction. In this study, they established that the methyl group protecting the 3' end of small RNAs in plants also lowers their ability to process RNAs. Interestingly, the presence in 3' of the small RNA of a stretch of uridines, which is linked in *A. thaliana* to the lack of methylation of the 3' end, also impedes with the activity of SDN (Ramachandran and Chen, 2008).

3. Experimental identification of miRNA-tailing enzymes?

The degradation of small RNAs by exoribonucleases represents the last step in the cascade of events that ultimately leads to the control of their stability. Our aim was therefore to identify an enzymatic activity responsible for the tailing of miRNAs and to look at the level of conservation of the mechanism across evolution.

In order to test the involvement of some of these enzymes in miRNA tailing, we decided to focus on two candidates, the mammalian gene Zcchc6 (referred to as Hs2 or Mm2 in Figure 18), a close ortholog of Zcchc11, and the *Arabidopsis thaliana* gene At2g45620 (referred to as At1 in Figure 9), a homolog of SpCID1. I will refer to the latter as TUT1.

3.1. Analysis of the role of TUT1 in miRNA degradation in *A. thaliana*

To assess the role of miRNA turnover in a physiologically relevant context, we aimed at establishing a role for the *Arabidopsis* TUT1 protein in miRNA tailing and degradation. This gene (At2g45620) is a close homolog of the Cid1 polyU polymerase that could be responsible for miRNA extension (Kwak and Wickens, 2007). In order to find out if TUT1 was indeed involved in miRNA tailing, we took advantage of the molecular phenotype of a particular *Arabidopsis* mutant. One of the major differences between plant and mammalian mature miRNAs is the presence of a methyl group at the 3' end of the plant miRNA. This modification

is performed by the methyl transferase HEN1. This methyl group has been proposed to protect miRNAs against 3' uridylation and to prevent further degradation. Indeed, *hen1* mutant plants show a low level of miRNA accumulation, and for some miRNAs, it is possible to detect by northern blot analysis a ladder corresponding to the addition of nucleotides at the 3' end of the small RNA (Li *et al*., 2005). Our idea was therefore to check whether we could prevent miRNA tailing and restore their accumulation in *HEN1/TUT1* double mutants.

3.1.1. Generation of *HEN1/TUT1* double mutant *Arabidopsis thaliana* plants

Arabidopsis thaliana is easily amenable to genetic manipulation due to the capacity of the pathogen *Agrobacterium tumefaciens* to introduce its DNA into the plant genome. A natural pathogen of most dicotyledons plants, *A. tumefaciens* is the causal agent of crown gall disease (Smith and Townsend, 1907). When it infects wounded plants, the virulent bacterium transfers one of its plasmids, the tumor inducing (Ti) plasmid, into the host cell. This plasmid encodes several virulence genes that code for enzymes responsible for the transduction of the Ti plasmid into plant cells, and enable the integration in the plant genome of a region (T-DNA) located between the left- and right-border of the plasmid. This region contains 3 genes coding for homologues of plant hormones (cytokinin, auxin) and enzymes causing the plant to synthesize opines, which will serve as carbon and nitrogen sources for the bacteria. The basis of *Agrobacterium*-mediated transformation of *Arabidopsis* relies on the use of a "disarmed" Ti-plasmid, where the virulence genes have been removed and the genes located between the left- and right-borders have been replaced by the gene of interest. For example, the gene of interest can be a reporter gene and/or a selection (antibiotic resistance) gene. The T-DNA sequence inserts itself randomly into the host genome. It is possible to perform this transformation on flowers simply by soaking them into an *Agrobacterium* culture; the resulting transformed seeds can then be grown and selected *via* the inserted antibiotic resistance. Since the insertion occurs randomly, it is also possible to use this approach to disrupt a coding sequence and generate knock-out mutants. A resource center located at The Ohio State University collects, reproduces, preserves and distributes seeds of *A. thaliana* mutants for virtually every gene (Alonso *et al*., 2003) (http://www.arabidopsis.org/).

We obtained mutants for the two genes *HEN1* and *TUT1*. For *HEN1*, the line *hen1-6* was available in the laboratory. In this mutant, the T-DNA has been inserted in an exonic sequence of the gene and the resulting expression and/or functionality of the protein is strongly reduced.

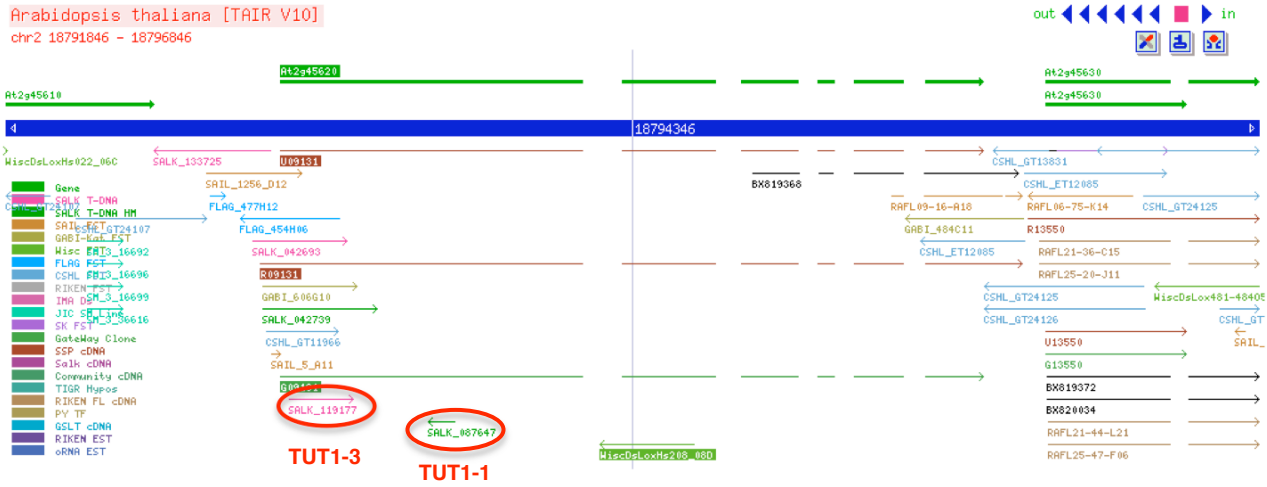


Figure 12: Localization of the T-DNA insertion in the insertion lines used to generate TUT1 / HEN1 crosses.

SIGnAL T-DNA Express, based on TAIR, version 10 (signal.salk.edu/cgi-bin/tdnaexpress.)

However, it could not be totally impaired to avoid the sterility of a complete knock-out of this gene. Indeed, the absence of HEN1 disturbs the stability of miRNAs, and of all small RNA species that are necessary for proper development (Chen *et al*., 2002).

We ordered insertion lines from the Arabidopsis Resource Center that disrupted the expression of the TUT1 gene product. Two mutants were ordered: *tut1-1* and *tut1-3*. In both cases, the T-DNA was inserted into exonic sequences, but in two different orientations: in antisense for *tut1-1* and in sense for *tut1-3* (Figure 12). Since no antibody is available for TUT1, we were not able to assess the accumulation of the endogenous protein in the *TUT1* mutants. However, analysis of the mRNA levels indicated that the *tut1-3* line might be an over-expressing line (D. Gagliardi, personal communication). Indeed, the inserted T-DNA contains a kanamycin resistance (neomycin phosphotransferase II) gene, which is under the control of its own promoter (Nopaline synthase (NOS)). It is possible that some recombination events might have triggered the excision of the NOS terminator or of part of the resistance gene, eventually leading to a constitutive expression of the *TUT1* gene from the NOS promoter. On the other hand we can be confident that the *tut1-1* insertion line is a knock-out line since the orientation of the T-DNA is antisense, thus avoiding the risk of alternative expression of the gene *via* the NOS promoter. Attempts to detect the messenger RNA indicated that its accumulation is indeed strongly reduced (D. Gagliardi, personal communication), confirming this assumption requires further ongoing analysis.

We generated double mutant lines by crossing *hen1-6* plants with either *tut1-1* plants or *tut1-3* plants by cross-fertilization of flowers. The seeds were collected, grown and the corresponding plants were genotyped by PCR analysis to verify the presence of the inserted T-DNAs.

3.1.2. Phenotypes of the different lines

All the plant lines used in this study are in a Columbia-0 ecotype background. Both *tut1-1* and *tut1-3* lines displayed the same phenotype as the Col-0 line, no particular feature was observed during the development of these plants (Figure 13). As described originally (Chen *et al*., 2002), HEN1 plays multiple roles in plant development and acts in specification of organ identity in the flower. Consistent with its broad implication in development, *HEN1* single mutants exhibit pleiotropic phenotypes during most stages of development, such as reduced organ size, altered rosette leaf shape and increased number of coflorescences (Figure 13).

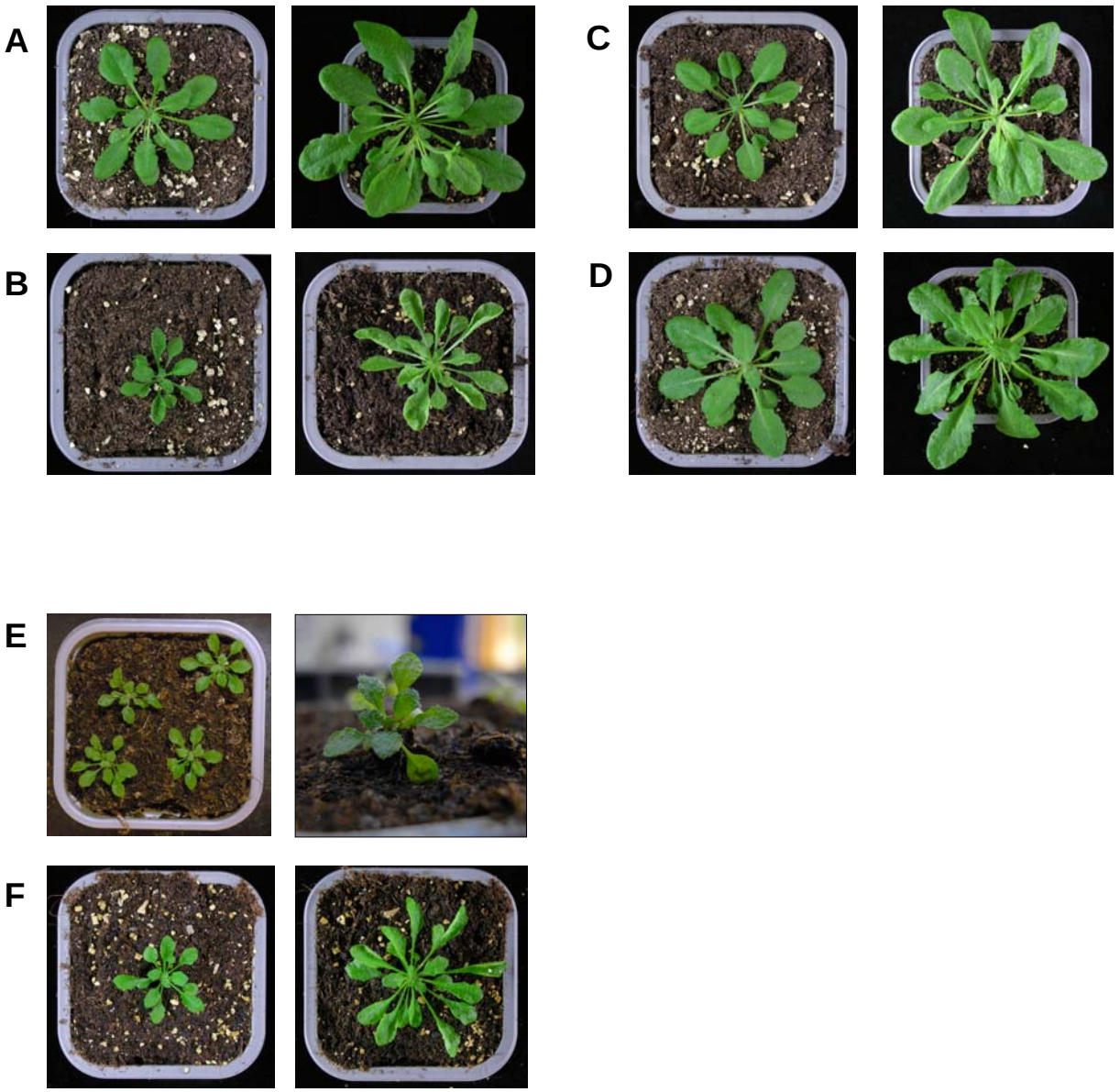


Figure 13 : Phenotypic appearance of the different plant lines used to make the cross. (A) Columbia0, (B) Hen1-6, (C) TUT1-3, (D) TUT1-1, (E) HEn1 / TUT1-3, (F) HEN1 / TUT1-1.

The *hen1-6/tut1-1* and *hen1-6/tut1-3* double mutants generated for this study did not display a phenotype different than that of *hen1-6* plants. With the *hen1-6/tut1-1* line, we expected to see a rescue of the hen1 phenotype upon deletion of a terminal uridyl transferase that could have been involved in small RNA tailing. Indeed, we postulated that the absence, or at least a reduced level of tailing, would preclude the degradation of the small RNAs and therefore rescue, at least partially, the developmental phenotypes linked to the absence of small RNA methylation. On the other hand, we thought that the overexpression of TUT1 in the *tut1-3* line would result in an aggravated "*hen1* phenotype" in the *hen1-6/tut1-3* double mutant. Unfortunately, the phenotypes observed in the double mutants were entirely similar to the phenotype of *hen1-6* single mutant plants (Figure 13).

3.1.3. Molecular analysis of small RNAs in the different genetic backgrounds

Even though we could not observe a clear difference in the phenotype of the *hen1/tut1* double mutant plants, we performed northern blot analysis of the accumulation of small RNAs (different miRNAs and one trans-acting siRNA) in different organs collected from these plants. Indeed, we could imagine that even in the absence of a macroscopic phenotype, some variations of the abundance of particular small RNAs species could provide a clue regarding the role of TUT1 in small RNAs tailing. We therefore extracted total RNA from young leaves (rosette stage) and flowers of each mutant analyzed. Due to the chronological order of events regarding the different genetic crosses, and since we first obtained the *hen1-6/tut1-3* double mutant, the analysis was first performed only with this mutant, which might be over-expressing rather than lacking TUT1.

3.1.3.1. Analysis of a *hen1-6/tut1-3* mutant

As can be seen in Figure 14 (right panel), the analysis revealed that the level of several miRNAs (miR167, miR156 and miR172) as well as the TAS3 tasiRNA accumulated as expected at comparable level in flowers from Col0, *tut1-3* and *tut1-1* plants. In the *hen1-6* plants, the accumulation of the same small RNAs is compromised. The detection of the expected ladder in the *hen1-6* plants is not clearly visible on these blots (although a smear is visible above the mature miRNA) but the accumulation of the small RNAs is strongly reduced compared to wild-type plants. Finally, the accumulation of small RNAs in the double mutant

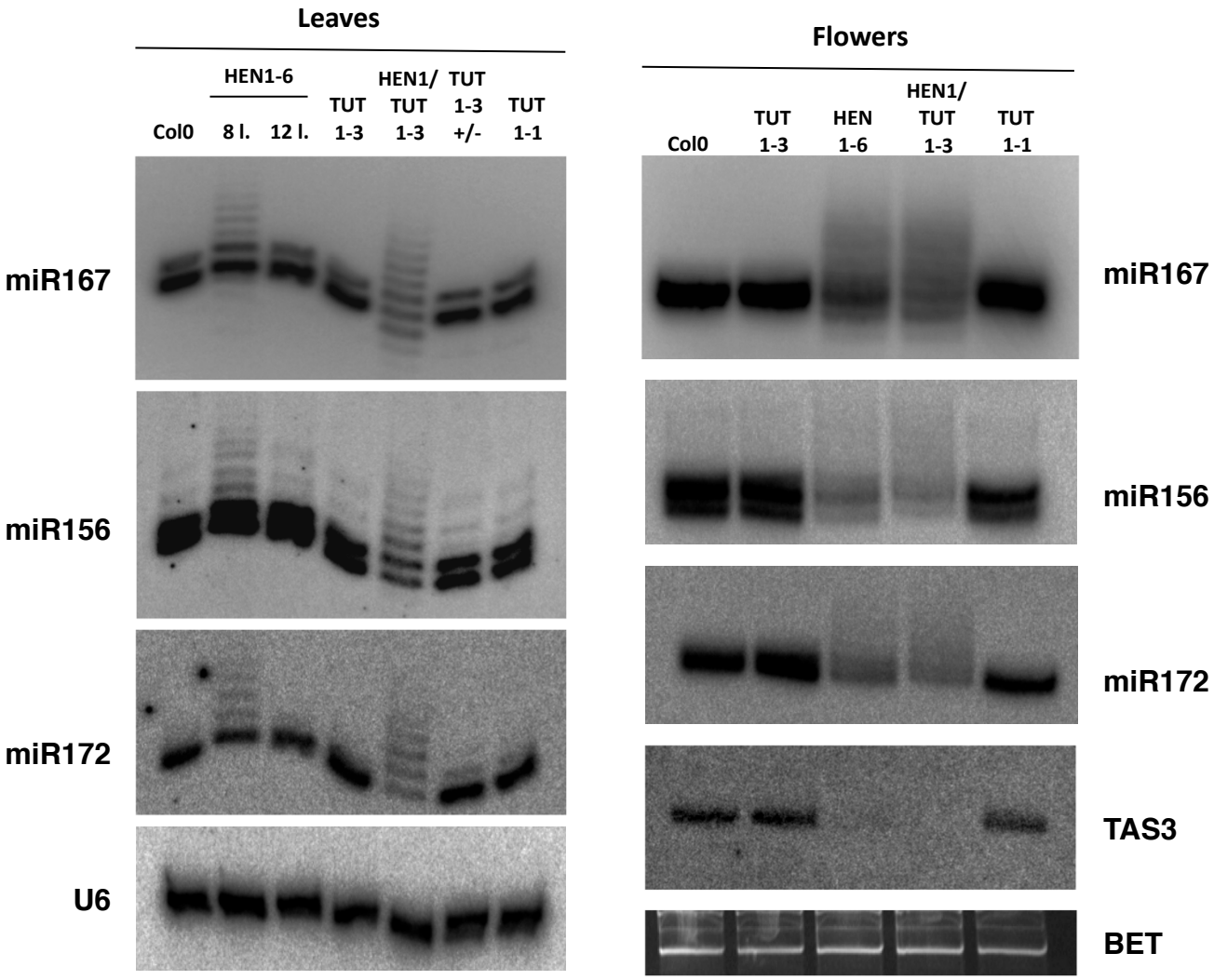


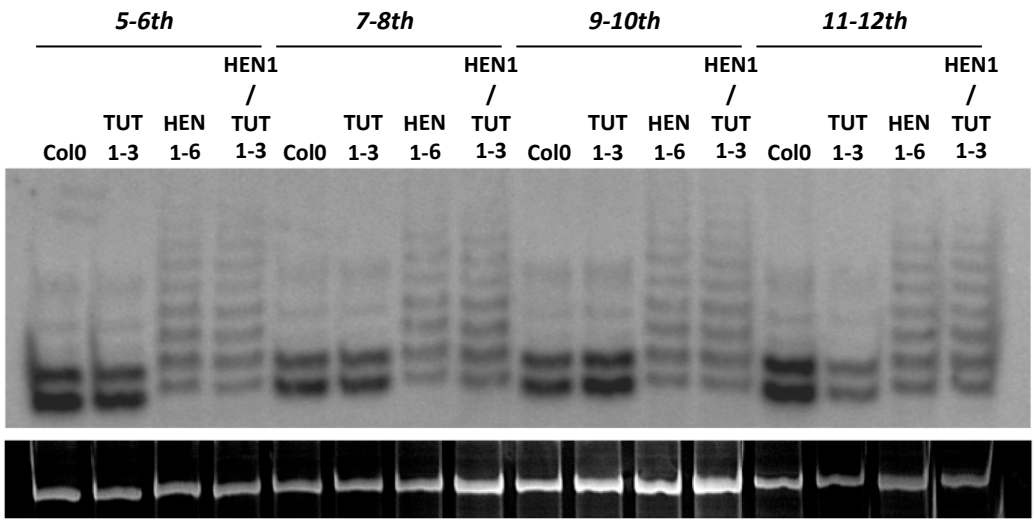
Figure 14: Pattern n°1 of expression of miRNAs in different mutant backgrounds in *Arabidopsis thaliana.*

Northern blot analysis using oligonucleotide probes for endogenous miR167, miR156, miR172 (in leaves and flowers) and TAS3 (flowers only). A loading control is provided either by U6 splicesosomal RNA probing or ethidium bromide staining of ribosomal RNA.

hen1-6/tut1-3 seems to be similar to the accumulation in the single *hen1* background, even though there seems to be a slightly lower accumulation in the double-mutant. Consistent with this observation, the level of the TAS3 tasiRNA, the biogenesis of which is dependent on a primary cleavage event triggered by miR390 (Allen *et al*., 2005), is almost undetectable in both *hen1-6* and *hen1-6/tut1-3* plants.

The miRNAs we analyzed all play well-described roles in *Arabidopsis* development. MiR156 is known to target a member of the squamosa promoter binding protein-like (SPL) transcription factor family. It regulates many developmental timing events like shoot development (Wu and Poethig, 2006), trichome distribution (Yu *et al*., 2010b) and flowering (Wang *et al*., 2009b). The juvenile-to-adult transition is correlated with an increase abundance of SPL3 mediated by a correlated decrease in miR156 levels (Wu and Poethig, 2006). Interestingly, the developmental timing of *Arabidopsis thaliana* is sequentially regulated by both miR156 and miR172. The decrease in miR156 accumulation also triggers an increase in the expression of another one of its targets, SPL9, which in turn directly promotes the expression of miR172b (Wu *et al*., 2009). MiR167 has been reported to target auxin response factors (ARFs) 6 and 8, and to be implicated in the control of flower maturation. Indeed, enhanced expression of miR167 leads to an increase in plant fertility of both ovules and anthers (Ru *et al*., 2006; Wu *et al*., 2006).

Given that these miRNAs are subjected to fine regulation of their accumulation during plant development, we also checked their accumulation in young vs. older leaves. Leaves were sampled from rosettes with a total leaf number of 8 or 12. For all genetic backgrounds, the level of accumulation of miR167, 156 and 172 was consistent with the observations made on flowers samples (Figure 14 left panel). However, we observed that the levels of miRNAs in the *hen1-6* simple mutant were strikingly higher than expected. In addition, the characteristic ladder usually seen in *HEN1* mutant plants could only be seen in 8-leaves stage plants. The presence of the T-DNA insertion in these plants has been confirmed by PCR genotyping. The stabilization of miRNAs levels in a *HEN1* genetic background is really an unexpected observation. Although most published data report on the effect of *HEN1* mutation on miRNA levels in flowers, it seems surprising that such a phenotype was never observed before. The only study that reported a somewhat similar observation was made in the laboratory of Xuemei Chen when they performed a screen for suppressors of the partial loss-of-function *hen1-2* allele (Yu *et al*., 2010a). In this screen, the authors identified mutations in subunits of the DNA-dependent RNA polymerase IV (NRPD1 or 2), which is essential for the biogenesis of 24 nt endogenous siRNAs, as well as in the RNA-dependent RNA polymerase 2 (RDR2), also essential for endogenous siRNA biogenesis. They showed that these mutations rescued the



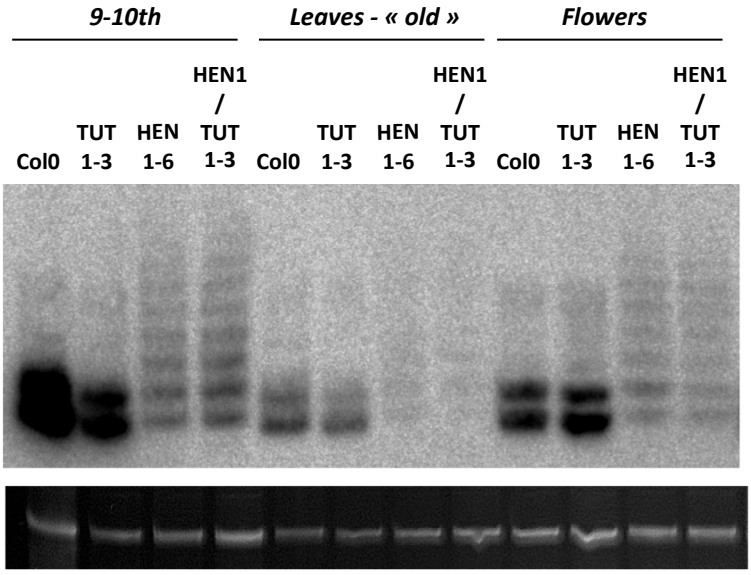


Figure 15: Pattern n°2 of expression of miRNAs in different mutant backgrounds in *Arabidopsis thaliana.*

Northern blot analysis using an oligonucleotide probe for endogenous miR172 in leaves of various age and flowers. A loading control is provided by ethidium bromide staining of ribosomal RNA.

methylated state of miRNAs and their accumulation, but only in the particular *hen1-2* background. They proposed that, in *NRPD1* or *RDR2* mutants, the absence of endogenous siRNAs (which represents more than 80% of the cellular small RNA population in wild type background and are methylated), could allow the remaining HEN1 protein production to efficiently methylate miRNAs, and thus protect them from uridylation and degradation. However, they were not able to reproduce this result in a stronger mutant allele of *HEN1*, like *hen1-1*, because the amount of remaining HEN1 protein was too low. Therefore, it could be that the discrepancy between leaves and flowers in our experiments is due to a difference in the accumulation of siRNAs, resulting in the increased availability of residual HEN1 to methylate miRNAs. This hypothesis remains highly hypothetical and we have no means to verify it other than performing deep-sequencing analysis of various tissues in different genetic backgrounds. In addition, the reason for this observation could be more complicated since we had difficulties in reproducing it (see below).

What was also striking in this particular situation was that in the *hen1-6/tut1-3* double mutants, we retrieved the expected accumulation pattern for the miRNAs analyzed (Figure 14 left panel, lane 5). The levels of the mature miRNAs were significantly weaker, and we could clearly see the accumulation of a ladder corresponding to the tailed miRNAs. This result is of special interest if we postulate that the TUT1 protein is overexpressed in the *tut1-3* mutant. We could hypothesize that this enzyme could compete with HEN1 for accessibility to miRNAs, in a situation were HEN1 is limiting, overexpression of TUT1 would then result in more efficient uridylation of miRNAs. This result has been observed in several technical and biological replicates of the experiment, but as we will see now, it was difficult to reproduce it in all independent biological replicates performed.

To check the possibility of a highly regulated developmental phenotype, we performed the same experiment, but instead of sampling all leaves of a plant at a defined stage, we only evaluated the accumulation of a miRNA in young leaves at several developmental stages. We thus harvested from different plants leaves 5 and 6, 7 and 8, 9 and 10, 11 and 12, “old” leaves and flowers. Unfortunately, in this experiment the molecular analysis of miR172 revealed no difference between *hen1-6* and *hen1-6/tut1-3* plants (Figure 15). Therefore, the reason for our initial observation remains unknown.

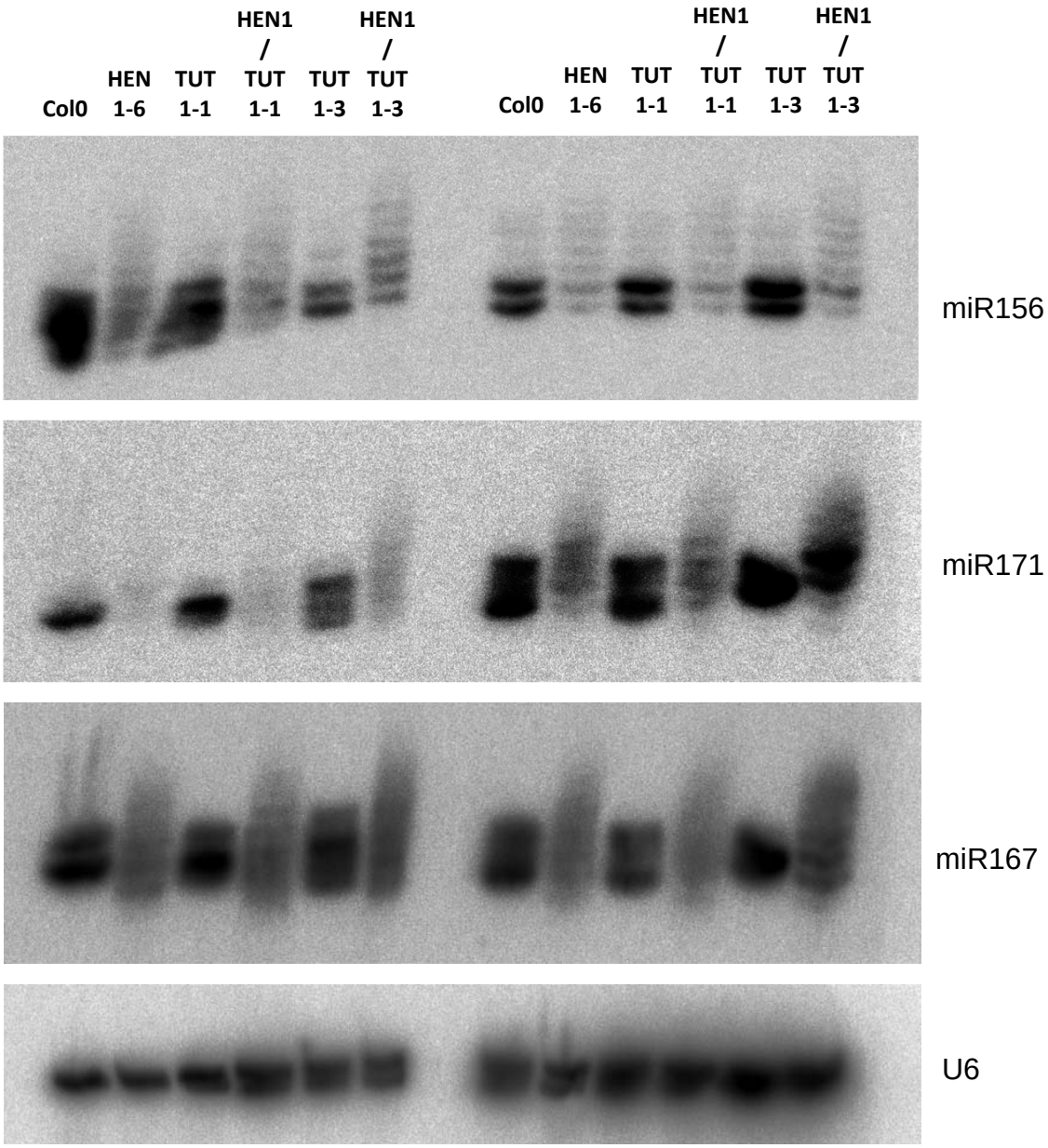


Figure 16: Northern blot analysis of miRNAs in leaves of TUT mutants using oligonucleotide probes for endogenous miR156, miR171 and miR167. A loading control is provided by U6 splicesosomal RNA probing.

3.1.3.2. Analysis of a *hen1-6/tut1-1* mutant

Finally, to clearly assess the role of TUT1, we performed similar molecular analysis using the *tut1-1* allele, which, according to the RNA analysis, is a true knock-out mutant. The northern blot analysis of miR156 and miR171 performed on RNA extracted from *tut1-1* plants did not reveal striking difference with Col0 plants. The double mutant *hen1-6/tut1-1* behaved similarly to that of the *hen1-6* plant, and did not show a reversal of miRNA regulation (Figure 16). From this experiment, we can therefore conclude that TUT1 does not seem to be involved in miRNA tailing in hen1 mutant plants.

One major limitation of this approach is the high probability that other members of the small RNA terminal nucleotide transferase family could act redundantly with TUT1, and would compensate for its activity when it is not expressed. This issue could have been solved by the analysis of a real overexpressor line, but further work is needed to formally prove that the *tut1-3* mutant overexpresses TUT1 at the protein level.

3.2. Role of the mouse Zcchc6 in miRNA activity

3.2.1. Description of the luciferase sensors and principle of luciferase assays

As a means to obtain insights into the involvement of the Zcchc6 protein in miRNA activity, we used an RNAi approach coupled to a luciferase-based miRNA reporter system. In this approach, the 3'UTR of the firefly luciferase gene is fused to a sequence perfectly complementary or to a sequence with 3 mismatches to a given miRNA. To facilitate the generation of such sensors, a gateway-cloning cassette (Invitrogen) was previously introduced into the commercially available dual luciferase-expression plasmid psiCHECK2 from Promega (Figure 17 A and see details in the material and methods section). The reporter expresses two luciferase enzymes, the firefly luciferase, which will be regulated by the miRNA of interest, and the renilla luciferase, which will serve as an internal reporter for transfection efficiency. The level of expression of the two proteins is assayed by measuring the luminescence emitted after addition of the dedicated substrates for both luciferases to a total cell lysate. HEK293 cells were cotransfected with luciferase sensors containing no binding site, a perfectly matching site or an imperfectly matching site for the MCMV-miR-M23-2 (cf. Figure 17 B), the corresponding miRNAs as duplexed oligonucleotides, and siRNAs against Zcchc6 or a negative control.

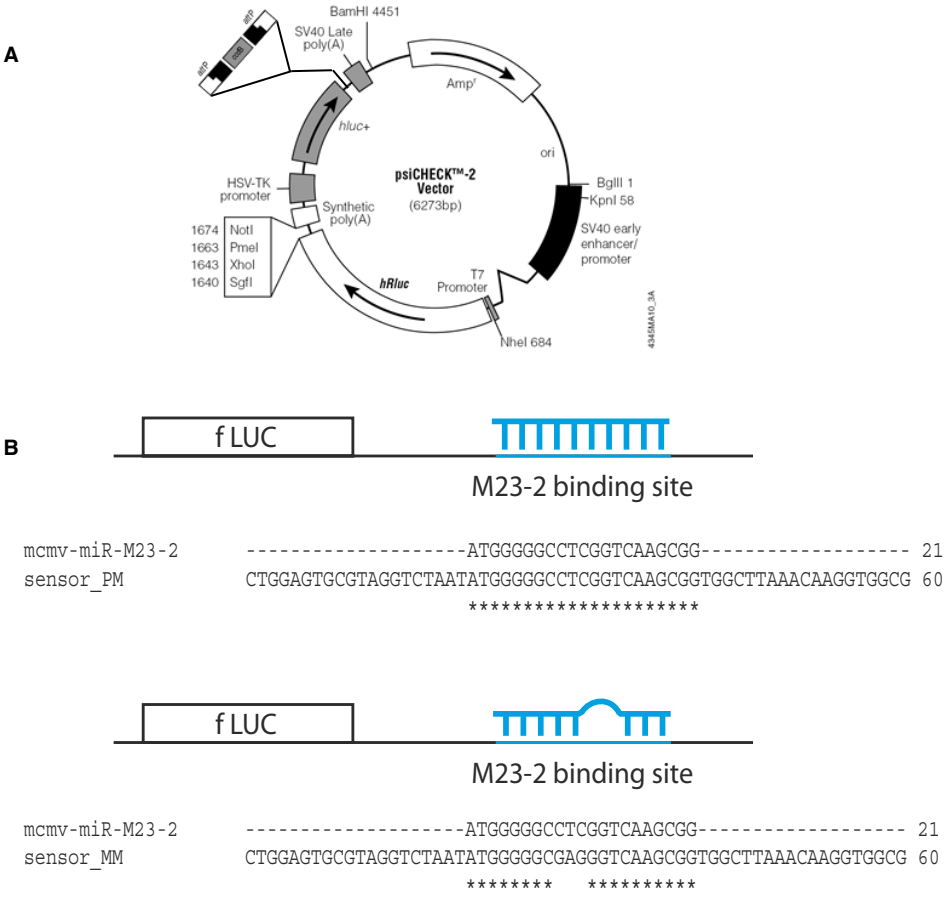


Figure 17 : Luciferase vector (A) and pairing of the small RNA on the 3'UTR of firefly luciferase transcript in the case of the perfect match sensor as well as the mismatch sensor (B).

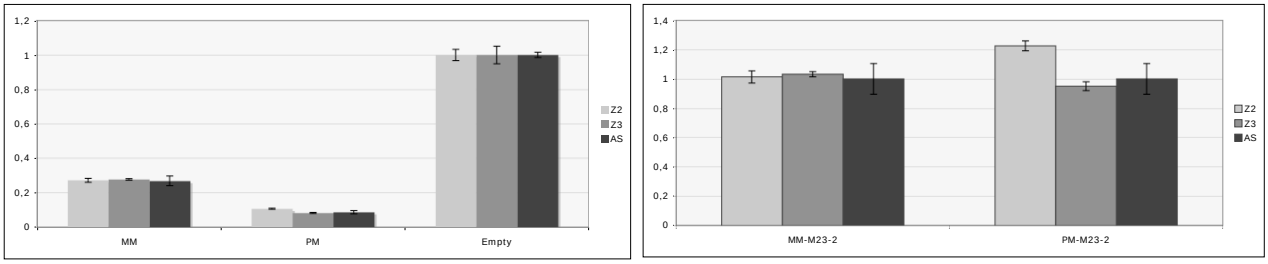


Figure 18 : Zcchc6 knockdown in HEK293 cells and luciferase assay of miRNA activity.

PsiCHECK2 sensors, miRNA mimic were transfected in HEK293 cells. siRNA against Zcchc6 or control siRNA were pre-transfected. Relative luciferase units (RLU) are represented and indicate the relative luminescence of the firefly and renilla luciferases normalized to an empty vector control (left panel). RLU normalized to the control siRNA condition are represented in the right panel.

3.2.2. Effect of Zcchc6 knock-down on the luciferase sensors

We performed the luciferase assay to monitor the activity of a miRNA mimic of miR-M23-2 in the presence of two different siRNAs directed against Zcchc6 (Z2 and Z3) or a control siRNA (AS). The commercially available control siRNA was purchased from QIAGEN and has been designed such that it does not target anything in the human genome. The two specific Zcchc6 siRNAs were also purchased from QIAGEN, and target the 3'UTR of the Zcchc6 transcript. In the control condition (AS siRNA), the presence of the miRNA mimics targeting the perfect match (PM) or mismatched (MM) sensor leads to a strong down-regulation of the firefly luciferase activity compared to the non-targeted Renilla luciferase (Figure 18, left panel). If the knock-down of Zcchc6 by siRNAs worked, and if Zcchc6 was involved in the destabilization of the transfected miRNA, then we could expect to see a stronger regulation of the firefly luciferase. However, we did not observe any significant difference in either sensor regulation by miR-M23-2 in the presence of Z2 and Z3 siRNAs, despite several replicates of the experiment. Figure 18 right panel, illustrates one of the experiments where the relative luciferase units (RLU) have been normalized to the control siRNA condition. There are several technical problems associated with this approach. For example, measuring an increased targeting when the basal regulation is already quite strong (almost 90% for the perfect match sensor) might prove difficult. In addition, there is no antibody available for the Zcchc6 protein, making it difficult to control for the efficiency of knock-down. For all these reasons, we decided to turn to an alternative approach in which we would overexpress the Zcchc6 protein instead of trying to deplete it.

3.2.3. Overexpression of Zcchc6

We obtained a cDNA IMAGE clone containing the greater part of the Zcchc6 coding sequence and cloned it into a mammalian constitutive expression vector where the protein is fused to a FLAG-HA tag located at its N-terminal (Nterm) end and a V5 epitope at its C-terminal (Cterm) extremity. Unfortunately, we were unable to clone the full-length coding sequence as described in the Pubmed accession NM_153538 (Figure 19) and no cDNA clone was available at that time. Even though the absence of one part of the protein could obviously jeopardize the identification of functional roles of the protein, the part we cloned contained the putative catalytic domain. A second nucleotidyl transferase domain is actually encoded at the Nterm of the protein but the lack of the aspartate triad necessary for cation coordination indicates that

>gi|254588107|ref|NM_153538.3| Mus musculus zinc finger, CCHC domain containing 6 (Zcchc6), mRNA

GGCTGAAGGAAAACACGGTGAGGAGCCGGGCCCGGACAGGAAGCGGCGGGGGACCGCAGCGGCGCTGGTA
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AATGAACTTCACAAGCCTTTCTGAAATAGTACAGAGTCAGAACTATGCCGGTTTATTTATCATTTTGAAA
TAAAATCGAACTTTGAACGTGAAAAAAAAAAAAAAAAA

Figure 19 : Zcchc6 coding sequence.

Sequence highlighted in grey has been cloned in the expression vector. The 5' and 3' UTRs are highlighted in yellow and the catalytic triad in red.

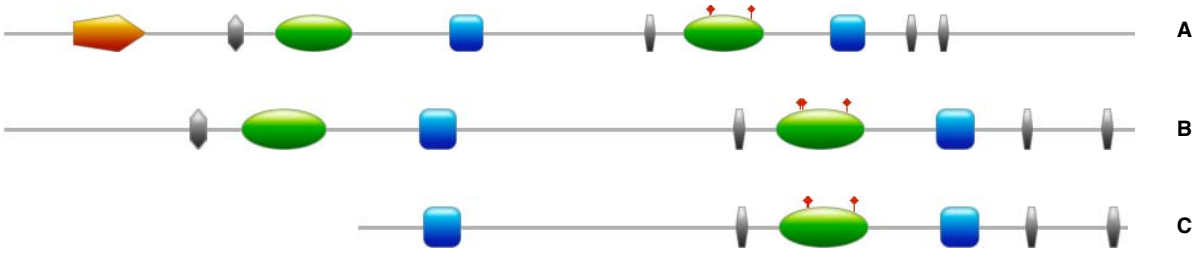

Figure 20 : Schematic representation of the domains encoded by Zcchc11 gene (A), Zcchc6 gene (B) and in the expression plasmid of Zcchc6 (C). The colour legends are the same as used in Figure 10 and the catalytic triads are indicated with red flags.

only the Cterm is catalytically active (Figure 20). Members of this protein family seem to have an intrinsic low stability in their N-terminal part. Jones and collaborators faced the same issue when trying to clone the Zcchc11 gene (Jones *et al*., 2009), and they confirmed that only the second catalytic domain of the protein is active (Figure 20). Considering the high level of conservation of the C-terminal part, we may be confident in the reality of the catalytic properties of the enzyme without ignoring a potential role of the N-terminal part of this protein to act as a docking platform for eventual co-factors or to regulate its enzymatic activity. For all these reasons, we therefore decided to further evaluate the role of this truncated protein.

We first verified that our expression plasmid was functional by checking the expression of the recombinant protein after transfection of HeLa and HEK293 cells. We performed a western blot analysis using an anti-HA antibody (Figure 21). The calculated size of the protein is around 110kD, which is consistent with the detection of a major band at approximately 140 kDa. The discrepancy between the expected and the observed size is likely due to the presence of the tags as well as eventual post-translational modifications.

We then performed the same luciferase reporter analysis described above in the presence of the Zcchc6 expression plasmid. In this case, we used two different strategies. First, we co-transfected in HEK293 cells the miR-M23-2 luciferase sensor together with miR-M23-2 or control miRNA mimics (*C. elegans* miR-67). Second, we used a sensor for an endogenous miRNA, miR-21, and co-transfected HeLa cells with 2'O methylated antisense oligonucleotides directed against miR-21 or a control miRNA (cel-miR-67). As a negative control for the Zcchc6 expression plasmid, we first used an empty vector, but due to the low reproducibility of our results, we generated a better control in the form of a catalytically inactive mutant version of the pDEST-Zcchc6 expression vector. To this end, we mutagenized each residue of the catalytic triad as described in Jones *et al*. by replacing the aspartic acids by alanines (Figure 22 and 23) (Jones *et al*., 2009). Forty-eight hours after transfection, the cells were lysed and the luciferases activities were measured.

We first assessed the effect of Zcchc6 expression on the regulation of the luciferase sensors PM miR-M23-2 and MM miR-M23-2 described previously, as a negative control we used a sensor with no site for miR-M23-2 inserted. We transfected these sensors in HEK293 cells together with miR-M23-2, or control miR-67 mimics with expression plasmids for the wild type (WT) or mutant (MUT) Zcchc6. As expected, a significant regulation of both the perfect match and

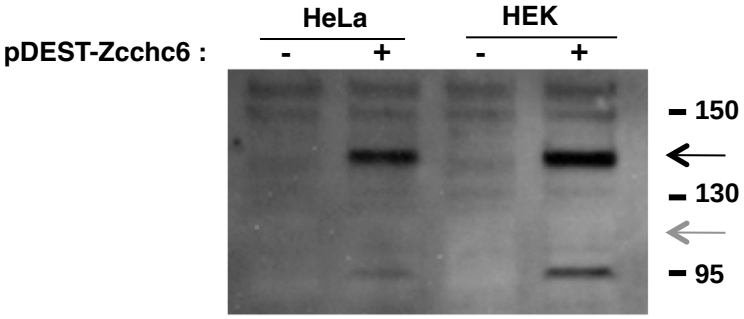

Figure 21 : Western blot detection of the fusion protein FLAG-HA-Zcchc6 transiently expressed in HEK cells and HeLa cells.

Black arrow indicates the signal corresponding to the FLAG-HA-Zcchc6 protein and grey arrow the expected signal.

Jones et al. Nature Cell Biology, 2009

Figure 22 : Conservation of the catalytic triad in Zcchc11 proteins from several species.

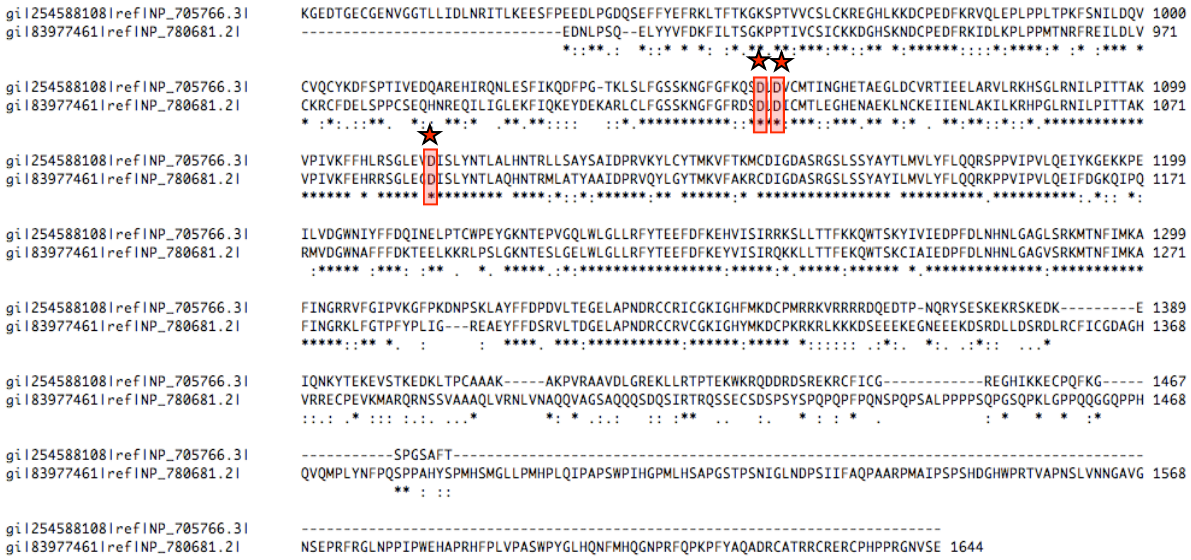

Figure 23 : Alignment of Zcchc6 and Zcchc11 proteins showing the conservation of the catalytic triad.

The three catalytic aspartic acids that were mutated to alanine are indicated by red stars.

mismatched sensors was observed only in the presence of the miR-M23-2 mimic, but not when the control miR-67 mimic was used. The down regulation was about 90% in the case of the perfect-match sensor for miR-M23-2 and about 70% in the case of the mismatch sensor. When measuring the effect of the over expression of Zcchc6, we observed that the firefly luciferase regulation by miR-M23-2 was reduced in the presence of the WT Zcchc6 when compared to the MUT Zcchc6 (Figure 24). The fold repression of the perfect match sensor by miR-M23-2 compared to miR-67 was 6-fold when the WT Zcchc6 was expressed, and 9-fold when the MUT was used. With the mismatch sensor, these values were 2 and 3-fold respectively. This effect could be observed in multiple independent experiments, of which two are shown in figure 24. These results indicate that over-expression of a catalytically active form of this enzyme might trigger either the degradation of a transfected miRNA or prevent its activity.

We then used a perfect match sensor for the cellular miR-21 that was transfected in HeLa cells rather than in HEK293 cells, as HEK293 cells do not highly express miR-21. Due to the high expression level of miR-21 in HeLa cells, miRNA mimics were not needed for this experiment. Instead, the "reverse" experiment was performed, i.e. we looked at the effect of Zcchc6 expression on the inhibitory effect of a 2'O methylated antisense oligonucleotide directed against miR-21. To this effect, the luciferase sensor for miR-21 was co-transfected in HeLa cells, along with 2'O methylated oligonucleotides against miR-21 (or the control miR-67) and with the expression plasmid for either WT Zcchc6 or MUT Zcchc6. As can be seen in figure 25, the regulation of the miR-21 sensor in the presence of the control miR-67 inhibitor was unchanged when either the WT or the MUT Zcchc6 was co-expressed. However, the inhibition of the luciferase regulation by the anti-miR-21 was more effective when the WT form of Zcchc6 was expressed. We estimated that the derepression by the miRNA inhibitor was 5-fold in the presence of a catalytically active Zcchc6 and only 4-fold when the mutated enzyme was used (Figure 25). It was published recently that transfection of a 2'O methylated antisense oligonucleotide could lead to tailing and trimming of the targeted miRNA (Ameres *et al.*, 2010). The fact that the overexpression of Zcchc6 enhances the inhibitory effect of an inhibitor against miR-21 could therefore imply that miR-21 is more efficiently tailed and therefore degraded. Because there was no measurable effect of Zcchc6 expression on the luciferase sensor regulation in the absence of the miR-21 inhibitor, we can conclude that the expression of the perfect match sensor alone is not sufficient to trigger tailing of the miRNA. This is probably due to the low level of the luciferase transcript compared to the high number of anti-miRNA molecules transfected. The missing experiment here would be to perform a northern blot

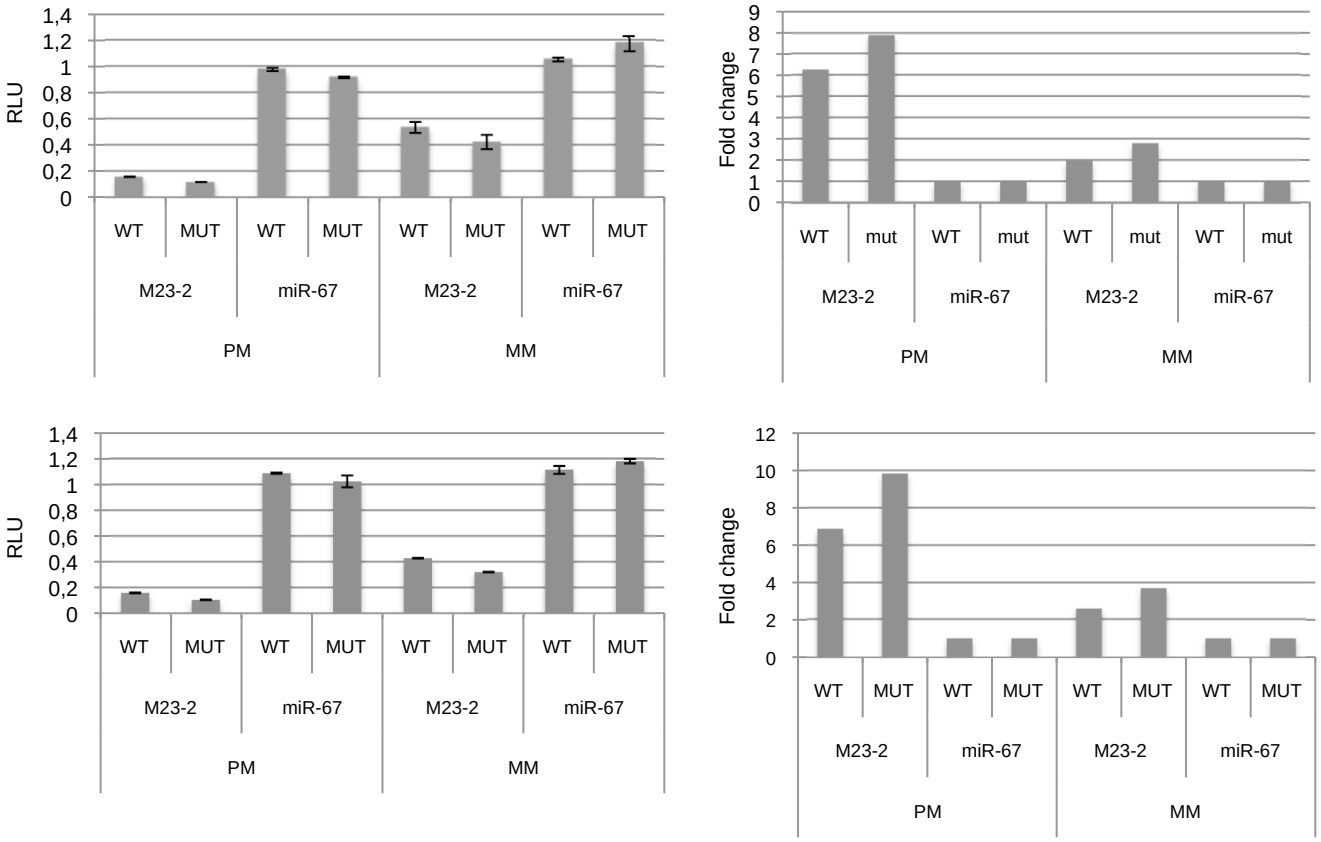

Figure 24 : Activity of a mimic of miRNA on perfect-match (PM) and mis-match (MM) luciferase sensors upon expression of a wild-type (WT) or mutated (MUT) version of Zcchc6 in HEK 293 Grip.

Two independant experiments are represented in this figure (upper and lower lanes). Relative luciferase unit (RLU) of firefly and renilla luciferases are represented after normalization to an empty sensor (left panels). Fold change repression in the presence of miR-M23-2 mimic compared to the control condition (miR-67 mimic) is represented for each condition (right panels).

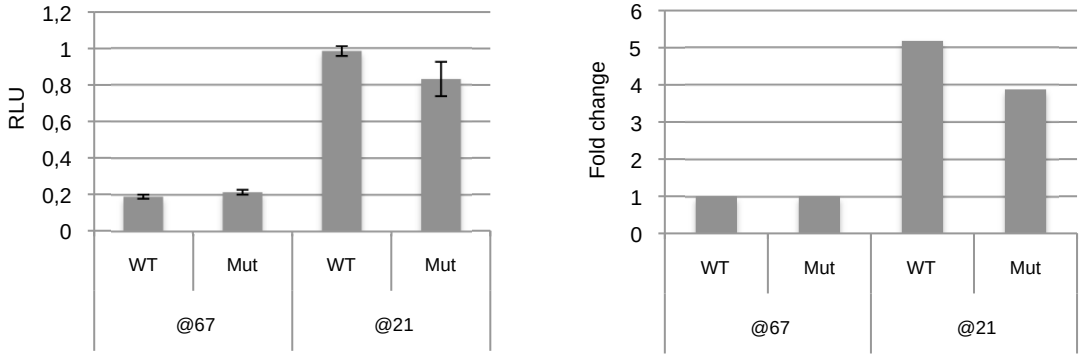

Figure 25 : Activity of an endogenous miRNA (miR-21) on a perfect-match luciferase sensor in HeLa cells. The luciferase activities were measured upon expression of a wild-type (WT) or mutated (MUT) version of Zcchc6 and in the presence of 2'O methyl antisens oligonucleotides against miR-21 (@21) or against a control miRNA, miR-67 (@67).

analysis of miR-21 in presence of the 2'O methylated oligonucleotide and with the WT or MUT Zcchc6 to see whether there is indeed a difference in tailing. We obtained preliminary data where we could confirm the degradation of miR-21 in presence of the anti-miRNA inhibitor, but we could not visualize a significant difference when Zcchc6 was co-expressed (data not shown). We need to repeat this analysis and to look at earlier time point to be able to see the tailing of the miRNA.

Chapter 2

Regulation of miR-27 by the mouse cytomegalovirus requires the abundant viral transcript m169 and the cellular protein HuR

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The next chapter will be presented as a draft for a manuscript that will be submitted soon.

1. Abstract

MicroRNAs (miRNAs) are small non-coding RNA molecules that play important regulatory roles in viral infections through their capacity to regulate the expression of both host and viral genes. We previously reported that during murine cytomegalovirus (MCMV) infection, the cellular miRNA miR-27a (as well as its isoform miR-27b) was strongly down-regulated. Furthermore, overexpression of these miRNAs has a negative impact on viral replication (Buck *et al*., 2010). Here, we show that MCMV encodes for a newly characterized transcript (m169), which is responsible for the targeting and rapid degradation of these cellular miRNAs during lytic infection. This function is mediated *via* a single binding site for miR-27, resulting in a modification of the mature miRNA within 4 hours of infection; accompanied by a 2-fold reduction in the level of the mature miRNA. Strikingly, following 2 days of infection, miR-27 is barely detectable by northern blot. Interestingly, this effect can be efficiently redirected to other miRNAs by modifying the respective miRNA binding site on the viral transcript. Moreover, we identified the cellular protein HuR as a putative candidate responsible for m169-mediated degradation of miRNAs.

It is well known that herpesviruses express a range of molecules, from proteins to small RNA, to modulate the cellular environment. Our findings highlights yet another viral strategy, the use of a long RNA to deregulate host miRNA expression.

2. Introduction

RNA silencing is a widespread phenomenon involved in the regulation of gene expression and is implicated in a broad range of biological pathways. This mechanism, present in organisms ranging from fungi to mammals, systematically relies on the presence of a small non-coding RNA of about 22 nucleotides (nt) in length. The small RNA regulates gene expression either *via* translation inhibition and/or destabilization of the targeted transcript after its assembly in a complex that invariably contains a member of the Argonaute protein family (Hock and Meister, 2008). In mammals a large part of these RNA silencing processes are mediated by the action of microRNAs (miRNAs). To date, more than 700 miRNAs have been identified in mouse, this figure stands at 1400 in humans (Kozomara and Griffiths-Jones, 2011). The biogenesis of miRNAs is a stepwise process where by an RNA pol II transcribed primary transcript is processed by the sequential action of the RNases III enzymes Drosha and Dicer. One strand of

the duplex is then loaded into an RNA-induced silencing complex (RISC), which can then bind to its target messenger RNA to regulate its translation (for review, see (Eulalio *et al*., 2008)). The other strand of the duplex, known as the passenger strand or star sequence (miRNA*), is often, but not always, actively degraded, while the loaded miRNA remains stable with a half-life in the range of days (Gatfield *et al*., 2009).

Although a picture of the mechanisms by which a miRNA regulates its target begins to emerge, much less is known about the regulation of small RNAs themselves. This regulation has been described to occur at the level of transcription, processing, or stability (reviewed in (Krol *et al*., 2010)). Several factors, which play a role in the regulation of miRNA transcription or processing, especially at the Drosha cleavage level have been identified, but information regarding the regulation of miRNA stability is much scarcer. Thus, it has been extensively reported that for example, the regulation of the maturation step of the let-7 precursor is subject to regulation via the interaction of the Lin28 protein with its terminal loop. After binding to the pre-miRNA, the Lin28 protein recruits the terminal uridyltransferase Zcchc11 protein, which will mediate tailing of the 3' end of the small RNA (Hagan *et al*., 2009; Heo *et al*., 2008; Lehrbach *et al*., 2009). The modification of small RNAs by nucleotide addition is not only observed for pre-miRNAs, mature miRNAs can also be readily modified. This has been initially reported in the plant model *Arabidopsis thaliana*, where miRNAs are usually methylated at their 3' end by the methyl transferase HEN1 (Yang *et al*., 2006b). In a HEN1 mutant background, the absence of the 2'O methyl group on small RNAs triggers their 3' end tailing, usually by uridylation to bring about their degradation (Li *et al*., 2005). It has thus been proposed that the methyl group on plant small RNAs actually acts as a protection against uridylation and subsequent degradation.

The regulation of miRNA expression is a natural response to biotic and abiotic stresses (Leung and Sharp, 2010). Among the former, viral infections represent a prominent part and are known to interact extensively with the RNA silencing machinery. In mammals, this is best exemplified by the hijacking of miRNAs by viruses, especially herpesviruses (Pfeffer *et al*., 2005). The use of small non-immunogenic RNA molecules to regulate their own set of genes but also to modify cellular gene expression to their own advantage is yet another illustration of the powerful adaptability of viruses. In addition to the expression of miRNAs, viruses also interact with cellular miRNAs. For example, miR-32 plays an antiviral role upon primate foamy virus (PFV) infection (Lecellier *et al*., 2005), and both miR-24 and miR-93 negatively regulate the vesicular stomatitis virus (VSV) (Otsuka *et al*., 2007). It is therefore not surprising that the expression level of some cellular miRNAs is deregulated during viral infection. We previously

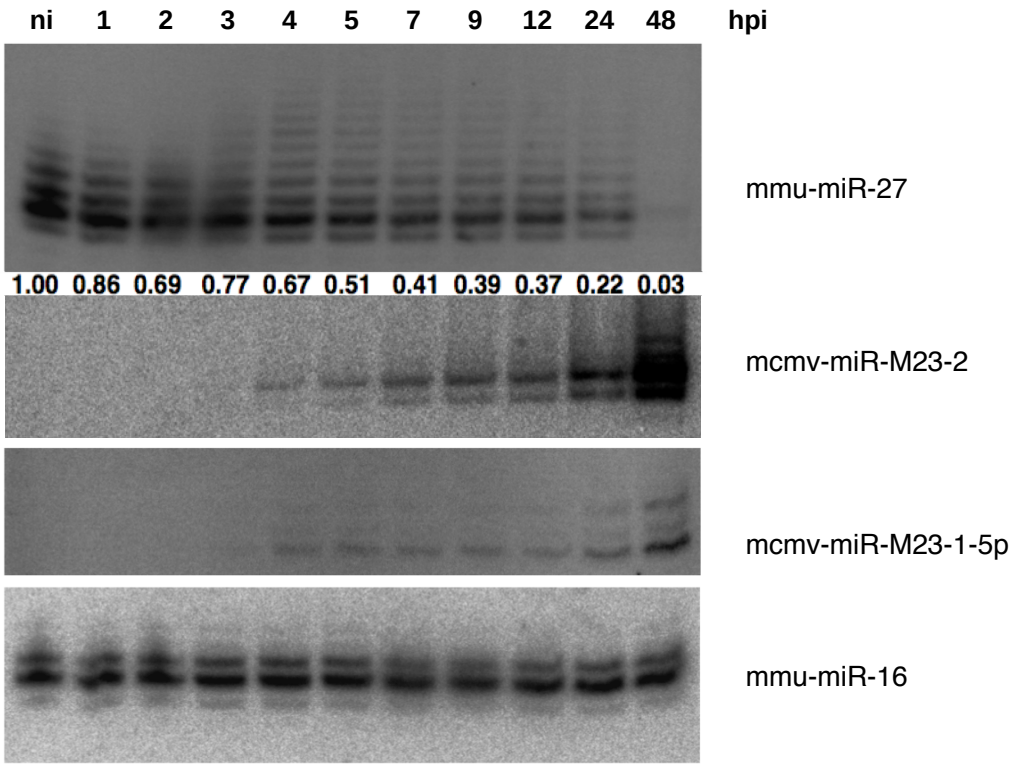


Figure 26: Detection by northern blot of the accumulation of viral and cellular miRNAs upon MCMV infection in 3T3 cells.

The relative quantification of the miR-27 major band, compared to the non-infected (ni) sample, is indicated under the corresponding lane.

showed that the cellular miRNA profile is not dramatically impacted upon following mouse cytomegalovirus (MCMV) infection. However, among the few miRNAs that show a significant alteration we found that one of them, miR-27a, almost disappears after 3 days of infection (Buck *et al*., 2010). Interestingly, we also reported that this miRNA seems to negatively impact MCMV replication *via* an unknown mechanism. MiR-27a is encoded within a miRNA cluster located on chromosome 8, along with miR-23a and miR-24-2. It possesses an isoform, miR-27b that is located on chromosome 13 and clustered with miR-23b and miR-24-1. Strikingly, only the level of the mature forms of miR-27a and 27b are impacted upon by the infection (Buck *et al*., 2010), and therefore it seems that the regulation has to occur at the level of stability of the mature miRNA. Given the fact

that blocking active transcription with Actinomycin D inhibits the destabilization of miR-27a, we hypothesized that a transcript either of viral origin, or a cellular transcript induced upon infection may be responsible for miR-27a degradation.

The role of the association between a small RNA and its target to mediate the degradation of the small RNA has been described recently. Ameres and colleagues found that the pairing of a miRNA to its target was responsible for its tailing and trimming (Ameres *et al*., 2010). Here, we report on the identification of a viral transcript involved in miR-27 binding and destabilization, and of the cellular factor likely to be involved in this mechanism.

3. Results

3.1. miR-27 is tailed and degraded upon MCMV infection

In order to get insights into the mechanism that triggers miR-27a degradation, we first looked if the post-transcriptional miRNA degradation was linked to modification of the mature miRNA. We performed a time course of MCMV infection of 3T3 fibroblasts. Cells were infected at a multiplicity of infection (MOI) of 10, and total RNA was harvested for northern blot analysis at 3, 5, 7, 9, 12, 24 and 48 hours post infection (hpi). As already described before, the level of miR-27a started to drop as soon as the infection is set up, leading to a diminution of the major form of miR-27a of about 50% after 5 h of infection, and 80% after 24 h (Figure 26). By assessing the accumulation of a control cellular miRNA, miR-16, we confirmed that MCMV infection does not lead to a general down regulation of the miRNA pathway in the cell. In parallel, we also measured the accumulation of virally encoded miRNAs (mcmv-miR-M23-2 and mcmv-miR-M23-1-5p). These two miRNAs accumulated as expected according to their

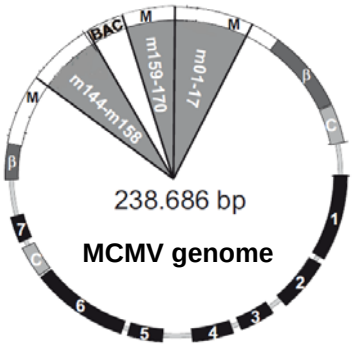


Figure 27: Schematic representation of the MCMV genome as present in the bacterial artificial chromosome (BAC).

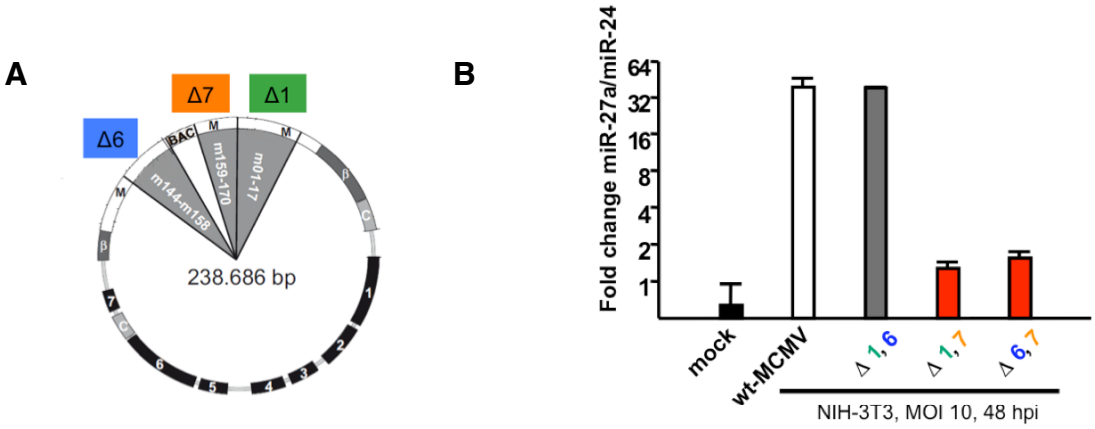


Figure 28: Effect of large deletion on miR-27 regulation by MCMV.

(A) Schematic representation of the deletion mutants of MCMV genome realized .

(B) The fold change represents the fold repression of miR-27 compared to the level of miR-24 as evaluated by qPCR on small RNAs.

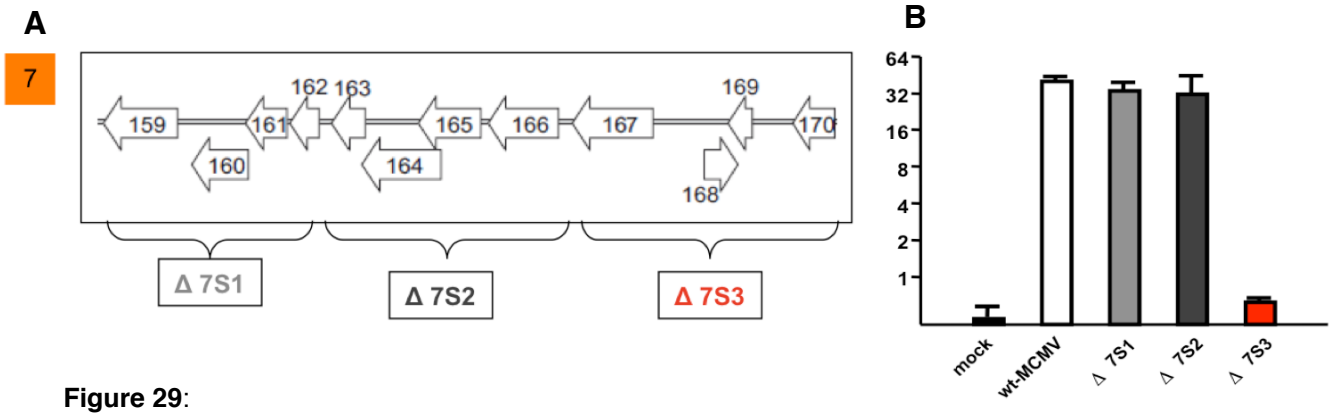


Figure 29:

(A) Schematic representation of the m159-m170 region of the MCMV genome and indication of the deleted region in three different mutant viruses analized.

(B) The fold change represents the fold repression of miR-27 compared to the level of miR-24 as evaluated by qPCR on small RNAs.

kinetics of expression (Buck *et al*., 2007; Dolken *et al*., 2007). Interestingly, the use of a locked nucleic acid (LNA) modified radiolabeled probe to detect miR-27 revealed the accumulation of several length isoforms (Figure 26). Based on the literature, it is very likely that these isoforms are due to heterogeneity at the 3' end of the miRNA. Two different reasons can explain this variability at the 3' end. In the first case, the cleavage event generating the 3' end is not precise, either at the level of Drosha processing if the mature miRNA is located on the 3' arm of the precursor, or at the level of Dicer processing if the mature miRNA is located on the 5' arm. In this case, the extra nucleotides found at the 3' extremity are of genomic origin. The second case occurs when non-templated nucleotides are added at the 3' extremity. These nucleotides are not encoded by the genome and are dependent on the catalytic activity of an enzyme. It is worth noting that miR-27a already shows several length isoforms in uninfected cells. The observation that the size of these isoforms increased over time indicates that at least partially, the tail might be composed of non-templated nucleotides added after miRNA processing. Due to the rapidity of appearance of the tailed forms of miRNAs, it is very unlikely that the modification of the maturation step could explain this phenomenon. According to the northern blot analysis, up to 10 nucleotides can be added on miR-27 after 7 hours of infection (Figure 26). The exact nature of the nucleotides that are added at the 3' end of miR-27 still needs to be determined.

3.2. Identification of a viral transcript involved in miR-27a regulation

Our previous observations, and the recent report by Cazalla *et al*. on the regulation of miR-27 during herpesvirus saimiri infection (Cazalla *et al*., 2010) prompted us to look for a viral transcript involved in miR-27 regulation. We took advantage of the existence of a MCMV genome cloned into a BACmid as well as a systematic collection of large deletion mutants available in the laboratory of U. Kozinowski (Figure 27). We tested several MCMV mutant strains for their ability to down-regulate miR-27a upon MCMV infection in 3T3 fibroblasts. Using qRT-PCR analysis to quantify the levels of miR-27 and miR-24 as a control, we first established that a virus with a large deletion encompassing the predicted trancripts m159 to m170 (deletion Δ7) was unable to regulate the level of miR-27a (Figure 28). We performed three infection experiments with three different mutants and after 2 days of infection at a MOI of 10, the down-regulation of miR-27a compared to miR-24 level is at least 20 times less efficient with the Δ7 mutant than with the wild-type virus. We then evaluated the role of three shorter deletion mutants inside the m159-m170 region. The first deletion removes the predicted

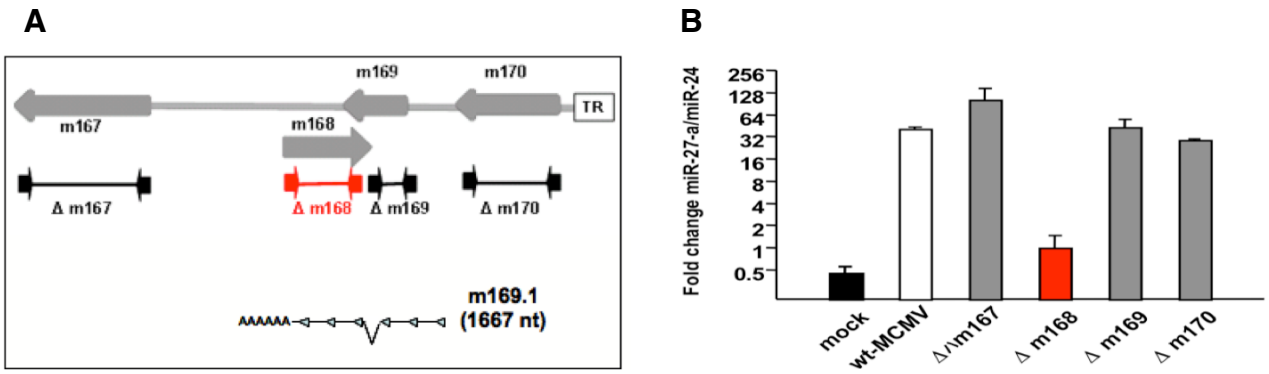


Figure 30: Identification of the transcript responsible for miR-27a degradation.

(A) Schematic representation of the m167-m170 region of the MCMV genome and indication of the deleted region in four different mutant viruses analyzed.

(B) The fold change represents the fold repression of miR-27 compared to the level of miR-24 as evaluated by qPCR on small RNAs.

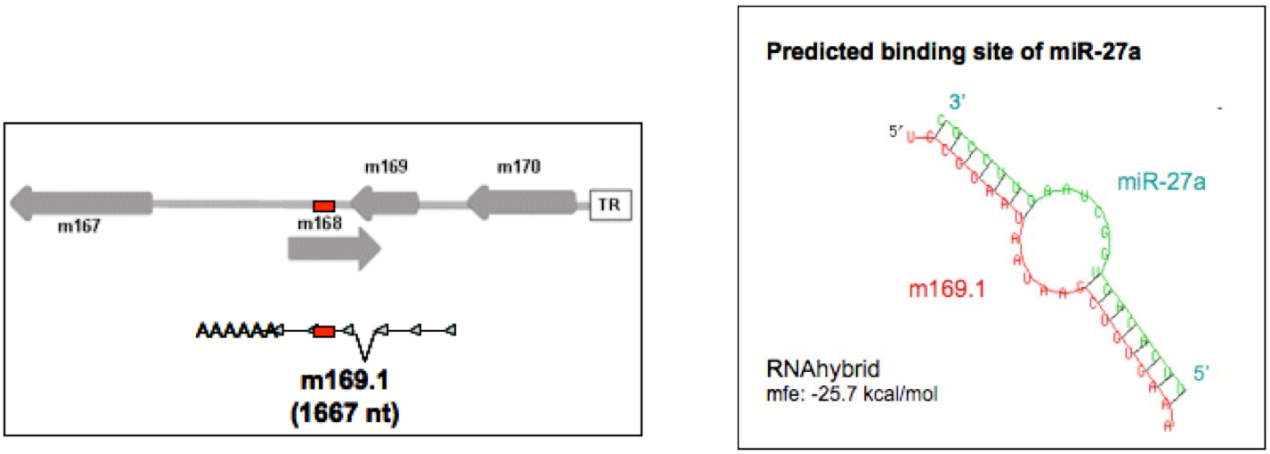


Figure 31: Localization of the predicted binding site for miR-27a in the 3'UTR of the m169.1 transcript and modelization of the binding as visualized by the RNAhybrid program. Mfe : minimal free energy, TR : terminal repeat.

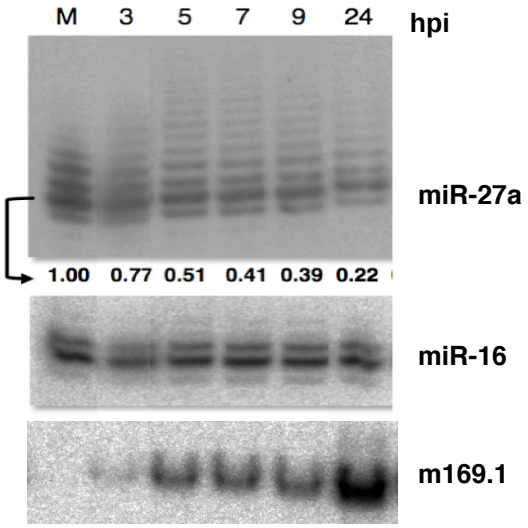


Figure 32: Expression of the m169.1 transcript upon MCMV infection in 3T3 cells. Accumulation of miR-16 and miR-27 are shown, as well as the quantification of the relative amount of the miR-27 major form as described in Figure 26.

open reading frames of m159 to m162 (Δ7S1), the second ORFs m163 to m166 (Δ7S2), and the third ORFs m167 to m170 (Δ7S3). Once again, we established the efficiency of miR-27 regulation by real time PCR analysis on mature miRNAs and we determined that the region 7S3 was required to maintain an efficient down-regulation of miR-27a (Figure 29). We finally used mutants lacking each individual ORF in this region, *i.e.* ORFs m167, m168, m169 and m170. The only mutant strain that lost the capacity to regulate miR-27a was the Δm168 mutant (Figure 30). The MCMV genome being double stranded DNA, the deletion of a predicted ORF on one strand systematically removes another coding or non-coding sequence on the other strand. In the case of the deletion of m168, it results in the removal of the 3'UTR of the m169.1 transcript located on the other strand of the genome. We therefore checked for a potential miR-27a binding site on both strands of the region deleted in the Δm168 mutant. Using the RNAhybrid program (Rehmsmeier *et al*., 2004), we predicted a potential binding site for miR-27a in the 3' UTR of the m169.1 transcript. The binding site has a ΔG of -25.7 kcal/mol and consists of an extensive 5' and 3' pairing of the miRNA with a central bulge of 6 unpaired nucleotides (Figure 31). We estimated the level of accumulation of the viral transcript by northern blot and we noticed that this transcript is already detectable after 3 hours of infection and accumulated at very high levels over the course of infection (Figure 32). An estimation of its abundance by real time PCR analysis indicated that after 1 day of infection, it is as abundant, if not more, as the IE1 transcript (data not shown).

3.3. Validation of miR-27a binding site within the m169 transcript

To verify the nature of this miRNA binding site, and its role in miR-27a regulation, we generated a virus with point mutations in the predicted seed-match region of the site. We inserted three point mutations, which either disrupted the base pairing, or lowered it by changing it to a G-U wobble. We then infected 3T3 cells with the WT or the ml69 mutant (mut169) and assessed the efficiency of miR-27a down regulation by qRT-PCR. As can be seen in Figure 33, the introduction of these point mutations was sufficient to lower the repression of miR-27a expression about 25 times. This result is especially striking considering the fact that only one introduced mutation out of three generated a real mismatch in the pairing, underlying the importance of perfect pairing in this region in the WT virus.

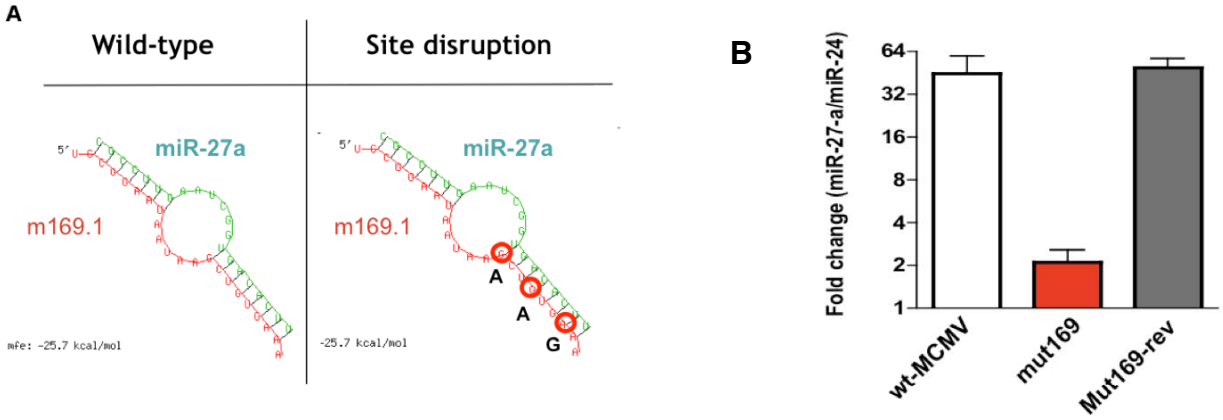


Figure 33: Validation of the miR-27 binding site by mutagenesis.

(A) Modelization of the miR-27 binding sites as visualized by the RNAhybrid program.

(B) The fold change represents the fold repression of miR-27 compared to the level of miR-24 as evaluated by qPCR on small RNAs in cells infected with the indicated viruses. As an additional control, the mut169-rev, revertant to mutations introduced in the mut169, was established and evaluated.

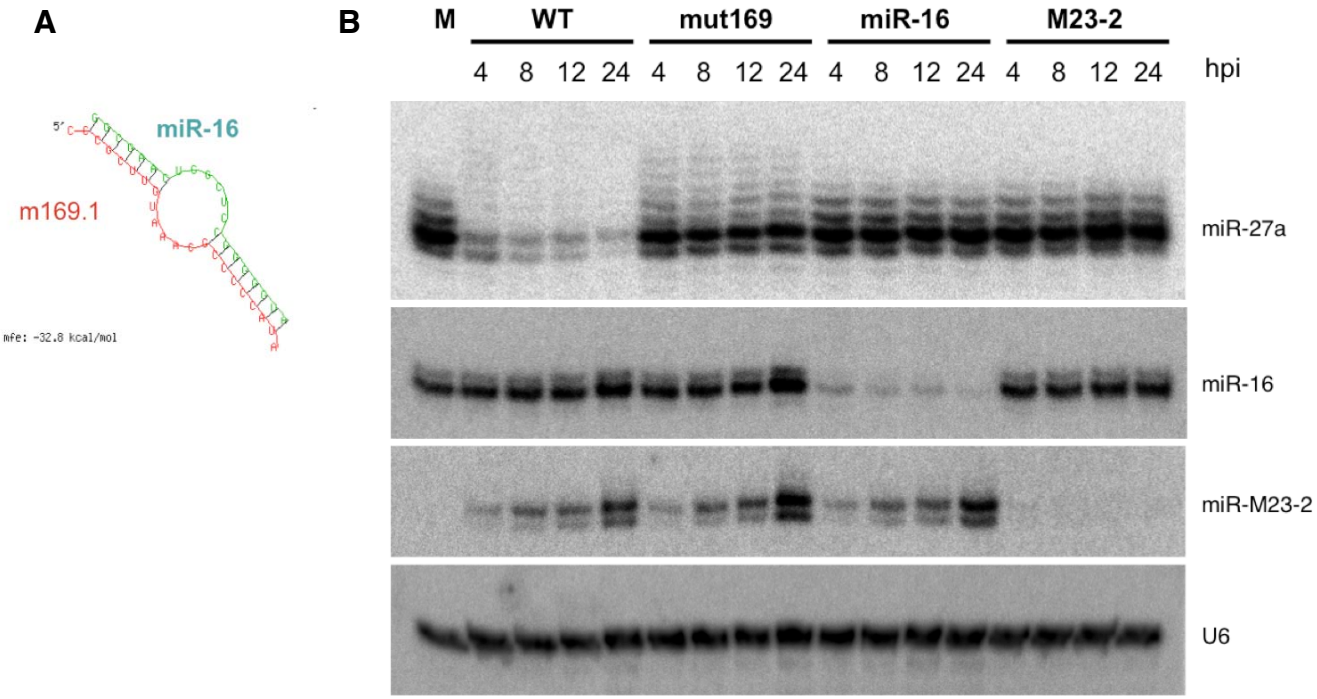


Figure 34:

(A) Model of site replacement, example shown for miR-16. The same type of binding was generated to target miR-M23-2 with the m169 transcript.

(B) Northern blot analysis of time courses of infection with four viruses containing either a WT, point-mutated (mut169), targeting miR-16 (miR-16) or MCMV-miR-M23-2 (M23-2) m169 transcript.

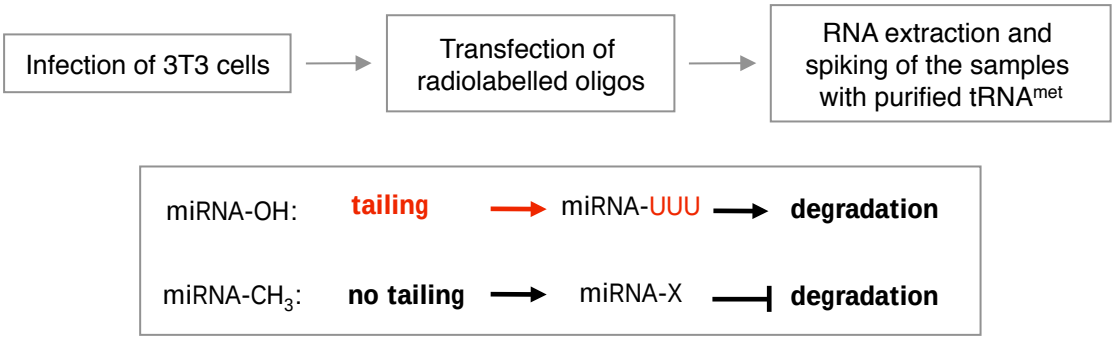
3.4. The m169 transcript can be engineered to regulate other miRNAs

In order to further validate the role of the m169 transcript in miRNA destabilization, we replaced in the BACmid the binding site for miR-27a with a binding site for miR-16, or for MCMV-miR-M23-2. We reconstituted viral stocks of these mutant viruses and used them to infect 3T3 cells. We then measured by northern blot the accumulation of the respective miRNAs in the infected cells. In all tested cases, we could efficiently redirect the m169 transcript to another miRNA, which was significantly degraded after 2 days of infection (Figure 34). However, we did not detect tailed forms for miR-16 or miR-M23-2 despite the fact that the miRNA is very efficiently degraded upon infection with their respective targeting viruses. This is probably due to the efficiency of the "trimming" process, and indicates that not all miRNAs respond similarly to the targeting by the m169 transcript. It could be that there are some sequence determinants that favor or inhibit the process. We also included in this northern blot analysis the point mutant version of m169 to confirm the results obtained previously by qRT-PCR. Although the level of mature miR-27a was clearly much higher in cells infected with this mutant than in cells infected with the WT virus, we could still observe a partial tailing of the miRNA. This might be due to the fact that the 3' end pairing of the binding site is intact, and might indicate that it is possible to partially uncouple tailing and degradation.

3.5. Requirements for tailing-dependent degradation of miRNAs

To further estimate the potential mechanism of the viral transcript induced degradation of miRNA, we went on to assess the requirement of the tailing step. We ran a pulse-chase experiment where infected cells where transfected with radiolabeled-oligonucleotides duplexes as miRNA mimics (the pulse) and estimated their rate of degradation (the chase) over time by gel analysis. For each miRNA evaluated, we used a mimic where the 3' proximal nucleotide possessed either a 2'OH or a 2'O Methyl. As the presence of a methyl group on the 3' end of plant small RNAs has been proposed to impede the activity of 3' modifying enzymes such as terminal nucleotide transferases, we thus expected to see an inhibition of the degradation of a 2'O methylated miRNA mimic, in the case of tailing being a mandatory step to mediate miRNA degradation. Indeed, we did observe that 2'O methylated mimics were not degraded as efficiently upon virus infection as non-modified mimics (Figure 35). However, these results need to be considered carefully. The effect is only marginal, but we did not expect to have a

A **Pulse-chase assay:**



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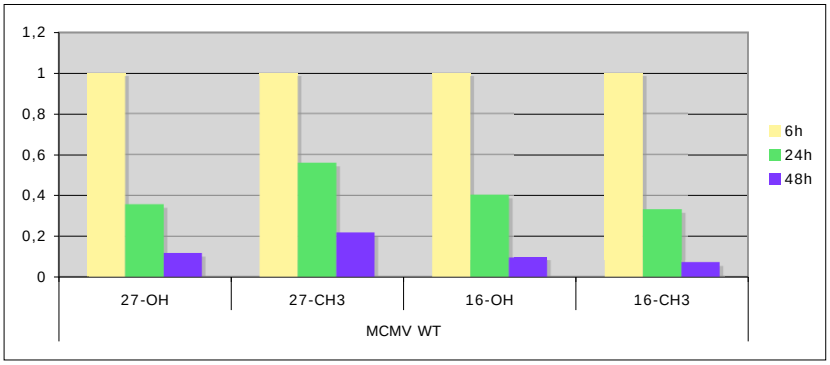


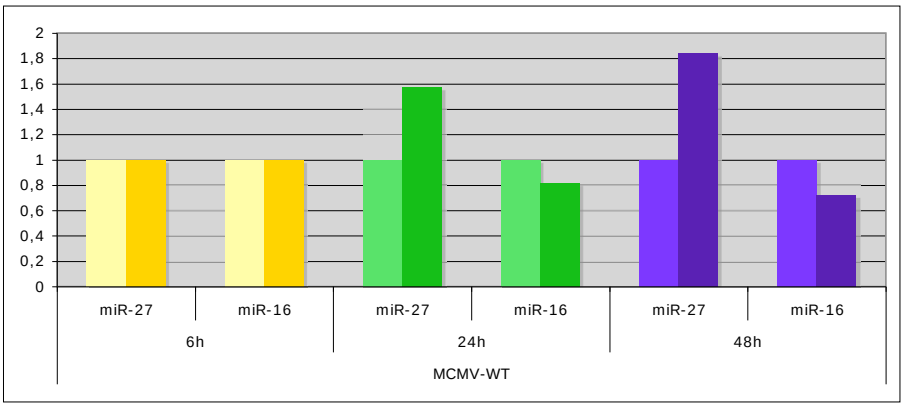


Figure 35: Role of the protection against 3' elongation on stabilization of the miRNA as assayed by pulse-chase assay..

(A) Principle of the pulse-chase experiment.

(B) Relative amount of miRNA mimics upon time. In the upper panel, a first normalization has been made with the earliest time-point (6h post transfection). Values from the lower panel have been normalized a second time to the level of the non-methylated mimic at each time-point.

strong stabilization of the methylated mimic. Indeed, the basal degradation rate of a non-targeted miRNA mimic is important. Nevertheless, there is a measurable difference since in this case the presence of a 3' methyl group does not seem to prevent the degradation of a mimic that is not a target of the virus (Figure 35). In conclusion, it appears that there is a link between the tailing of the miRNA and its degradation.

3.6. At which level do the tailing and degradation occur?

Since the degradation of miR-27a depends on its interaction with a viral transcript, the mechanism involved must occur after the loading of the miRNA into RISC. We first confirmed that the m169 transcript was indeed recruited by Ago2 by performing a qRT-PCR on RNA isolated after Ago2 immunoprecipitation from total extracts of MCMV infected or non-infected 3T3 cells. As can be seen in Figure 36, m169 RNA was significantly enriched in Ago2 IP compared to a control IP, at levels similar to Irf1, a known miRNA target that we used as a positive control. We then tried to assess if the tailed form of miR-27a could be detected in association with Argonaute 2. We thus performed a northern blot analysis on RNA isolated after Ago2 IP. The results show that we cannot detect modified miR-27a loaded into Ago2 (Figure 37). There are two possibilities to explain this data, it could be that the miRNA is not available for tailing when it is loaded in Ago2, and that the binding to the m169 transcript somehow results in its unloading followed by tailing and degradation. Alternatively, the tailing can occur within Ago2, but will trigger the unloading of the miRNA, which explains that the tailed miRNA cannot be detected in Ago2 IP. The latter hypothesis would be consistent with the crystal structure data of a ternary Ago2/guide RNA/target RNA complex, which shows that a conformational change is induced by the presence of the target transcript, resulting in the release of the miRNA 3' extremity from Ago2 (Wang *et al*., 2009c)

3.7. The cellular protein HuR is necessary for miR-27 degradation

Among the different candidates that could be involved in miR-27 tailing and degradation, the terminal uridyl transferases were well positioned. Since Zcchc11 is responsible for both the uridylation of miRNA and their precursors, leading to their degradation, we checked its potential role in miR-27 tailing. Zcchc11 has a close homolog in mouse termed Zcchc6, so we

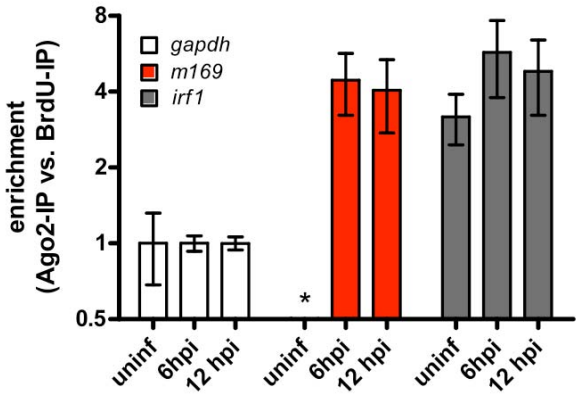


Figure 36: m169 is recruited to Ago2-complexes during early MCMV infection.
qPCR data for gapdh, m169 and irf1 (a known cellular miRNA target) are shown.
* = unspecific signal

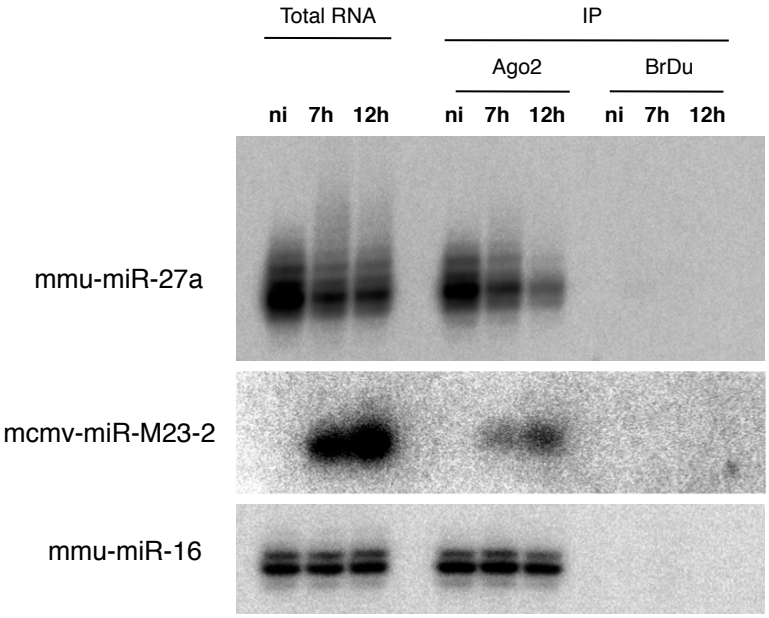


Figure 37: Northern blot detection of miRNAs from immuno-precipitation of Ago2 or control at early time points of infection. 2μL of the total IP have been loaded for both Ago2 IP and a control (BrDu) IP. As a control 5μg of total RNA extracted from infected cells have been loaded.

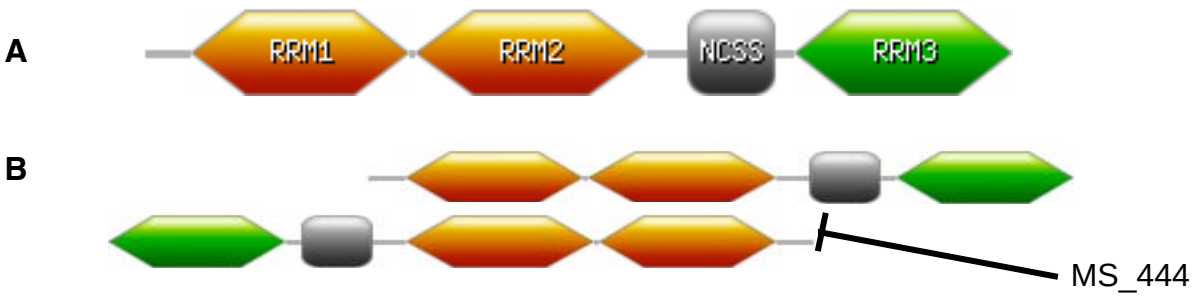


Figure 38: Conserved domains found in HuR.
(A) RNA recognition motif (RRM) 1 and RRM2 allow the homodimerization of HuR, the nucleo-cytoplasmic shuttling signal (NCSS) and RRM3 mediates the shuttling of the protein.
(B) MS_444 is a small molecule able to prevent RRM1/2 homodimerization.

performed a knockdown by RNAi of these two proteins and assayed the efficiency of miR-27 tailing and degradation upon MCMV infection. However we did not detect any effect of their knockdown on miR-27 regulation (L. Dölken, personal communication).

Other proteins could be involved in small RNAs modifications (see introduction part 6). Among these, the ubiquitously expressed RNA binding protein, HuR, has been described as being responsible for the regulation of numerous mRNAs. Its interaction with targeted mRNAs is mediated by binding to an A-U rich element (ARE) in the 3'UTR of the mRNAs and is thought to mediate their stabilization. HuR protein possesses 3 different RNA Recognition Motif (RRM1, 2 and 3). Two of them (RRM1 and RRM2) are implicated in the dimerization of the protein as well as in ARE binding whereas the third (RRM3) has been proposed to possess a terminal adenosyl transferase activity and to be involved in the shuttling of the protein between the cytoplasm and nucleus (Figure 38). HuR has also been linked previously to miRNA, when it was shown that its binding to miRNA targets could affect the fate of the transcript. In one case, the regulatory effect of miR-122 on the cationic amino acid transporter 1 (CAT1) mRNA could be reversed upon binding of HuR. Indeed, Bhattacharyya *et al*. showed that amino-acid starvation resulted in the relocalization of HuR from the nucleus to the cytoplasm and in its binding within CAT1 3'UTR. In turn this interaction resulted in the redirection of the mRNA from P-bodies to polysomes, where it could then be actively translated (Bhattacharyya *et al*., 2006). In another example, the binding of HuR within the 3' UTR of a let-7 target, c-Myc, had the opposite effect and resulted in the repression of the target by the miRNA by promoting the recruitment of the let-7-loaded RISC to the 3'UTR of c-Myc (Kim *et al*., 2009). HuR has also been described to bind the 3'UTR of p53 in response to ultraviolet light irradiation to enhance its translation (Mazan-Mamczarz *et al*., 2003). The mechanism involved could also be due to the action on miR-122 and its target CPEB or eventually by the joint action of HuR, Gld2 (stabilizing miR-122) and Gld4 (stabilizing p53 mRNA) (Burns *et al*., 2011). Cytoplasmic polyadenylation is a regulator of translation induced in many pathways as germ cell development, cellular senescence... The cytoplasmic polyadenylation element binding protein (CPEB) is necessary for the regulation of p53 at the mRNA level in the control of cellular senescence in primary human fibroblasts. As CPEB is able to nucleate several factors including Gld2, a direct role on p53 mRNA stabilization was first considered. Instead, it has been shown that Gld2 depletion induces p53 translation and in turn a premature senescence and that another non-canonical poly(A) polymerase, Gld4 was actually responsible of the stabilization of p53 mRNA. Interestingly, the role of Gld4 is counterbalanced by the action of Gld2 of miR-122. Because miR-122, also present in primary

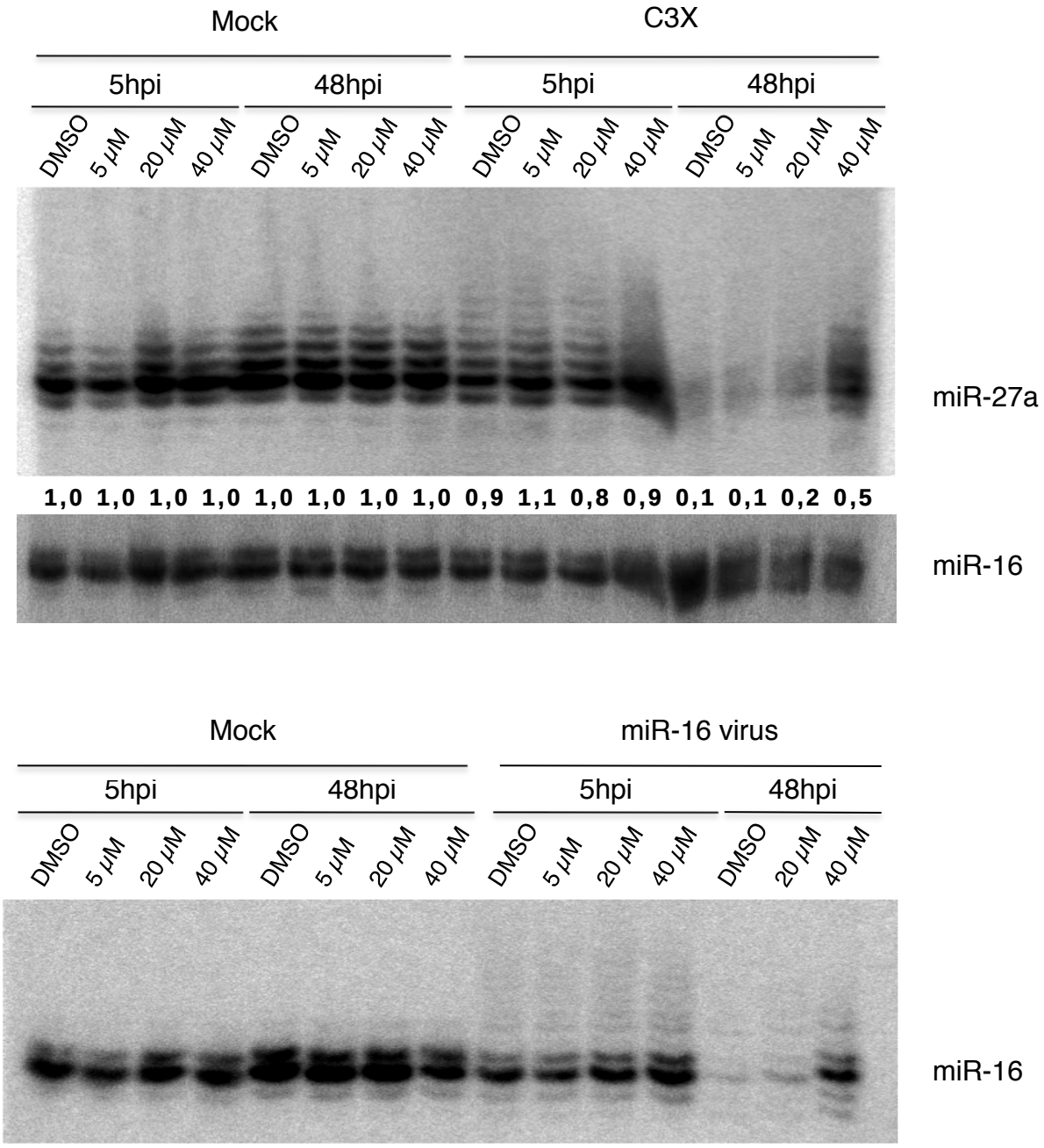


Figure 39: Effect of HuR inhibition on miR-27 regulation.
Northern blot analysis of 3T3 cells infected by MCMV m169-WT (C3X) or MCMV m169-miR16. The cells have been treated for one hour before infection with MS_444, an inhibitor of HuR protein at various concentrations, or DMSO only. After 1 hour of infection, the inhibitor is again added to the culture medium and stays until the indicated time of RNA extraction.

fibroblasts, targets the 3'UTR of the CPEB mRNA and is stabilized by Gld2, a coordinated action of Gld2 and Gld4, and also HuR is required to maintain a steady state level of p53 mRNA.

In addition, HuR has been implicated in numerous viral infections. It has thus been proposed to play a proviral role during hepatitis delta virus (HDV) replication *via* association with the mRNA of the small and large delta antigens, two forms of the only protein products of the HDV RNA genome (Casaca *et al*., 2011). It also seems that HuR is able to bind the 3' extremity of hepatitis C virus (HCV) RNA (Spangberg *et al*., 2000) and the 3' UTR of late transcripts from human papillomavirus type 1 (Sokolowski *et al*., 1999). HuR can also functions as a negative regulator of the HIV-1 IRES and a positive regulator of HCV IRES. These opposing roles are probably linked to the ability of HuR to shuttle between the cytoplasm and the nucleus (Rivas-Aravena *et al*., 2009).

The mechanism by which HuR can repress the action of some miRNAs is currently unknown. In collaboration with the laboratory of Nicole-Claudia Meisner (Novartis Institute for Biomedical Research, Basel), we obtained unpublished information that actually HuR would be able to degrade miRNAs associated with their mRNA targets and that this degradation seems to involve the tailing of the small RNA followed by its degradation an intrinsic exonuclease activity of the protein. Since a small molecule inhibitor of HuR was available ((Meisner *et al*., 2007) and Figure 38), we decided to test the involvement of HuR in the regulation of miR-27a during MCMV infection. We therefore used various amount (5, 20 or 40μM) of the HuR inhibitor MS_444 to treat 3T3 fibroblasts prior to their infection with WT MCMV, or the mutant virus engineered to target miR-16. We incubated 3T3 cells with the different concentrations of MS_444 or DMSO as a control for 1 hour before infection, and then changed the medium during the first hour of the infection. After one hour of incubation with the virus, we changed the medium and re-added the inhibitor or DMSO appropriately. After 48 hpi, it was obvious that miR-27a was almost totally degraded (90%) in cells infected with the WT virus and treated with DMSO, and expressed at 50% of the control level in infected cells treated with 40μM of MS_444 (Figure 39). Interestingly, the same effect could be observed in cells infected with the miR-16 targeting virus. In the presence of 40μM of HuR inhibitor, miR-16 accumulated to an almost normal level at 48 hpi, but not when cells were treated with DMSO alone. It thus appears that blocking HuR activity with this chemical compound is sufficient to prevent miRNA destabilization by the MCMV m169 transcript.

4. Discussion

Nucleotide addition is important both to stabilize and destabilize RNAs. Similarly to the role played by the polyA tailing of mRNAs, nucleotide addition on small RNAs can act as a stabilization signal as observed with adenosine addition by GLD2 on miR-122 (Katoh *et al*., 2009). Nevertheless, tailing of small RNAs was initially described as a destabilization factor, especially in HEN1 mutant plants (Li *et al*., 2005). The link between uridylation and degradation is not restricted to plants as both precursor and mature miRNAs in mammals can be subjected to 3' tailing, either triggering inhibition of processing or direct degradation (Hagan *et al*., 2009; Heo *et al*., 2008; Heo *et al*., 2009; Jones *et al*., 2009; Lehrbach *et al*., 2009). Tailing-mediated degradation seems to be dependent on the degree of pairing of the small RNA to its target. Indeed, it has been shown that a perfect pairing could induce the tailing and further degradation of small RNA (Ameres *et al*., 2010). However, this observation may not explain all the possible outcomes of a miRNA pairing to its target, as others have shown that miRNAs could be protected from degradation upon binding to their target transcript (Chatterjee *et al*., 2011; Katoh *et al*., 2009).

We showed that post-transcriptional degradation of miR-27a during MCMV infection was linked to 3' modification on the small RNA. These modifications seem to consist of the addition of nucleotides resulting in a "ladder" that can be visualized by northern blot analysis. We hypothesize that this tailing is a prerequisite for the degradation of the mature miRNA, as it increases in size and consecutively the level of the predominant miRNA form decreases in abundance. We found that the modification and degradation of miR-27 required its pairing to an abundant viral transcript, m169, which contains a single binding site for miR-27 in its 3'UTR. The specific degradation of miR-27a can be reverted by introducing mutations in the miR-27 seed-match in the binding site of m169. Interestingly, we also showed that we could redirect the effect onto another miRNA by inserting the corresponding binding site into the viral genome.

From our observations, it seems that the sequence of miR-27 has some special features. Indeed, even in non-infected cells, the miRNA already accumulates several length isoforms as shown by northern blot analysis. One possible explanation of this observation could be that miR-27 is by default bound by an endogenous target, which could trigger its modification, but whose abundance may not be sufficient to induce its degradation. We also observed that the miR-27 binding site m169 point mutant is not totally impaired in its regulatory function. Indeed, the level of miR-27 goes down in cells infected with this mutant (although to a lesser extent

compared to cells infected with the wild type virus), and tailing still seems to proceed as seen in the northern blot analysis. These observations indicate that there might be separate tailing and trimming activities.

From our analysis, it also seems that the sequence of the targeted miRNA impacts the efficiency of the process. Indeed, we did not detect tailed forms for miR-16 in cells infected with a virus targeting this miRNA. Nevertheless, it seems to be degraded very efficiently. This could be explained by a difference in the kinetics of degradation depending on the targeted miRNA.

We obtained solid preliminary evidence that the cellular factor involved in miRNA degradation by the m169 transcript could be HuR. According to the data obtained by the Meisner laboratory, it seems that, *in vitro* at least, HuR is involved in both tailing and trimming of the miRNA. In our case, we observed that chemical inhibition of the HuR enzyme restored the stability of at least two tested miRNAs targeted by the corresponding viruses.

The down-regulation of miR-27a upon murine cytomegalovirus infection is not the sole example of the degradation of a cellular miRNA degradation triggered by a viral infection. Cazalla and collaborators showed that T cells transformed by herpesvirus saimiri (HVS) expressed highly abundant non-coding U-rich transcripts (HSUR). These RNAs are small and conserved among HVS subgroups, especially HSUR-1 and 2. They enclose binding sites for several cellular miRNAs. Among them, miR-27 has been shown to be post-transcriptionally degraded in a HSUR sequence-specific and binding-specific manner (Cazalla *et al*., 2010). Although they did not assess a physiological role of miR-27a degradation upon infection with HVS, we previously reported that miR-27a has an antiviral role during MCMV infection, leaving open a possibility for a general involvement of miR-27 as well as HuR protein in herpesvirus infection.

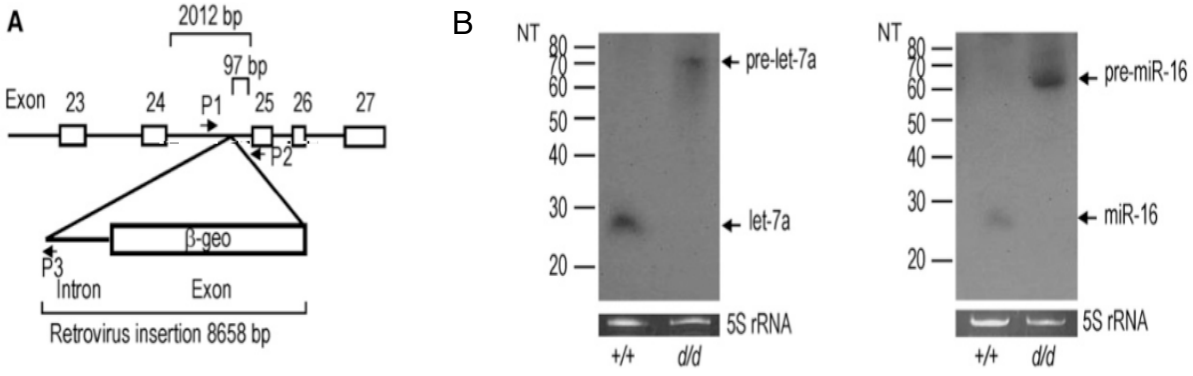
Chapter 3

Roles of miRNAs in MCMV infection

In order to get a broader view of the global involvement of miRNAs upon viral infection, we decided to investigate the role of Dicer during MCMV infection. To this end, we used a mouse model that has already been used to study other viruses (Otsuka *et al*., 2007). These Dicer-deficient animals were obtained from the Han laboratory, then at the Scripps Institute, and a mouse colony was established in the animal facility of the Institute of Immunology in Strasbourg. These mutants were generated using a gene-trap method (Otsuka *et al*., 2007; Stanford *et al*., 2001). Heterozygous Dicer1-deficient mice were generated from ES cells containing a retroviral gene trap in the Dicer1 locus within the 97 base pairs upstream of exon 25. The inserted fragment, lying in an intron, contains a promoterless reporter gene flanked by a 3' acceptor-splicing site and codes for its own transcriptional termination sequence. The transcription of the gene followed by the splicing of the transcript leads to the expression of a Dicer1-ß-galactosidase-neomycin (ß-geo) fusion molecule lacking the second RNase III domain and double-stranded RNA-binding domain. Deletion of these two domains abolishes Dicer's activity (Bernstein *et al*., 2003), so mice homozygous for this mutation can be considered to be null with respect to Dicer1 activity (Figure 40). The genotype of these Dicer1-deficient mice was verified by PCR with primers located at each side of the gene trap and in the ß-geo cassette. Most homozygotes for this variant Dicer1 allele died *in utero* owing to defects in angiogenesis (Yang *et al*., 2005). Nevertheless, one male Dicer1-deficient homozygote that appeared to be healthy was obtained, and was used to produce more viable Dicer1$^{d/d}$ mice, therefore giving the possibility to establish a Dicer-deficient line. It is worth noting that a low abundance of wild-type Dicer mRNA can still be detected in embryo, which is most likely required to bypass the embryonic lethality of a complete absence of Dicer. The residual expression of Dicer1 in Dicer1$^{d/d}$ mice is most likely to be caused by a low-frequency alternative splicing event that removes the gene-trap-vector sequence, resulting in the processinf of a wild-type- transcript.

1. Effect of the Dicer mutation on viral infections

Otsuka and collaborators assessed the role of several viral infections on the Dicer deficient mice. They showed that infection of peritoneal macrophages with either an RNA virus,



Otsuka *et al.*, Immunity, 2007

Figure 40 : Constitutive hypomorphic mutant from B. Beutler and J. Han (San Diego) built by gene trap vector insertion (A). The expression of the protein is almost not detectable in macrophages as detected by western blot and is correlated with a defect in mature miRNA processing (B).

vesicular stomatitis virus (VSV), or with that of a DNA virus, herpes simplex virus type 1 (HSV-1), led to the production of a significantly higher amount of viral particles in the Dicer$^{d/d}$ background. They further investigated the role of Dicer upon VSV infection. They found out that the increased virus production was not due to the absence of production of any virally derived siRNA. They also assessed the potential alteration of the interferon-mediated response in Dicer$^{d/d}$ cells because these cytokines are known to play an essential role in the innate immune response against viral infections in mammals (Basler and Garcia-Sastre, 2002). It appeared that neither type I interferon (IFN) proteins nor the efficiency of the IFN response were affected. However, they determined that two ubiquitously expressed miRNAs, miR-93 and miR-24, targeted sequences within, respectively, the VSV L gene and P gene. The increased susceptibility to VSV in Dicer$^{d/d}$ mice was due to the impaired production of these two miRNAs in the Dicer$^{d/d}$ background (Otsuka *et al*., 2007).

Regarding MCMV infection, it has previously been demonstrated that the global miRNA profile is not dramatically affected by MCMV infection, with the notable exception of miR-27a (Buck *et al*., 2010). This observation does not preclude the possibility that, similar to VSV, MCMV could be directly targeted by cellular miRNAs. Therefore, acting on the miRNA machinery by removing Dicer is expected to have two possible outcomes. Since MCMV encodes its own set of miRNAs, the lack of a functional maturation machinery might negatively impact its infectivity, if indeed viral miRNAs provide an advantage to the virus. On the other hand, cellular miRNAs will be affected as well, and provided that they control the virus, the Dicer$^{d/d}$ animals should therefore be more susceptible to MCMV infection. The two outcomes are of course not mutually exclusive, and we might expect a combination of the two. However, Otsuka and colleagues showed that HSV-1, a miRNA-encoding virus, was more potent in a Dicer deficient background. Therefore, it seems that the cellular miRNAs activity is more important than viral miRNA production, at least in respect to HSV-1.

2. Role of Dicer in MCMV infection

I contributed in the early steps of the Dicer$^{d/d}$ mutants characterization and in the analysis of the impact of the mutation on viral miRNAs production. We first tried to assess the level of accumulation of the Dicer protein in the mutant background. However, despite several attempts to develop optimal conditions to detect the accumulation of the protein, the lack of a good available antibody against mouse Dicer protein at that time did not allow us to successfully

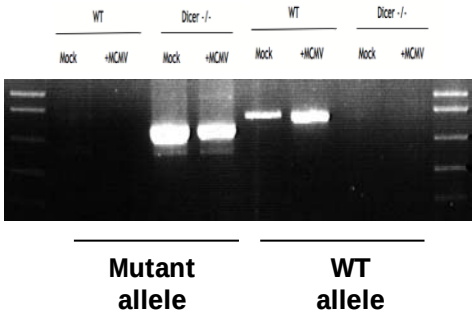


Figure 41 : Detection of the gene insertion in macrophage genomic DNA.

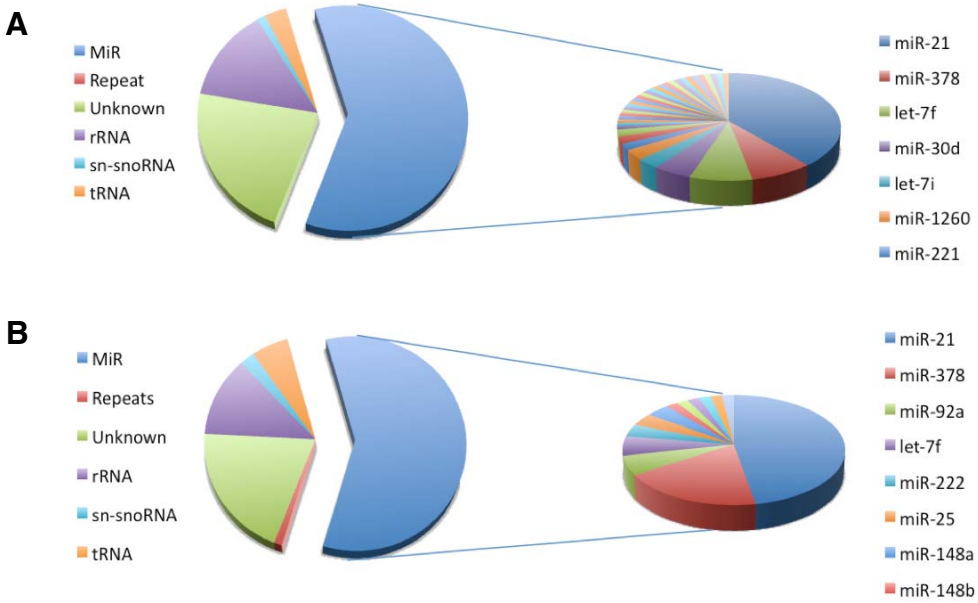


Figure 42 : Expression of miRNAs in peritoneal macrophages either WT (A) or Dicer deficient (B). Macrophages were infected at a MOI of 1 and RNA were extracted after 48h of infection with MCMV.

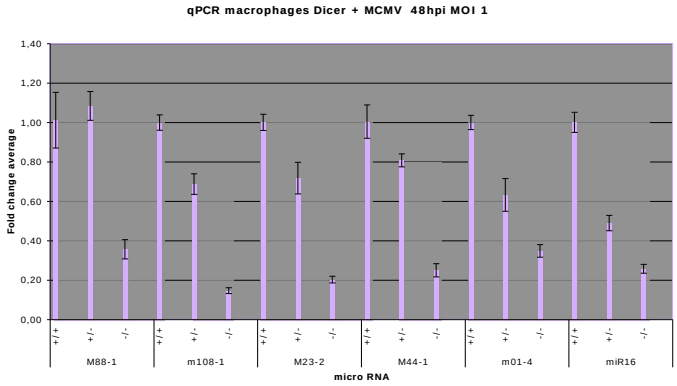


Figure 43 : Expression of miRNAs in Dicer deficient peritoneal macrophages. Macrophages were infected at a MOI of 1 and RNAs were extracted after 48h of infection with MCMV.

measure Dicer expression. However, we checked the genomic DNA content from peritoneal macrophages isolated from Dicer$^{d/d}$ mice, and we confirmed that the retroviral trap sequence was present (Figure 41).

We next analyzed the accumulation of mature miRNAs by generating small RNAs libraries from WT and Dicer$^{d/d}$ peritoneal macrophages. By cloning and small scale sequencing of these samples, we noted that some miRNAs were still accumulating in both cases, as expected upon the hypomorphic nature of the mutant, but the level of sequencing did not allow us to get further insights on this point (Figure 42). However, and because sequencing is not qualitative for the number of miRNAs in a cell, we checked by real-time RT-PCR the levels of some mature miRNAs in infected macrophages isolated from Dicer d/d, Dicer d/+ and wild-type mice, and we did in fact observe a significant difference in their accumulation (Figure 43)**.**

Although I switched fully to the study of miRNA modifications after these initial results were obtained, it is worth noting that this approach has enabled successful further study. Some preliminary results obtained in the laboratory of P. Georgel seem to indicate that the Dicer$^{d/d}$ mutation results in increased infectivity of MCMV in newborn mice, and that this might involve the regulation of the innate immune response by cellular miRNAs.

3. Importance of individual viral miRNAs in MCMV infection

As an annex to this chapter, a publication termed "Cytomegalovirus microRNAs facilitate persistent virus infection in salivary glands" will be presented.

For this work, we generated mutant virus strains in order to remove a single miRNA locus. Due to the presence of miRNA precursors on both strand of the DNA genome, a large deletion of the genome (276bp) abolishes the expression of mcmv-miR-M23-2, as well as mcmv-miR-m21-1 wich is located antisense to this miRNA. A second virus strain was generated by insertion of point mutations to disrupt the formation of the hairpin structure, leading once again to the absence of expression of both these miRNAs.

We tested the accumulation of these viruses in mouse embryonic fibroblasts (MEF), and it appears that the absence of these two miRNAs does not affect viral replication.

Two mouse strains were tested for an eventual attenuation of the viral replication in the absence of the miRNAs. C57BL/6 are able to efficiently control acute infection due to the strong NK cell activation elicited by direct recognition of the viral protein m157 *via* the Ly49H receptor. This receptor is absent on the NK cell surface of balb/c mice.

No differences were observed at 3 days post-infection (dpi), probably due to a too short period of time necessary to express viral miRNAs and set up the regulation of their targets. Interestingly, at 14dpi, the viral replication in salivary glands of C57BL/6 mice was strongly attenuated with the mutant virus. The same observations have been realized in balb/c but only at 25dpi following a subacute dose of virus. This is likely due to the inability of the immune system to control the infection at a higher dose of infection in these mice. This assumption is well illustrated because depletion of NK and $CD4^+$ T cells lifted the attenuation observed for the mutant virus. In line with this, one of the top bioinformatics target predictions for miR-M23-2 is the CXCL16 chemokine, the targeting of which by M23-2 has been validated by luciferase assays.

In conclusion, this work represents the first example of a phenotype associated with a single deletion mutant of miRNA, reinforcing the role of miRNA as key regulators of a viral infection.

4. Annex : « Cytomegalovirus microRNAs Facilitate Persistent Virus Infection in Salivary Glands »

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Cytomegalovirus microRNAs Facilitate Persistent Virus Infection in Salivary Glands

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Abstract

Micro (mi)RNAs are small non-coding RNAs that regulate the expression of their targets' messenger RNAs through both translational inhibition and regulation of target RNA stability. Recently, a number of viruses, particularly of the herpesvirus family, have been shown to express their own miRNAs to control both viral and cellular transcripts. Although some targets of viral miRNAs are known, their function in a physiologically relevant infection remains to be elucidated. As such, no *in vivo* phenotype of a viral miRNA knock-out mutant has been described so far. Here, we report on the first functional phenotype of a miRNA knock-out virus *in vivo*. During subacute infection of a mutant mouse cytomegalovirus lacking two viral miRNAs, virus production is selectively reduced in salivary glands, an organ essential for virus persistence and horizontal transmission. This phenotype depends on several parameters including viral load and mouse genetic background, and is abolished by combined but not single depletion of natural killer (NK) and $CD4^+$ T cells. Together, our results point towards a miRNA-based immunoevasion mechanism important for long-term virus persistence.

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Introduction

The human cytomegalovirus (HCMV), a member of the β-herpesvirus family, is an important pathogen in immunocompromised patients and the leading cause of congenital birth defects with about 1/1,000 newborns affected [1]. After primary infection, herpesviruses establish a life-long latent infection, leaving the infected host at risk of subsequent reactivation and disease. During their co-evolution with their hosts, cytomegaloviruses have encountered a broad array of immune defense mechanisms, and have thus developed multiple strategies to counteract them (reviewed in [2]). Recently, both viral and cellular miRNAs have been identified as new players in the complex interaction between viruses and their hosts, providing interesting new candidates for targets of urgently needed antiviral drugs (for review see [3]). These small, ~22 nucleotides long non-coding RNAs regulate the expression of their targets through both translational inhibition and regulation of target RNA stability (for review see [4]). While a single miRNA can regulate the expression of a large number of target genes, the extent of regulation of protein levels usually does not exceed two to three fold [5,6]. Although a number of viruses (particularly of the herpesvirus family) have been shown to express miRNAs during productive infection and latency [3], the function of viral miRNAs in a physiologically relevant infection remains to be elucidated. Sullivan *et al.* showed that a miRNA mutant murine polyomavirus was not impaired during *in vivo* infection [7], and thus no phenotype of a viral miRNA knock-out mutant has been described so far. Here, we report on the first functional phenotype of a mouse cytomegalovirus (MCMV) lacking two miRNAs. During subacute infection, miRNA mutant virus production was selectively reduced in salivary glands, the major source of persistent CMV infection and virus spread from host-to-host [8,9]. This phenotype depended on several parameters including viral load and mouse genetic background, and was abolished by combined depletion of natural killer (NK) and $CD4^+$ T cells. Together, our results point towards a miRNA-based immunoevasion mechanism in an organ essential for long-term virus persistence and host-to-host transmission.

Results/Discussion

Generation of mutant viruses

During productive lytic infection a variety of virally encoded miRNAs are expressed by both HCMV (11 miRNAs) and MCMV (18 miRNAs), which accumulate to high levels throughout the course of infection [10,11,12,13]. This was seen for a number of

Author Summary

The recent discovery of miRNAs of viral origin has dramatically changed our view on virus-host interaction. Viral miRNAs have been shown to regulate genes of both cellular and viral origin, contributing to a favorable environment for the virus. However, the real importance of virus-encoded miRNAs during infection of their hosts remains elusive. In this manuscript, we report on the first functional phenotype of a miRNA knock-out mutant of the mouse cytomegalovirus *in vivo*. We show that the mutant virus is attenuated specifically in the salivary glands of infected mice, an organ essential for long-term persistence of the virus and host-to-host spread. Interestingly, this attenuation revealed a striking dependence on genetic background of the mice under study. Only combined depletion of natural killer and T cells abolished the phenotype. These results indicate that, by regulating the immune system, viral miRNAs may play an important role in an efficient persistent infection.

different cell types upon HCMV and MCMV infection. In MCMV infected fibroblasts, viral miRNAs constitute as much as two thirds of the total miRNA pool at three days post infection (dpi) as assessed by small RNA cloning [12]. At this time-point, two viral miRNAs, namely miR-M23-2 and miR-m21-1, together constituted ~25% of the overall miRNAs in small RNA libraries. Both miRNAs belong to the m21/m22/M23 miRNA cluster and consist of two pairs of pre-miRNAs, which are expressed antisense to each other at the same genomic locus (Fig. 1A). Of note, this peculiar localization does not hinder their relative expression. We recently reported on an MCMV knock-out mutant lacking both of these viral miRNAs [11]. In this mutant (ΔmiR-M23-2), pre-miR-M23-2 was replaced by 276 nt of stuffer DNA resulting in the knock-out of both miR-M23-2 and miR-m21-1. We now constructed a second independent mutant (miR-M23-2-mut) by traceless mutagenesis [14] inserting 17 point mutations into the miRNA precursor sequence to disrupt hairpin formation and pre-miRNA processing (Fig. 1A). In addition, revertants were created for both viruses (ΔmiR-M23-2-rev and miR-M23-2-mut-rev) by fully restoring the native pre-miRNA locus. Expression of the respective miRNAs was measured by northern blot (Fig. 1B), confirming efficient knock-out and repair of miRNA expression in the mutant and revertant viruses, respectively. Expression of the neighboring genes and miRNAs of the m21/m22/M23 miRNA cluster were quantified by qPCR (for m21 and M23 mRNAs) (Fig. S1A, B) and northern blot (for mcmv-miR-M23-1-3p and miR-m22-1) (Fig. S1C). In both mutants and their respective revertants, no significant effect on expression levels of neighboring transcripts or miRNAs was observed. To demonstrate that miR-M23-2 is functional and can repress luciferase activity of a sensor target during lytic MCMV infection, we created a reporter construct expressing firefly luciferase containing a perfect match binding site for miR-M23-2 in its 3′UTR. Dual luciferase assays demonstrated that miR-M23-2 is functional and can repress luciferase activity of a sensor target during lytic MCMV infection with wild type (wt) MCMV and the revertant virus, but not with miR-M23-2-mut mutant virus (Fig. 1C).

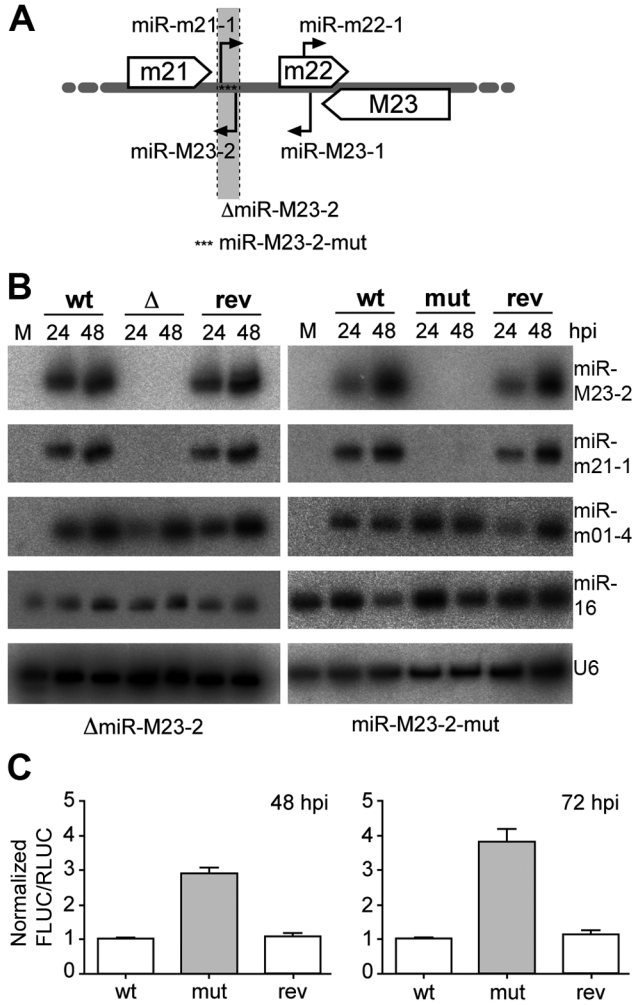

Figure 1. Obtention and characterization of miRNA mutant MCMV. A. Schematic illustration of MCMV miRNAs expressed from the m21/m22/M23 MCMV miRNA cluster. Open arrows indicate the coding regions of the m21, m22 and M23 genes. Black arrows indicate pre-miRNAs and their orientation. The region deleted and replaced with stuffer DNA in the ΔmiR-M23-2 mutant MCMV is indicated (light grey and dotted lines), whereas asterisks indicate the point mutations in the miR-M23-2-mut mutant. **B.** Expression levels of miR-M23-2 and miR-m21-1 in NIH-3T3 fibroblasts infected at an MOI of 10 for 24 and 48 h with either wild type (wt) MCMV, MCMV-ΔmiR-M23-2 (Δ) and its revertant (rev) or wt MCMV, MCMV-miR-M23-2-mut (mut) and its corresponding revertant (rev) were determined by northern blot. The viral miRNA mcmv-miR-m01-4, the cellular miR-16 and the snRNA U6 were used as controls. M, mock. **C.** MiR-M23-2 is able to repress target gene expression late in infection. NIH-3T3 cells were infected either with wt MCMV, MCMV-miR-M23-2-mut or its revertant. 24 hpi cells were transfected with a dual luciferase reporter construct containing a perfect match for miR-M23-2 in the 3′-UTR of firefly luciferase (FLUC). *Renilla* luciferase (RLUC) activity was used as a transfection control. Luciferase activity was measured at 48 and 72 hpi.
doi:10.1371/journal.ppat.1001150.g001

Both MCMV-ΔmiR-M23-2 and MCMV-miR-M23-2-mut are specifically attenuated in salivary glands during subacute infection

Neither of the two miRNA knock-out mutants showed any attenuation on murine embryonic fibroblasts *in vitro* ([11] and data not shown) indicating that the effect of miR-M23-2 mutation on the virus did not affect viral replication in general. It also confirmed that no second site mutation gravely impaired any essential genes of the mutant viruses. We thus started to infect C57BL/6 and BALB/c mice. While C57BL/6 mice are able to efficiently control acute MCMV infection, BALB/c mice are more susceptible. This is due to the activating NK cell receptor Ly49H,

present in C57BL/6 but not BALB/c mice, which directly recognizes the MCMV m157 protein resulting in robust NK cell activation and enhanced virus control [15,16]. Following three days of infection with either wt MCMV, ΔmiR-M23-2 or its revertant, no difference in virus titers were observed in lungs, spleen or kidney (Fig. S2). The lack of attenuation in any organ of both C57BL/6 and BALB/c at 3 dpi indicates that time might be an important factor to consider when investigating the function of viral miRNAs. As miRNAs are only able to repress *de novo* protein synthesis but have no effect on existing proteins, their effect depends on the decay of existing proteins unless their targets are significantly induced upon infection. Relevant levels of most viral miRNAs are probably not reached before 12 to 24 hours post infection (hpi), and it might take even longer until they are able to recruit significant amounts of their target RNAs to RISC complexes. As first viral progeny are released from infected cells as early as 24 hpi [17], viral miRNAs probably do not have sufficient time to regulate the majority of their targets during early stages of infection.

We therefore studied mice at 14 dpi. At this time, the immune system already controls the virus in most organs, except in lungs and salivary glands. Interestingly, infection with both mutants resulted in an attenuation of ~100-fold in salivary glands of C57BL/6 mice. However, only minimal attenuation (<2-fold) was detectable in salivary glands of BALB/c mice (Fig. 2A and B). In contrast, all five viruses under study replicated to similar titers in lungs in both strains. The complete lack of any attenuation in lungs of C57BL/6 mice was surprising, but confirmed that all mice had been infected with an equal virus load. As this phenotype was comparable for both mutants and completely lifted by the two revertants, we can be confident that the knock-out of the two miRNAs was responsible for the observed attenuation. As a consequence, only the second set of mutants (MCMV-miR-M23-2-mut and its revertant) were used in further experiments. The lack of attenuation of the miRNA knock-out mutants at 3 dpi as well as the selective attenuation in C57BL/6 but not BALB/c mice at 14 dpi following intravenous (i.v.) infection, argues for a specific function of these two MCMV miRNAs in supporting persistent infection in salivary glands, and against an impairment of virus spread to this site. A few MCMV genes have been identified to be specifically required to maintain spread as well as persistent infection in salivary glands, but their mode of action has remained questionable [18].

Level of attenuation is dependent on mouse strain and viral load

In order to test whether the miRNA knock-out mutant would require more time for attenuation to become apparent in BALB/c mice, we infected BALB/c mice for 25 days. While at 25 dpi virus titers in lungs had significantly dropped compared to 14 dpi, virus titers in salivary glands were very similar to those seen after 14 days. Only a very small, yet significant attenuation by ~2-fold was observed in salivary glands (Fig. 2C), while no attenuation was observed in lungs. This clearly less pronounced attenuation of mutant virus in salivary glands in BALB/c mice could be the consequence of the inability of the host immune system to overcome high virus load in this strain, due to the less stringent innate immune control, as compared to C57BL/6 mice. Indeed, virus titers observed in salivary glands of BALB/c mice at both 14 and 25 dpi were significantly higher than in C57BL/6 mice. To test whether viral load in salivary glands had any effect on the observed phenotype, we repeated the infection of BALB/c mice with 20-fold less virus. Remarkably, virus dose reduction now resulted in a significantly greater attenuation (~5 fold) of the mutant in salivary glands at 14 dpi (Fig. 2C). The attenuation of the mutant virus could also be seen in BALB/c mice infected intraperitoneally with 5×10^4 PFU (Fig. S3). To further characterize the effects of host genetics on the observed phenotype, we tested three other mouse strains with different susceptibility to MCMV infection (CBA/J, DBA/2 and 129/SvJ mice). In addition, we also included 129/SvJ.IFNγR$^{-/-}$ mice [19], which lack the IFNγ receptor and are thus even more susceptible to MCMV infection than their parental strain. Interestingly, miR-M23-2-mut was attenuated in salivary glands of all four strains at 14 dpi (Fig. 3). Despite very high viral load, attenuation was also observed in 129/SvJ.IFNγR$^{-/-}$ mice. Therefore, both host genetics and viral load are contributing factors involved in attenuation of the miRNA knock-out mutant in salivary glands.

Notably, control of MCMV in salivary glands is completely different than in any other tissue. The virus counteracts host defenses in this sentinel organ by means that are still not fully understood. Virus persistence in salivary gland tissues appears mainly to reflect the situation in acinar glandular epithelial cells. In these cells, MCMV replicates to high titers with a distinct morphogenesis and without causing gross tissue damage [20]. Infectious particles are stored and secreted from large cytoplasmic vacuoles, orientated towards excretion ducts, filled with high numbers (~1,000) of virions [21]. Interestingly, for unknown reasons, virus isolated from salivary glands of naive mice at 14 dpi is also several fold more virulent than virus coming from any other organ or from cell culture. This gain in virulence is lost after a single round of replication in tissue culture [22]. As MCMV infection in salivary glands results in prolonged production of virus with only minimal cytopathic effects, viral miRNAs most likely gain more time to affect target protein levels in this setting. In contrast, severe tissue damage in lungs caused by prolonged and uncontrolled MCMV infection is the main cause of death in animals infected with a lethal dose of MCMV [23]. We speculate that the lack of attenuation in lungs after 14 and 25 days of infection may be explained by the dominance of lytic virus production over virus persistence. However, selective attenuation of the miRNA knock-out mutant in salivary glands may also reflect viral miRNAs targeting immune control mechanisms of particular importance in this organ or miRNA-mediated effects which, in other organs, are exerted by other viral genes.

Attenuation of MCMV-miR-M23-2-mut in salivary glands is reverted by combined but not single depletion of NK- and CD4-T-cells

In salivary glands MCMV infection is almost completely resistant to immunological control by $CD8^+$ T cells. Only the concerted action of $CD4^+$ T cells and cells with a natural killer (NK) cell-like phenotype finally results in termination of productive infection after many weeks or even months of infection [8,9]. As such, $CD4^+$ T cell deficient mice establish persistent infection for many months, which is restricted to salivary gland [21]. In addition, cytokine signaling is known to play an important role (reviewed in [18]). How the virus persists for a long time in salivary glands, in spite of fully primed immune control, remains an open question. In order to test whether these two MCMV miRNAs are involved in immunological control in salivary glands, we depleted both NK and $CD4^+$ T cells in C57BL/6 mice. While the mutant virus was attenuated without NK- and T-cells depletion, combined depletion resulted in significantly higher virus titers (Fig. 4A). When we performed single depletion experiments for NK, $CD4^+$ or $CD8^+$ T cells in C57BL/6 mice, the depletion of NK and $CD4^+$ T cells but not $CD8^+$ T cells resulted in a ~100-fold increase in virus titers (Fig. 4B). This is

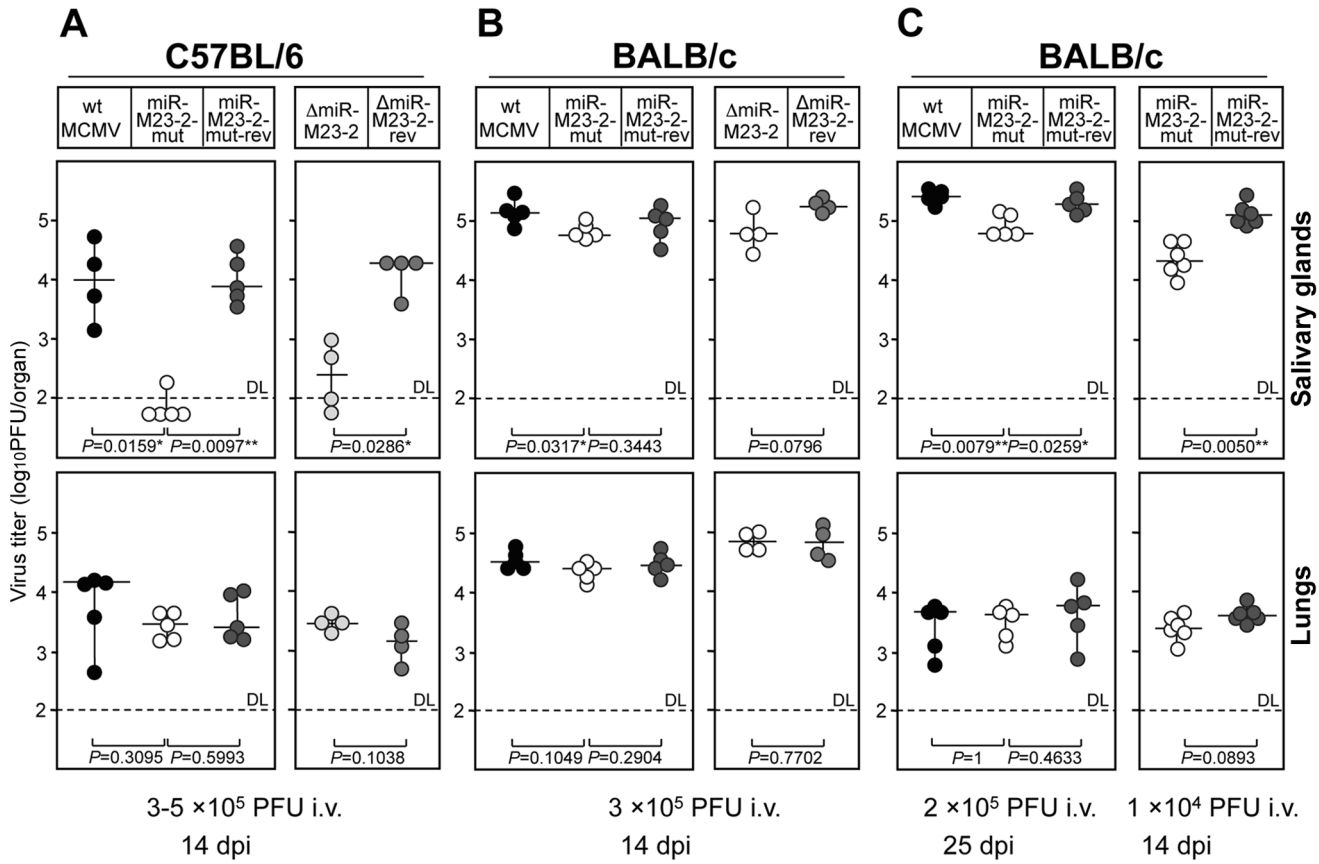

Figure 2. Phenotype of miRNA mutant and revertant MCMV in salivary glands and lungs. A. C57BL/6 mice were injected intravenously (i.v.) with 3×10^5 PFU (left panel) or 1×10^5 PFU (right panel) of indicated viruses. Virus titers in salivary glands and lungs were determined 14 days post infection. There were significant differences in virus titers in salivary glands between the groups of mice infected with miR-M23-2-mut and wt MCMV, miR-M23-2-mut and miR-M23-2-mut-rev, as well as between the groups of mice infected with ΔmiR-M23-2 and ΔmiR-M23-2-rev. **B.** BALB/c mice were injected i.v. with 3×10^5 PFU of indicated viruses. Virus titers in salivary glands and lungs were determined 14 dpi. **C.** BALB/c mice were injected i.v. with 2×10^5 PFU (left panel) or 1×10^4 PFU (right panel) of indicated viruses. Virus titers in salivary glands and lungs were determined 25 days (left panel) or 14 days (right panel) post infection. There were significant differences in virus titers in salivary glands between the groups of mice infected with miR-M23-2-mut and wt MCMV, miR-M23-2-mut and miR-M23-2-mut-rev (left panel), as well as between the groups of mice infected with miR-M23-2-mut and wt MCMV, and miR-M23-2-mut and miR-M23-2-mut-rev (right panel). Titers in organs of individual mice (circles) and median values (horizontal bars) are shown. DL = detection limit; * $p<0.05$; ** $p<0.01$.
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consistent with previously published data showing that $CD4^+$ cells and NK cells control MCMV infection in salivary glands and prevent virus spread [8]. However, although depletion of NK cells or $CD4^+$ T cells resulted in significant increase in titers of miR-M23-2-mut, the differences between equally depleted groups of mice infected with wt MCMV or miR-M23-2-mut-rev still remained significant.

Salivary glands are a privileged site for prolonged CMV replication in spite of a fully primed immune response. Virus excretion in saliva is an important mechanism for host-to-host transmission of both murine and human cytomegaloviruses [1]. In mice, salivary glands also represent the first site to produce virus after reactivation [8]. In fact, MCMV was originally isolated from a salivary gland of a persistently infected mouse and was named salivary gland virus of the mouse [24]. Altogether, our results indicate that at least one of these two MCMV miRNAs supports persistent MCMV infection in salivary glands. We speculate that this particular function of viral miRNA has evolved to allow virus spread to new hosts *via* saliva. Interestingly, although the knock-out of both miRNAs resulted only in a very mild attenuation in BALB/c mice, attenuation was substantially increased by reducing the dose of infection. Dependence on viral load in BALB/c mice perhaps implicates cross-talk between infected cells and thus implies the involvement of cytokines and/or chemokines. The fact that only combined depletion of NK cells and $CD4^+$ T cells abolished the attenuation may also argue for cytokine dependent attenuation. Attenuation was preserved even in $IFN\gamma R^{-/-}$ mice, indicating that the mechanism is IFNγ independent. Upon severe immunosuppression, infection of connective tissue fibroblasts is also observed in salivary glands [21]. Therefore, we cannot exclude that insufficient immunological control following combined depletion of NK and $CD4^+$ T cells simply masked the phenotype of viral miRNAs in persistently infected cells due to the contribution of lytic infection in surrounding cell populations like stromal fibroblasts.

Prediction of cellular targets of miR-M23-2

In order to get a hint about the possible mechanism behind the observed phenotype of our mutant virus, we predicted the potential cellular targets of miR-M23-2 and miR-m21-1 using the RepTar algorithm [25]. From these predictions, we extracted all genes relevant to the immune response, and found 166 and 200

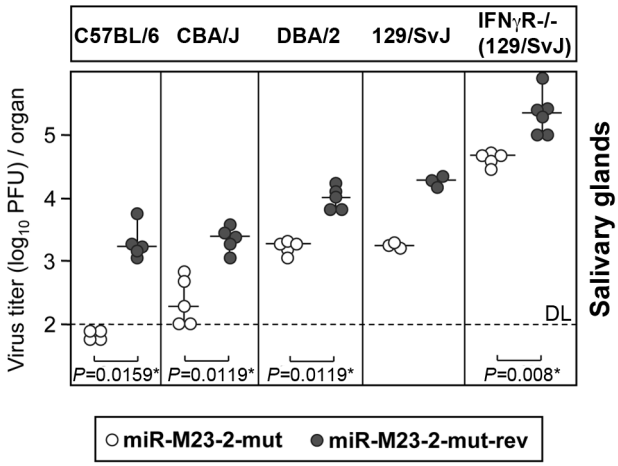

Figure 3. Attenuation of miRNA mutant MCMV in various mouse genetic backgrounds. C57BL/6, CBA/J, DBA/2, 129/SvJ. and 129/SvJ IFNγR$^{-/-}$ mice were i.p. injected with 2×10^5 PFU of MCMV-miR-M23-2-mut or MCMV-miR-M23-2-mut-rev. Virus titers in salivary glands were determined 14 dpi. Titers in organs of individual mice (circles) and median values (horizontal bars) are shown. There were significant differences in virus titers in salivary glands between mice infected with MCMV-miR-M23-2-mut and MCMV-miR-M23-2-mut-rev for C57BL/6, CBA/J, DBA/2 and 129.SvJ IFNgR$^{-/-}$ mice. DL = detection limit; * $p<0.05$; ** $p<0.01$.
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putative targets for miR-m21-1 and miR-M23-2 respectively (Table S1). Among these predictions, 77 genes were predicted to be targeted by both miRNAs. The chemokine CXCL16 was among the top predicted targets with multiple putative binding sites for both miR-m21-1 and miR-M23-2 (Fig. S4). The predicted binding of miR-M23-2 was more favorable than that of miR-m21-1 in terms of free energy of pairing. CXCL16 is a recently discovered chemokine that is expressed in both soluble and transmembrane forms, ligates to CXCR6 chemokine receptor and guides migration of activated Th1 and Tc1 cells [26], as well as NK cells [27]. It is mainly expressed by dendritic cells and macrophages, but also by fibroblasts and endothelial and epithelial cells [28,29]. We cloned the full length 3′UTR of CXCL16 in a luciferase reporter vector, and tested its regulation by both miR-M23-2, and miR-m21-1. While no measurable regulation was observed for miR-m21-1, miR-M23-2 readily regulated the CXCL16 reporter (Fig. S5A). In addition, using 2′-O-methylated antisense oligonucleotide, we confirmed that the CXCL16 reporter regulation by miR-M23-2 could be inhibited in a sequence specific manner (Fig. S5B). In order to test whether miR-M23-2 could also regulate the CXCL16 reporter in the context of virus infection, we transfected the CXCL16 luciferase reporter in cells infected either with the miR-M23-2-mut virus or its revertant. The repression of a luciferase reporter containing either a perfect match sensor, a mismatched sensor for miR-M23-2 or the 3′UTR of CXCL16, was only detectable in cells infected with the revertant, but not the mutant virus (Fig. S5C).

Both cellular and viral miRNAs are known to target a large number of different genes. As such, hcmv-miR-UL112-1 targets a number of viral transcripts including the major viral transactivator IE1 as well as the host's NK cell activating ligand MICB [25,30,31]. Knock-out of both miR-M23-2 and miR-m21-1 probably resulted in the loss of regulation of several genes. Therefore, although preliminary observations indicate that CXCL16 is a target of miR-M23-2 but not of miR-m21-1, it is very unlikely that its regulation is the sole reason for the attenuation of miR-M23-2/m21-1 mutant viruses. This concept is also supported by our findings that attenuation of the miR-M23-2/m21-1 mutant viruses revealed dependence on host genetics and viral load, indicating a multifactorial rather than a single mode of regulation. Indeed, our target predictions contained numerous other genes involved in innate immune response (Table S1). Although the exact mechanism by which miR-M23-2 and miR-m21-1 contribute to an efficient accumulation in salivary glands remains to be identified, we hypothesize that these viral miRNAs contribute to the generation of a microenvironment in this organ that is favorable for the virus to persist. Additional mechanisms such as the regulation of viral gene expression might also contribute to the observed phenotype. In this context, it is not surprising that lifting only one type of control, e.g. depletion of CD4$^+$ T cells, was not sufficient to completely revert viral miRNA function.

Viral miRNAs have been implicated in the control of viral latency and reactivation although formal proof *in vivo* is still lacking [3]. Our data not only provide the first *in vivo* phenotype of viral miRNAs but also demonstrate that viral miRNAs are important in chronic infection in a site well known for its significance in host-to-host transmission.

Materials and Methods

Ethics statement

All of the protocols used for breeding of mice and different kinds of treatments were approved by the Ethical Committee of the Faculty of Medicine University of Rijeka and were performed in accordance with Croatian Law for the Protection of Laboratory Animals, which has been harmonized with the existing EU legislation (EC Directive 86/609/EEC).

Construction of mutant viruses

As pre-miR-M23-2 and pre-miR-m21-1 are located on opposing strands of the MCMV genome and are 98% complementary (the predicted 5′-end of pre-miR-m21-1 extends the 3′-end of pre-miR-M23-2 by one nucleotide), deletion of one miRNA always results in concordant knock-out of the other. To exclude unexpected effects on the genomic locus by the miRNA knock-out we constructed two independent mutants and revertants. The generation of the first mutant (MCMV-ΔmiR-M23-2) has been described [11]. The revertant virus was created by replacing the 276 bp insert with an expression cassette for galactokinase (GalK) and kanamycin resistance (Kn) in DH10B E.coli. The linear DNA fragment was generated by PCR on pGPS-GalK/Kn using primers H5-miR-M23-2-galK/Kn and H3-miR-M23-2-galk/Kn. Sequences of these and all other primers are provided in Table S2. After transferring the recombinant BAC to SW102 bacteria by electroporation, the wt pre-miR-M23-2 sequence was restored by traceless mutagenesis using a linear DNA fragment generated by PCR on pSM3fr using the PCR primers H5-miR-M23-rev and H3-miR-M23-rev [14]. The second mutant (MCMV-miR-M23-2-mut) was constructed solely in SW102 bacteria. First, the whole m21/m22/M23 locus of pSM3fr was replaced using homologous recombination by a GalK/Kn cassette amplified from pGPS-GalK/kn using primers H5-GalK/Kn-m22 and H3-GalK/Kn-m22. Next, the whole m21/m22/M23 miRNA cluster containing 4 miRNAs and part of the m22 gene was subcloned into pGPS1.1 (resulting in pGPS-m22) by PCR cloning using primers m22-for and m22-rev. To generate the mutant pre-miR-M23-2 template, 17 point mutations were inserted into the pre-miR-M23-2 within pGPS-m22 (resulting in pGPS-m22-miR-M23-2-mut) by oligonu-

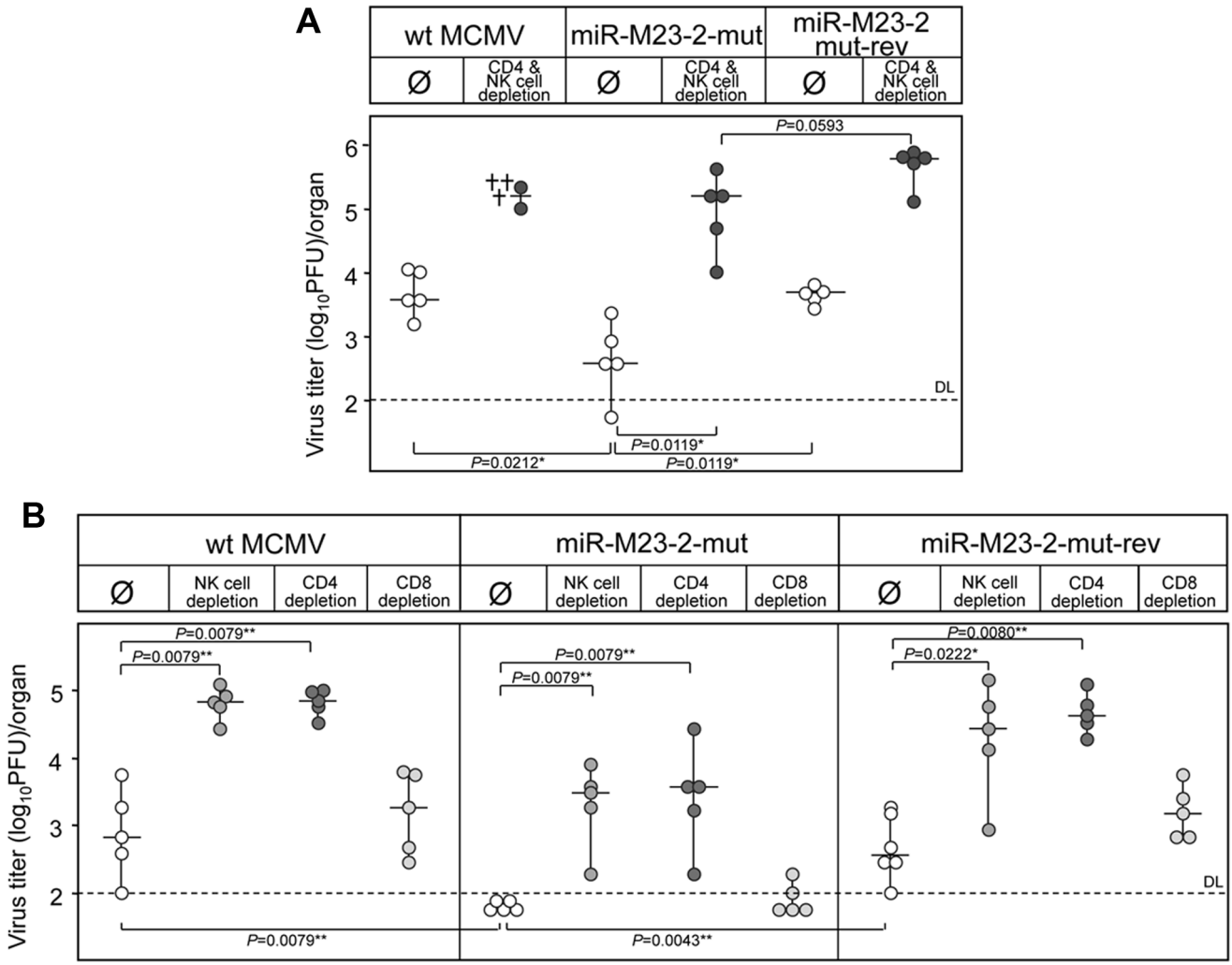

Figure 4. Reversion of the miRNA mutant phenotype by immune cells depletion. A. C57BL/6 mice depleted for both NK and $CD4^+$ T-cells or mock treated were i.v. injected with 3×10^5 PFU of either wt MCMV, MCMV-miR-M23-2-mut or its revertant. Virus titers in salivary glands were determined 14 dpi. Differences in virus titers were significant between groups of undepleted mice infected with miR-M23-2-mut and wt MCMV, as well as with miR-M23-2-mut and miR-M23-2-mut-rev. Depletion of $CD4^+$ T cells and NK cells resulted in significant increase in the titer of miR-M23-2-mut as well as miR-M23-2-mut-rev. Differences in virus titers between groups of depleted animals infected with miR-M23-2-mut and miR-M23-2-mut-rev were not significant. The crosses for the group of wt MCMV infected and CD4 and NK cell depleted group indicate animals that died a day before virus determination. **B.** C57BL/6 mice, undepleted or depleted of either $CD4^+$ T cells, $CD8^+$ T cells or NK-cells, were i.p. injected with 2×10^5 PFU of wt MCMV, MCMV-miR-M23-2-mut or its revertant. Virus titers in salivary glands were determined 14 dpi. Titers in organs of individual mice (circles) and median values (horizontal bars) are shown. DL = detection limit; * $p<0.05$; ** $p<0.01$.
doi:10.1371/journal.ppat.1001150.g004

cleotide cloning with the two oligonucleotides pre-miR-M23-2-mut-s and pre-miR-M23-2-mut-as and an ApaL1 and PvuI digest. The m21/m22/M23 locus with the mutated pre-miR-M23-2 was excised from pGPS-m22-miR-M23-2-mut using EcoRV and inserted into pSM3fr-m22-galK/Kn by traceless mutagenesis resulting in the MCMV-M23-2-mut BAC. To generate the revertant, the GalK/Kn cassette was reinserted again as described above followed by reinsertion of the wt m21/m22/M23 miRNA cluster using the EcoRV fragment of pGPS-m22.

All viruses were reconstituted by transfecting the recombinant BACs into murine embryonic fibroblasts as described [23]. Virus titers were determined on MEFs by standard plaque assay [23].

Northern blot analysis

RNA was extracted using TRIzol and northern blotting was performed on 10 µg of total RNA as described before [11,32]. Probes were 5′ ^{32}P-radiolabelled oligodeoxynucleotides perfectly complementary to the miRNA sequence or to part of the U6 snRNA sequence. Blots were analyzed and quantified by phosphorimaging using a FLA5100 scanner from Fuji.

Quantitative PCR analysis of viral transcripts

Real time PCR analysis of m21 and M23 transcripts was performed as described before [12] using primers m21for and m21rev and M23for and M23rev respectively as indicated in Table S2.

Target prediction by RepTar algorithm

RepTar is a miRNA target prediction algorithm that is independent of evolutionary conservation considerations and is not limited to seed pairing sites. It is based on the finding that in some targets the miRNA binding site repeats in the 3′UTR. It

identifies high scoring repetitive elements in each 3′UTR, matches them to the miRNA sequences and evaluates them as candidate miRNA binding sites. Based on the information gained from these repetitive sites, RepTar then searches for non-repetitive sites as well. The final set of Reptar predictions includes targets with conserved or non-conserved single or multiple binding sites of several types including: seed binding sites, seed wobble sites (seed sites that include G:U pairing in the seed), 3′ compensatory binding sites and full-match binding sites. The special properties of the algorithm make it advantageous for predicting targets of the less conserved viral miRNAs.

Dual luciferase experiments

The perfect match sensor for miR-M23-2 was obtained by annealing the oligonucleotides PM-M23-2-for and PM-M23-2-rev. The miR-M23-2 mismatch sensor was obtained by annealing the oligonucleotides MM-M23-2-for and MM-M23-2-rev. The 3′UTR of CXCL16 (position 16–1100) was PCR amplified from NIH-3T3 genomic DNA using primers CXCL16-for and CXCL16-rev. To each product, AttB1 and AttB2 sites were incorporated by PCR using primers AttB1-for and AttB2-rev (see Table S2). The resulting PCR products were then recombined sequentially in pDONR/Zeo and in psiCHECK-2 plasmids using Gateway technology (Invitrogen). NIH-3T3 cells were infected with wt MCMV, MCMV-miR-M23-2-mut as well as with MCMV-miR-M23-2-mut-rev at an MOI = 10 using centrifugal enhancement. 24 h post infection cells were transfected with psiCHECK-PM-M23-2 using the TransIT-3T3 Transfection kit (Mirus) following the manufacturer's instructions. At 48 and 72 h post infection luciferase activities were measured employing the Dual-Luciferase Reporter Assay (Promega).

For CXCL16 dual luciferase assays, constructs were co-transfected into NIH-3T3 cells with the indicated concentrations of miRNA mimic or Allstars negative control siRNA (Qiagen), using lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Luciferase activities were measured 48 h post transfection (Glomax, Promega). The inhibition of miRNA activity was performed by co-transfection of 100 nM 2′-O-methylated oligonucleotides specific for miR-M23-2 or for *C. elegans* miR-67 as a control. For CXCL16 luciferase assays in MCMV-infected cells, NIH-3T3 cells were infected with MCMV-miR-M23-2 mut or MCMV-miR-M23-2-mut-rev at an MOI = 10, using centrifugal enhancement. 24h post infection cells were transfected with psiCHECK-PM-M23-2, psiCHECK-MM-M23-2 or psiCHECK-CXCL16 using lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Luciferase activities were measured 48 h post transfection (Glomax, Promega).

Mice

BALB/c (H-2^d), C57BL/6 (H-2^b), CBA/J (H-2^k), DBA/2 (H-2^d), 129/SvJ (H-2^b) and 129/SvJ IFN$\gamma R^{-/-}$ (H-2^b) mice were housed and bred under specific-pathogen-free conditions at the Central Animal Facility, Faculty of Medicine, University of Rijeka.

Infection conditions and detection of infectious MCMV in tissues, depletion of lymphocyte subsets, and statistical evaluation

Mice were injected either intraperitoneally or intravenously with indicated doses (1×10^4 PFU to 5×10^5 PFU) of tissue culture-grown wt MCMV or recombinant viruses in 0.5 ml of diluent. Organs were collected either 14 or 25 days after infection and virus titers were determined by a standard plaque-forming assay [33]. In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocyte subsets and NK cells was performed by intraperitoneal injection of the mAbs to CD4 (YTS191.1), to CD8 (YTS 169.4) molecules [34] and to NK1.1 (PK136) [35]. Statistical significance of the difference between experimental groups was determined by the Mann-Whitney exact rank test.

Supporting Information

Figure S1 Quantitative analysis of miR-M23-2 flanking transcripts and miRNAs. **A and B.** qRT-PCR analysis of m21 and M23 transcripts accumulation in wt MCMV, ΔmiR-M23-2, miR-M23-2-mut and their revertants infected NIH-3T3 cells at 48 hpi. Data was normalized to IE1 expression levels. **C.** Northern blot analysis of miR-M23-1-3p and miR-m22-1 accumulation in cells infected with wt MCMV, ΔmiR-M23-2, miR-M23-2-mut and their revertant viruses at 48 hpi. M, mock infected cells; EtBr, Ethidium Bromide.
Found at: doi:10.1371/journal.ppat.1001150.s001 (0.24 MB TIF)

Figure S2 Virus titers of wt MCMV, ΔmiR-M23-2 and miR-M23-2-mut in various organs at 3 days post infection. C57BL/6 and BALB/c mice were injected i.v. with 5×10^5 or 3×10^5 PFU, respectively with wt MCMV, ΔmiR-M23-2 and miR-M23-2-mut, as well as with their respective revertants. Virus titers in organs were determined 3 days post infection. Titers in organs of individual mice (circles) and median values (horizontal bars) are shown. DL = detection limit.
Found at: doi:10.1371/journal.ppat.1001150.s002 (0.12 MB TIF)

Figure S3 Virus titers of wt MCMV, miR-M23-2-mut and miR-M23-2-mut-rev in BALB/c salivary glands and lungs at 14 days post intraperitoneal infection. Titers in organs of individual mice (circles) and median values (horizontal bars) are shown. DL = detection limit; * $p<0.05$; ** $p<0.01$.
Found at: doi:10.1371/journal.ppat.1001150.s003 (0.11 MB TIF)

Figure S4 Schematic representation of miR-M23-2 and miR-m21-1 binding sites within the CXCL16 3′UTR. Shown are the sites predicted by RepTar version 1.1, based on the statistical profiles of repeating elements in the 3′UTR. The miRNA is indicated in red on top of the alignment. For miR-M23-2 there is an additional full seed match at position 221, which was not detected by the version of the algorithm used at the time of the analysis. Loc: position in 3′UTR (location is zero based). ΔG: free energy of pairing in Kcal/mol.
Found at: doi:10.1371/journal.ppat.1001150.s004 (0.31 MB TIF)

Figure S5 Identification of a putative cellular target of miR-M23-2. **A.** CXCL16 is regulated by miR-M23-2, but not by miR-m21-1. NIH-3T3 fibroblasts were co-transfected with a bulged luciferase sensor for miR-M23-2 or a luciferase reporter construct containing the entire CXCL16 3′UTR, and with the indicated miRNA oligonucleotides mimics or negative control siRNA. Dual luciferase assays were performed 48 h post-transfection (n = 5). FLUC to RLUC ratios were first normalized to the values obtained for the empty reporter vector and then to the values obtained with the negative control siRNA, which were set to 1. **B.** Regulation by miR-M23-2 oligonucleotide mimic of both mismatched sensor for miR-M23-2 and CXCL16 3′UTR luciferase reporter can be reverted by co-transfection of a 2′-O-methylated (2′O Me) antisense oligonucleotide directed against miR-M23-2, but not by a control (Ctrl) 2′-O-methylated antisense oligonucleotide directed against the *C. elegans* miRNA miR-67. Dual luciferase assays were performed 48 h post-transfection (n = 6). FLUC to RLUC ratios were first normalized to the values obtained for the empty reporter vector and then to the values obtained with the control 2′-O-methylated oligonucleotide, which

were set to 1. **C.** Regulation of perfect match (PM), mismatch (MM) sensors for miR-M23-2 or CXCL16 3′UTR luciferase reporters in cells infected with MCMV-miR-M23-2-mut or its corresponding revertant. Dual luciferase assays were performed 48 h post-transfection (n = 3). Shown here is a representative example of four independent experiments.
Found at: doi:10.1371/journal.ppat.1001150.s005 (0.46 MB TIF)

Table S1 Immuno-related predicted targets of miR-m21-1 and miR-M23-2. Gene annotations were extracted from http://cgap.nci.nih.gov/Genes/GOBrowser. All genes with the GO Biological Process category: "immune system process" were considered immuno-related. For each target gene the number of predicted binding sites and the binding site with minimal free energy upon miRNA:3′UTR pairing are reported. The minimal free energy value and the pattern of base-pairing for this site are shown (computed by the program RNAcofold of Vienna package). miRNA and mRNA are displayed as the upper and lower string, respectively. A solid line represents canonical Watson-Crick base pairing, and a colon represents G:U base pairing.
Found at: doi:10.1371/journal.ppat.1001150.s006 (1.04 MB PDF)

Table S2 List of PCR primers and oligonucleotides.
Found at: doi:10.1371/journal.ppat.1001150.s007 (0.23 MB PDF)

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Author Contributions

Conceived and designed the experiments: LD AK UHK SJ SP. Performed the experiments: LD AK SK LT MT LM NE YA. Analyzed the data: LD AK LT ZR NE YA HM UHK SJ SP. Contributed reagents/materials/analysis tools: ZR HM UHK SJ. Wrote the paper: LD SJ SP.

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DISCUSSION

In the course of my PhD project, I have been involved in the study of the role and regulation of miRNAs during viral infections. More specifically, I initiated a project aimed at deciphering the global role of miRNAs during MCMV infection, and I contributed to the molecular analysis of mice infected with miRNA-mutant MCMV strains. In the latter case, we showed that the deletion of a single miRNA locus within the MCMV genome was sufficient for the appearance of a phenotype that was deleterious for the virus. Indeed mcmv-miR-M23-2 seems to be required for the accumulation of viral particles in salivary glands of infected mice (an important site of persistence), which indicates that the regulatory network involving this miRNA serves to promote cell immune defense, as well as maintenance of persistent infection that, in theory would benefit the virus for host-to-host transmission.

In addition to the work on MCMV miRNAs, I dedicated the greater part of my research to the study of factors involved in the regulation of miRNAs themselves. Given the essential role of miRNAs in virtually every biological pathway, the capacity to actively regulate these small molecules appears to be an essential feature for all organisms containing miRNAs. The role of miRNA 3' end modification has already been described to be a potential way to regulate their action and accumulation. Along that line, we established that the cellular miRNA miR-27, which displays some antiviral properties against MCMV, was actively degraded in cells infected with this virus. We showed that down-regulation of miR-27 is strikingly rapid, correlating with early times points in MCMV infection. Its degradation is linked to a 3' tailing of the small RNA, and we obtained solid evidence about the role of the cellular protein HuR in this process. Finally we assessed the role of terminal nucleotide transferases on miRNA degradation. We established that it is unlikely that TUT7 (Zcchc6) in mammalian cells, as well as TUT1 (At2g45620) in *Arabidopsis thaliana* are involved in miRNA degradation in our experimental conditions.

This work established a number of indications that argue against the fact that small RNAs and specifically miRNAs are passive molecules whose activity would be regulated only *via* their basal level of transcription and decay rate. Instead, they are extensively subjected to modifications that in turn are capable of modifying their accumulation and degradation.

In this part, I will put our results in perspective with the current state of the art.

1. Importance of miRNAs in stress regulation

There is a raising number of studies pointing out to the role of miRNAs in stress situations, as we observed with the role on miR-M23-2 upon MCMV infection. Among the main roles of miRNAs during stress situations, there is an increasing number of evidences pointing toward the importance of regulatory networks related to immune system recognition/activation. One important aspect of the immune response in unusual stress conditions is the activation of innate immunity, for example *via* Toll-like receptor (TLR) recognition, which is actively regulated by miRNAs (for review see (O'Neill *et al*., 2011)). Other important players of innate immunity include natural killer (NK) cells. NK cells are able to sense potentially deleterious modifications of the cells by diverse mechanisms of signal recognition involving cell surface markers. For example, tumor cells are known to down-regulate the level of expression of the class I major histocompatibility complex (MHC) molecules, that will in turn activate NK cells *via* the "*missing-self recognition"* mechanism (Watzl, 2003). This activation relies on the absence of a negative regulatory signal, but other signals, such as the NKG2D receptor, can activate NK cells (for review see, (Raulet, 2003)). Recognition of NKG2D ligands triggers not only the activation of NK cells, but also that of T cells and macrophages. The ligands are various: MHC class I chain related (MIC), retinoic acid early inducible (Raet) and UL16 binding proteins (ULBP) (Raulet, 2003). These ligands are usually up regulated specifically in tumor cells, virus-infected cells and more generally upon stress. As masters of the evasion of the host immune responses, herpesviruses have developed strategies to counteract the exposure of these ligands at the cell surface of infected cells. For example, MCMV regulates the NKG2D ligands MULT-1, RAE-1 and H60 through the m145, m152 and m155 proteins (Hasan *et al*., 2005; Krmpotic *et al*., 2002; Krmpotic *et al*., 2005; Lodoen *et al*., 2003; Lodoen *et al*., 2004).
MICB has been especially reported as one of the major NKG2D activators upon herpesvirus infection (Raulet, 2003) and in turn is actively regulated by viral miRNAs to relieve stress induced activation of NK cells. MICB is regulated not only by an HCMV miRNA, miR-UL112 (Stern-Ginossar *et al*., 2007), but also by cellular miRNAs, allowing the cell to maintain MICA and MICB expression under a certain threshold in the absence of any stress signal and to escape mis-recognition by NK cells. The inappropriate downregulation of MICA and MICB can promote tumor escape (Stern-Ginossar *et al*., 2008). This efficient stress induced activation of the innate immunity response is also regulated by two other herpesvirus miRNAs, EBV-miR-BART2-5p and KSHV-miR-K12-7 (Nachmani *et al*., 2009).

Consistent with these observations, the potential role in the regulation of CXCL16 by mcmv-miR-M23-2 is not surprising and is also consistent with a general viral induced control of the host immune response.

1.1. The impact of miRNAs depends on the cellular state

The absence of stressful conditions may prevent the observation of the role of some miRNA in certain situations. In this respect, it becomes apparent as to why single miRNA mutants do not always present a phenotype, leading to the idea that miRNAs are dispensable for development and viability under standard laboratory conditions. As far as viral miRNAs are concerned, we showed that the deletion of the viral miRNA miR-M23-2 in the genome of MCMV led to a specific phenotype during the course of infection in mouse. This study was the first one to link a single miRNAs locus to a physiological effect in the context of an *in vivo* infection. Interestingly, this phenotype seems to be dose-dependent. In salivary glands of BALB/c mice, the absence of miR-M23-2 seems to prevent an efficient virus production only when the animals are infected with a low dose of virus, leading to propose that the immune response may be controlled in normal conditions by this miRNA (Dolken *et al*., 2010).

The stress-dependent appearance of miRNA roles is not limited to viral infections. I will describe several examples that show the implication of miRNAs in response to environmental changes. The first report describing a stress-induced phenotype in a miRNA mutant was reporter by Xu and collaborators in 2003. They described that although flies lacking miR-14 expression were viable, they showed greater sensitivity to stress stimuli and reduced viability than WT flies, owing to a deficiency in cell death suppressor activity (Xu *et al*., 2003). Another fly miRNA, miR-7, has recently been described as an important element to promote robustness, especially in the context of sensory organ development. Consistent with this role, a homogenous developmental environment does not specifically require the presence of miR-7. However, upon environmental perturbation, like temperature fluctuation, the presence of this miRNA is required for optimal development of the eye (Li *et al*., 2009). The roles of miRNAs during stress are not restricted to flies. Indeed, the mammalian heart-specific miRNA, miR-208, is required to regulate stress-dependent cardiomyocyte growth (van Rooij *et al*., 2007). Likewise, even if miR-8 knock-out zebrafish embryos do not present a developmental phenotype, they do not respond properly to osmotic stress (Flynt *et al*., 2009). Finally, in *Arabidopsis thaliana*, the stress induced by low-phosphate condition is at least partially

regulated by the action of miR399 (Bari *et al*., 2006). In conclusion, in every organism encoding miRNAs, it is clear that these regulatory molecules can play key roles in stress-induced gene regulation.

1.2. Adaptation to physiological changes also involves miRNAs

The robustness conferred by miRNAs is not only essential for the organism during stress conditions, miRNAs are also involved in the regulation of changes linked with cell type specificity or cell fate. Thus, it has been shown that neuronal miRNAs generally have a shorter half-life than in other tissues. Indeed, miRNAs in retinal as well as non-retinal neurons present a half-life of about 1 hour (Krol *et al*., 2010), strikingly shorter than the average 5-day half-life in other cell types (Gantier *et al*., 2011). Interestingly, the miRNA turnover is tightly connected to the activity of these neurons and is controlled in a feedback loop manner to manage the right level of decay/expression. Among these miRNAs, some are sensitive to light to dark adaptation. The down-regulation of miR-183/96/182 cluster, miR-204, and miR-211 occurs really fast in dark conditions and the transcriptional activation of these miRNAs is again really fast when switching back to light conditions (Krol *et al*., 2010).

2. How to regulate miRNA expression?

The discovery that some dedicated cellular pathways have evolved to regulate the stability of miRNAs is not surprising. It would indeed be hard to imagine that such powerful and abundant gene regulators would always be present at high levels in the cell. These molecules need to fine-tune a network of gene expression corresponding to every different state at the cellular, but also at the tissue and whole organism level. Of course, small RNAs are regulated at the transcriptional level like any RNA polymerase II product; they are subject to the same regulatory pathways as any messenger RNA in the cell. Nevertheless, the reactivity required to control the cell homeostasis in response to danger signals involves an active regulation, not only during their biogenesis, but also at the level of the stability of the active form.

2.1. Conservation of enzymatic activities involved in miRNAs regulation

Several enzymes have been characterized that are involved in the regulation of miRNA stability, via their ability to tail their 3' extremity. Interestingly, such enzymatic activities have been noted in evolutionary distant organisms. In *Arabidopsis thaliana*, even if the enzyme responsible for this activity has not yet been identified, the existence of a tailing mechanism is commonly observed in the absence of HEN1-mediated miRNA methylation (Li *et al*., 2005). In mammals, the role of the TUT4 (Zcchc11) protein has been assessed for both the uridylation of pre-miRNAs and mature miRNAs (Hagan *et al*., 2009; Jones *et al*., 2009). The link between uridylation of miRNAs and siRNAs and their degradation has also been made in the unicellular photosynthetic alga *Chlamydomonas Rheinhardtii*, where the MUT68 terminal nucleotidyltransferase and RRP6 exosome subunit respectively perform the uridylation and degradation of miRNAs and siRNAs (Ibrahim *et al*., 2010). The reality of 3' modification of small RNAs has thus been assessed in several species. Furthermore, a recent publication indicates that not only it represents a widespread process, but also that the activity might be performed by several enzymes, that are probably not only cell type but also sequence specific (Wyman *et al*., 2011).

It would indeed be surprising that only one mechanism triggers the regulation of miRNAs, according to the diversity of mechanisms that they are involved in. It is more than likely that several pathways regulate the stability of one given miRNAs, and that would implies that several proteins as well as accessory factors are involved.

The likelihood of the redundancy between several members of the TUT family has probably been a barrier to assess our working hypothesis in Arabidopsis. Indeed there is a strong possibility, even in the case where TUT1 would have been responsible for the tailing of small RNAs, that alternative members of the same family can be recruited when the major factor involved is missing.

It is also worthy to note that not only miRNAs are regulated *via* tailing. Initially, the miRNA targeting of mRNA, in the case of pairing leading to cleavage of the target, had been linked with the terminal modification of the targeted messenger in plants but also in mammals and viral infections (Shen and Goodman, 2004).

2.2. Role of accessory/associated proteins

We have already seen that several stress-related miRNA regulations involved protein partners. The activation or repression of transcription factors upon stress is known to participate in the regulation of miRNA biogenesis. P53 is activated upon DNA damage, and in turn can act at the level of pri-miRNA transcription together with an associated action with p68 (DDX5 helicase) on Drosha processing to enhance the maturation of several miRNAs with growth-suppressive functions (Suzuki *et al*., 2009). The regulation of let-7 by Lin28 is also induced by stress. The first inflammation event that triggers NFκB activation of Lin28 transcription leads to the down-regulation of let-7, releasing the repression of Il-6 production. Finally, Il-6 can enhance NFκB expression resulting in a positive feed back loop (Iliopoulos *et al*., 2009).

In plants, the development of the shoot apical meristem (SAM) and its maintenance is under control of class III homeodomain-leucine zipper (HD-ZIP III) transcription factors. Interestingly, Ago10 has been described to be a regulator of SAM maintenance by sequestering miR166/165 and preventing their loading into the Ago1 complex. The preferential loading of miR166/165 in Ago10 is mediated by the structure of these particular miRNA duplexes. Ago10 loaded miR166/165 is not anymore able to target HD-ZIP III transcription factors, therefore maintaining the undifferentiated state of the shoot apical meristem (Zhu *et al*., 2011).

Thus, the identification of enzymatic activities involved in miRNAs stability, as well as the global regulation of miRNA activity, should not be considered as a simple relationship between one miRNA, one target, one regulator. It should rather be a complex combination of events with some partners probably having various if not conflicting roles at the first look.

The relief of miR-122 repression on the CAT-1 mRNA upon stress involves yet another accessory protein, the 3' untranslated region trans-acting factor Hu protein R (HuR). This is mediated by the translocation of HuR from the nucleus to the cytoplasm, followed by the release of the mRNA from miRNP and its exit from P-bodies and recruitment to polysomes (Bhattacharyya *et al*., 2006). Recently, N. Meisner and colleagues described that HuR possessed a terminal adenosyl transferase activity (Meisner *et al*., 2009). We set up a collaboration with the group of Dr. Meisner following their discovery that HuR might be involved in miRNA tailing and trimming (N. Meisner personal communication). Our observations confirmed these results since we found that miR-27 degradation upon MCMV infection could be blocked upon HuR inhibition by a small molecule. It might be that the relief of CAT-1 mRNA from miR-122 regulation also involves tailing and trimming of the miRNA

by HuR, without excluding the possibility of a competition for decay factors on ARE binding sites.

Of note, HuR has also been reported to promote the repression of mRNA. It can recruit let-7-loaded RISC on c-Myc mRNA, down-regulating c-myc expression in normal conditions (Kim *et al*., 2009). It was also reported to block c-Myc mRNA translation in the case of hypoxia and cancer (Talwar *et al*., 2011).

2.3. Post-translational modifications

The regulation of miRNA activity or stability can also involve post-translational modifications of essential factors. In human cells, the phosphorylation of a highly conserved tyrosine residue at position 529 of Ago2 prevents the efficient binding of the 5' phosphate of small RNAs and P-body localization (Rudel *et al*., 2011). Phosphorylation has also been shown to affect the cytoplasmic localization of Ago in GW bodies if it occurs at serine-387 (Zeng *et al*., 2008). Interestingly, the phosphorylation of AGO triggered by MAP kinase signaling has been proposed to be induced upon stress (Adams *et al*., 2009; Zeng *et al*., 2008). Not only phosphorylation, but also hydroxylation seems to be a post-transcriptional modification that can affect Ago2 stability and the efficiency of RNA interference (Qi *et al*., 2008).

In addition to phosphorylation and hydroxylation, polyADP-ribosylation has also been proposed to play a role in the regulation of small RNA activity (Leung *et al*., 2011). PolyADP-ribose (PAR) is known for its function in the nucleus on chromatin structure and transcription. The PAR polymerases (PARP) family is involved in caspase-related and unrelated programmed cell death, but also intracellular trafficking of important transcription factors such as NFκB and p53 (Elmageed *et al*., 2011). The study by Leung and collaborators showed that argonaute proteins are modified by polyADP-ribosylation upon stress and that this is probably linked to their association with mRNAs. Furthermore, PARP proteins and PAR hydrolases accumulate in stress granules (SG) upon stress conditions together with Ago2, where they are likely to play a role in assembly and maintenance of SG and alleviate miRNA-mediated silencing activity.

It is highly probable that the basal partner requirement for biogenesis and action of miRNA is now yet well defined. However many other regulatory modes will be discovered, putting to a high importance to discover new factors, such as obviously small RNA modifying enzymes, but also others, such as transcripts like MCMV-m169.

2.4. Implication of the RNA target

Before they were found to naturally occur in viruses such as MCMV, miRNA sponges were artificially engineered to down regulate the accumulation of a given small RNA in the cell. They have been designed to surpass the limitations of sequence specific inhibitor (like 2'O methyl antisense oligonucleotides). The expression of these decoys can be easily driven by a promoter of choice such as classical strong, constitutive promoters, to specifically target a given miRNA family or individual miRNA. Furthermore they can potentially be used to generate cell lines with constitutive expression (Ebert *et al*., 2007). Actually, the concept of providing a small RNAs decoy to prevent the recognition of the "real" target is a natural biological feature inside the organism. Upon phosphate starvation in plants, miR399 is induced, as is the IPS1 (induced by phosphate starvation 1) non-coding RNA, triggering a binding site for miR399 in its 3'UTR with a bulge at the miRNA cleavage site. The accumulation of both the natural sponge (IPS1) and the miRNAs upon stress, first allows the down-regulation of the miR399 target PHO2. Once IPS1 (and the related At4) level is abundant enough to sequester miR399, the regulation of PHO2 is relieved (Franco-Zorrilla *et al*., 2007). This mechanism allows an elegant way to fine-tune the control of phosphate starvation response.
Similarly, miRNA sequestration by a decoy RNA has also been described in the case of the expression of pseudogenes. The PTEN1P pseudogene derives from the PTEN gene likely from a retrotranscription event that created a gene containing a mutated start codon. It seems that the binding of oncogenic miRNAs in the 3'UTR of both transcripts alleviates the down regulation of PTEN protein expression to control human cell proliferation (Poliseno *et al*., 2010).
Viral sponges are an effective rapid way to alter the level of a miRNA to modulate the cellular environment. The relationship between an induced sponge-transcript and its related miRNA is akin to the regulation of miR-27 by the MCMV m169 transcript. In a highly similar manner, the herpesvirus saimiri also induces the down-regulation of the same miRNA by expressing non-coding viral RNAs, U rich RNAs (HSUR) 1 and 2, that bind miR-27 and lead to its degradation (Cazalla *et al*., 2010).

This mode of regulation appears to be an efficient way to redirect miRNAs from one transcript to the next, or prevent their binding to an undesirable target. However, the question or relative amounts of the target RNA and the miRNA is critical here, especially when one considers the huge difference that can exist within a single cell between one miRNA, that can accumulate thousands to hundreds of thousands molecules, and an mRNA, which is never more expressed

than in the range of a dozen molecules per cell. In this situation, even providing twice as much decoy target might not be sufficient to fully distract the miRNA from its natural targets. This problem has been overcome in the case of MCMV, since the m169 transcript is expressed at very high levels. Finally, the other solution, that is used by both MCMV and HSV, is to trigger miRNA degradation upon its binding to its pseudo targets, which allows the transcript to be recycled. Nevertheless, how the cell differentiates between the pairing of a miRNA to a 'real' target, and its pairing to a sponge or decoy target remains an open and challenging question.

MATERIAL and METHODS

1. Material

1.1. Cell lines

- Hela (CCL-2) is a human epithelial cell line derived from a cervix adenocarcinoma. HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences.
- Human embryonic kidney (HEK) GripTite™ 293 MSR Cell Line (Invitrogen) is a genetically engineered HEK 293 cell line that expresses the human macrophage scavenger receptor and strongly adheres to standard tissue culture plates.
- NIH 3T3 (CRL-1658) is a mouse fibroblastic cell line derived from embryo.
- 3T3 balb/c clone A31 (CCL-163) is a cell line developed by S.A. Aaronson and G.T. Todaro in 1968 from disaggregated 14- to 17-day-old BALB/c mouse embryos (Aaronson and Todaro, 1968).
- M2-10B4 (ATCC CRL-1972) is a stromal cell line derived from the bone marrow stromal cells from a (C57BL/6J X C3H/HeJ) F1 mouse.
- Peritoneal macrophages were obtained two days after activation by thioglycollate injection.

1.2. Plants

Arabidopsis thaliana is a small flowering plant from the *Brassicaceae* family. It possesses a relatively small genome that has been sequenced in 2000. The life cycle is rapid and the production of seed is prolific and easy, making this organism a good model for plant biology. Thanks to the high efficiency of transformation using *Agrobacterium tumefaciens*, several mutant lines are available.

The lines used in this study were:

One *Arabidopsis thaliana* natural accession originating from Columbia, USA, Col-0.

The mutants lines TUT1-1 and TUT1-3 (At2g45620) as well as the HEN1-6 (At4g20910) are T-DNA insertion mutants (Alonso *et al*., 2003) (respectively SALK_087647C, SALK_119177 (exonic insertions) and SALK_090960). They all originate from *Arabidopsis thaliana* natural ecotype Columbia and were obtained from the Arabidopsis Biological Resource Center (ABRC).

1.3. Bacterial strains

Escherichia coli Top 10 (Invitrogen)

F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ

The galE mutation confers high competence to transformation (defect in LPS formation resulting in an higher binding and/or uptake of DNA).

Escherichia coli DB3.1

F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1.

The gyrA462 mutation confers the capacity to these cells to propagate ccdB-containing plasmids. This strain is used to amplify all non-recombinated gateway plasmids.

1.4. Viruses

- MCMV Perth strain (used to infect peritoneal macrophages)
- MCMV C3X Smith Strain
- Wild type and mutant MCMV viruses derived from the Smith strain were reconstituted by transfection of the respective bacterial artificial chromosome (BAC) DNA into MEFs using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. All virus stocks were prepared on M2-10B4 cells, and virus titers were determined by the standard plaque assay.

The corresponding genomes can be found under the accession numbers NC_004065 (Smith strain) and AM886412 (Perth strain/ strain K181).

2. Methods

2.1. Cell culture

2.1.1. Culture conditions

Cells were grown in a humidified cell culture incubator and maintained at 37 °C in 5 % CO2. All media and reagents used for cell culture were obtained from Gibco, Invitrogen, unless

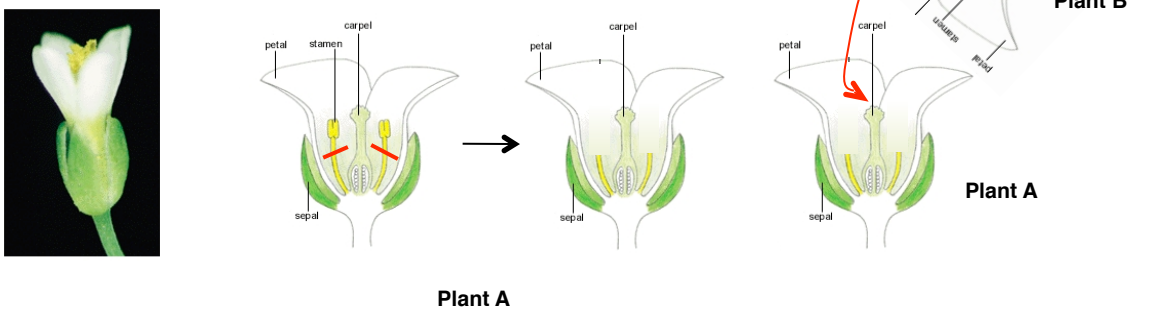

Figure 44: Overview of cross principle in *Arabidopsis thaliana*.

Original pictures from Alberts *et al.*, Molecular Biology of the Cell. 3rd edition, Garland Science.

stated differently. Cell lines were cultured in Dulbecco's modified Eagle Medium (DMEM) GlutaMAX, or RPMI supplemented with 10 % fetal bovine serum (FBS) (Eurobio).

2.1.2. Cell splitting

After aspirating spent media, monolayer cell cultures were briefly rinsed in sterile 1x phosphate buffered saline (PBS) prepared from powder DPBS (USBiological). The volume of PBS used is dependent on the size of the flask being split. The cells were treated with enough 0.05 % Trypsin-EDTA 4Na to cover the base of the flask, then aspirated, and returned to the incubator until the monolayer detached from the flask/well base. Detached cells were re-suspended in an appropriate volume of fresh medium and split according to cell type and experimental requirements. Cells were counted using a hemocytometer prior to plating at a specific density for transfection experiments.

2.2. Arabidopsis thaliana

2.2.1. Genetic crosses

Floral dips of *Arabidopsis* are emasculated to avoid self-fertilization before crossing. 1 day later, these flowers are fertilized with the pollen of a genetically different plant by scraping the pollen sacs on the prepared carpels of the initial plant (Figure 44). About 3 weeks after, the siliques originating from the cross are harvested and the seed are next sown.

2.2.2. Soil seeding

Arabidopsis seeds are sown on soil, and grew with a photoperiod of 12 hours at 20°C. After three weeks, they are transplanted and, after 2 or 3 extra weeks, are transferred in long day conditions (photoperiod day/night of 16/8 hours, at 18-20°C).

2.3. Microbiology

2.3.1. Preparation of competent bacterial cells

Rubidium chloride thermo-competent cells

A 10mL culture is grown overnight in LB. 500mL of LB medium are inoculated with 5mL of the starter culture, and next grown at 37°C to an absorbance (595nm) of 0.5. The bacterial

culture is cooled 15 min on ice, transferred to ice cold centrifuge bottles and spinned 10 min at 5000 rpm at 4°C. The supernatant is poured off and the pellet is resuspended on ice in 30 mL of cold Tfb I for 15 minutes. The solution is spinned 10 min at 5000 rpm and 4°C and resuspended on ice in 6mL of ice cold Tfb II. Finally, aliquots of 100μL are prepared in prechilled microtubes and freezed in liquid nitrogen.

LB medium :

Bacto-tryptone	10g
Yeast extract	5g
NaCl	10g
dH_2O	800mL

Adjust pH to 7.5 with NaOH.

Adjust volume to 1L with dH_2O and sterilize by autoclaving

Tfb I (30mM KOAc, 50mM $MnCl_2$, 100mM RbCl, 10mM $CaCl_2$, 15% Glycerol)

RbCl	6,046g
$MnCl_2$-$4H_2O$	4,948g
KOAc	1,472g
$CaCl_2$-2H2O	0,735g
Glycerol 100%	75mL

Adjust pH to 5.8 with acetic acid

Add water to 500mL

Tfb II (10mM NaMOPS, pH 7.0, 75mM CaC1$_2$, 10mM RbC1, 15% Glycerol)

MOPS 0.2M	20mL
RbCl	0,483g
$CaCl_2$-$2H_2O$	4,411g
Glycerol 15%	60mL
MOPS	20,926g

Adjust pH to 6.5 with HCl. Add water to 500mL.

2.3.2. Bacterial transformation

Heat-shock transformation is performed by mixing the plasmid DNA and chemically competent bacteria in a Eppendorf tube. After equilibration on ice for 15 minutes, the mixture is incubated 35 seconds at 42°C, and briefly cooled-down on ice. Then 600μL of pre-warmed SOC medium are added and the bacteria are incubated between 40 and 60 minutes at 37°C in a

water-bath or a dry-bath with shaking. 100 to 300μL are spread on LB agar plates containing the appropriate antibiotic and grown overnight at 37°C.

LB agar

15g agar (Roth) to 1L of LB medium

SOC medium:

H_2O	900mL
Bacto Tryptone	20g
Bacto Yeast Extract	5g
NaCl 5M	2mL
KCl. 1M	2.5mL
$MgCl_2$ 1M	10mL
$MgSO_4$ 1M	10mL

Adjust to 1L with distilled H_2O and sterilize by autoclaving.

Add sterile glucose so that the final concentration is 20mM.

2.4. Virology

2.4.1. Virus amplification

M2-10B4 cells were seeded in 10 cm plate and infected at a confluency of 70% with 2 mL of virus stock (approx 10^6 PFU) to reach a multiplicity of infection of about 0.1.

After 5 days to 1 week, when all cells show the typical phenotype of infection (extended cytoplasm) and start to detach, the cells were scraped and collected with the supernatant to infect six 225 cm^2 flasks containing M2-10B4 cells at 70% of confluency.

After one week of infection, cells start to detach. The growing medium of all flasks is pooled and frozen with addition of 1% DMSO, from a stock of DMSO 10% diluted with DMEM+ FBS 10%. Virus stocks are aliquoted in 2 mL Eppendorf tubes and stored at -80°C.

2.4.2. Titration of viral stocks on M2-10B4 cells

M2-10B4 cells (or 3T3 balb/c cells) were seeded into 24-well plates so that they are 80% confluent the day of infection. The day of infection, the medium is changed and 150 μL of fresh medium is added. Serial dilutions of the viral stock are prepared up to 10^{-8} in 96 wells plate. 100 μL of each dilution is added to each well and the virus is adsorbed to the cells for 4

to 6 hours at 37°C in the incubator. After adsoption of the virus, the wells are overlaid with 1mL of warm DMEM+ FBS 10 % with methylcellulose. Plates stayed in the incubator until plaques are large enough for scoring.
After 5 to 7 days, the medium is removed and the cells are washed at least twice with D-PBS. To fix the assay, 500 μL of formamide crystal violet are added to the cells for 15 minutes. After washes with tap water, the plates are air-dried and scoring of plaques is done manually.

2.4.3. Infection of NIH-3T3 cells

Cells are split 24 hours before infection, counted and plated at $0.5x10^5$ cells per well of a 6 well plate. The next day, aliquot of virus are thaw on ice, the medium is removed from wells and 3 mL of fresh medium containing virus are applied on the cells. The plate are centrifuged in swinging rotor centrifuge for 30 minutes at 800 g at 20°C to 37°C and then incubated at 37°C for 1 hour. The cell culture medium is then replaced with fresh medium. The centrifugation leads to an increase of infectivity of the supernatant by at least 10-fold.
In order to infect virtually all cells on a plate you need to use a multiplicity of infection (MOI) of 5. Using an MOI of 1 you will only infect <30% of cells or so. Unless otherwise stated, cells are infected at a MOI of 10.

2.4.4. Infection of peritoneal macrophages

After activation of peritoneal macrophages followed by peritoneal washes with PBS, the cells are collected and plated in 6 well plates. They are infected as previously described.

2.5. Molecular biology

2.5.1. Nucleic acids quantification

DNA is quantified based from 260 nm absorbance using a Thermo Scientific NanoDrop™ 1000 spectrophotometer and its corresponding software.
RNA is quantified using a Thermo Scientific NanoDrop™ 1000 spectrophotometer and its corresponding software. The quality of RNA is assessed with the 260/280-absorbance ratio. A value between 1.8 and 2 is considered as a good indicator for good quality of RNA sample.

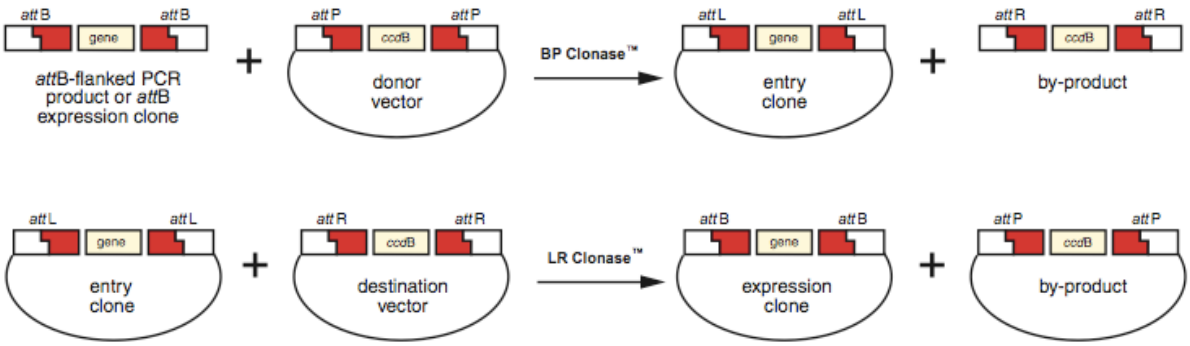

Figure 45 : Gateway cloning principle and gateway compatible expression plasmid with a N-terminal HA flag.

2.5.2. Plasmid DNA preparation

All plasmid DNAs have been prepared from liquid LB medium cultures with the appropriate antibiotic. After overnight growth, the plasmid DNA is extracted following manufacturer's instruction with the appropriate kit, according to the initial volume of bacterial culture: NucleoBond Xtra Midi (Macherey-Nagel) or GenElute HP Plasmid Miniprep kit (Sigma-Aldrich).

2.5.3. DNA purification

PCR reaction were purified either directly or on agarose with NucleoSpin Extract II kit (Macherey-Nagel). Alternative gel purification of DNA relied on low-melting agarose gel (Agarose type LM-3, Euromedex) separation followed by basic phenol-chloroform purification.

2.5.4. Gateway cloning

The Gateway technology is a cloning method based on the site-specific recombination properties of bacteriophage lambda when integrating into the *E. coli* chromosome. The components of the lambda recombination system are modified so that the recombination reactions maintain orientation and reading frame. Briefly, recombination occurs between specific attachment (att) sites on the interacting DNA molecules. AttB sites (usually on the *E. coli* chromosome) and attP on the lambda chromosome can recombinate upon lambda integration to give rise to attL and attR sites (Figure 45). This reaction can be reversed. The Gateway cloning approach relies on the generation of a attB-PCR product where the sequence of interest is amplified by nested PCR to introduce half attB sites at each extremities. A second nested PCR is realized to generate the two full-size attB sites. The attB-flanked PCR product is then recombined to an attP substrate (donor vector, pDONRZeo, Invitrogen) to create an attL-containing entry clone (pDEST or modified psiCHECK2, Promega). This reaction is mediated by the BP clonase mix (Invitrogen) between the attB sites on the PCR products and the aatP sites located at each extremities of a ccdB cassette upon manufacturer's instructions. The ccdB cassettes contain a ccdB gene for negative selection and a chloramphenicol resistance gene for counter-selection. The entry clone is then recombined to the destination vector containing an attR flanked ccdB cassette to create an attB-containing expression clone. This reaction is catalyzed by LR Clonase enzyme mix.

To generate luciferase reporters plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C.1 (Invitrogen) at the 3'-end of the firefly luciferase gene into the Xba *I*

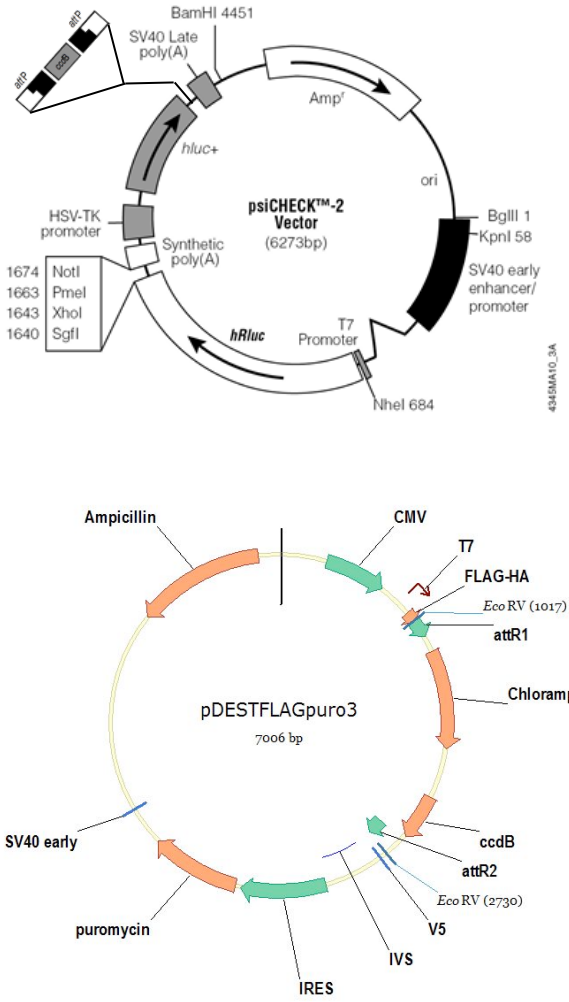

Figure 46 : Modified psiCHECK-2 vector (Promega) with an inserted gateway cloning cassette (Invitrogen).

site of psiCHECK-2. attB-PCR products were cloned into pDONR/Zeo (Invitrogen) and then recombined in the modified psiCHECK-2 vector by Gateway cloning. PDEST-FLAGpuro3 already contains a gateway compatible cassette (Figure 46).

2.5.5. PNK labelling

Probes were 32P-radiolabelled oligodeoxynucleotides perfectly complementary to the miRNA sequence or to part of the U6 snRNA sequence

Oligodeoxynucleotide 20μM	1 μL
PNK buffer 10x	2 μL
32P-gamma-ATP (110 Tbq/mmol ; 10 μCi/μL)	2.5 μL
T4 polynucleotide kinase (10u/μL)	1 μL
H2O	13.5 μL

The unincorporated radiolabelled nucleotides are removed from reaction mixture by Sephadex G25 columns (GE Healthcare).

2.5.6. Protein detection

Total proteins from HeLa cells and HEK293 cells transfected with the pDEST-Zcchc6 were extracted in lysis buffer (150 mM NaCl; 50 mM HEPES, pH 7.4; 5 mM EDTA and 0.1% NP40), 30 µg of proteins were resolved by SDS-PAGE and transferred by electro-blotting onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Peroxidase-conjugated antibodies directed against the HA tag (Sigma-Aldrich) were used at a dilution of 1/6000 and revealed directly through enhanced chemiluminescence (Roche Lumi-light PLUS) and visualized on SuperRX film (Fuji)

2.5.7. Dual luciferase experiments

The perfect match sensors for miR-M23-2 and miR-21 were obtained by annealing the oligonucleotides PM-M23-2-for and PM-M23-2- rev or PM-m21-for and PM-m21-rev. The miR-M23-2 and miR-21 mismatch sensors were obtained by annealing the oligonucleotides MM-M23-2-for and MM-M23-2-rev as well as the oligonucleotides MM-m21-for and MM-m21-rev. AttB1 and AttB2 sites were incorporated by PCR using primers attB1-for and attB2-rev. The resulting PCR products were then recombined sequentially in pDONR/Zeo and in a modified psiCHECK-2 plasmids using Gateway technology (Invitrogen).

2.5.8. Luciferase assays

Cells were seeded in 48-well plates at 10^5 cells/well and then incubated overnight. The following day plasmid, miRNA mimics or siRNAs were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer protocol. Eventually, cells were subject to a second transfection the next day. In this case, the lipid complexes were removed after 6h post transfection and the cells were incubated overnight. After 24 or 48 h, cells were washed with D-PBS and lysed with 65 μL passive lysis buffer (Promega). 10 μL of the lysate were assayed for firefly and *Renilla* luciferase activity, using the dual-luciferase reporter assay system (Promega) and a luminescence module (Glomax, Promega). The relative reporter activity was obtained by first, normalizing to the transfection efficiency with the *Renilla* activity, and then, to the firefly activity obtained for an empty control reporter, in presence of control construct, to normalize for the effect of transfection of these expression vectors.

2.5.9. Macrophages genotyping

Peritoneal macrophages are subject to a second genotyping (a first genotyping has been made on living animals). Genomic DNA is precipitated after Tri-Reagent (MRC) extraction from the organic phase and interphase by addition of 0.3 mL of 100% ethanol per 1 mL of TriReagent used. The DNA is sedimented by centrifugation at 2000g for 5 minutes at 4°C. The pellet is then washed with a solution of 0.1 M sodium citrate in 10% ethanol. After two washes, the pellet is resuspended in 75% ethanol, centrifuged and air dried in an open tube. The DNA is resuspended into 300 μL of 8mM NaOH. The pH is ajusted to 8.4 with 22.2 μL of 0.1 M Hepes and 2.5 μL are used in the following PCR mix :

Buffer 10x	2.5 μL
dNTP 10 mM	0.5 μL
primer A 10 μM	0.5 μL
primer B 10 μM	0.5 μL
Dream Taq (Fermentas)	0.12 μL
DNA	2.5 μL
H2O	18.38 μL

P1 (mDicer1 intron 24) : 5' ccttgcgtggtcagcattagcatt

P2 (mDicer1 exon25) : 5' ttctcctcatcctcctcggatctc

P3 (retrovirus intron) : 5' cactccaacctccgcaaactccta

Amplicon WT (P1 / P2) : 802 bp

Amplicon KO (P1 / P3) : 547 bp

PCR program :

a : 30" 94°C

b : 30" 94°C

c : 30" 58°C

d : 1' 72°C

e : 7' 72°C

repeat b to d 35 times

2.5.10. *A. thaliana* insertion lines genotyping

The genotyping of the different Arabidopsis thaliana insertion line was realized with the REDExtract-N-Amp PCR Ready Mix (Sigma-Aldrich) upon manufacturer's instructions. Briefly, one leave of a rosette stage plant is added directly to 50 μL of extraction buffer and denaturated for 10 minutes at 95°C. Then 50 μL of a dilution buffer are added. 4 μL of the mix are used to run a PCR with pairs of primers corresponding to the WT gene or T-DNA borders sequence (LBa1) according to the following mix

PCR mix (including dNTP, Taq, Buffer)	10 μL
Template	4 μL
Oligonucleotide A 10μM	1 μL
Oligonucleotide B 10μM	1 μL
H_2O	4 μL

10 μL of the PCR reaction are resolved and analyzed on a 1% agarose gel.

2.6. RNA analysis

2.6.1. Extraction of RNA

Monolayer cells were lysed by TRIreagent (Molecular Research Center) addition directly to the wells. Organs and plants were grinded in tubes plus beads, or in a mortar. RNA was then extracted following manufacturer's instruction and additionally purified with at least one acidic

phenol/chloroform step. RNA is subjected to precipitation with 3 volumes of 100% ethanol and -20°C storage for at least one hour. The pellet is resuspended in 50% deionized formamide.

2.6.2. Northern blot analysis

Northern blotting was performed on 10 μg of total RNA diluted in 1 volume of loading solution (Urea 8M, EDTA 50mM, bromophenol blue). The samples migrate on a 17.5% acrylamide gel. For 200 mL, 84 gr urea have been dissolved in 87.5 mL of a concentrated solution of acryl-bisacrylamide 40% (Rotiphorese 19 :1, Roth) and 10 mL of tris borate EDTA buffer 10x (Euromedex). The polymerization is achieved by addition of 32μL of APS 25% and 4 μL of TEMED (Roth) per 10mL volume. The gel is run in an Owl P10DS tank (Thermo Scientific) at 500 volts so that the loading dye reaches the bottom of the gel.

Semi dry transfert is then performed using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-rad) at 25 volts for one hour.

Cross-link is made either with UV at 260 nm or by EDC cross linking as described in Pall and Hamilton, Nature protocol, 2008 (Pall and Hamilton, 2008). Briefly, for 1 g of EDC, 325 μL of 1 methylimidazol are diluted in 20 mL milliQ water. The pH is adjusted to 8 with Hcl. Then EDC is added and the volume is completed to 31,9 mL. The membrane is carefully positioned on a EDC solution pre-wet Watman paper and then incubated for 1h30 at 60°C. The membrane is rinsed with water before hybridization.

Hybrizations were done at 50°C overnight in a rotating incubator. Washings were twice 10 min with 5x saline-sodium citrate (SSC) buffer, 0.5% SDS and an additional 10 min wash with 1x SSC, 0.5%.

Equal loading of the gels was confirmed by ethidium bromide staining of the tRNA band or/and by reprobing the blot for U6 snRNA using 32P-labeled 5' GCAGGGGCCATGCTAATCTTCTCTGTATCG oligodeoxynucleotide.

Blots were analyzed and quantified by phosphorimaging using a FLA5100 scanner from Fuji.

Blots were stripped and reprobed several times. Complete stripping of the blot was confirmed by phosphorimaging of the membrane before reprobing.

2.6.3. qPCR

Quantitative real-time PCRs were established using a Roche LightCycler 480 Real-Time PCR System. For mature miRNAs, total RNA was poly-adenylated and reverse transcribed using the miScript Reverse Transcription kit (QIAGEN) as per the manufacturer's instructions with 1 mg

of total RNA per reaction. The obtained cDNA (1 mL of a 1/5 dilution) was then PCR-amplified using the miScript SYBR Green PCR kit (QIAGEN) in 10 mL reaction mixtures consisting of Quantitect SYBR Green PCR master mix and 0.625 mM of the miRNA-specific primer and the miScript universal primer. PCRs were subjected to 15 min of 95°C hot-start enzyme activation, and 40 cycles of 95°C denaturation for 15 sec, 55°C annealing for 30 sec, and 72°C elongation for 30 sec, including subsequent melting curve analysis. The data was analyzed using the ΔΔCt method (Bookout *et al*., 2006) using U6 snRNA as an endogenous reference, and the mock-infected sample as a calibrator. The amplification efficiency as determined with the LinReg software (Ramakers *et al*., 2003) was used to calculate fold changes relative to mock. Primers were the mature miRNA sequences for the forward primers, and the universal miScript primer provided by QIAGEN for the reverse primer.

2.6.4. Small RNAs cloning

2.6.4.1. Adenylation of 3'adapter for small RNA cloning

In order to adenylate the 3' adapter, the following mix is heated 3 minutes at 95°C and then incubate on ice for 5 minutes:

Phosphorylated adapter (500μM)	5 μL
Adapter antisens (100μM)	75 μL
Tris pH 7,5 (2M)	0.25 μL
NaCl (0,3M)	5 μL
EDTA (1mM)	10 μL
Water (milliQ)	19,75 μL

The following ingredients are added to the mix :

MOPS (0,2M)	40 μL
$MgCl_2$ (1M)	2 μL
DTT (0,1M)	20 μL
ATP (100mM)	20 μL
Concentrated T4 DNA Ligase	18 μL

The mixture is incubated 24 hours at 25°C.

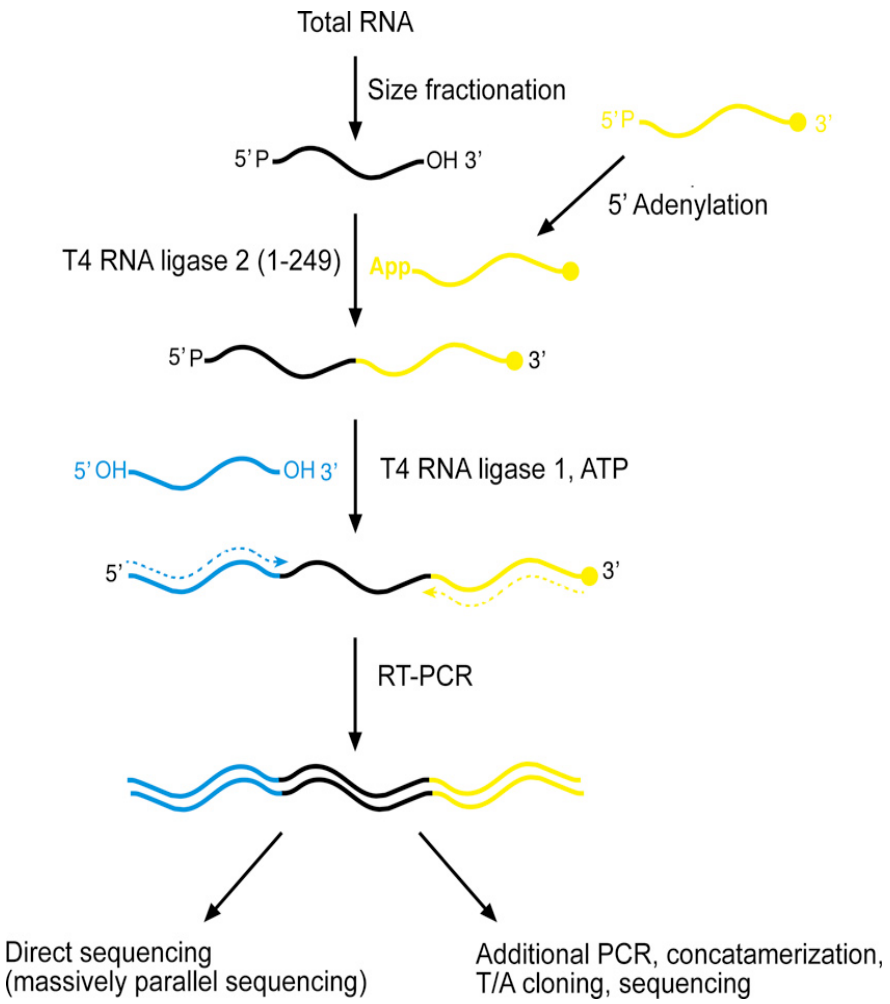

Figure 47 : Schematic representation of the small RNA cloning protocol steps.

After 24 hours, one volume of loading solution (EDTA 50mM, formamide 99%, bromophenol blue 0.5 mg/mL) is added and the total reaction is loaded on acrylamide gel 17.5%, with the phosphorylated adapter as size control.

After 1 to 2 hours run at 500 V, 30 W, the band of the adenylated adapter is revealed by UV-shadowing and excised from the gel with a blade. The gel slices are cut in little pieces and 2 to 3 volume of NaCl 0.3M are added. After over night incubation with shaking (800 rpm) at 4°C, the eluate is transferred to a new tube and 2.5 volumes of 100% ethanol are added. After precipitation and centrifugation, the supernatant is carefully removed and the pellet is resuspended in 20μL of water.

The absorbance of the RNA is evaluated at 260 nm and the RNA concentration is calculated with the formula:

[μM] = [(OD260 x dilution) / (11000 x (base number))]x 10^6

Finally, the concentration is adjusted to 50 μM with water.

2.6.4.2. Preparation of size markers

19nt and 24nt long oligonucleotides containing a PmeI restriction sites were radiolabeled by PNK labeling and then purified on a 15% acrylamide gel (acrylamide 15%, Urea 7M, TBE 0.5x) After migration of the sample at 500V, 30W for 1h30, the gel is wrapped in Saran and exposed an autoradiographic film for about 1 minute to visualize the labeled oligonucleotides.

The bands corresponding the full length oligonucleotide are cut in small pieces and stored into a 1.5mL silicon tubes. The oligonucleotides are eluted overnight at 4°C under agitation in NaCl 0.3M. The next day, the aqueous phase is precipitated by addition of 2.5 volumes of 100% ethanol. After centrifugation, the pellet is resuspended in 30 μL of water.

2.6.4.3. Small RNAs cloning

We spiked 50 μg total RNA with 0.2 nM radiolabeled size markers (5'-CGUACGCGGGUUUAAACGA-3' and 5'-CGUACGCGGAAUAGUUUAAACUGU-3'). Total RNA is next size-fractionated and ligated to a 5'-adenylated 3' adapter oligonucleotide (5'-AppTTTAACCGCGAATTCCAG-L-3'; Ap, Adenylate; p, phosphate; L, C6-amino linker (Dharmacon)) using the Rnl2(1–249) ligase in the absence of ATP. The reaction is performed in a final volume of 20 μl containing 0.1 μg of the Rnl2(1–249) ligase, 5 μM of the 3' adapter oligonucleotide, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.05 M Tris-HCl (pH 7.6) and 0.1 mg acetylated bovine serum albumin. After gel purification, the ligation product is directly

joined to the 5' adapter chimeric DNA/RNA oligonucleotide (5'-ACGGAATTCCTCACTrArArA-3'; r, ribonucleotide) using T4 RNA ligase 1 (NEB) in the presence of ATP and in the same reaction solution as above. The final ligation product is reverse-transcribed using the primer 5'-GACTAGCTGGAATTCGCGGTTAAA-3' and PCR amplified using the reverse transcription primer and the primer 5'-CAGCCAACGGAATTCCTCACTAAA-3'. After purification, the PCR product is digested with *Pme*I to eliminate size marker sequences and an additional PCR is performed to introduce the nonpalindromic *Ban*I restriction sites with primers 5'-CAGCCAACAGGCACCGAATTCCTCACTAAA-3' and 5'-GACTAGCTTGGTGCCGAATTCGCGGTTAAA-3'. The *Ban*I-digested PCR products are then concatemerized using T4 DNA ligase and later ligated into the commercial T/A cloning vector pCR2.1 (Invitrogen). Individual colonies are screened for the presence of inserts by PCR and the insert-containing PCR products are sequenced (Figure 47).

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APPENDIX

The Properties and Roles of Virus-encoded MicroRNAs

Mélanie Tanguy and Sébastien Pfeffer

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The Properties and Roles of Virus-encoded MicroRNAs

3

Mélanie Tanguy and Sébastien Pfeffer

Abstract

The discovery that viruses could encode micro (mi)RNAs, similarly to the eukaryotic organisms they infect, has opened new perspectives in the study of host–virus interactions. These small regulatory RNAs, which are critically involved in an ever-increasing number of biological processes, have revolutionized the way we used to see gene regulation. Some mammalian viruses, mainly from the herpesvirus and polyomavirus families, have hijacked this mechanism in order to help them achieve the infection of their host. In this chapter, we will present the diversity of known viral miRNAs, their specific properties, their viral and cellular targets and the roles they play during the course of infection. We will see that more and more it appears that virally encoded miRNAs seem to be critically involved in every step of the virus life cycle.

Introduction

The prime role of RNA silencing during a viral infection seems to act as a defence system to protect the organism. This is commonly admitted in plant and insect organisms, where viruses are both effectors and targets of the silencing machinery (see Chapter 2). However, the situation is much less clear in mammals where it remains to be formally demonstrated that there really is an antiviral role of RNAi in the context of a physiologically relevant infection. The missing indisputable proof for this peculiar protection mechanism would be the identification of siRNAs of viral origin in infected cells or tissue. Yet, in the search of these elusive viral siRNAs, what has been surprisingly found instead was that some viruses do indeed express small RNAs, but these rather resemble micro (mi)RNAs and they appear to be beneficial for the virus rather than part of a degradation or protection scheme.

Among the different classes of small regulatory RNAs, miRNAs are one of the most represented and studied in vertebrates. These 21–24 nucleotides (nt) long molecules are key managers of gene expression and are involved in a very large number of biological processes. Although they were discovered in the nematode *Caenorhabditis elegans* in 1993 (Lee *et al.*, 1993; Wightman *et al.*, 1993), they were only shown to be present in other eukaryotes in 2001 (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001). Their biogenesis has been under intense scrutiny since, and has been reviewed extensively elsewhere (for example see (Bartel, 2004; Du and Zamore, 2005; Kim, 2005; Meister and Tuschl, 2004; Okamura *et al.*, 2008)]. Fig. 3.1 depicts the critical steps and enzymes involved in this pathway. Briefly, miRNAs are typically transcribed by RNA polymerase II, and in rare occasions by RNA pol III, in the nucleus of the cell. The large pri-miRNA transcript is then processed by the microprocessor complex, where the type III ribonuclease Drosha is the active component, to give rise to the stem–loop precursor miRNA (pre-miRNA). In few cases, pre-miRNAs named mirtrons can be excised in a Drosha-independent manner by direct splicing. The pre-miRNA is then exported to the cytoplasm via the exportin 5 factor. Once in the cytoplasm, the pre-miRNA is processed into an RNA duplex, very similar to an siRNA duplex, by a second RNase named Dicer.

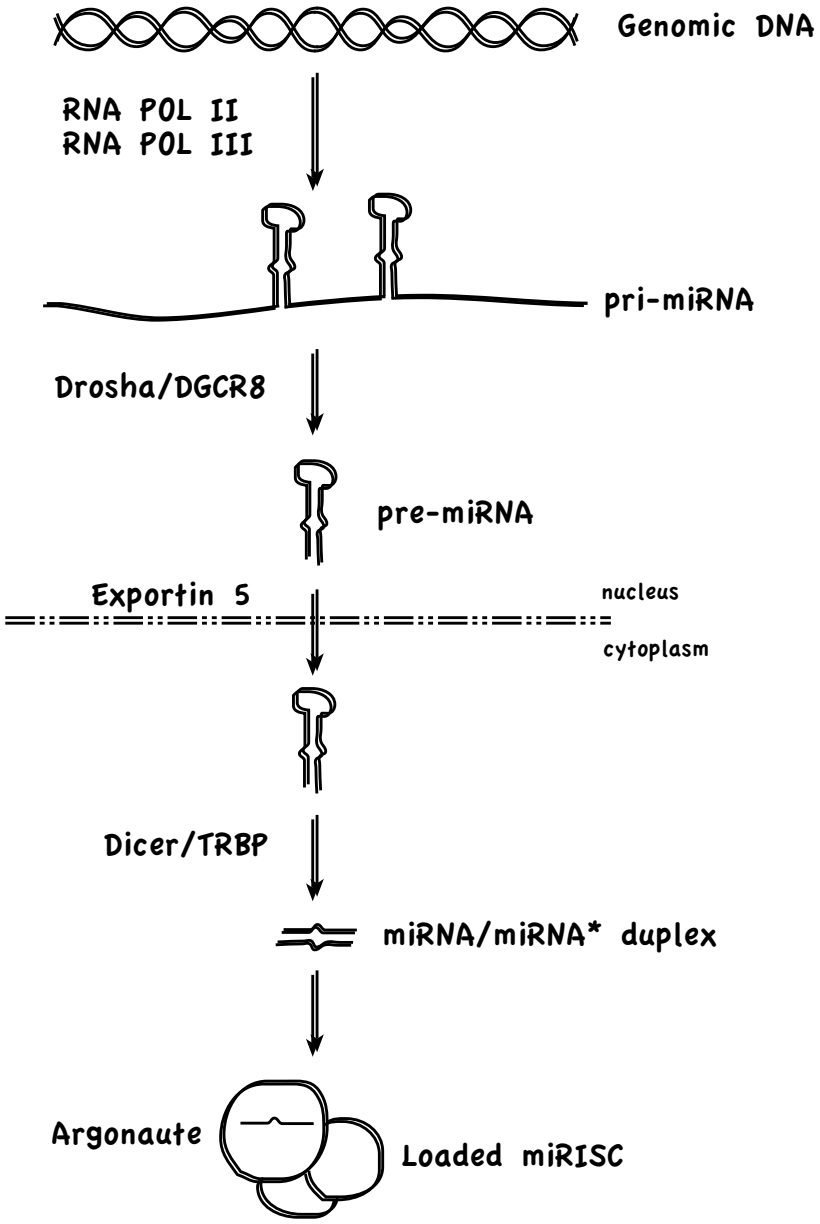

Figure 3.1 Key factors involved in the biogenesis of cellular and viral miRNAs. DGCR8, DiGeorge critical syndrome region 8; TRBP, Tat-responsive element RNA-binding protein.

After this step, either the mature strand or both strands are incorporated in the RNA-induced silencing complex (RISC), which invariably contains a member of the Piwi/Argonaute family of proteins. The active RISC will be directed to target sequences by the loaded mature miRNA. The targeted transcript will afterward undergo two possible fates that, at least partially, depend on its degree of complementarity with the small RNA. In case of a perfectly matched binding site and only when RISC contains Argonaute 2, the targeted transcript will be cleaved and degraded by exonucleases. When the pairing is only incomplete, with stretches of paired nucleotides

and bulges, the translation of the target mRNA will be inhibited. This translation block can be achieved in different ways from the inhibition of initiation to the destabilization of the RNA by de-adenylation and decapping (reviewed in Eulalio *et al.*, 2008; Pillai *et al.*, 2006). Often, the miRNA target will be translocated to so-called cytoplasmic processing or GW182 bodies where it will not be available to the translation machinery. Interestingly, this sequestration is reversible in certain stress conditions (Bhattacharyya *et al.*, 2006). Most targets that have been identified up to known are recognized by miRNAs through binding sites in their 3′-UTR (Lim *et al.*, 2005), although it appears that miRNAs can also attach to other parts of the mRNA (Orom *et al.*, 2008; Tay *et al.*, 2008). Some of the miRNA nucleotides appear to be more important than others in the recognition of the target. In particular, several publications have pointed to the importance of the so-called seed of the miRNA, which is composed of nucleotides 2–8, with some variations depending of the reports (Lewis *et al.*, 2005).

Viral miRNAs that have been described up to now mostly belong to three distinct virus families: Herpesviridae, Polyomaviridae and Adenoviridae. We will review here the current knowledge on virus-encoded miRNAs from the extensive description of the great diversity of known viral miRNAs to their possible functions during the infectious cycle.

The diversity of viral miRNAs

To date, more than 140 miRNAs of viral origin have been identified (Table 3.1). In this section, we will present the viruses in which they have been found, their genomic localization and their mode of expression. The vast majority of viral miRNAs are not evolutionary conserved between distant virus relatives or with their host. However, there are a few notable exceptions where some limited sequence homologies can be found between a viral miRNA and a cellular miRNA, but this rather seems to be due to a co-evolution in function. As we will see below, there also are a number of cases where the role of the miRNA has been conserved between distantly related viruses with no conservation at the primary sequence level.

Gammaherpesviruses

Epstein–Barr virus (EBV) was the first human virus shown to encode miRNAs. Pfeffer *et al.* initially identified 5 miRNAs in the BL41 cell line latently infected with the B95.8 strain of EBV (Pfeffer *et al.*, 2004), before an additional 18 were found, among which 13 were localized in a region of 12 kb that is deleted in this particular strain (Cai *et al.*, 2006; Grundhoff *et al.*, 2006). EBV primo-infection causes infectious mononucleosis in young patients before it goes for a lifelong latent infection – up to 95% of people are latently infected with this virus in the Western world. EBV was the first oncogenic virus identified in humans, and was primarily reported to be the aetiological agent of Burkitt's lymphoma. Additionally, EBV is known to be associated with a number of other neoplastic disorders such as B and T cells lymphomas, as well as nasopharyngeal and gastric carcinomas (Kieff and Rickinson, 2007). The miRNAs in EBV are located in three different clusters: two within the BART (BamHI A rightward transcripts) locus, which coding potential is uncertain and another one within the BHRF1 (Bam H1 rightward open reading frame 1) transcript, an early lytic transcript associated with the replicative cycle and a distant homologue of Bcl2 (Oudejans *et al.*, 1995). This transcript is also involved in the translation of EBNA proteins (EBV nuclear antigens). MiR-BHRF1-1 to 3 are potentially located within the 5′ and 3′-UTR of BHRF1 transcript, but some have reported that they could be expressed from the large EBNA1 transcript, and not from the shorter BHRF1 mRNA. The BART clusters include miR-BART-1 to miR-BART-20, which map to intronic regions of the BART transcript. A recent study has shown that the BART miRNAs are produced prior to splicing and that a specific form of the spliced precursor transcript is favouring miRNAs production (Edwards *et al.*, 2008). Interestingly, EBV miRNAs have been shown to accumulate differently in different types of carcinomas. Thus, BART miRNAs, but not BHRF1 miRNAs, are highly expressed in gastric carcinoma cell lines and tumour samples (Kim do *et al.*, 2007), which has an important implication for their putative role in the development of this disease.

Cai and coworkers cloned small RNAs from another, distant member of the

Table 3.1 Known virus-encoded miRNAs

Virus family, genus	Name	No. of pre-miRNAs	Names	References
Herpesviruses				
α/Simplexvirus	HSV1	6	miR-H1 to H6	Cui *et al*. (2006), Umbach *et al*. (2008)
	HSV2	3	miR-I to III	Tang *et al*. (2008a,b)
α/Mardivirus	MDV1	14	miR-M1 to M13, miR-M31	Burnside *et al*. (2006, 2008), Yao *et al*. (2008)
	MDV2	17	miR-M14 to M30	Yao *et al*. (2007)
β/Cytomegalovirus	HCMV	11	miR-UL22A, UL36, UL70, UL112, UL148D, US4, US5-1, US5-2, US25-1, US25-2, US33	Dunn *et al*. (2005), Grey *et al*. (2005), Pfeffer *et al*. (2005)
	MCMV	18	miR-m01-1 to m01-4, m21-1, m22-1, M23-1, M23-2, M44-1, M55-1, m59-1, m59-2, M87-1, m88-1, M95-1, m107-1, m108-1, m108-2	Buck *et al*. (2007), Dolken *et al*. (2007)
γ/Lymphocryptovirus	EBV	23	miR-BART1 to 20, BHRF1-1 to 3	Cai *et al*. (2006), Grundhoff *et al*. (2006), Pfeffer *et al*. (2004)
	rLCV	16	miR-rL1-1 to 16	Cai *et al*. (2006)
γ/rhadinovirus	KSHV	12	miR-K12-1 to 12	Cai *et al*. (2005), Grundhoff *et al*. (2006), Pfeffer *et al*., (2005), Samols *et al*. (2005)
	RRV	7	miR-rR1-1 to 7	Schafer *et al*. (2007)
	MHV68	9	miR-M1-1 to 9	Pfeffer *et al*. (2005)
Polyomaviruses	SV40	1	miR-S1	Sullivan *et al*. (2005)
	SA12	1	No name	Cantalupo *et al*. (2005)
	BKV	1	miR-B1	Seo *et al*. (2008b)
	JCV	1	miR-J1	Seo *et al*. (2008b)
	MCV	1	miR-M1	Seo *et al*. (2008a)
Adenoviruses	hAV	1	No name	Andersson *et al*. (2005), Aparicio *et al*. (2006), Sano *et al*. (2006), Xu *et al*. (2007)
Ascoviruses	HvAv	1	miR-1	Hussain *et al*. (2008)

gammaherpesvirus genus, the rhesus lymphocryptovirus (rLCV) (Cai *et al.*, 2006). Notably, the 16 identified miRNAs precursors mapped to regions similar to those found in EBV, and eight mature miRNAs were conserved in sequence suggesting that an evolutionary pressure maintained these sequences.

Kaposi's sarcoma-associated herpesvirus (KSHV), another gammaherpesvirus, is the primary aetiological agent of Kaposi's sarcoma, an endothelial tumour developing in

immunocompromised individuals. KSHV is also associated with aggressive lymphomas such as body-cavity-based lymphoma, pleural effusion lymphoma and multicentric Castleman's disease (Ganem, 2007). It encodes 12 miRNAs (Cai *et al.*, 2005; Grundhoff *et al.*, 2006; Pfeffer *et al.*, 2005; Samols *et al.*, 2005). These miRNAs are clustered in one segment located at the vicinity of the major KSHV latency transcript K12. The K12 locus encodes three isoforms of Kaposin proteins, which role is not well known yet, although the A isoform of Kaposin has been shown to have transformation properties (Kliche *et al.*, 2001). KSHV-miR-K12-1 to miR-K12-9 and miR-K12-11 are located in the intron of the larger kaposin transcript, while miR-K12-10 maps to the coding region and miR-K12-12 resides within the 3′-UTR of the K12 coding sequence. One of KSHV miRNA, miR-K12-10, is edited at position 2 of the miRNA sequence. The two variants presenting either an adenosine or a guanine at this position seem to accumulate to similar level in the latently infected BCBL1 cell line (Pfeffer *et al.*, 2005). The role of this editing is not known, but its location at a critical residue within the miRNA seed region suggests that it could be important during target recognition.

Similarly than rLCV and EBV, miRNAs have been identified in a monkey model of KSHV, the rhesus rhadinovirus (RRV), and seem to have been maintained when these viruses diverged million years ago. However, in this case, the miRNA sequences are not conserved, but their genomic location is in a locus similar to the KSHV one. The RRV pri-miRNA could be expressed from the ORF73 gene as a unique transcript. Eleven RRV-derived miRNAs were discovered in latently infected epithelial cells. These 11 functional miRNAs derive from 7 precursors, and, as for KSHV, seem to be mostly latent genes. However, at least three of them are strongly induced during the virus lytic cycle, indicating that the pre-miRNAs could be transcribed from two different promoters depending on the latent vs. lytic state of infection (Schafer *et al.*, 2007).

The last gammaherpesvirus in which miRNAs have been identified is the mouse gammaherpesvirus 68 (MHV68). This virus is evolutionary related to the human gammaherpesviruses EBV and KSHV and is often referred to as the small animal model for the biology of these viruses (Speck and Virgin, 1999). In 2005, Pfeffer *et al.* identified nine miRNAs (miR-M1-1 to M1-9) encoded by MHV68 in a latently infected mouse B lymphoma cell line. Interestingly, all of them mapped to a 6-kb region near the M1 ORF, and were located downstream of previously identified tRNA genes. The function of these tRNAs had remained an enigma since it had been shown that none of them was aminoacylated. Upon discovery of the miRNAs, it became clear that these tRNAs acted as RNA pol III promoters for their expression in a non conventional manner (Pfeffer *et al.*, 2005).

Betaherpesviruses

The betaherpesvirus human cytomegalovirus (HCMV) has the largest genome of all herpesviruses. Acute disease occurs only in a small fraction of infected individuals, and only when the ability to mount an efficient immune response is jeopardized, i.e. transplacental transmission or primary infection of immunocompromised patients (Mocarski *et al.*, 2007). HCMV miRNAs were first described by Pfeffer *et al.* who cloned and sequenced small RNAs from lytically infected primary fibroblasts and identified nine miRNAs (Pfeffer *et al.*, 2005). Dunn *et al.* also described in 2005 2 HCMV-encoded miRNAs, miR UL23, leading to two mature miRNAs (hcmv-miR-UL23-5p and UL23-3p, now reported as miR-UL22A and UL22A*) and hcmv-miR-US24 (now reported as miR-US25-1). They are expressed, respectively, at early and immediate-early stage of infection (Dunn *et al.*, 2005). Finally, Grey *et al.* described 5 HCMV miRNAs among which were two novel miRNAs (miR-US4-1 and miR-UL70-1) (Grey *et al.*, 2005). The later were discovered by looking for stem–loop structures conservation between the HCMV and the chimpanzee cytomegalovirus (CCMV). Out of 110 HCMV stem loop sequences conserved in CCMV, Grey *et al.* predicted 13 potential miRNAs. Again, these miRNAs are expressed with an immediate early (UL70-1) or early kinetics (US4-1, US5-1, US5-2, UL36-1). HCMV miRNAs are spread throughout the viral genome and are located either in non-coding regions, intronic regions or complementary strand of known ORF of viral genes. As an example, miR-UL-112–1 is

located antisense to the viral uracil DNA glycosylase UL114. One of these miRNAs, miR-US36-1, is located in the intron of the UL36 gene, but does not seem to follow the immediate-early expression pattern of this gene. This indicates that there could be an alternate promoter driving its expression or that there is a stabilization of the intron after UL36 transcription.

The murine cytomegalovirus (MCMV) encodes 18 pre-miRNAs that are both early and late gene products (Buck *et al.*, 2007; Dolken *et al.*, 2007). No virally encoded miRNAs has been described during latency, probably due to the lack of an amenable system to study MCMV latency phase. Similar to HCMV these miRNAs are spread along the genome and expressed either individually or in cluster, but none of them is conserved at the primary sequence level. Also, their genomic localization differs between the two viruses, with the notable exception of miRNAs in the M23 region of MCMV corresponding to the UL23 region of HCMV. Notably, it has been shown that MCMV miRNAs are expressed from both strands of the viral DNA genome. Thus, the star sequence of miR-M23-2 is complementary to 16 nucleotides of the miR-m21-1 sequence. The same sequence complementarity exists for miR-M23-1–5p and miR-m22-1, and for miR-m107-1 and miR-m108-2-3p. These miRNAs are all detectable by Northern blot so we should admit that these genomic localizations do not interfere with the miRNAs accumulation. In addition, some MCMV miRNAs were described to be polyuridylated (Dolken *et al.*, 2007), although the physiological relevance of this modification is not yet understood. One hypothesis is that it could be a signal for degradation, as plant miRNAs have been shown to be degraded following polyuridylation when their 3′ end is not protected by a methyl group (Yu *et al.*, 2005). However, another recent report claims that a uridine stretch at the 3′ end of a miRNA could have a protective role against an *Arabidopsis* exonuclease (Ramachandran and Chen, 2008), which indicates that the exact role of this post-transcriptional modification is far from being elucidated.

Alphaherpesviruses

The Alphaherpesvirinae subfamily includes several miRNAs coding viruses. Two simplexviruses, HSV1 and HSV2, and two mardiviruses, MDV1 and MDV2, have been shown to use this way of modulating gene expression.

Herpes simplex virus type 1 (HSV1), the aetiological agent of oral herpes encodes six microRNAs, miR-H1 to miR-H6 (Cui *et al.*, 2006; Umbach *et al.*, 2008). Except for miR-H1 that was detected primarily in lytically infected cells (Cui *et al.*, 2006), the other HSV1 miRNAs are expressed in latently infected cells. MiR-H1 is a late gene product transcribed upstream of the latency-associated transcript (LAT) transcription start site. Interestingly, miR-H6 was found to be expressed from the same genomic location as miR-H1, but on the complementary strand (Umbach *et al.*, 2008). The LAT transcript is the source of the remaining four miRNAs, which are all located in the second exon of the 6.3kb spliced LAT transcript. When cloned by the Cullen lab from a vector expressing the LAT transcript, the most abundant miRNAs were mir-H2-3p and miR-H4-5p. The star sequences of these miRNAs were also detected (miR-H2-5p and miR-H4-5p). Among these latently expressed miRNAs, miR-H2 is transcribed in an antisense orientation to ICP0, a viral regulator important for productive HSV-1 replication and probably in reactivation from latency (see below). HSV1-miR-H3 and miR-H4-5p are located antisense to ICP34.5.

The close relative herpes virus simplex 2 (HSV2), the aetiological agent of genital herpes, is also a source of miRNAs. This virus encodes a LAT associated miRNA named miR-I. This miRNA is expressed *in vivo* under the control of LAT promoter in the latency model used, i.e. the guinea pig, and it is located on the strand antisense to the ICP34.5 transcript, a key viral neurovirulence factor (Tang *et al.*, 2008a). It has been shown recently that HSV-2 LAT exon 2 encodes two additional miRNAs, miR-II and miR-III, which are located in an antisense orientation to, respectively, the 5′-UTR of ICP34.5 and the coding region of ICP0-exon3 (Tang *et al.*, 2008b). These miRNAs are less abundant than miR-I. Interestingly, this novel study reinforces the LAT promoter independent transcription of these miRNAs. Indeed several other promoters active during productive infection could also be involved in miRNAs expression. Thus, miR-I and miR-II were detected early after inoculation,

implying that the LAT promoter was not involved in their initial expression. Consistent with this postulate, the deletion of the LAT promoter did not totally abolish the presence of miR-I but strongly reduced its level of expression (Tang *et al.*, 2008a). MiR-I has also been shown to be expressed during acute infection in sensory ganglia. By luciferase assay and Northern blotting experiments, Tang and coworkers showed that ICP4 is down-regulating the LAT and also the ORF O promoters activity (Tang *et al.*, 2008b).

Two other alphaherpesviruses, Marek's visease virus (MDV) 1 and 2, express miRNAs. Marek's disease (MD) is a chicken lymphoproliferative disorder associated with aggressive T-cell lymphomas. The serotype 1 of MDV has been reported to be oncogenic but the serotype 2 is not. By high-throughput sequencing of small RNA cloned from infected chicken embryotic fibroblasts, Burnside *et al.* first found that MDV1 encode eight different miRNAs (Burnside *et al.*, 2006). Yao *et al.* who worked with an MDV-transformed CD4 T-cell line (MSB-1) identified an additional six miRNAs in MDV1 genome (Yao *et al.*, 2008). Some arguments led to think that the MDV miRNAs were transcribed from three independent primary clusters: one in the LAT region and two upstream and downstream of the Meq transcript. Meq, which is indeed transcribed in tumours and transformed cells, is a protein with oncogenic potential and one of his spliced variant has been reported to be the major MDV oncoprotein (Liu and Kung, 2000). Eight of the miRNA precursors are clustered in the Meq region and show the same expression pattern. While miR-M2 to M5 and M11 are located upstream of the Meq promoter and lies antisense to RLORF8, miR-M1 is found within the intron of Meqsp, the spliced variant of Meq. The remaining miRNAs map to the LAT region of the MDV genome. LAT in MDV is expressed in an antisense orientation to ICP4 and, up to now, no translation product of the corresponding transcript has been found.

The other Marek's disease virus, MDV2, encodes 17 miRNAs, 16 of which are clustered in a 4.2-kb-long repeat region that encodes R-LORF2 to R-LORF5 putative open reading frames. The single miRNA outside of this cluster is located in the short repeat region, within the C-terminal region of a major transcriptional regulatory protein, an ICP4 homologue (Yao *et al.*, 2007).

Polyomaviruses

The simian virus 40 (SV40) encodes one precursor of miRNA that was discovered by Sullivan *et al.* in 2005. The authors used a computational approach to predict likely precursors of miRNAs, and validated their prediction by Northern blotting. Using probes complementary to each strand of the predicted precursor, they showed that both arms of the pre-miRNA were expressed as functional miRNAs, miR-S1-3p and miR-S1-5p. SV40 miRNAs accumulate at late times of infection. This is totally consistent with the late expression of the viral transcript that encloses the precursor. These miRNAs are perfectly complementary to early T antigen mRNAs and are involved in their cleavage (see below) (Sullivan *et al.*, 2005).

Interestingly, the function of miRNAs in polymaviruses seems to have been conserved, although not at the primary sequence level. Using an approach similar to the one they used for SV40, Sullivan and co-workers showed that the human BK and JC polyomaviruses do each express a single miRNA in a genomic localization similar to SV40. SV40, JC and BK viruses' miRNAs show some limited nucleotides identities notably in their seed sequences. The most abundant miRNA of JCV (jcv-miR-J1-5p) is detectable *in vivo* in tissues of infected patients. Despite these sequences divergence, all three miRNAs are expressed in the late phase and target their complementary early mRNAs, thus allowing these viruses to shut down the expression of early viral genes in late infection (Seo *et al.*, 2008b). Similarly, a miRNA with conserved functions has been found in Merkel cell polymavirus, a recently identified human virus associated with cancer (Seo *et al.*, 2008a). Finally, a report from Cantalupo *et al.* in 2005 argues that the simian agent virus 12 (SA12) also encodes miRNAs. SA12 is closely related in sequence to the human BK virus, raising the possibility that both viruses circulate between baboon and human. Bioinformatics analyses showed that the pre-miRNA described in the other viruses of this family is also conserved in SA12 genome. Northern blotting experiments confirmed the presence of miRNAs derived from both strand of the stem loop precursor but the

functional activity of SA12 miRNAs has not been investigated (Cantalupo *et al.*, 2005).

Other viruses

The genomes of adenovirus type 2 and 5 encode two non-coding virus-associated (VA) RNAs, VAI RNA and VAII RNA. VA RNAs are non-coding RNAs transcribed by RNA polymerase III that accumulate in very large amounts in the late phase of infection. The VAI RNA, via its highly structured stems, blocks the PKR-dependent anti-viral pathway by binding PKR and preventing the inhibition of protein synthesis (Berk, 2007). These VA RNAs are exported to the cytoplasm via exportin 5, the same export factor as for pre-miRNAs, and have been shown to function as inhibitors of siRNA and miRNA function (Andersson *et al.*, 2005; Lu and Cullen, 2004). Additionally, it was recently reported that VA RNAs could be processed into small RNAs (Aparicio *et al.*, 2006; Sano *et al.*, 2006). Although their processing by Dicer is quite inefficient (only 2–5%), given the very large amount of VA RNA molecules this results in a significant quantity of svaRNAs. In fact, these small RNA are efficiently assembled in the RISC, and even compete with cellular miRNAs for Argonaute 2 binding (see below) (Aparicio *et al.*, 2006; Xu *et al.*, 2007). One could speculate that a complete processing of VA RNA would be deleterious for its other activities. It is not totally clear whether adenovirus small RNA have any cellular targets, but given their characteristics and their assembly into RISC, they can be seen as a form of viral miRNAs.

The insect virus *Heliothis virescens* ascovirus (HvAV) also encodes one single miRNA that was identified by bioinformatics prediction, and validated by Northern blot analysis. HvAv-miR-1 is located in the major capsid protein gene. It was reported to be present at late point after infection (Hussain *et al.*, 2008).

The presence of miRNAs in retroviruses such as HIV remains a matter of debate that will not be developed here. Although some have reported that regions such as Nef (Omoto and Fujii, 2005) or Tar (Ouellet *et al.*, 2008) could be a potential source of miRNAs, others failed to clone miRNAs from HIV-infected cells (Pfeffer *et al.*, 2005).

Targets of viral miRNAs: finding the needle in a haystack

The precise functions of individual viral miRNAs remain to be determined, but an increasing number of studies are starting to unveil some of the roles that these small RNAs might be playing. Intuitively, one would imagine that the involvement of miRNAs in the viral life cycle could be at two main levels: to modify the cellular environment so as to provide an optimized setting for the infection to take place, or to act directly on the virus to fine-tune the expression of its genes. In the first case scenario, viral miRNAs would for example help the virus escape the immune response, or they could participate in the oncogenesis induction to immortalize the cells producing the virus. In the second hypothesis, the miRNAs would regulate the expression of viral genes at key steps of the virus like cycle (for example, the transition from lytic to latent stage). The regulation of their targets by miRNAs can be done in several manners that depend on the level of complementarity of the small RNAs and the targeted transcripts. Like cellular miRNAs, viral miRNAs can engage in different possible interactions with their targets, from a fully complementary binding, to a more canonical binding involving the seed of the miRNA (Fig. 3.2).

Viral targets and modes of recognition

The first targets of viral miRNAs that have been identified are of viral origin, and an increasing number of reports confirms that it is a widespread phenomenon (Table 3.2). In the original paper describing the identification of virus-encoded miRNAs, it was proposed that one of EBV miRNAs, namely miR-BART2, was potentially targeting the viral DNA polymerase BALF5. This hypothesis was inferred from the fact that miR-BART2 gene was expressed from a position opposite to the genomic location of BALF5 transcript (Pfeffer *et al.*, 2004). There was also some support that miR-BART2 was involved in the cleavage of BALF5 transcript from an earlier study that showed this RNA to accumulate in two forms of distinct lengths, and that mapped precisely the site of divergence between the two to a locus that latter turned out to correspond exactly to the position opposite to the middle of miR-BART2 (Furnari *et al.*, 1993).

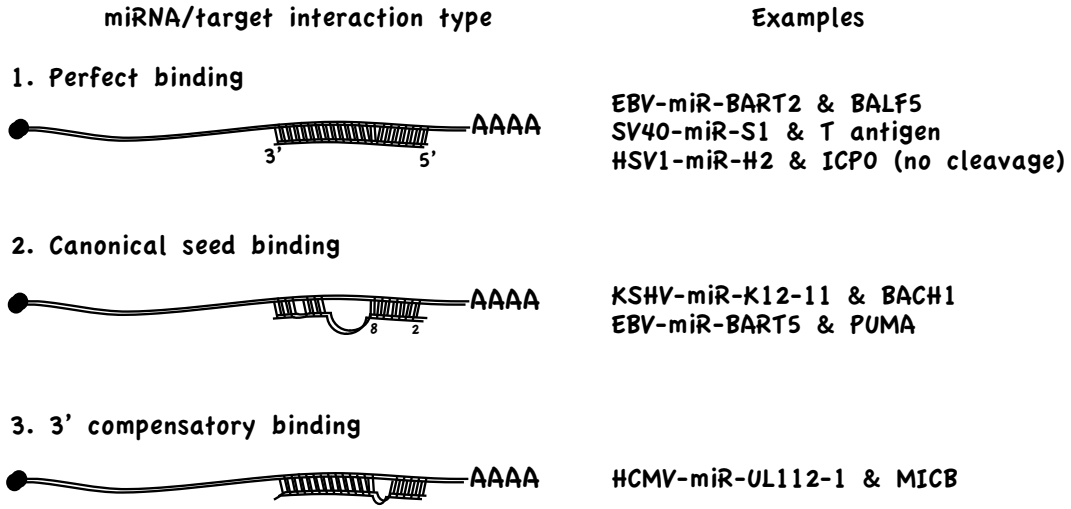

Figure 3.2 Modes of target recognition by viral miRNAs. The targeted transcript is represented with the bound miRNA in the different configurations possible. See text for details.

A recent report has confirmed the involvement of miR-BART2 in that process (Barth *et al.*, 2008). This mode of regulation by cleavage, which is not predominant among animal miRNAs, is also found in the polyomavirus SV40. Indeed, SV40 miRNA miR-S1 is also localized antisense to the early T antigen (Ag) transcript, and engages its cleavage (Sullivan *et al.*, 2005). Interestingly, the regulation of T Ag expression by a viral miRNA has been conserved between the simian virus and the human BK and JC viruses (Seo *et al.*, 2008b), as well as in the Merkel cell polyomavirus (Seo *et*

Table 3.2 Viral targets of viral miRNAs

Virus	miRNA	Target	Function	References
SV40	miR-S1	T antigen	Early protein	Sullivan *et al*. (2005)
BKV	miR-B1	T antigen		Seo *et al*. (2008b)
JCV	miR-J1	T antigen		Seo *et al*. (2008b)
MCV	miR-M1	T antigen		Seo *et al*. (2008a)
EBV	miR-BART2	BALF5	DNA polymerase	Barth *et al*. (2008), Pfeffer *et al*. (2004)
	miR-BART1, 16	LMP1	Signalling molecule	Lo *et al*. (2007)
HCMV	miR-UL112-1	UL112/113, UL120/121	Immediate early protein	Grey *et al*. (2007)
		UL123 (IE72, IE1)	Immediate early protein	Grey *et al*. (2007), Murphy *et al*. (2008)
HSV-1	miR-H2-3p	ICP0	Immediate early protein	Umbach *et al*. (2008)
	miR-H6	ICP4	Immediate early protein	Umbach *et al*. (2008)
HSV-2	miR-I	ICP34.5	Pathogenicity factor	Tang *et al*. (2008a)
	miR-II, miR-III	ICP34.5, ICP0		Tang *et al*. (2008b)
HvAv	miR-1	ORF1	DNA polymerase	Hussain *et al*. (2008)

al., 2008a). The conservation is not at the level of the sequence of the miRNA itself, but in all cases, the miRNA gene is located antisense to the large T-antigen transcript and is responsible for its cleavage by RISC.

It is noteworthy that the recognition of these viral transcripts occurs via cleavage after a perfect binding of the target (Fig. 3.2). However, it should be noted that at least in one case, the perfect complementarity between a viral miRNAs and its target does not lead to regulation, and in another example, the regulation exists but does not involve cleavage and degradation of the transcript. In the first case, Grey *et al.* used a computational approach to identify the putative viral target of HCMV miR-UL112-1. By restricting their search to targets that were conserved between the human and the chimpanzee cytomegaloviruses, they came up with a list of 14 candidate 3′ UTRs, of which they could validate three (UL112/113, UL120/121 and UL123) by luciferase assay (see below). Surprisingly, UL114, which is perfectly complementary to miR-UL112-1 because it lies antisense to it on the genome, could not be validated as a target (Grey *et al.*, 2007). Although this was only measured by reporter assay, this finding could indicate that the degree of complementarity between a miRNA and its target cannot explain everything. It could be that in this particular case, the binding site is not accessible due to a strong local secondary structure, or to the interaction with an RNA-binding protein. The second scenario features a miRNA encoded by HSV1, Cullen and colleagues identified the miRNAs of HSV1 by small RNA cloning and deep sequencing (see above), and showed that three of them lied antisense to genes of known function. Thus, miR-H2 could perfectly basepair with the transcript coding for ICP0, and both miR-H3 and H4 are antisense to the ICP34.5 gene. The targeting of ICP0 by miR-H2 was confirmed by Western blot analysis, but interestingly, although there was a very strong effect of miR-H2 at the protein level, the level of ICP0's messenger RNA was not affected (Umbach *et al.*, 2008). This observation is in agreement with a recent report by Brodersen *et al.* who demonstrated that the predominant mode of regulation by small RNAs in plants was at the level of translation inhibition, even in the cases of a perfectly matched miRNA/transcript pair (Brodersen *et al.*, 2008). Umbach *et al.* did not examine the regulation of ICP34.5 by miR-H2 and H3, but Tang *et al.* from the laboratory of P. Krause showed that HSV2 was also encoding a miRNA, named miR-I, which genomic localization was on the strand opposite to the one of ICP34.5. They confirmed both by reporter assay and Western blot analysis that ICP34.5 was indeed targeted by this miRNA, however they did not measure the effect at the RNA level (Tang *et al.*, 2008a). In another report, this group latter found that two other miRNAs, miR-II and miR-III, expressed at a lower level by HSV2, were respectively targeting ICP34.5 and ICP0 with a perfect complementarity similarly to HSV1 miRNAs (Tang *et al.*, 2008b). Together with the data obtained with polyomaviruses (Seo *et al.*, 2008b), this is further evidence that some function of viral miRNAs are conserved between distantly related viruses without a conservation at the sequence level.

Other targets of viral origin that do not require perfect binding of the viral miRNA have been identified. In these examples, the interaction is more canonical and occurs via a classical 'seed-type' binding (Fig. 3.2). We have already mentioned the recognition of three viral transcripts coding for immediate early proteins by HCMV miR-UL112-1 (Grey *et al.*, 2007). Murphy *et al.* also used a bioinformatics approach to predict viral targets of viral miRNAs. They validated the regulation of HCMV UL123 (IE1) by the same miRNA, miR-UL112-1, using luciferase reporter assays and Western blot analysis (Murphy *et al.*, 2008). Among HSV1 miRNAs, miR-H6 has also been shown to be involved in the regulation of ICP4 via a canonical interaction. Co-expression of a plasmid encoding ICP4 and of a synthetic oligonucleotide mimicking miR-H6 results in the down-regulation of ICP4 expression from the plasmid, whereas a mutant version of ICP4 in which the miRNA binding site has been mutated is not affected (Umbach *et al.*, 2008). Besides targeting BALF5 for cleavage, some of EBV miRNAs have been shown to deregulate the expression of LMP1 through imperfect binding. Lo *et al.* have reported that miR-BART1-5p, miR-BART16 and miR-BART-17-5p all have potential binding sites within LMP1 3′-UTR, and a luciferase reporter fused to LMP1 3′-UTR is down-regulated in

the presence of these miRNAs. Additionally, the level of LMP1 protein could be lowered following overexpression of either miRNAs, or up-regulated following blockage of the miRNAs by antisense oligonucleotides (Lo *et al.*, 2007). Interestingly, among the putative binding sites of these BART miRNAs, some displayed some mismatches within the seed region, an observation that has also been made for cellular targets of viral miRNAs (see below). This should attract our attention to the fact that in the search of miRNA targets we should not restrict ourselves to candidates that strictly follow pre-established rules. In fact, even cellular miRNAs can in some cases act through non-perfect seed/seed-matches pairing, including the very first identified miRNA, Lin-4 that binds to its target lin-14 with a bulge in its seed. Amazingly, this bulge even seems to be required for the regulation mediated by the small RNA (Ha *et al.*, 1996).

Finally, the last virus for which a viral target has been identified is the insect DNA virus *Heliothis virescens ascovirus*. HvAv miRNA miR-1 can partially basepair with the viral DNA polymerase transcript. Interestingly, it seems that the effect on the targeted transcript is at the RNA level, although some more experiments are needed to confirm the initial observation (Hussain *et al.*, 2008).

Cellular targets

The number of validated cellular targets of viral miRNAs is still lower than for viral targets (Table 3.3), although we should expect this tendency to be reversed in a near future. The identification of cellular targets remains a challenge due to the lack of amenable high-throughput validation method. What holds true for cellular miRNAs is true for viral miRNAs as well. Actually, finding targets for viral miRNAs can prove even more difficult than for host miRNAs. Indeed, a number of people have relied on bioinformatics prediction to predefine candidates to be followed on. In order to increase the signal-to-noise ratio in these predictions, people have used the propensity of miRNAs to be conserved between species to look for binding sites in regions that were also conserved cross-species (Rajewsky, 2006). This approach cannot be directly transposed to viral miRNAs, which are, for a very large proportion, not conserved with the host miRNAs or between distantly related viral species. Nevertheless, there are notable exceptions to this observation. The most striking of these exceptions is the KSHV miRNA, miR-K12-11, which has partial homology with the cellular miRNA, miR-155. Moreover, this conservation is somewhat restricted to the seed region of the miRNA. This observation inspired two groups to postulate that the functions of miR-155 and of miR-K12-11 were conserved, and that the two miRNAs must share a common set of targets. To challenge their hypothesis, Skalsky *et al.* and Gottwein *et al.* turned to a global approach for target identification that relies on the use of transcriptomic analyses to determine which transcripts are down-regulated upon a given miRNA overexpression in a transgenic cell line (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). This method has initially been used to identify the targets of tissue-specific miRNAs after their misexpression in HeLa cells. Lim and co-workers showed that overexpression of the neuron specific miR-124, or

Table 3.3 Cellular targets of viral miRNAs

Virus	miRNA	Target gene	Function	References
KSHV	miR-K12-6–3P and others	THBS1	Angiogenesis regulator	Samols *et al*.(2007)
	miR-K12-11	BACH1, Fos	Transcription factors	Gottwein *et al*. (2007), Skalsky *et al*. (2007)
EBV	miR-BHRF1-3	CXCL-11	Chemokine	Xia *et al*. (2008)
	miR-BART5	PUMA	Pro-apoptotic protein	Choy *et al*. (2008)
HCMV	miR-UL112-1	MICB	NK cell ligand	Stern-Ginossar *et al*. (2007)
MDV	miR-M4	PU.1	Transcription factor	Zhao *et al*. (2008)

muscle specific miR-1, led to the down-regulation of a large number of target mRNAs, and that among the deregulated transcripts, there was a significant enrichment in seed match motifs (Lim *et al.*, 2005). The down-regulation of transcripts accumulation upon miRNA recognition can be a consequence of their de-adenylation and/or decapping, although this does not occur systematically (Eulalio *et al.*, 2008). In the case of KSHV miR-K12-11, the two reports showed that Affymetrix analyses of cell lines expressing either miR-K12-11 or miR-155 could identify a common set of targets that had a repeated occurrence of the conserved seed match motif. Among the targets that were validated by luciferase reporter assays, and by Western blots, were the transcription factors Fos and Bach-1 (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Very interestingly, the conservation of the seed region between a cellular and a viral miRNA has been predicted in a few other cases (Gottwein and Cullen, 2008), and the functionality of this conservation has been confirmed for another viral orthologue of miR-155. Indeed, MDV1 miR-M4 also has the same seed that the chicken miR-155, and has been shown to target PU.1, a known target of miR-155 (Zhao *et al.*, 2008). The microarray analysis approach has also been used in a broader attempt at finding cellular targets for ten out of the known twelve miRNAs encoded by KSHV. The fact that all of KSHV miRNAs are clustered on the same genomic locus helped Samols *et al.* to express them in HEK293 cells. After transcriptomic analysis of these cells, they identify a number of putative targets of which they could validate THBS1, a protein playing a role in angiogenesis (Samols *et al.*, 2007). Intriguingly, all 10 miRNAs were predicted to target THBS1 3′ UTR, although this observation was not truly validated.

Despite the underlying problem of the lack of viral miRNAs conservation, computational target predictions have been used successfully in some instances. To bypass the problem, Stern-Ginossar *et al.* designed a novel algorithm for target prediction that did not rely on cross-species conservation. Rather, they looked for the occurrence of repeated motifs within 3′ UTRs and searched for their potential interactions with HCMV miRNAs. This approach enabled them to predict and validate the NK cell ligand MICB as a target of HCMV miR-UL112-1 (Stern-Ginossar *et al.*, 2007). Interestingly, the binding of miR-UL112-1 to MICB 3′-UTR occurs in a non-canonical manner with a two-nucleotide bulge within the seed, and a stronger binding of the 3′ end of the miRNA (Fig. 3.2). Intriguingly, a closely related isoform of MICB, MICA, which differs only by one nucleotide in the putative miR-UL112-1 binding site, is not a target of this miRNA.

The use of algorithms initially designed to predict cellular miRNAs targets has also proved useful. Thus, Choy *et al.* used two different programs, namely MiRanda and RNAhybrid, to predict targets for each EBV BART miRNA. By using luciferase assays, they could validate one cellular target of miR-BART5, namely PUMA (P53 upregulated modulator of apoptosis), which is involved in the control of apoptosis (Choy *et al.*, 2008). It should be noted however, that this was the only target validated out of nine short listed predicted targets for this miRNA, which is not too bad but also shows the limits of this approach. Another cellular target has been validated for EBV miR-BHRF1-3. Xia *et al.* took on to validate a prediction done with the program MiRanda and previously published for this miRNA (Pfeffer *et al.*, 2004), the chemokine CXCL-11. The authors showed that transfection of miR-BHRF1-3 resulted in a decrease in CXCL-11 protein level, and that conversely, blocking miR-BHRF1-3 in infected cells resulted in an increase of protein level (Xia *et al.*, 2008). One word of caution though, the authors state in their article that miR-BHRF1-3 is 100% complementary to its target, but actually the alignment is only imperfect with some mismatches and GU wobbles. However, there are three predicted binding sites for this miRNA in CXCL-11 3′-UTR (Pfeffer *et al.*, 2004).

Roles and implications of viral and cellular targets regulation

We portrayed before the viral and cellular targets that have been identified so far, and the different means by which a miRNA can recognize and regulate its targets. We will now focus more precisely on the functions that the regulation of these targets play, and the implications in context of the viral life cycle. Although there is a large diversity

of both viral and cellular targets, some unifying themes are emerging.

Regulation of latency

A key step in all herpesviruses life cycle is the transition from a productive lytic infection to a latent infection where the virus will remain dormant in the infected host. A number of evidences now point to a strong involvement of viral miRNAs in this transition through the regulation of lytic viral proteins. Thus, the regulation of EBV DNA polymerase BALF5 by the miRNA miR-BART2 was shown to play a role in the control of virus accumulation. Through cleavage of the BALF5 transcript, this miRNA might degrade aberrantly transcribed BALF5 messenger RNA to keep the virus replication in check. However, the control is not entirely mediated by miRNA because blocking their expression by chemically modified antisense oligonucleotides does not induce lytic replication in latently infected cells (Barth *et al.*, 2008). A striking parallel with the role of miR-BART2 in regulating EBV DNA polymerase was reported recently in the insect virus HvAv. Here also, the viral DNA polymerase expression seems to be controlled in a temporal manner by a virus-encoded miRNA. However in the latter case, the miRNA expression alone seems sufficient to control virus replication (Hussain *et al.*, 2008).

The viral DNA polymerase is not the only essential gene during lytic infection to be targeted by miRNAs. The human cytomegalovirus is another fascinating illustration of how critical small RNAs can be for the virus replication. Two reports have showed that one of HCMV miRNAs, miR-UL112-1, can target immediate-early viral genes (Grey *et al.*, 2007; Murphy *et al.*, 2008). Grey *et al.* first identified UL112/113, UL120/121 and UL123 (encoding the IE72 or IE1 protein) as targets of miR-UL112-1, and both UL112/113 and IE72 encode products important for the virus replication. They also showed that miR-UL112-1 overexpression during infection could inhibit immediate-early gene expression and viral DNA replication. Since IE72 is necessary for an efficient replication of the virus through transactivation of early and late viral genes, one could imagine that its repression by a miRNA could play an important role for the establishment of a latent or persistent infection. These results were confirmed by Murphy *et al.* who independently predicted the same binding site for miR-UL112-1 in the 3′-UTR of IE72 (IE1). They also confirmed the involvement of this miRNA in IE1 down-regulation, and constructed a mutated virus in which miR-UL112-1 expression was impaired. Although the mutant virus replication was not perturbed in fibroblasts, a significant higher level of IE1 accumulated in cells infected by this virus compared to the wild type. Very interestingly, Murphy *et al.* also predicted that other viral miRNAs could potentially target IE genes in four herpesviruses, for example KSHV miR-K12-6-3p was predicted to target Zta and Rta, which share a 3′-UTR and are strong trans-activators of lytic gene expression (Murphy *et al.*, 2008).

The alphaherpesviruses also use miRNAs to regulate the expression of immediate-early genes. Strikingly, all the miRNAs that have been identified in this family of viruses are localized in latency-associated transcripts. For both HSV genera, a miRNA has been found to be expressed antisense to the ICP0 transcript, a key immediate-early transcriptional activator that might be important for the entry into the productive lytic cycle. For the two viruses, down-modulation of ICP0 expression by their respective miRNAs (i.e. miR-H2-3p and miR-III) was confirmed (Tang *et al.*, 2008b; Umbach *et al.*, 2008). Interestingly, Tang *et al.* showed that HSV2 miRNAs expression was controlled by the viral factor ICP4. Since in HSV1, ICP4 is itself regulated by the viral miRNA miR-H6, it appears that the switch to latency is very tightly controlled at multiple levels where miRNAs play a central role. Even though there are some notable differences between HSV1 and HSV2 regarding the level of expression of each viral miRNAs, and the sequences of the miRNAs themselves, their genomic localization, and mode of action seem to have been partially conserved between the two viruses. The implication of MDV1 and 2 miRNAs in the control of latency has not been explored yet, however given their localization in the latency associated transcript of this virus, and their high abundance in latently infected cells, it is likely that they will also play a prominent role in this process (Burnside *et al.*, 2006; Yao *et al.*, 2008).

Controlling the immune response

An important role of virus-encoded miRNAs is also to help the virus escape the immune response and remain unnoticed in the infected host for long period of time. The use of small RNAs for that purpose is a perfect example of how viruses causing persistent infection have co-evolved with their hosts. It is indeed remarkable that some viruses have been able to hijack the cellular miRNA biogenesis machinery, which enables them to express short non-immunogenic RNA molecules with potential deleterious effects to the host. As an example, the polyomavirus SV40 miRNA miR-S1 regulates the expression of the large T antigen transcript by cleavage, but this regulation does not seem to impact on the virus replication. Rather, it has been shown by using a mutant virus not expressing the miRNA that the role of this control was to lower the antigen load in infected cells to prevent recognition by cytotoxic T cells. Host cells infected with the miRNA mutant virus were much more efficiently killed by cytotoxic T lymphocytes (CTL) than cells infected with the wild-type virus (Sullivan *et al.*, 2005). As we noted previously, the regulation of T Ag by miRNA has been conserved in the human polyomaviruses BK, JC and Merkell cell (Seo *et al.*, 2008a,b), and most probably in the baboon polyomavirus SA12 (Cantalupo *et al.*, 2005). Even though the direct effect on CTL recognition has not been measured for these viruses, it is very likely that the T Ag regulation plays the same role than during SV40 infection.

The same HCMV miRNA that was shown to play important roles in the control of viral replication is coincidently also involved in the regulation of a cellular gene very important for keeping HCMV infected cells in check. Indeed, Stern-Ginossar *et al.* showed that the major histocompatibility complex class I-related chain B (MICB) gene was targeted by HCMV-miR-UL112-1. MICB is a stress-induced ligand of a natural killer (NK) cell activating receptor, and is known to be critical for NK cell killing of virus-infected cells. The regulation of MICB by miR-UL112-1 also takes place in the real context of infection, and cells expressing miR-UL112-1 ectopically are less sensitive to specific killing by NK cells. Moreover, a mutant virus not expressing miR-UL112-1 results in cells being more effectively killed by NK cells after infection, very much like what has been previously observed with SV40 virus and CTL (Stern-Ginossar *et al.*, 2007). It is worth noting that the virus UL16 protein already lowers MICB expression at the cell surface (Cosman *et al.*, 2001), thus there seems to be a cumulative effect due to miRNA expression that helps the virus achieve the difficult task to escape the immune response of the host.

The last example of immune surveillance regulation by viral miRNAs is illustrated by one of EBV miRNA, miR-BHRF1-3, which regulates the expression of the chemokine CXCL-11, also known as the IFN-inducible T-cell attracting chemokine (I-TAC). The level of this viral miRNA is high in type III latency cell line, and in primary EBV-associated AIDS-related diffuse large B-cell lymphomas (DLBCL). Conversely, primary Burkitt lymphoma and primary effusion lymphoma cell lines express high level of BART miRNAs, but no BHRF1-3 miRNA. The level of CXCL-11 in these different cell types is inversely correlated with the level of miR-BHRF1-3, and blocking this miRNA expression results in an increase in CXCL-11 level (Xia *et al.*, 2008). These results indicate that the immunosuppressive effect via regulation of the chemokine CXCL-11 is highly dependent of the immune status of the infected person and imply that the involvement of viral miRNAs in these processes is not a simple task to address.

Roles in oncogenesis

EBV emerged as the first candidate human tumour virus in the sixties and is now the prototype for the oncogenic gamma-herpesviruses subfamily, which is associated with a number of lymphomas, carcinomas and sarcomas (Kieff and Rickinson, 2007). KSHV is also tightly associated with a number of oncogenic disorders. For both of these viruses, recent evidences pointed to an implication of viral miRNAs in the development of cancer linked with the viral infection. The involvement of viral miRNAs in oncogenesis can be seen both due to the targeting of viral genes and of cellular genes. Hence, some of EBV BART miRNAs down-regulate the expression of the viral LMP1, a well-described viral oncogene, which explains the lack of correlation between LMP1 mRNA and protein expression in different

EBV infected epithelial cells, and nasopharyngeal carcinomas (NPC). Indeed, there is an inverse correlation of BART miRNAs and LMP1 protein levels in NPC tissues (Lo *et al.*, 2007). LMP1, a constitutively active tumour necrosis factor receptor, is a multifaceted protein that is both capable of inducing cell growth and transformation, and, when overexpressed, growth inhibition and sensitization to apoptosis (Eliopoulos *et al.*, 1996; Liu *et al.*, 2002). Therefore, BART miRNAs might act to fine-tune the expression of this protein during different steps of the infection, and as a consequence, drive LMP1 in inducing cell transformation rather than apoptosis. It is also intriguing to note that LMP1 is known to induce, through the action of NF-κB, the expression of a cellular miRNA, miR-146a, which is an important factor in lymphocyte signalling pathways (Cameron *et al.*, 2008). The latest observation adds yet another layer of complexity in the already intricate interplay between viruses and the miRNA machinery.

Control of apoptosis is an efficient way to drive cell into proliferation, and it seems that it is being used extensively by viral miRNAs. Accordingly, EBV miRNA miR-BART5 targets PUMA, both a P53-dependent and independent pro-apoptotic gene, and blocking miR-BART5 activity results in an increase in etoposide-induced apoptosis (Choy *et al.*, 2008). Upon induction of its expression by P53, PUMA can bind to Bcl-2, localizes to the mitochondria to induce cytochrome c release, and activate the rapid induction of programmed cell death (Nakano and Vousden, 2001). The regulation of a downstream target of P53 by a viral miRNA also adds to the growing complexity of interactions between P53 and miRNAs, as P53 has been shown to be a transcription factor for the cellular miRNAs of the miR-34 family (He *et al.*, 2007). The anti-apoptotic effect mediated by the repression of PUMA by miR-BART5 completes a panel of tools that EBV can employ to inhibit cell death, such as the viral Bcl-2 homologue BHRF1 (Henderson *et al.*, 1993).

KSHV miRNAs could also play a role in the development of cancer. Their genomic localization in one of KSHV most transforming region (Muralidhar *et al.*, 2000) is already an indication that they could be directly involved in oncogenesis. More concrete evidence came with the finding that KSHV miRNAs were targeting THBS1 in 293 cells (Samols *et al.*, 2007). A gene involved in the regulation of angiogenesis, THBS1 codes for a matricellular protein playing important roles in cell–cell and cell–matrix adhesion, and is down-regulated in numerous cancers. It possesses both anti-proliferative and anti-angiogenic activity (de Fraipont *et al.*, 2001). Although some have reported THBS1 to be poorly expressed in Kaposi's sarcoma lesions (Taraboletti *et al.*, 1999), to date there is no direct evidence about the involvement of KSHV miRNAs in this regulation in context of a real infection and not only in an artificial system using 293 cell lines. Other cellular genes, which are targeted by KSHV miR-155 orthologue, miR-K12-11, have been identified, such as the transcription factors BACH1 and Fos (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Although there is no direct connection to oncogenesis, it is interesting to note that miR-155 has been implicated in B-lymphoproliferative disorders (Kluiver *et al.*, 2005) and in normal B cell development (Thai *et al.*, 2007), and it could be that there are additional cellular targets for miR-K12-11 that will explain its role in KSHV-induced pleural effusion lymphomas (PEL). In fact, PEL do not express miR-155, but they do express miR-K12-11 at high levels. Therefore, the viral miRNA could complement the lack of miR-155 and contributes to lymphomagenesis. Intriguingly, EBV does not express an orthologue of miR-155, but EBV infection has been shown to induce the expression of this cellular miRNA (Gatto *et al.*, 2008). Thus, these two gammaherpesviruses use different strategies to attain a similar objective. Finally, the chicken Marek's disease virus also expresses an orthologue or miR-155 that was shown to target PU.1 (Zhao *et al.*, 2008) and could also be involved in the virus-induced lymphomagenesis.

Other functions

Besides the evident functions that we described in the previous paragraphs, there are a number of other ways viral miRNAs can use to perturb the normal functioning of the cell or subtly modifying its environment. One should also consider the interactions that viruses engage with cellular miRNAs, as we saw above for a few cases. This can take

place either through modulation of cellular miRNAs expression following infection, or through direct interactions of cellular miRNAs with the viral genome or viral transcripts. Although there have been a number of reports describing the latter interactions, we will not describe those into details. Rather, we refer the reader to Chapter 5. It is also possible that virus-encoded miRNAs can act indirectly by saturating, or modifying the miRNA biogenesis machinery. Hence, the adenovirus non-coding VA RNAs are expressed at very high levels and have been shown to saturate the exportin 5 factor (Lu and Cullen, 2004), and to compete for Dicer with endogenous miRNAs or transfected hairpin RNAs (Andersson *et al.*, 2005) thus acting as a suppressor of RNA silencing. Therefore, these non coding RNAs could very well act by preventing the function of cellular miRNAs, and not by directly targeting viral or cellular transcripts. Nevertheless, although Dicer processes VA RNAs very inefficiently, a non-negligible number of small RNAs can be assembled into RISC, and the possibility that they do act as true miRNAs remains open. Although no target has been identified to date, two groups showed that VA-derived small RNAs are bound to Argonaute 2, and that they can regulate the expression of artificial reporter genes (Aparicio *et al.*, 2006; Xu *et al.*, 2007). The work by Aparicio *et al.* also reported that blocking VA small RNAs affects efficient adenovirus production, indicating that they play important roles during the virus infectious cycle.

Similarly, miRNAs identified for the mouse CMV during lytic infection of fibroblasts accumulate in very high amount compared to cellular miRNAs, and it could be that they do partially function by saturating the machinery (Dolken *et al.*, 2007). However, the situation encountered upon *in vitro* infection of cells in culture with a high dose of virus, and where each individual cell will be infected is very remote from what happens during infection of the natural host. Nevertheless, it could very well be that during *in vivo* infection, the virus will express high levels of viral miRNAs in some cell types, and lower amount in others, thereby specifying a distinct mode of action in each situation.

Concluding remarks

Viral miRNAs have been discovered only recently, but their importance in the study of host–virus interactions has risen exponentially since. We cannot overlook the fact that these tiny RNA molecules might be central in a number of processes during the different steps of the infection. Actually, some observations that could not be readily explained upon their discovery turned out to involve miRNAs. Thus, the cleavage of EBV BALF5 transcripts in two forms of different lengths, the precise role of the large EBV BART RNA, or the stabilization of the LAT intron in HSV1 and 2, are a few examples of data that became clearer once miRNAs were taken into account. The existence of virally derived miRNAs also points to fundamental differences in the relationship between viruses and RNA silencing in mammals. Although the debate whether RNA silencing can be seen as an antiviral defence in mammals like in plants and insects remains controversial but open, it is evident that some viruses have evolved to utilize this cellular machinery for their own good. The positive regulation of HCV replication by a cellular miRNA is another instance of viral hijacking of the miRNA pathway. What clearly lacks to formally prove that RNA silencing is (or is not) a true antiviral system in mammalian organisms would be the detection of siRNAs of viral origin in infected tissues. So far, there have been only non-conclusive or negative data (Lin and Cullen, 2007; Pfeffer *et al.*, 2005). However, recent evidence has emerged that endogenous siRNAs deriving from repeats can be cloned from germline specific tissues, such as oocytes and testes (Tam *et al.*, 2008; Watanabe *et al.*, 2006), or from ES cells (Babiarz *et al.*, 2008). These are clearly distinct from the previously described piwi-associated small RNAs (piRNAs) (Aravin *et al.*, 2006; Girard *et al.*, 2006), and point to a possible involvement of siRNAs in some processes during mammalian development. Thus, we cannot completely exclude that in some particular conditions, siRNAs of viral origin could arise, although it remains to be shown. Nevertheless, there is an involvement of small RNAs in antiviral defence, as it has also been shown that miRNAs of cellular origin can have deleterious effects on viruses. Hence, miR-32 negatively regulates the primate foamy virus (Lecellier *et al.*, 2005),

and Dicer-deficient mice are hypersusceptible to vesicular stomatitis virus due to the lack of peculiar miRNAs accumulation (Otsuka *et al.*, 2007), and interferon-induced miRNAs have a negative impact on HCV (Pedersen *et al.*, 2007). The latest case nicely exemplifies that miRNAs can have both a positive and a negative impact on the very same virus, and it could be that for some DNA viruses encoding miRNAs that are beneficial for the virus, there will be cellular miRNAs, that will have direct or indirect detrimental effects to the infection. Actually, this hypothesis is not only fictional, as Pellett and coworkers nicely showed that two cellular miRNAs, miR-100 and miR-101, regulate the mTOR pathway, which is important for HCMV infection. In turn, HCMV down-regulates the expression of these two miRNAs, indicating that the virus can counteract the potential problem (Wang *et al.*, 2008).

Finally, one can wonder whether viral miRNAs have positive effects only for the virus they derive from. It is puzzling to note that most of the viral miRNAs have been found in viruses that have co-evolved with their host, and that cause persistent infection most of the time without the development of any symptom. A provocative view could be that these viruses are in some respect beneficial to their host, at least when the host is immunocompetent. This vision gained some support recently in a report that showed that herpesvirus latency confers a surprising benefit to the host. Indeed, Barton *et al.* (2007) reported that mice latently infected by either MHV68 or MCMV were resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis*. These results suggest that latency could be seen as a symbiotic relationship rather than a potentially harmful ticking bomb. In this respect, viral miRNAs have clearly a potential to be beneficial for the host through the modulation of genes important in, for example, innate immunity, or other yet unknown processes.

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Regulation of cellular and viral microRNAs accumulation upon mouse cytomegalovirus infection

Abstract

Viruses from the *herpesviridae* family, such as the mouse cytomegalovirus (MCMV), are known for expressing their own micro (mi)RNAs, but they can also perturb the expression of cellular miRNAs. Although the cellular miRNA profile is only modestly impacted in MCMV infected cells, one particular miRNA, miR-27a is dramatically down-regulated upon infection. Interestingly, the regulation of this miRNA seems to be at the level of the stability of the mature form. The goal of this thesis was to identify viral and cellular factors involved in miR-27 regulation. In addition, we also looked more broadly for potential enzymatic activities involved in the regulation of the stability of miRNAs. Therefore, we studied the role of the terminal uridyl transferase TUT1 in Arabidopsis thaliana and of one of its homolog, Zcchc6, in mammalian cells. Although our efforts to establish the role of these proteins in the modification of miRNAs were not fully conclusive, we could get insights into the mechanism by which miR-27 is degraded via pairing to an abundant viral transcript.

Keywords: MCMV, miRNA, post-transcriptional regulation, small RNA decay.

Régulation de l'accumulation de microARN viraux et cellulaires au cours de l'infection par le cytomégalovirus murin.

Résumé

Les virus de la famille des *herpesviridae*, tels que le cytomégalovirus murin (MCMV), peuvent exprimer leurs propres micro-(mi)ARN, ils sont également capables de déréguler l'expression des miARN cellulaires. Bien que le profil global d'expression des miARN cellulaires ne soit que très modestement perturbé dans les cellules infectées par le MCMV ; l'accumulation d'un miARN particulier, miR-27a, est très fortement régulée au cours de l'infection. Il est intéressant de noter que cette régulation semble se dérouler au niveau de la stabilité de la forme mature du miARN. Ainsi, le but de ce travail de thèse fut l'identification de facteurs à la fois viraux et cellulaires impliqués dans la régulation de miR-27. De plus, nous avons cherché à identifier de manière globale de potentielles activités enzymatiques impliquées dans la régulation de la stabilité des miARN. Nous avons à ce titre étudié le rôle de la terminal uridyl transférase TUT1 chez *Arabidopsis thaliana* ainsi que celui d'un de ses homologues chez les mammifères, Zcchc6. Bien que nos efforts pour établir le rôle de ces protéines dans la modification des miARN ne furent pas complètement concluants, nous avons établi les bases du mécanisme par lequel miR-27 est dégradé *via* son appariement à un abondant transcrit viral.

Mots-clés : MCMV, miARN, régulation post-transcriptionnelle, dégradation des petits ARN.