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Étude des mécanismes de régulation de biogenèse des microARN exprimés par l'Herpèsvirus associé au sarcome de Kaposi

(Study of mechanisms regulating the biogenesis of microRNAs expressed by Kaposi's sarcoma-associated herpesvirus)

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Abbreviations :

AGO: Argonaute

BCBL1: body cavity-based lymphoma 1 cell line

Cas9: CRISPR associated protein 9

circRNA: circular RNA

CMV: cytomegalovirus

CRISPR: clustered regularly interspaced short palindromic repeats

DGCR8: DiGeorge syndrome critical region 8

DNA: deoxyribonucleic acid

EBV: Epstein Barr Virus

Exp5: Exportin 5

gRNA: guide RNA

HCT116: human colorectal carcinoma 116 cell line

HEK293FT: human embryonic kidney 293FT cell line

HNRL1/hnRNPUL1: heterogeneous nuclear ribonucleoprotein U-like protein

hnRNPL: heterogenous nuclear ribonucleoprotein L

hnRNPK: heterogenous nuclear ribonucleoprotein K

KD: knock-down

KO: knock-out

KS: Kaposi's Sarcoma

KSHV: Kaposi's Sarcoma Associated Herpesvirus

miRNA: micro RNA

mRNA: messenger RNA

ORF: open reading frame

PEL: primary effusion lymphoma

Pol II: RNA-polymerase II

Pol III: RNA-polymerase III

pre-miRNA: precursor miRNA

pri-miRNA: primary miRNA

RBD: RNA-binding domain

RBM45: RNA-binding protein 45

RBP: RNA-binding protein

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RNAi: RNA interference

RNase: ribonuclease

RT-qPCR: reverse-transcription quantitative PCR

RTA: replication and transcription activator

shRNA: short hairpin RNA

siRNA: small interfering RNA

SUGP1: SURP and G-patch domain-containing protein 1

UTR: untranslated region

I. Foreword

The recognition that non-coding RNAs may play important regulatory functions within cells launched a revolution in our understanding of biology. For what was before thought useless junk, forged completely new paradigms and became a large field of study. Last decades brought to light remarkable and unforeseen aspects of cellular life related to non-coding RNAs. Indeed, while only 1.5-2% of human genome has a protein-coding potential, more than 75% is actively transcribed (Djebali et al., 2012). Along with the well appreciated ribosomal and transfert RNAs, the transcriptome harbors plenty of other functional RNA species. With continuous advance in technology, we not only discover new classes of non-coding RNAs, but we are amazed by the diversity of biological functions carried out by these molecules.

Among the best studied non-coding RNAs in animals are microRNAs (miRNAs). They have emerged as potent post-transcriptional regulators of gene expression controlling virtually all biological processes (Bartel, 2018). We have now an increased appreciation of miRNA importance for development, cell differentiation, proliferation, metabolism and stress response. Needless to say that impairment of miRNA expression and activity has been linked to diseases such as cancer and some of them are recognized as proto-oncogenes and tumor suppressors (Rufino-Palomares et al., 2013). The specificity of miRNAs expression in normal and diseased cells make them reliable biomarkers and numerous efforts are ongoing to use them as therapeutic molecules (Rupaimoole and Slack, 2017).

The ability to shape gene regulatory networks makes miRNAs interesting tools for viruses too (Cullen, 2011; Kincaid and Sullivan, 2012). Contrary to other micro-organisms, viruses are unable to replicate independently from their host. Therefore, these obligatory parasites need to hijack and domesticate host cell functions to their own benefit. Viral expression of non-coding regulatory RNAs such as miRNAs represent the ultimate adaptation to host cell environment, since these are a sophisticated way to fine-tune host-pathogen relationship.

Non-coding RNAs in viral infections are at the heart of research in the laboratory led by Dr. Sébastien Pfeffer, where I carried out my PhD project. This manuscript aligns with one of the main topics, which has been extensively studied by the research group, namely the functions

and regulation of miRNAs expressed by the Kaposi's sarcoma associated herpesvirus (KSHV). KSHV miRNAs play an important part in its infectious cycle and participate in optimizing conditions within infected cells, so that the virus can escape immune surveillance and establish life-long infection. As a bystander effect, sustained viral miRNA expression leads to tumorigenic processes and ultimately to cell transformation and cancer. Hence it is essential to understand the mechanisms that allow KSHV to produce and regulate its miRNAs. Driven by this perspective, the aim of the work presented here was to shed light on molecular mechanisms underlying the control of KSHV miRNA expression. We focused in particular on the biogenesis of a cluster of ten viral miRNAs, studying the impacts of their genomic arrangement and trans-acting factors on their expression.

II. Introduction

1. Small non-coding RNAs and RNA interference

The idea that small RNA molecules are key regulators of gene expression was first postulated by the Ambros and Ruvkun labs in 1993. Both groups observed a peculiar downregulation of LIN14 protein concomitant with the expression of a small non-coding RNA coined lin-4. Lin-4 was able to bind to *LIN14* mRNA due to sequence complementarity and this process was essential for the developmental timing in *C. elegans* (Lee et al., 1993; Wightman et al., 1993). Around the same time, a new pathway of inhibitory small RNAs started to be uncovered, it turned out to be a universal phenomenon shared by almost all eukaryotes and gained the generic name of RNA interference (RNAi). RNAi revealed to be one of the fundamental principles in post-transcriptional gene regulation. Very soon after its characterization, it opened ways for unprecedented laboratory tools allowing to turn down genes at will (Doench et al., 2003; Elbashir et al., 2001a, 2002). In addition to research purposes, it also seeded exciting and innovative ideas such as RNAi-based therapies (Gavrilov and Saltzman, 2012). The recognition of the impact RNAi had in research laboratories and beyond, was endorsed by the Nobel prize awarded to the RNAi pioneers Craig C. Melo and Andrew Z. Fire in 2006.

Three major classes of small regulatory RNAs involved in RNAi-related processes have been defined, small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs). While their origins and biological functions vary, they share a common molecular mechanism for their activity. This involves the small RNA serving as a guide for an effector protein to direct its function towards a complementary target transcript (Meister, 2013). While piRNAs are involved in the control of transposable elements, inhibiting their dangerous activation in germ line, siRNAs are produced upon recognition and cleavage of long double-stranded RNA molecules of endogenous or exogenous origin. In invertebrates, siRNAs function as the primary antiviral defense mechanism, attacking viral genomes upon detection of their replication intermediates (Ketting, 2011; Ozata et al., 2019). Finally, microRNAs are ubiquitous and expressed in all kinds of physiological contexts. They fine-tune expression of

protein-coding genes and they represent an important layer of post-transcriptional regulation participating to cell homeostasis (Bartel, 2018).

2. microRNAs

2.1 Discovery and nomenclature

After the initial observations of heterochronic small RNAs lin-4 and let-7 coordinating the development of *C. elegans* (Lee et al., 1993; Reinhart et al., 2000), let-7 was found to be conserved across evolution (Pasquinelli et al., 2000) and many other similar genes were identified from invertebrates to human (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). It became clear that these small RNAs and their activity is not restricted only to the worm biology. Indeed, initially coined microRNAs solely owing to their small size, this novel class of regulatory RNAs raised considerable excitement regarding their function and the extent of their regulatory potential. The ubiquitous expression in almost all eukaryotes and the essential character of the main components of the miRNA pathway point out the vital part they play in cell biology (Bernstein et al., 2003; Bertomeu et al., 2018; Schaefer et al., 2007). Today, it is well appreciated that miRNA-mediated regulation is involved in virtually all biological processes, and we are probably only starting to understand the complexity of gene regulatory networks at the level of the cell, but also the entire organism.

Since the early cloning of cDNA sequences from size-fractionated RNA allowed to find the first miRNAs, the advent of high-throughput approaches has allowed the identification of thousands of putative miRNAs. Very soon, the need for a unified and systematic annotation led to the implementation of a common nomenclature (Ambros et al., 2003). This was based simply on the name “miR” (with a capital R for the mature miRNA and a lower case r when referring to the gene) followed by a number attributed according to the order of discovery or with respect to homologous miRNAs already identified in other species. The only exceptions are the very first miRNAs which were identified and named due to their mutant phenotype such as let-7 (Lethal 7,(Reinhart et al., 2000)), thus their homologs in other species retained the same name. To discriminate individual miRNAs between species, a three-letter prefix is used, e.g. cel-let-7 and hsa-let-7 (let-7 in *C. elegans* and *H. sapiens* respectively). More extended annotation is sometimes needed, when miRNAs are evolutionary related. If miRNAs belong to the same family (same seed sequence), they can be given a letter in suffix (e.g. hsa-miR-451a and hsa-

miR-451b). In addition, one miRNA can sometimes be encoded by several genes, in this case a number is added after the gene name (e.g. hsa-miR-16-1 and hsa-miR-16-2). Viral miRNAs do not completely comply with these rules. They usually integrate also the name of a previously identified proximal coding or non-coding genomic element (e.g. miR-BART-1 expressed by Epstein Barr Virus).

All miRNAs reported so far are listed in the database miRbase, which has been curated and maintained until 2019. The last version of the miRBase (v.22.1) lists more than 38 000 entries, among which are 1917 human miRNA genes (https://www.mirbase.org/) (Kozomara et al., 2019). However, with the increased depth of sequencing techniques used to detect putative miRNAs, many of the registered sequences are unlikely to be authentic miRNAs with genuine biological functions, but rather degradation products or technical artifacts (Fromm et al., 2022; de Rie et al., 2017). More recently, miRBase has been integrated in the more general Rfam RNA database (https://rfam.xfam.org/) and will gradually stop being updated.

2.2 Genomic and transcriptional context

Metazoan miRNA genes are found at various genomic locations. About half of miRNA loci are intergenic, while the second half reside within introns or exons of other coding or non-coding transcripts (Rearick et al., 2011; Rodriguez et al., 2004). Transcription of intergenic miRNAs is driven by their own promoters while intronic and exonic ones usually rely on the host gene promoter (Baskerville and Bartel, 2005; Berezikov et al., 2005; Rodriguez et al., 2004). However, a number of studies have shown that the miRNA transcriptional landscape can be more complex due to alternative splicing events or presence of alternative promoters activated in particular physiological contexts (see review in section 4.2). For instance, some intronic miRNAs have independent promoters and transcription start sites. In this way, their expression can be uncoupled from their host gene, e.g. in response to stress conditions (Monteys et al., 2010; Ramalingam et al., 2014).

2.3 miRNA biogenesis

The canonical miRNA biogenesis pathway in animal cells entitles sequential maturation of a long primary miRNA (pri-miRNA) transcribed by the RNA Polymerase II (Cai et al., 2004; Lee et al., 2004). The first processing step takes place in the nucleus where the pri-miRNA folds-back into a stem-loop structure. This is recognized by the heterotrimeric Microprocessor complex, composed of the endonuclease Drosha and its cofactor DGCR8 (DiGeorge syndrome

critical region 8, named Pasha in invertebrates) (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Lee et al., 2003). Drosha recognizes and binds to the lower stem of the pri-miRNA, but requires the assistance of a dimer of DGCR8 that interacts with the apical loop, thus helping to position and stabilize the complex in a functional conformation (Nguyen et al., 2015; Partin et al., 2020). Drosha's catalytic core contains two type III RNase domains that each cleaves one strand of the pri-miRNA hairpin, approximately 11 nt from the basal junction and 20-22 nt from the apical loop. Efficient processing of the pri-miRNA by the Microprocessor relies on structural and sequence elements present on the pri-miRNA which improve the recognition and allow optimal cleavage of the miRNA precursor. These features are described in more detail in the review in part 4.2.

Since Microprocessor complexes have been found to localize to sites of active transcription and Drosha has been shown to interact with elongating Pol II complex, it is believed that pri-miRNA processing occurs co-transcriptionally (Ballarino et al., 2009; Morlando et al., 2008; Pawlicki and Steitz, 2008; Yin et al., 2015). What is more, it has been shown that Drosha interacts with the splicing machinery and this interaction can favor some intronic miRNA maturation even though both processes, splicing and pre-miRNA cropping, can be completed independently (Agranat-Tamir et al., 2014; Kim and Kim, 2007; Mattioli et al., 2014).

Microprocessor cleavage liberates a ~70nt stem-loop called precursor miRNA (pre-miRNA) with a typical 5' phosphate, 3' hydroxyl group (OH) and a 2nt overhang on the 3' end (Lee et al., 2003). This is important for the recognition by Exportin 5 (Exp5) that protects the pre-miRNA from degradation and mediates its nuclear export in a RAN-GTP-dependent manner (Bohnsack et al., 2004; Yi et al., 2003; Zeng and Cullen, 2004).

Once in the cytoplasm, pre-miRNAs are processed by another type III RNase, Dicer, associated to its cofactor TRBP (TAR RNA-binding protein) (Bernstein et al., 2001; Chendrimada et al., 2005; Ketting et al., 2001; Wilson et al., 2015). Dicer crops the apical loop leaving, similarly to Drosha, a 2 nt 3' overhang. The resulting 20-22 nt long RNA duplex containing a 5p and a 3p strand (Elbashir et al., 2001b; Macrae et al., 2006) is then transferred to one of the Argonaute (AGO) family proteins in a process referred to as RISC loading (Kobayashi and Tomari, 2016). RISC stands for RNA-induced silencing complex and it mediates the miRNA inhibitory function (Hutvágner and Zamore, 2002). However, before RISC can act on its targets, one of the strands has to be eliminated by AGO itself. It has been

shown that most miRNA duplexes exhibit asymmetry, i.e. one of the two strands is preferentially selected to become the mature miRNA, while the other, the "passenger strand", is removed and degraded. Strand selection reflects the mechanistic ease for AGO to open and unwind the duplex, based on the relative thermodynamic stability at the 5' end of each strand. In other words, AGO selects the strand less stably paired at its 5'end. As a result, one precursor gives rise to systematically one mature miRNA, even though a minor proportion of passenger strand can be still incorporated into AGO (Khvorova et al., 2003; Schwarz et al., 2003). On the other hand, certain miRNA duplexes do not exhibit such asymmetry and both strands can give rise to equally abundant and functionally relevant miRNAs. In such cases, the mature miRNAs are distinguished by the suffix -5p and -3p according to the strand they derive from.

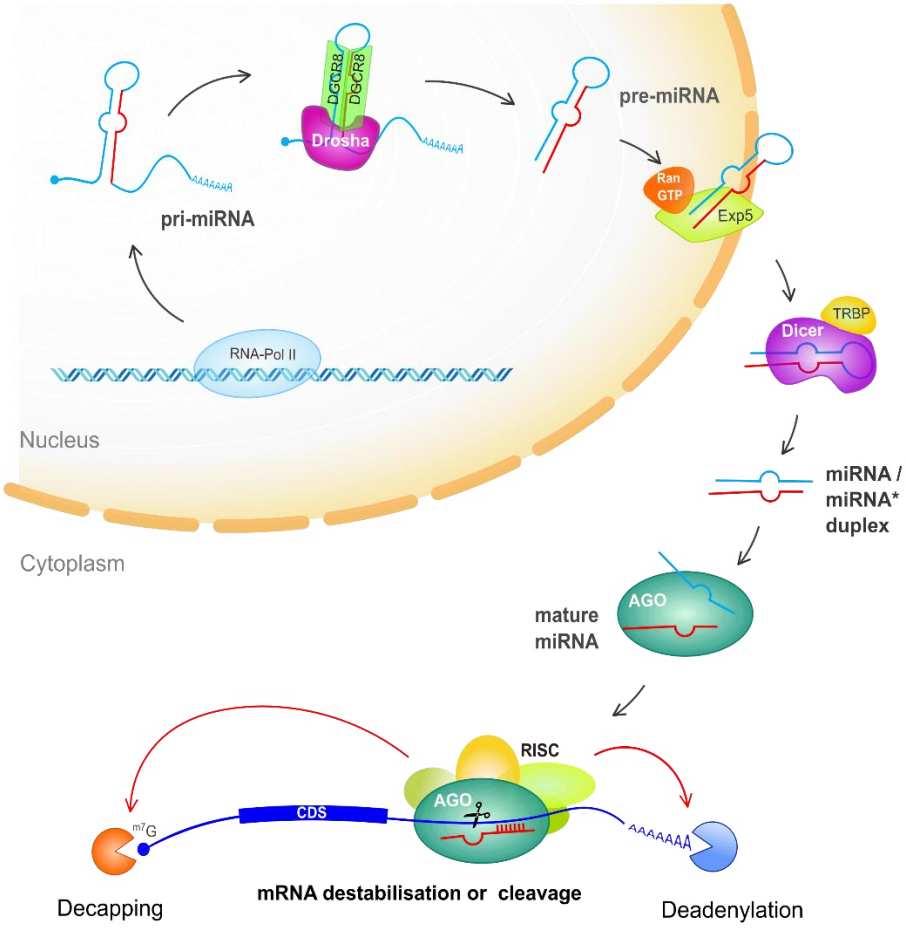

Figure 1 : Canonical miRNA biogenesis and mode of action. Transcription by RNA Polymerase II generates primary miRNA transcripts (pri-miRNAs) which are recognized and cleaved by the Microprocessor complex (Drosha/DGCR8). Precursor miRNA stem-loops (pre-miRNAs) are exported to the cytoplasm with the help of exportin 5 (Exp5) and Ran-GTP. Dicer/TRBP cleaves the apical loop resulting in a 20-22 nt duplex that is passed on to one of the Argonaute (AGO) proteins. AGO discards the passenger (miRNA*) strand and mediates the interaction between the miRNA and its target mRNA (complementary to the miRNA seed sequence). Assemby of the RNA-induced silencing complex (RISC) leads to translation impairment through mRNA destabilization or direct cleavage (by AGO2). CDS: coding sequence.

2.4 IsomiRs

The biogenesis pathway may sometimes generate alternative miRNA isoforms also termed IsomiRs (Ameres and Zamore, 2013). Imprecise Drosha and Dicer cleavage of the pri/pre-miRNA can produce species with heterogenous 3' and 5' extremities that accumulate in addition to the major miRNA isoform. These alternative miRNA forms may expand the regulatory potential of miRNA genes (Kim et al., 2016b; Ma et al., 2013; Umbach and Cullen, 2010; Wu et al., 2009). In addition, it has been shown that editing of miRNA precursors by adenosine-to-inosine (A-to-I) conversion by adenosine deaminases acting on RNAs (ADARs) can generate isomiRs with potentially divergent functions to the original miRNA (Kawahara et al., 2007; Pfeffer et al., 2005). Finally, many miRNAs present heterogeneities at their 3'ends. These arise from untemplated nucleotide addition by terminal-nucleotide-transferases (TUTs) or from trimming by 3'-exonucleases (Ha and Kim, 2014).

2.5 Mode of action

Upon being loaded with a guide miRNA, AGO scans the mRNA pool within the cell for sequences complementary to the miRNA (Bartel, 2009; Kobayashi and Tomari, 2016). The interaction between the miRNA and its target mRNA occurs usually within the 3'UTR, even though coding sequence and 5'UTR can be also targeted. Following the assembly of RISC complex, the target transcript is downregulated through two main molecular mechanisms based on the extent of sequence complementarity and the AGO protein involved. First, if the pairing between miRNA/target is perfect and extensive, and if they are associated within AGO2, the latter is able to directly cleave the target mRNA due to its slicing activity (Becker et al., 2019; Liu et al., 2004; Meister et al., 2004). However, the three remaining AGOs have lost their catalytic activity, although it should be noted that AGO3 does possess some residual slicing activity in specific conditions (Park et al., 2017). As a result, the major outcome of miRNA activity in mammalian cells is translational block and mRNA destabilization. This happens through the recruitment of an adaptor protein from the GW182 family (TNRC6 in mammals), which in turn engages the CCR4/NOT deadenylation complex initiating the 3'-5' degradation. Upon shortening of the poly-A tail, the helicase DDX6 represses translation and recruits the decapping complex DCP1/DCP2 to the 5'end. Finally, the mRNA decay is completed by the 5'-3' exoribonuclease 1 (XRN1) (Béthune et al., 2012; Djuranovic et al., 2012; Eichhorn et al., 2014; Mathys et al., 2014).

The vast majority of miRNA/mRNA interactions occurs through binding of only a limited region of the miRNA. It is called the "seed" and corresponds to nucleotides 2 to 8 from the 5' end of the miRNA (Brennecke et al., 2005). The limited base pairing driving target specificity implies theoretical targeting of tens, if not hundreds of potential mRNA targets. It was indeed estimated that up to 60-75% of human mRNAs are directly regulated by miRNAs (Bartel, 2009; Friedman et al., 2009). What is more, if a miRNA regulates a transcription factor or a hub protein integrating more signaling networks, the impact can be much broader. This supports the idea that miRNA-dependent regulation virtually spans all physiological processes within a cell. However, in order to achieve efficient downregulation, the miRNA concentration within a cell must be high enough to induce real physiological changes. As a general rule, the miRNA abundance can be used as a measure of miRNA activity (Denzler et al., 2016). On the other hand, a finer regulation of miRNA targetome can be achieved by the presence of several target sites for one or several miRNAs on one single transcript. Thus less abundant miRNAs might still be physiologically relevant if they act in concert with others (Grimson et al., 2007).

The requirement for tiny seed-match interaction, as well as the potential synergistic relationships between different miRNAs makes target prediction and validation a laborious process. In fact, experimental evidence is necessary for each target and each miRNA individually. Finally, it is important to bear in mind that the validated interactions might only be relevant in particular physiological contexts.

2.6 Non-canonical miRNAs

Earlier studies looking for novel miRNAs typically relied on the biogenesis criteria. Identifying genomic loci susceptible to generate precursors that can enter the known miRNA processing pathway was the standard procedure to detect candidate miRNA genes. However, deep-sequencing of total or AGO-enriched small RNA libraries has revealed a variety of miRNA-like molecules consistently accumulating within cells and within RISC complexes. These did not always align to what was expected miRNA genes, i.e. precursors transcribed by Pol II and folding into a typical stem-loop substrate for the Microprocessor. Sometimes, they even originated from other small non-coding RNA precursors. In parallel, experiments depleting the major components of the miRNA biogenesis machinery revealed expression of miRNAs that can bypass the main processing routes, revealing that functional miRNAs are not only products of the major biogenesis pathway (Babiarz et al., 2008; Kim et al., 2016b).

The largest group to date of such non-canonical miRNAs are the mirtrons (Berezikov et al., 2007; Ruby et al., 2007). Mirtrons are Drosha-independent since they correspond to small introns that are excised during splicing. The splicing reaction generates a lariat that is debranched and structurally rearranged into the form of a pre-miRNA (Schamberger et al., 2012). This can be directly recognized by Exp5 if the pre-miRNA extremities overlap precisely the pre-mRNA splice junctions. If one of the splice is located further apart from the pre-miRNA end, this creates a 3'- or 5'-tailed mirtron, which has to be first trimmed by the nuclear exosome or other yet-to-be-identified exonucleases (Flynt et al., 2010). Interestingly, some mirtrons have been shown to be processed also when splicing is impaired, requiring Drosha, but not DGCR8. These particular precursors have been termed simtrons (splicing-independent mirtron-like miRNAs) (Havens et al., 2012). Later, Hansen *et al.* demonstrated the existence of another class of molecules originating from short introns and presenting miRNA activity. The latter were termed agotrons since they readily associate with AGO, but are processed independently of both Drosha and Dicer (Hansen et al., 2016).

Another case of non-canonical miRNAs are endogenous short hairpin RNAs (Babiarz et al., 2008). It has been shown that Pol II can generate short transcripts corresponding directly to pre-miRNAs. Their 5'end aligns to the transcription start site and their 3' end to transcription termination signal. Thus, after being exported to the cytoplasm by Exp1, these precursors become direct substrates for Dicer (Xie et al., 2013). Similarly, short transcripts containing pre-miRNAs hairpins generated by Pol III, were also reported (Babiarz et al., 2008; Burke et al., 2014; Kincaid et al., 2012).

Several classes of non-canonical miRNAs have been shown to derive from chimeric precursors transcribed by Pol III and originating from other small non-coding RNAs such as small nucleolar RNAs (snoRNAs) (Ender et al., 2008) or tRNAs (Babiarz et al., 2008; Haussecker et al., 2010). As an example, murine γ-herpesvirus MHV68 produces its miRNAs from hairpins adjacent to tRNA precursors (Pfeffer et al., 2005). Bogerd and colleages have further studied these molecules and found that the tRNA and pre-miRNA are first separated by cellular tRNAse Z and then join the canonical biogenesis pathway (Bogerd et al., 2010).

Finally, there is one example known to date of a *bona fide* miRNA processed by Drosha but bypassing Dicer. miR-451 is highly expressed in erythrocytes and it is produced from an unusually short stem-loop processed by the Microprocessor (however requiring the presence of a helper hairpin, see review in section 4.2). After being exported to the cytoplasm, pre-miR-

451 enters directly AGO2, which uses its slicer activity and recruits the PARN (Poly(A)-specific ribonuclease) to produce the mature miRNA (Cifuentes et al., 2010). This enables a sustained expression of miR-451 during erythrocyte differentiation, which is associated with a global miRNA shutdown due to Dicer downregulation (Kretov et al., 2020).

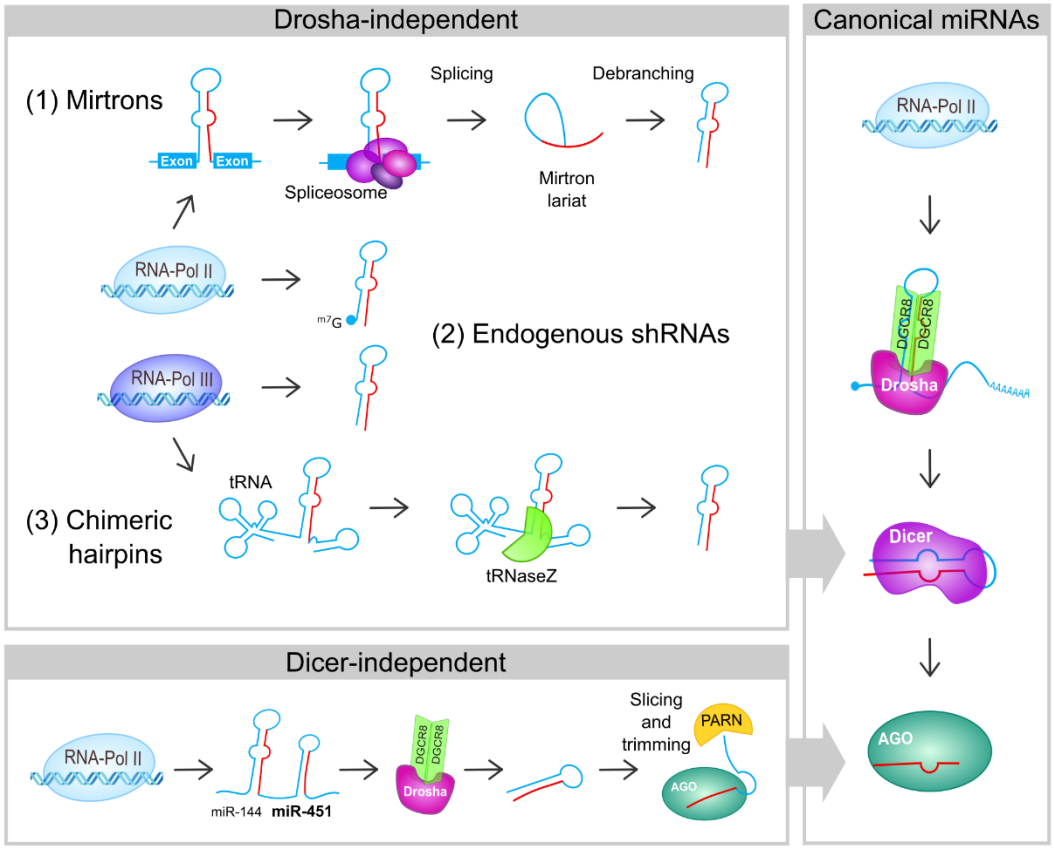

Figure 2: Examples of non-canonical miRNA biogenesis. Alternative processing pathways can generate pre-miRNA-like molecules from mirtrons, by direct transcription of short hairpin RNAs (shRNAs) or from chimeric molecules harboring also other non-coding RNAs. These pre-miRNAs can be further maturated by Dicer and act though the canonical pathway. A particular case is represented by miR-451 that is cleaved by the Microprocessor, however requiring the presence of the neighboring miR-144 on the pri-miRNA. Pre-miR-451 is then directly processed by AGO and PARN exonuclease in order to generate functional miRNA.

3. miRNA regulation

The fundamental role of miRNAs within a cell is to fine tune its proteome adapting it to different contexts and respond to various internal and external cues. However, this also means that miRNA expression itself must be in turn tightly controlled in space and time. Otherwise, dysregulated miRNA levels and activity can deviate towards pathologic conditions.

3.1 Transcriptional regulation

As for coding genes, the first layer of miRNA regulation occurs at the transcriptional level. Many miRNA present tissue-specific expression (e.g. miR-1 in muscle and nerve tissue, miR-122 in liver), which results from the activity of particular transcription factors and epigenetics (Landgraf et al., 2007; Sempere et al., 2004). What is more, they can be expressed as a part of various signaling pathway activation, in response to stress or environmental stimuli (Gulyaeva and Kushlinskiy, 2016; Krol et al., 2010).

Interestingly, some miRNAs can form negative feedback loops by targeting their own transcription factors, thereby auto-regulating their steady-state levels and maintaining cell homeostasis (Kotaki et al., 2017).

3.2 Regulation of miRNA biogenesis

Besides mechanisms in control of transcriptional activity at miRNA promoters, post-transcriptional regulation takes place at each step of the miRNA biogenesis pathway. The activity of the Microprocessor, nucleo-cytoplasmic export, Dicer or AGOs as well as the accumulation of processing intermediates along the entire process is tightly controlled by accessory factors and processes that fine-tune the final functionally relevant levels of mature miRNAs. These post-transcriptional regulatory mechanisms can act in a specialized fashion, modulating the biogenesis of a single miRNA, or they can impact more globally larger subsets, if not the entire pool of miRNAs expressed at a given moment.

3.2.1 Modulation of biogenesis factor activity

Certain physiological conditions such as hypoxia or activation of stress response may require broader remodeling of miRNA activity. Various signaling pathways were shown to widely affect miRNA production through stimulatory or inhibitory post-translational modifications (PTMs) of the core biogenesis factors. This allows fast response and transition between cell states. As an example, phosphorylation events of DGCR8 and TRBP stimulate their function and increase global miRNA levels as a result of MAPK/ERK signaling (Herbert et al., 2013; Paroo et al., 2009). On the other hand, phosphorylation of specific amino acids of Drosha and AGO2 under stress conditions was shown to decrease their interactions with DGCR8 and Dicer respectively, thus negatively affecting miRNA biogenesis (Shen et al., 2013; Yang et al., 2015). Besides direct stimulatory or inhibitory effect on the processing pathway

effectors, other PTMs can also impact their stability or subcellular localization (Herbert et al., 2013; Qi et al., 2008; Tang et al., 2010; Treiber et al., 2019).

Interestingly, the miRNA pathway is also directly involved in the regulation of the biogenesis machinery. The procesing enzymes are also sensitive to post-transcriptional silencing by some miRNAs (Martello et al., 2010; Tokumaru et al., 2008). Another interesting phenomenon is also the Microprocessor-mediated regulation of DGCR8. A pre-miRNA-like stem loop forms in the 5'UTR of DCGR8 mRNA and can be cleaved by the Microprocessor when the complex is abundant enough. This leads to DGCR8 mRNA decay and a retroactive feedback loop in order to preserve optimal levels of the Microprocessor within a cell (Han et al., 2009).

3.2.2 Regulation by RNA-binding proteins (RBPs)

While Drosha and DGCR8 form the minimal and sufficient enzymatic core required for pri-miRNA processing (Denli et al., 2004), Gregory and coworkers brought attention to the fact that in cells, most of Microprocessor resides within larger complexes formed by a number of accessory proteins (Gregory et al., 2004). The latter belong, for the biggest part, to a large group of proteins designated as RNA-binding proteins (RBPs). RBPs are involved in all aspects of RNA metabolism (processing, transport, localization, decay, post-transcriptional regulation) and they interact with RNA molecules through one or more RNA-binding domains (RBD). RBPs often form highly dynamic ribonucleoprotein complexes and facilitate crosstalk between various cellular pathways. Mammalian cells express more than one thousand of different RBPs and a number of them has been shown to engage in miRNA biogenesis, not only at the level of Microprocessor, but during the entire process (reviewed in (Creugny et al., 2018; Michlewski and Cáceres, 2019; Treiber et al., 2019)).

The mode of action of RBPs acting as biogenesis co-factors can be general, affecting all (or large groups) of miRNAs, or limited to one or several miRNAs sharing a particular feature. The outcome of co-factor activity can be either positive/stimulatory or negative/inhibitory towards the given miRNA generation. There have been numerous studies investigating the implication of RBPs in miRNA biogenesis, most of them focused on a given RBP affecting one or a small subset of miRNAs. However, taking advantage of high-throughput approaches, large scale studies were also carried out and broadened the repertoire of known biogenesis co-factors

(Nussbacher and Yeo, 2018; Treiber et al., 2017). Yet, there are probably many more to be discovered.

3.2.3 Examples of co-factors involved in miRNA biogenesis

One of the most prominent examples of miRNA biogenesis modulation exerted by co-factors is the regulation of let-7 by Lin28A and Lin28B. Both proteins bind to the stem loop of pre-let-7 and negatively affect its processing, but each of them use a distinct mechanism. While Lin28B binding inhibits the recognition of the pri-miRNA by the Microprocessor (Piskounova et al., 2011), Lin28A acts in the cytoplasm and recruits terminal-uridylyltransferases (TUT4 or TUT7). The latter add a stretch of Us to the 3'end of the pre-let-7. This not only impedes further processing by Dicer, but also marks the RNA for degradation by the exoribonuclease DIS3L2 (Thornton et al., 2015; Ustianenko et al., 2013; Viswanathan et al., 2008). Let-7 being one of the most studied miRNAs, numerous other co-factors have been associated with its processing. hnRNP A1 and KSRP can both bind to pri-let-7 apical loop, however with opposite outcomes, resulting in inhibition or stimulation of pri-let-7 processing respectively (Michlewski and Cáceres, 2010). The case illustrates that the processing of one miRNA can be balanced by the competitive binding of proteins with antagonistic effects, according to the physiological context driving the expression of the latter.

To further complicate the picture, one co-factor can also regulate different miRNAs by inducing opposite effects. An example of such double functionality is hnRNP A1. As mentioned above, it inhibits the processing of let-7, but at the same time, it is required for optimal processing of miR-18a. Extensive biochemical and functional assays have demonstrated that binding of hnRNP A1 induces relaxation of the pri-miR-18a stem-loop structure thereby facilitating Microprocessor-mediated cleavage (Guil and Caceres, 2007; Kooshapur et al., 2018; Michlewski et al., 2008).

Besides binding to and mediating interactions between miRNA precursors and biogenesis machinery, co-factors may also bring into play distinct catalytic activities. This is the case of the immune modulator Monocyte chemotactic protein-1-induced protein-1 (MCPIP1), which has been shown to bind numerous pre-miRNAs in the cytoplasm. MCPIP1 not only compete with Dicer for pre-miRNA substrates, but also directly cleaves them due to its RNase activity (Happel et al., 2016; Kook and Ziegelbauer, 2021; Suzuki et al., 2011). MCPIP1-cleaved pre-miRNAs cannot be further processed by Dicer and are quickly degraded

by other enzymes. Another enzyme involved in miRNA biogenesis is the adenosine deaminase acting on RNA 1 (ADAR1). Given that sequence and structure elements are important for correct processing of miRNA hairpins, adenosine-to-inosine editing of miRNA precursors performed by ADAR1 can specifically impact their maturation at various levels (Chawla and Sokol, 2014; Kawahara et al., 2008; Luciano et al., 2004; Yang et al., 2006). What is more, if editing occurs within the mature miRNA sequence, it can affect the given miRNA targetome (Iizasa et al., 2010; Kawahara et al., 2007). Interestingly, while the dimeric form of ADAR1 is responsible for editing events, monomeric ADAR1 has been also identified in complex with Dicer. This interaction has been shown to globally enhance Dicer-mediated miRNA processing and RISC loading (Ota et al., 2013). Several other examples of co-factors are presented in

Table **1**.

3.2.1 Regulation by other RNAs

Proteins are not the only molecules that can exert regulatory functions during miRNA biogenesis. Various RNA molecules, notably long non-coding RNAs (lncRNAs) can bind to the precursors in *trans* and either directly modulate the core processing activities or recruit additional regulatory factors (see review in part 4.2). Mature miRNAs in turn can also interact and modulate the processing of pri-miRNAs in autoregulatory loops. This has been shown with mouse miR-709 whose binding to pri-miR-15~16 inhibits the processing of both miRNAs, whereas binding of mature let-7 can stimulate the processing of its own pri-miRNA in *C. elegans* (Tang et al., 2012; Zisoulis et al., 2012). Recently, a study by Hennig and colleagues brought to light a peculiar regulatory mechanism involving the binding of a mature miRNA expressed by the human herpesvirus 6 (HHV6) to the apical loops of several members of the miR-30 family. This directly interfered with their processing and had surprisingly broad impact on viral and cell physiology (Hennig et al., 2022). These examples also illustrate some of the alternative functions of miRNAs described so far.

Table 1: Protein co-factors with known function in miRNA biogenesis.

Co-factor	miRNA(s)	Impact on miRNA biogenesis	Mode of action	Reference
BCDIN3D	miR-145	negative	Inhibition of Dicer processing through methylation of the pre-miRNA	(Xhemalce et al., 2012)
BRCA1	let-7, miR-16, mir-34, miR-145	positive	Drosha stimulation in complex with DDX5, p53 and DDX9	(Kawai and Amano, 2012)
DDX3X	miR-183~182, subset of miRNAs	Negative or positive	Binding to pri-miRNA and regulation of Microprocessor activity	(Krol et al., 2015; Zhao et al., 2016)
DDX5 (p68)	Subset of miRNAs	positive	Interaction with Drosha and recruitment of additional co-factors such as p53 and SMADs	(Davis et al., 2008; Suzuki et al., 2009; Wang et al., 2012)
DDX9	Global impact on miRNAs	positive	Enhancement of RISC loading	(Robb and Rana, 2007)
DDX17 (p72)	miR-26a~b	positive	Pri-miRNA binding and splicing regulation	(Lambert et al., 2018; Ngo et al., 2019)
EWS	Global impact on miRNAs	positive	Drosha recruitment to pri-miRNA	(Ouyang et al., 2017)
FUS	miR-9, miR-125, miR-200	positive	Drosha recruitment	(Dini Modigliani et al., 2014; Morlando et al., 2012)
HUR	miR-7	negative	Together with MSI2, the protein complex inhibits Drosha cleavage	(Choudhury et al., 2013)
MBNL1	miR-1	positive	Binds topre-miRNA apical loop and prevents binding of LIN28 (negative regulator)	(Rau et al., 2011)
RBFOX2 and RBFOX3	Subset of miRNAs	Negative and positive	Depending on the pri-miRNA, modulate Microprocessor recruitment through binding to pri-miRNA apical loop	(Chen et al., 2016; Kim et al., 2014)
SRSF3	Subset of miRNAs	positive	Binding to CNNC motif on pri-miRNA downstream of pre-miRNA stem-loop	(Auyeung et al., 2013)
TRIM25	let-7	negative	Binding to the apical loop of pre-let-7 helps Lin28a to recruit TUT4 for efficieng pre-let-7 uridylation	(Choudhury et al., 2014)
TDP-43	miR-132, miR-143, miR-558, miR-574	positive	Binding to apical loops of pri-miRNAs and Microprocessor recruitment	(Chen et al., 2018)
YB-1	miR-29b-2	negative	Inhibition of Drosha binding	(Wu et al., 2015)

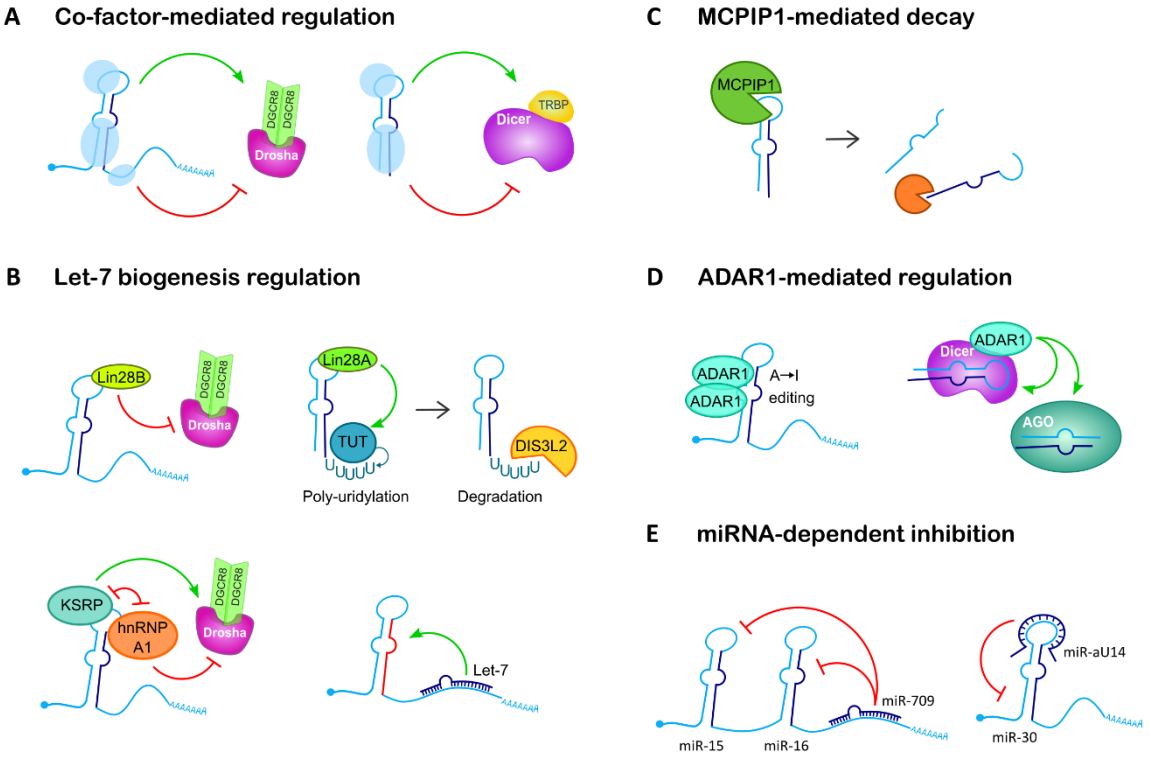

Figure 3: Regulation of miRNA biogenesis in *trans*. A) Binding of protein co-factors to various regions of pri/pre-miRNA hairpins can have positive or negative effects on their processing. B) Different modes of regulation of let-7 biogenesis. Lin28B inhibits pri-let-7 nuclear processing by the Microprocessor, whereas Lin28A recruits TUTs to pre-let7 inducing its polyuridylation and degradation. Competitive binding by KSRP and hnRNP A1 to the apical loop of pri-let7 stimulate or impede its processing. Mature let-7 has been shown to increase pri-let-7 maturation in *C.elegans*. C) MCPIP1 cleaves numerous pre-miRNAs at the level of their apical loop, thus leading to their degradation instead of processing by Dicer. D) Precursor editing by dimeric ADAR1 can impact miRNA maturation. Monomeric ADAR1 can bind to Dicer and stimulate its processing as well as RISC loading. E) Mouse miR-709 can bind to the bicistronic pri-miRNA containing pre-miR-15 and pre-miR-16 and decrease their processing. miR-aU14 expressed by HHV-6A can inhibit the processing of several members of the miR-30 family by binding to precursor apical loops.

3.3 Regulation of miRNA activity and turnover

Next to the regulation taking place during miRNA biogenesis, differential turnover of these molecules also contributes to their steady-state levels. MiRNAs are believed to be unusually stable as compared to other longer RNA molecules, such as mRNAs. This is in part due to the protective function of AGO, which can retain the miRNA for extended periods in order to be recycled from one mRNA target to another. The half-life of a miRNA associated with RISC can expand to several days (Gantier et al., 2011). In addition, AGO overexpression leads to globally higher levels of miRNAs within cells, which can be explained by their slower degradation (Diederichs and Haber, 2007). However, not all miRNAs possess the same level of stability and various decay mechanisms can take place to control their turnover. First, miRNAs

can be sequestrated from their targets by other non-coding RNAs containing miRNA binding sites. Several long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) were described to act as sponges of miRNAs in different physiological contexts (Gong et al., 2021; Sun et al., 2018; Zhang et al., 2020, 2018). This sponging effect can in some cases be followed by active miRNA degradation. Secondly, elevated levels of highly complementary targets can trigger active degradation of miRNAs through the mechanism of target-directed miRNA degradation (TDMD) (Ameres et al., 2010; Cazalla et al., 2010). This relies on nucleotide addition (tailing) and exonucleolitic degradation (trimming) by TUT1 and DIS3L2 respectively (Haas et al., 2016; Yang et al., 2020). In some cases, TDMD can be accomplished through selective ubiquitination and proteasome-mediated degradation of AGO instead of miRNA tailing and trimming (Han et al., 2020; Shi et al., 2020). Finally, sequence determinants at the 5' and 3'end of the miRNA were also proposed as a factor for increased decay of some miRNAs (Bail et al., 2010; Zhou et al., 2018).

4. miRNA clusters and their regulation

An important feature in miRNA biology, yet somewhat underestimated, is the fact that many of them are grouped into genomic clusters and co-expressed on polycistronic pri-miRNAs. In the light of recent findings, this aspect has gained more attention and we now realize how important this might be for miRNA expression.

4.1 Clustered miRNA evolution

Different types of clusters are recognized frequently reflecting the evolutionary relationships between the different miRNAs. First, genomic amplifications gave rise to tandems of miRNAs belonging to the same miRNA family that tend to retain important sequence homology, or at least identical seed sequence. These arrangements are classified as homo-clusters. On the contrary, hetero-clusters comprise various miRNAs with no sequence similarity that were shaped by *de novo* formation (Marco et al., 2013). Finally, combination of the two classes is also common. As the apparent evolutionary links suggest, clusters are a driving force allowing appearance of new miRNA genes, as well as miRNA evolution. This is not only related to duplications followed by sequence divergence, but also through *de novo* formation. It was indeed suggested that birth of new miRNAs occurs preferentially within transcripts already bearing pre-existing miRNAs (Malnou et al., 2019; Marco et al., 2013; Wang et al.,

2016b). Random miRNA-like hairpins arise stochastically by transcription of various genomic loci (Djebali et al., 2012). Dynamic sequence alterations however could make it difficult for a new miRNA to acquire a relevant function, if it is located in a functionally less important region and its transcription is not maintained. On the other hand, if there is another essential element regularly transcribed in the vicinity, the new miRNA would have a better chance to be retained through evolution. If this element is a proximal pre-existing miRNA, this becomes even more obvious since the new hairpin could benefit not only from the transcription, but also from the nearby Microprocessor activity. Thus, miRNA clusters were also called "miRNA nurseries" because they can shelter young miRNAs and provide the time necessary for their functional adaptation.

4.2 Post-transcriptional regulation of miRNA polycistrons

Besides the selective advantage in terms of evolution, polycistronic miRNA expression might provide additional benefits over stand-alone miRNAs. One of them is innovation to the spectrum of regulatory mechanisms controlling the expression of individual cluster members (Figure 4). On this additional layer of regulation within miRNA polycistronic transcripts, I have written a review with Dr. S. Pfeffer, which is hereby included (Vilimova and Pfeffer, 2022).

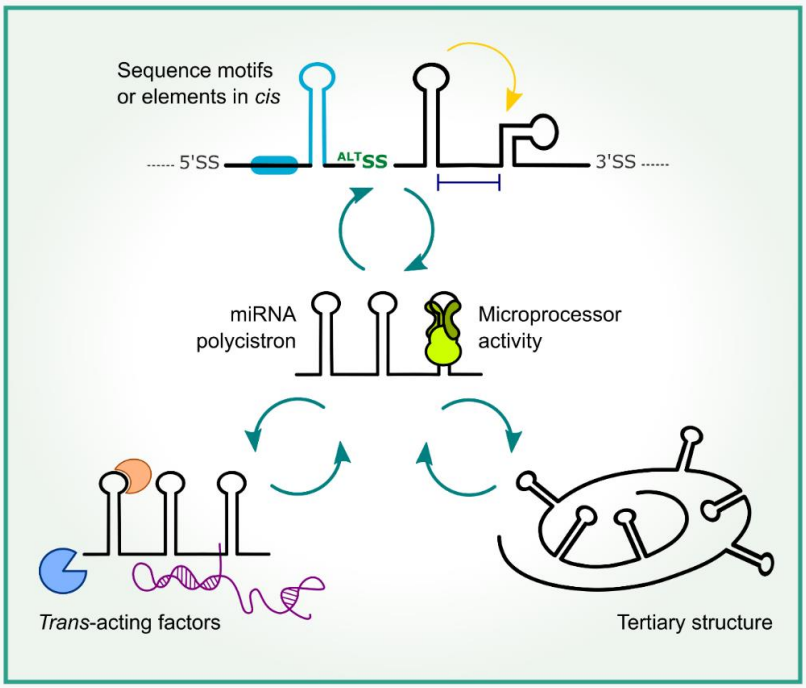

Figure 4: Graphical abstract from the review Post-transcriptional regulation of polycistronic microRNAs. Microprocessor activity within polycistronic microRNA transcripts is tightly regulated and can lead to differential expression of miRNAs organized in clusters. This regulation involves elements in cis-, trans-acting factors as well as the three-dimensional structure of the transcript.

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ADVANCED REVIEW

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Post-transcriptional regulation of polycistronic microRNAs

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Abstract

An important proportion of microRNA (miRNA) genes tend to lie close to each other within animal genomes. Such genomic organization is generally referred to as miRNA clusters. Even though many miRNA clusters have been greatly studied, most attention has been usually focused on functional impacts of clustered miRNA co-expression. However, there is also another compelling aspect about these miRNA clusters, their polycistronic nature. Being transcribed on a single RNA precursor, polycistronic miRNAs benefit from common transcriptional regulation allowing their coordinated expression. And yet, numerous reports have revealed striking discrepancies in the accumulation of mature miRNAs produced from the same cluster. Indeed, the larger polycistronic transcripts can act as platforms providing unforeseen post-transcriptional regulatory mechanisms controlling individual miRNA processing, thus leading to differential miRNA expression, and sometimes even challenging the general assumption that polycistronic miRNAs are co-expressed. In this review, we aim to address the current knowledge about how miRNA polycistrons are post-transcriptionally regulated. In particular, we will focus on the mechanisms occurring at the level of the primary transcript, which are highly relevant for individual miRNA processing and as such have a direct repercussion on miRNA function within the cell.

This article is categorized under:

RNA Processing > Processing of Small RNAs

Regulatory RNAs/RNAi/Riboswitches > Biogenesis of Effector Small RNAs

RNA Interactions with Proteins and Other Molecules > RNA-Protein Complexes

KEYWORDS

biogenesis, cluster, Drosha, microRNA, polycistron, post-transcriptional regulation

1 | INTRODUCTION

MicroRNAs (miRNAs) belong to small noncoding RNAs acting as essential regulators of gene expression. The importance of miRNAs in maintaining the homeostasis of the cell and the link between their dysregulation and the

development of diseases have been extensively studied and recognized (Bartel, 2018; Kabekkodu et al., 2018; Rufino-Palomares et al., 2013). Yet, we are still discovering new mechanisms that post-transcriptionally regulate the accumulation of miRNAs themselves, which in turn directly influence the activity of each of these important regulatory molecules.

In animals, the canonical miRNA biogenesis pathway (Figure 1) entitles the sequential maturation of a long RNA Polymerase (Pol) II primary transcript (pri-miRNA) by two type III ribonucleases. In the first step, the pri-miRNA folds into a stem-loop structure, which is recognized and cleaved by the Microprocessor complex composed of the RNase III Drosha and a dimer of Pasha/DGCR8 (DiGeorge syndrome critical region 8), to generate the precursor miRNA (pre-miRNA). Then, the pre-miRNA is exported to the cytoplasm by the Exportin 5 factor and processed by another RNase III, called Dicer. The resulting 20–24 nucleotide (nt) long RNA duplex is loaded into one of the Argonaute (AGO) proteins and after unwinding the mature miRNA remains associated with AGO to form the effector RNA-induced silencing complex (RISC). The miRNA acts as a guide for the AGO protein to recognize its target RNAs. Upon base-pairing between the miRNA seed sequence (i.e. nt 2 to 7 or 8) and a complementary mRNA target sequence, RISC will recruit additional factors to either destabilize the target mRNA or impede its translation (see Bartel, 2018 for an extensive review on miRNA biogenesis and mode of action). Each step of this pathway is tightly controlled by a wide range of mechanisms, from regulating the transcription of the pri-miRNA to triggering mature miRNA decay, that ultimately fine-tune the accumulation of individual mature miRNAs (Treiber et al., 2019).

Since the first miRNA genes were discovered in animal genomes, researchers noticed their propensity to be localized in proximity and form groups of 2 or more tandemly arranged miRNAs, henceforth referred to as miRNA clusters. This observation was instrumental in helping in the systematic discovery of novel miRNAs. Based on the hypothesis that clustering is frequent and that novel miRNA genes can be located in the vicinity of previously known miRNAs, scanning of proximal genomic regions allowed to increase the miRNA repertoire (Altuvia, 2005; Sewer et al., 2005).

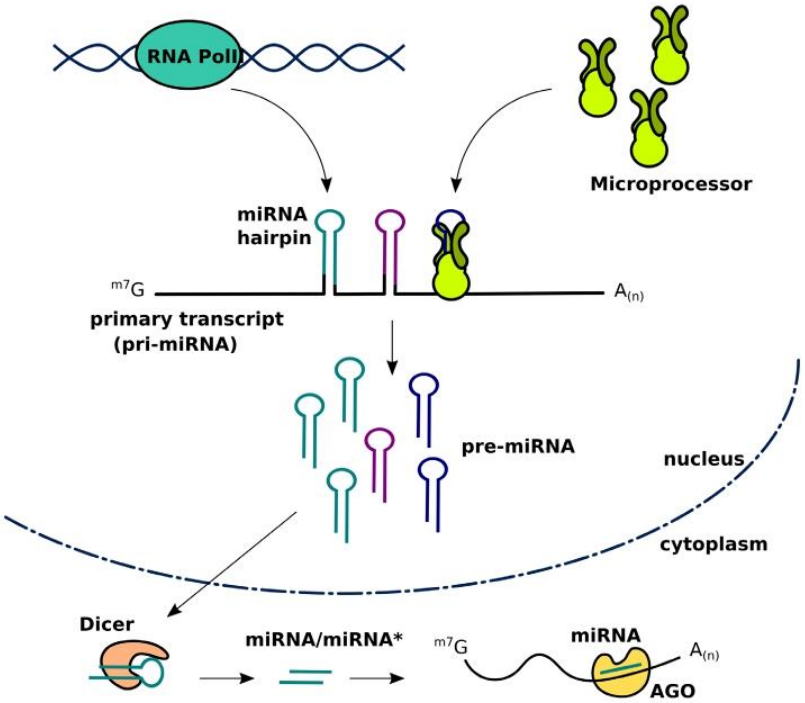

FIGURE 1 Canonical pathway for miRNA biogenesis. miRNA genes are transcribed by the RNA-polymerase II. Clustered miRNAs are transcribed on a single primary transcript harboring individual miRNA hairpins. The latter are recognized by the heterotrimeric Microprocessor complex composed of one molecule of Drosha and two molecules of DGCR8. Microprocessor cleaves out the stem-loop structures or pre-miRNAs that are exported to the cytoplasm and further processed by dicer. miRNA/miRNA* duplexes are recognized by Argonaute proteins (AGO) which eliminate the miRNA* and scan potential target mRNAs for sites complementary to mature miRNA. Interaction between the mature miRNA and the target transcript leads to translation initiation blockage and mRNA decay

Another in silico approach aimed to predict miRNA clusters with no need for a pre-existing miRNA in their proximity. The main prerequisite for this algorithm was the clustered feature of paralogous miRNA genes (Mathelier & Carbone, 2013). With the annotation of miRNA genes in various animal species nearing completion, or already final as in human (Fromm et al., 2022), it appears that the original observations can be confirmed and that genomic clustering of miRNA genes is indeed higher than expected at random (Chaulk et al., 2016; Guo et al., 2014). According to Wang and colleagues who did an exhaustive analysis of miRNA gene distribution in six animal models, the proportion of miRNAs organized in clusters ranges from 17.3% in chicken to 52% in zebrafish (Wang, Luo, et al., 2016).

A miRNA cluster is usually defined by the presence on the same genomic locus of adjacent pre-miRNA sequences that are transcribed in the same orientation and are not separated by another transcription unit. Most of the clusters are small, ranging from 2 to 6 precursors, while some large clusters may contain dozens of miRNA genes such as the chromosome 19 miRNA cluster (C19MC) in human, miR-430 cluster in zebrafish and Sfmb2 miRNA cluster in mouse, which encompass 46, 58 and 72 pre-miRNAs, respectively (Malnou et al., 2019; Thatcher et al., 2008). Given their genomic proximity, they are believed to be transcribed as polycistrons (Baskerville & Bartel, 2005; Ozsolak et al., 2008; Saini et al., 2007) with one pri-miRNA carrying all the pre-miRNAs. This indeed makes sense given that polycistronic transcription allows several miRNAs to benefit from the same regulation by transcription factors and their co-expression allows to control several target genes at once (Lataniotis et al., 2017; O'Donnell et al., 2005; Segura et al., 2017; Sylvestre et al., 2007; Wee et al., 2012). Along the same line, several groups have shown that clustered miRNAs tend to target genes involved in the same biological process or pathway (Kim et al., 2009; Li et al., 2021; Wang, Luo, et al., 2016). This allows for a simultaneous and cooperative miRNA activity and may induce stronger response at the cellular level. Furthermore, many clusters contain miRNAs from the same miRNA family (with the same seed sequence), which share a set of common targets. Their simultaneous expression would therefore have an additive effect and potentiate a more robust target downregulation. Thus, polycistronic transcription has advantages that resemble the ones found in prokaryotic operon systems. Finally, it has been estimated that about 50% of conserved vertebrate miRNAs are located within miRNA clusters thereby indicating an evolutionary pressure to maintain these genes and their organization (Altuvia, 2005; Wang, Luo, et al., 2016).

Interestingly, although there is coordinated transcription of miRNAs within a cluster, many reports point to the fact that the mature miRNAs originating from this transcription unit can be expressed at very different levels and can follow different expression patterns in response to physiological stimuli (Contrant et al., 2014; Edwards et al., 2008; Gu et al., 2013; Hooykaas et al., 2016; Kotaki et al., 2017; Pratt et al., 2009; Ryazansky et al., 2011; Sempere et al., 2004; Welten et al., 2014; Yu et al., 2006; Zhou et al., 2018). This clearly indicates that there are regulatory mechanisms put in place at the post-transcriptional level to achieve differential expression of individual cluster members. Indeed, it has been shown that the same miRNA can be expressed to strikingly different levels if expressed individually or within a cluster (Chakraborty et al., 2012; Contrant et al., 2014). What is more, the relative expression can be also impacted by the pre-miRNA position within the polycistron. This indicates that there are features within a polycistronic pri-miRNA transcript that directly influence the coordinated processing of individual miRNAs. This in turn allows to achieve expression patterns perfectly tailored for their function. Hence, the long primary transcript can for example serve as a higher-order scaffold providing structural and sequence elements for auto-regulatory intramolecular interactions and/or help in the recruitment of external accessory factors. In addition, increasing evidence shows that within one cluster, individual pre-miRNAs do not behave as independent units, but show unexpected relationships making the whole cluster a dynamic and interdependent system.

In this review, we will focus on polycistronic miRNAs expressed in animals and in some of their viruses, since the latter strictly depend on the host cell machinery to express their miRNAs. We aim to summarize the current evidence of the post-transcriptional molecular mechanisms orchestrating the differential processing of pre-miRNAs within polycistronic primary transcript. Eventually, we hope to provide the reader with a comprehensive view of these events, which are crucial for the accumulation of clustered miRNAs at the right level.

2 | IMPORTANCE OF SEQUENCE, SECONDARY STRUCTURE, AND CLUSTER ASSISTANCE

Although there are examples of noncanonical, Drosha-independent, mechanisms of miRNA processing (e.g. mirtrons [Ruby et al., 2007]), polycistronic miRNAs do rely on the Microprocessor complex for separating the cluster members. It is well recognized that local secondary structure and primary sequence motifs present on the precursor hairpins play

essential roles for efficient recognition by Drosha/DGCR8 (reviewed in Creugny et al., 2018; Michlewski & Cáceres, 2019). The presence of several of these features has an additive effect, while their absence can prevent a given pre-miRNA processing (Fang & Bartel, 2015). Among the structural requirements, we can cite the length of the stem, the presence of a bulge in the stem and the size of the apical loop. Sequence-wise, a UG motif at the base of the stem-loop, a UGU/GUG motif in the apical loop and a CNNC motif downstream of the pre-miRNA were all shown to positively contribute to the miRNA biogenesis (Figure 2a). Therefore, various combinations of these pre-requisites on the different pre-miRNAs within a polycistron can influence the efficiency of their respective processing and account for their differential expression. Indeed, after resolving the secondary structure of a long miRNA cluster (mir-K10/12) expressed by the Kaposi's sarcoma associated herpesvirus (KSHV) by SHAPE (Selective 2′ Hydroxyl Acylation) analyzed by Primer Extension analysis, Contrant et al. examined the features of the 10 miRNA precursors within the cluster and concluded that some of the hairpins are more prone to be processed than others. Importantly, a good correlation was found between the presence of the predefined characteristics cited above and the relative expression levels of the individual miRNAs (Contrant et al., 2014).

Interestingly, suboptimal hairpins have been shown to be enriched within polycistrons (Hutter et al., 2020; Shang et al., 2020). Given their consistent accumulation, this led to the hypothesis that since they are not ideal substrates for the Microprocessor, there must be other mechanisms to specifically boost their processing in an indirect manner.

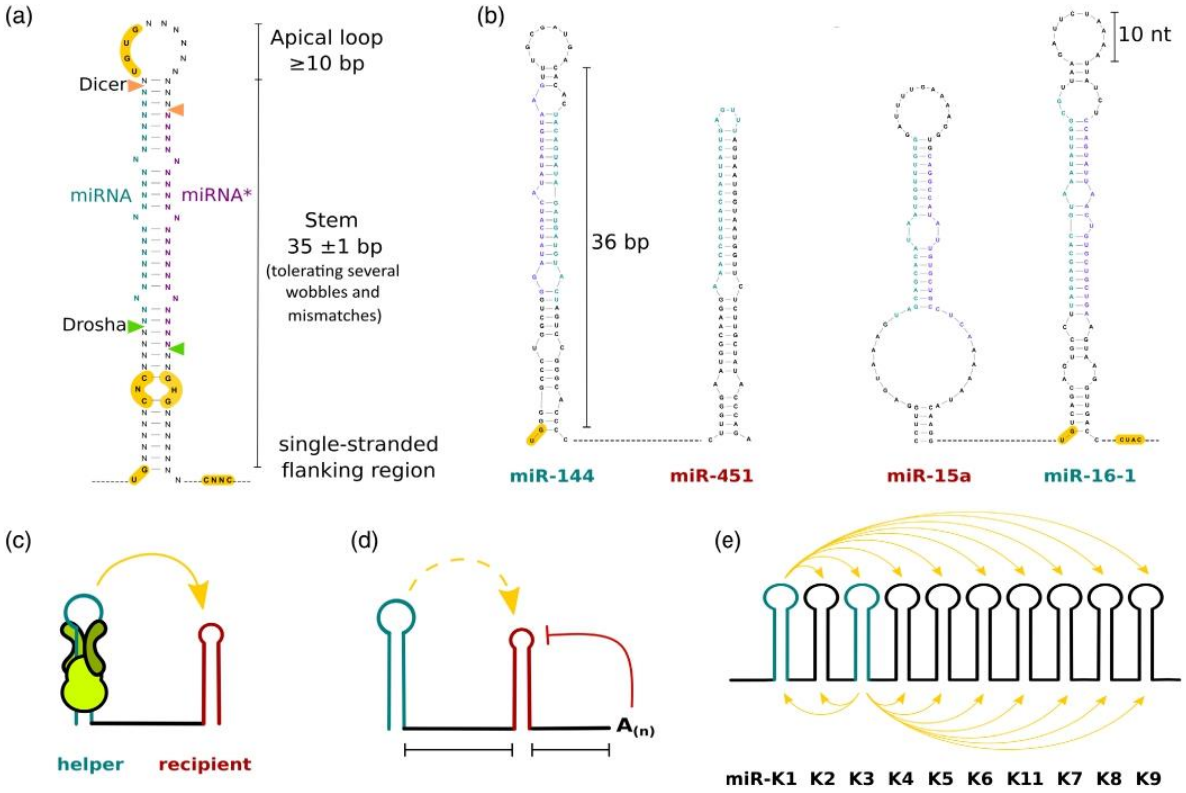

FIGURE 2 Importance of primary sequence and *cis*-acting elements for pri-miRNA cleavage. (a) Hypothetical miRNA hairpin presenting the sequence motifs (highlighted in yellow) and structural features known to enhance Microprocessor recognition/activity. Drosha and dicer cleavage sites are represented by green and orange triangles respectively. (b) Examples of miRNA pairs involved in cluster assistance. The main characteristics optimizing recognition of the helper hairpins or hindering the processing of recipient hairpins are featured. Structures are derived from available SHAPE data (Shang et al., 2020) or predicted with RNA structure (https://rna.urmc.rochester.edu/). (c) Principle of cluster assistance. Presence of the helper hairpin on the primary transcript enhances the processing of the recipient hairpin. (d) In some cases, the helper hairpin not only enables, but simultaneously limits the recipient expression (dashed arrow). Distance between the hairpins and other sequences such as poly(A) sites can also inhibit cluster assistance. (e) Schematic view of KSHV miRNA cluster with pri-miR-K1 and pri-miR-K3 having a positive regulatory effect on the remaining miRNAs within the cluster

Focusing on bi- and tri-cistronic clusters in different animal species, several groups have noticed that deletion of one specific member of the cluster can severely impede the processing of another member, while deleting the other might have no effect (Fang & Bartel, 2020; Feederle et al., 2011; Haar et al., 2016; Hutter et al., 2020; Lataniotis et al., 2017; Shang et al., 2020; Truscott et al., 2016). A common feature of the precursors that fail to be processed in the absence of the other partner is that they present suboptimal characteristics and are therefore poor Microprocessor substrates per se. As such, they require the presence of a proximal helper precursor which meets canonical requirements for optimal Microprocessor processing. This phenomenon has been referred to as cluster assistance (Fang & Bartel, 2020; Hutter et al., 2020) (Figure 2b,c). Strengthening the evidence that optimal folding of the helper hairpin is key for cluster assistance, mutations designed to impact its recognition by the Microprocessor, such as disturbing proper base-pairing within the stem or reducing the size of the stem or of the apical loop, not only reduce the expression of the helper itself, but also of the recipient (Fang & Bartel, 2020; Hutter et al., 2020; Shang et al., 2020). Conversely, when the structure of the recipient hairpin is optimized, it can be processed on its own, even in the absence of its partner on the primary transcript (Fang & Bartel, 2020; Hutter et al., 2020; Shang et al., 2020; Truscott et al., 2016). In addition, cluster assistance is maintained in chimeric constructs where the helper is replaced by another heterologous miRNA stem-loop, unrelated in sequence, but with better hairpin characteristics (Fang & Bartel, 2020; Haar et al., 2016; Hutter et al., 2020; Shang et al., 2020; Truscott et al., 2016). Thus, the impact of the helper hairpin on its neighbor is not solely related to its sequence, structure or potential downstream function.

Other aspects of the phenomenon were also studied in detail. Namely, it was shown that cluster assistance does not operate directionally since swapping the order of helper and recipient stem-loops does not hinder it (Fang & Bartel, 2020; Hutter et al., 2020; Shang et al., 2020; Truscott et al., 2016). However, it seems to be mostly found in clusters where hairpins directly follow one another. Thus, when two suboptimal hairpins are placed immediately downstream of a helper pre-miRNA, there is a strong improvement in processing only for the proximal pre-miRNA while the distal one is only moderately impacted. However, placing the helper hairpin in between the two suboptimal ones provides efficient assistance to both of them (Shang et al., 2020). As to the maximal distance between the neighbor hairpins, it can be increased up to 1200 nt without lowering the potentiating effect (Hutter et al., 2020; Shang et al., 2020). It has been shown that interaction with RNA Polymerase II can be implicated in the enhancement of some pre-miRNA processing (Church et al., 2017). However, it does not seem to play a prominent role in cluster assistance since both RNA Polymerase II and III can synthesize the primary transcript without impacting the phenomenon (Fang & Bartel, 2020; Shang et al., 2020).

Deeper investigation of the molecular mechanism driving cluster assistance led to a model, in which the helper hairpin efficiently recruits the Microprocessor complex and then facilitates its transfer to the recipient stem-loop. The cleavage of the helper pre-miRNA might occur first, but is not necessarily required for the completion of the recipient cropping, since selective tethering of Microprocessor via the B-box system can mimic helper-driven assistance (Fang & Bartel, 2020). In addition, it has been proposed that the mechanism might act as an evolutionary driver of novel miRNAs emergence. Indeed, a recent miRNA, from an evolution point of view, might benefit from the presence of a nearby optimal pre-miRNA even though it has not yet acquired all the characteristics required for its own efficient processing. Taken together, these findings indicate that certain miRNA hairpins might possess additional function than to solely serve as precursors of mature miRNAs, which would include the stimulation of biogenesis within miRNA clusters.

However, not every case of cluster assistance fully complies with these principles. Thus, results obtained by the Delecluse and Frolov groups, studying the Epstein–Barr virus (EBV) mir-BHRF1 ~ 3 and the *Drosophila* mir-11 ~ 998 clusters respectively, indicate that the presence of optimal helper stem-loops can not only potentiate, but simultaneously limit aberrant overexpression of their neighbors (Figure 2d). This would contribute to a more sophisticated fine-tuning effect relevant for physiological needs of each given mature miRNA (Haar et al., 2016; Truscott et al., 2016). Similarly, further sequences surrounding the hairpins or the presence of other elements in *cis* may weaken the stimulatory effect. For example, the presence of a downstream poly(A) signal limits the cluster assistance phenomenon, presumably due to competition between the Microprocessor and transcription termination machineries (Haar et al., 2016).

In the case of clusters containing more than two or three miRNAs, interdependence in processing between the hairpins reveals more complicated schemes. This has been shown by our group in the context of the viral cluster mir-K10/12 expressed by KSHV and containing 10 miRNA precursors. The presence in *cis* of two particular stem-loops, pre-miR-K1 and pre-miR-K3, within the cluster is necessary for efficient expression of the remaining miRNAs (Vilimova et al., 2021). They are both very efficiently processed by Drosha and the deletion of any of them globally impedes the entire cluster expression (Figure 2e). Moreover, insertion of a heterologous miRNA precursor in lieu of pre-miR-K1

efficiently rescues the expression, excluding the possibility that the sequence or the downstream function of this particular miRNA is required. While these observations are in adequation with cluster assistance in smaller clusters detailed above, in this particular case, the regulation seems to have broader implication. The stimulatory effect is neither reserved only to closest neighbors, nor to suboptimally folded hairpins. What is more, the local context on the transcript and the order of hairpins is of importance given that swapping the stem-loops impacts their processing efficiency (Contrant et al., 2014). While this is not excluded also for smaller clusters, the biogenesis of larger polycistronic pri-miRNAs likely relies on a synergy of mechanisms that may combine efficient Microprocessor recruitment by optimal hairpins, structural remodeling and implication of other factors in trans, as we will discuss below.

3 | INVOLVEMENT OF *TRANS*-ACTING FACTORS

In addition to *cis* regulatory elements, external factors interacting with the primary transcript also play an important role in modulating the processing of clustered miRNAs. Selective processing of specific pre-miRNAs within a cluster may rely on the activity of various RNA-binding proteins. These can either enhance the maturation by recruiting the Microprocessor or by remodeling suboptimal secondary structure, or on the contrary, compete with the cropping machinery or mask important recognition sites on the pri-miRNA. Numerous studies were dedicated to the discovery of such co-factors involved in miRNA biogenesis resulting in a collection of proteins known to be involved in the processing of specific miRNAs or more generally modulating the function of the Microprocessor itself. As an example, the protein hnRNPA1 has been shown to selectively bind to the terminal loop of miR-18a within the mir-17~92a cluster. This induces a relaxation of the stem-loop structure and facilitates the processing of miR-18a, while the other miRNAs within the cluster remain insensitive to hnRNPA1-mediated regulation (Guil & Cáceres, 2007; Kooshapur et al., 2018; Michlewski et al., 2008). Another example comes from the studies of the 14q32/12F1 miRNA cluster, where binding of different proteins (Mef2a, HADHB, and CIRBP) to particular pre-miRNAs from the cluster seems to specifically modulate their conversion into mature miRNAs (Downie Ruiz Velasco et al., 2019; Welten et al., 2017).

Besides these co-factors, whose functions were recently reviewed elsewhere (Michlewski & Cáceres, 2019), we would like to discuss a particular group of *trans*-acting factors, which were studied specifically in the context of miRNA polycistrons. More particularly, some proteins were shown to participate in the above-mentioned mechanism of cluster assistance. The Bartel and Herzog groups hypothesized that given the fact that the cleavage of the helper hairpin is not required, the mechanism is probably related either to the recruitment of the Microprocessor or of other factors that may help in transferring its activity on the recipient hairpin. When a purified Microprocessor was incubated *in vitro* with a primary transcript containing a helper and a recipient stem-loop, the latter failed to be efficiently processed. However, the phenomenon was functional if whole cell extract was used in the assay (Fang & Bartel, 2020). This demonstrated the need of additional factors for the assistance to occur. Functional screens allowed to identify two proteins, ERH (enhancer of rudimentary homolog) and SAFB2 (scaffold attachment factor B2), whose depletion selectively impacts the processing of recipient hairpins. What is more, both proteins directly bind to the Microprocessor (Fang & Bartel, 2020; Hutter et al., 2020; Kwon et al., 2020) and can interact with each other (Drakouli et al., 2017). In addition, both proteins are known to dimerize and dimerization of SAFB2 is likely to be necessary for efficient cluster assistance (Arai et al., 2005; Hutter et al., 2020). This led to a model in which these proteins mediate association of two or more Microprocessors, thereby allowing the simultaneous recognition and cleavage of neighboring hairpins (Figure 3a).

Protein co-factors are not the only *trans*-acting regulators of polycistronic miRNA maturation. Several reports have shed light also on the implication of long noncoding (lnc)RNAs. Through partial complementarity with regions on the primary transcript they can either inhibit correct folding, mask important sequences or help reposition the RNA scaffold for better recognition and processing by the Microprocessor, similarly to snRNAs during splicing. Among these lncRNAs are the so-called Transcribed ultra-conserved regions (T-UCRs) which are perfectly conserved between mouse, rat and human genomes (Bejerano et al., 2004). Such striking evolutionary retention suggests that they may play key functions in physiological or stress conditions. Liz et al. have shown that the T-UCR uc.283+A can directly bind to a complementary sequence in the lower stem of the miR-195 hairpin and selectively impede its recognition by the Microprocessor while the expression of the second miRNA within the cluster, miR-497, is not impacted (Liz et al., 2014). Interestingly, two other T-UCRs, uc.372 and uc.173, were shown to inhibit the processing of miR-195 (J. Guo et al., 2018; Xiao et al., 2018) (Figure 3b). Unfortunately, the expression level of miR-497 was not measured in these two studies, making it difficult to conclude whether the inhibitory effect is unique to miR-195 or impacts the entire cluster. An opposite situation was described by Soler et al., they showed that the binding of uc.160 to complementary sequences

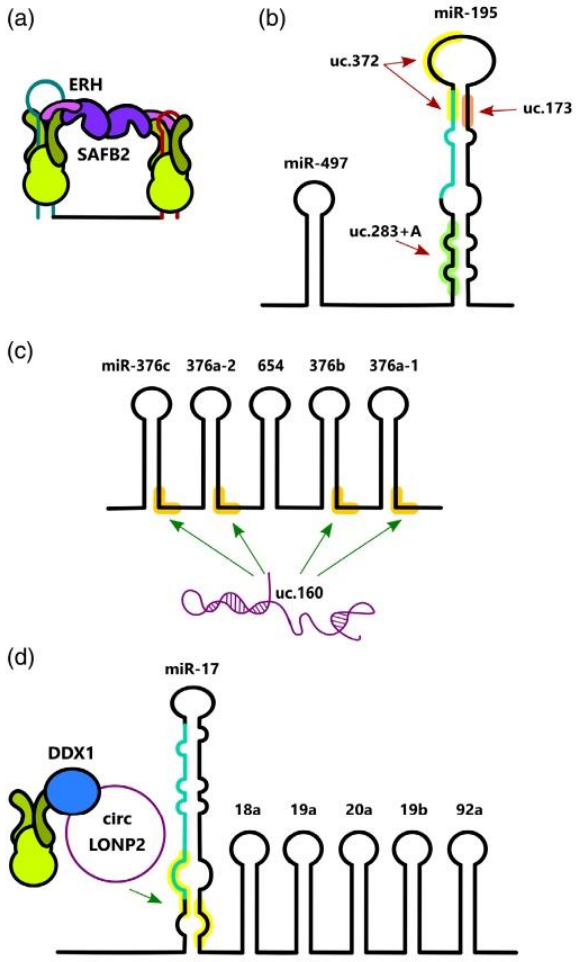

FIGURE 3 Contribution of *trans*-acting factors for miRNA processing. (a) Model of cluster assistance through Microprocessor dimerization involving ERH and SAFB2. (b,c) T-UCRs can bind to pri-miRNAs through complementary sequences highlighted in different colors. Inhibitory or stimulatory effect of this interaction on pri-miRNA processing is indicated by red and green arrows, respectively. Mature miRNA sequence is drawn in light blue. (d) circLONP2/DDX1-mediated recruitment of the Microprocessor to miR-17 hairpin

at the basal junctions of four out of the five members of the mir-376c~376a-1 cluster lead to an increased level of their processing (Soler et al., 2021) (Figure 3c). Finally, a recent paper by Han et al. reported a novel function of a circular RNA, circLONP2, in the processing of miR-17. CircLONP2 helps to recruit the Microprocessor to the miRNA hairpin by simultaneously binding to its stem and to the RNA helicase DDX1, which in turn directly binds DGCR8 (Figure 3d). This then leads to miR-17 upregulation in colorectal carcinoma (Han et al., 2020). However, how this impacts the processing of the remaining five members of the mir-17~92a cluster was not investigated.

4 | TERTIARY STRUCTURE OF THE PRIMARY TRANSCRIPT

Tridimensional folding of RNA molecules is essential for their proper function. Since the primary transcript of miRNA clusters may span over several kb, it is easily conceivable that these long RNA molecules are prone to higher-order structural arrangements and intra-molecular interactions. Thereby, a specific 3D architecture might explain differences in the processing efficiency of individual miRNA hairpins depending on their position within the molecular scaffold. This was best demonstrated for the mir-17~92a cluster, which contains six miRNA precursors within ~800 nt.

Several biochemical and imaging studies have shown that the primary transcript of mir-17~92a folds into a compact globular structure in vitro (Chakraborty et al., 2012; Chaulk et al., 2011; Du et al., 2015). It was hypothesized that a tight tertiary structure could represent a barrier for Drosha cleavage. To test this, Chakraborty et al. split the cluster in two halves and rearranged their organization. This led to a loosen RNA structure and had an impact on the overall accumulation of the processing intermediates and mature miRNAs both in vitro and in vivo (Chakraborty et al., 2012). Interestingly, all the pre-miRNAs on the shuffled cluster were better processed indicating that in such relaxed conformation, they might be more accessible to the Microprocessor. The wild-type primary transcript would thus impose structural constraints to limit its processing and auto-regulate the amounts of produced miRNAs. Submitting the primary transcript to structural probing by SHAPE also revealed that nucleotides within certain apical loops and segments between the stem-loops are solvent-inaccessible indicating that they might be implicated in tertiary-structure contacts (Chakraborty & Krishnan, 2017). What is more, phylogenic comparisons of these regions have shown that they are well conserved supporting their functional importance for the maintenance of this compact conformation (Chakraborty et al., 2012).

Similar conclusions were made by Chaulk et al., who also studied the impact of tertiary structure on mir-17~92a processing. Using single particle electron microscopy, they not only confirmed the compact folding of the primary transcript in a globular structure, but also noticed the presence of a dense core consisting of the 3′ region of the cluster (Chaulk et al., 2011). Buried within this core by the 5′ extremity of the transcript, miR-19b and miR-92a hairpins are less exposed to the Microprocessor complex. Indeed, upon deletion of the 5′ part of the pri-miRNA, the sequestrated hairpins can be processed with increased efficiency. This indicates that the core miRNAs can only be processed after the cleavage of the more exposed 5′ end which would liberate the remaining miRNA precursors. However, shortly after being released, the core domain seems to be targeted by exonucleolytic degradation limiting the timeframe during which it can be processed (Chaulk et al., 2011). Together, these mechanisms might explain the lower expression levels of the 3′ miRNAs compared to the 5′ miRNAs seen in some cell lines. Further investigation of the internal core architecture has provided evidence that miR-19b interacts with a non-miRNA stem-loop located between miR-19b and miR-92a hairpins (Figure 4a). This interaction is important to maintain the compaction of the core domain, since its disruption specifically increases the expression of miR-92a (Chaulk et al., 2014). Thus, tertiary structure-based regulation of differential expression of the individual miRNAs within the mir-17~92a cluster is achieved by limiting the accessibility of certain miRNAs.

Another model to explain the auto-inhibitory structure found in the mir-17~92a cluster was proposed by Du et al. They identified two *cis*-regulatory complementary sequences within the primary transcript that can directly bind to

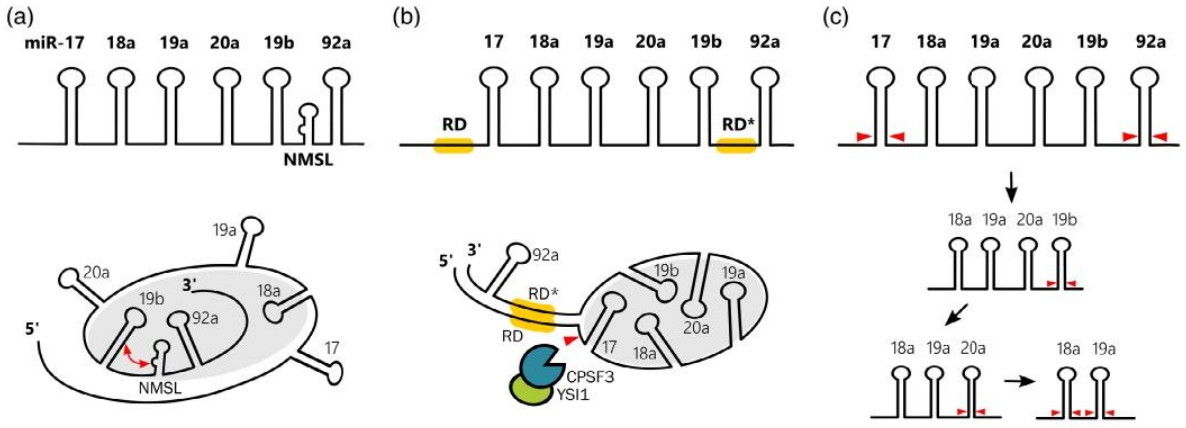

FIGURE 4 Role of the three-dimensional structure in the regulation of mir-17~92a processing. (a) miRNA stem-loops internalized within the 3′ core (highlighted in gray) are less accessible to the microprocessing machinery. Tight structure of the internal core is maintained by the interaction between the stem of pre-miR-19b and the non-miRNA stem-loop (NMSL) (Chaulk et al., 2011, 2014). (b) Binding of the repressive domain (RD) and its complementary sequence RD* within the primary transcript forms an auto-inhibitory structure which limits the processing of all the miRNAs except miR-92a. The structure is resolved upon the cleavage (red arrowhead) of the transcript by the endonuclease CPSF3 and ISY1 (Du et al., 2015). (c) Mir-17~92a progressive hierarchical biogenesis model (Donayo et al., 2019)

each other. This higher order conformation hinders the processing of the miRNAs on the cluster, except for miR-92a, which is located outside of the structure and thus remains insensitive to this steric inhibition. Interestingly, the structure imposed by the regulatory sequences is resolved by the action of the endonuclease CPSF3, assisted by the ISY1 protein, upstream of the miR-17 stem-loop. The processing intermediate termed "pro-miRNA" is then processed with higher efficiency compared to the noncleaved molecule. This results in an increase in the expression of five out of the six miRNAs within the cluster. Interestingly, there is a direct correlation between the expression of these five miRNAs and of CPSF3 and ISY1 during differentiation of mouse embryonic stem cells, the experimental model used for this study (Du et al., 2015) (Figure 4b).

The mir-17~92a cluster has been coined an "oncomiR" owing to the fact that it plays important roles in cell cycle, proliferation and apoptosis (reviewed in Olive et al., 2013). In numerous cancers of hematopoietic origin, the cluster undergoes genomic amplification leading to its aberrant expression. Donayo et al. studied the differential maturation of the individual miRNAs in the cluster in this particular oncogenic context. In contrast with the previous reports, the authors observed an accumulation of several processing intermediates indicating that there is a hierarchy in the primary transcript maturation, which occurs in a well-defined series of consecutive processing events. According to their model, the pre-miRNAs are cropped progressively from both extremities, leaving miR-18a and miR-19b as the last processed precursors, which correlates with their lower expression levels (Figure 4c).

Although they are all focused on the same miRNA polycistron, the above-presented models are not often compatible, if not contradictory. They are however consistent with the idea that the differential processing of miRNAs in a cluster is regulated structurally and does not necessarily rely on a uniform biogenesis process. The various conformational and processing patterns observed might be attributed to different experimental systems, given that the miRNAs originating from the cluster are known to present a dynamic and variable relative accumulation levels as a function of both developmental stage and tissue-type (Abasi et al., 2017). Moreover, different mechanisms controlling the maturation might be rewired between physiological and pathological conditions and most probably depend on the presence of potential co-factors that might be required to unlock a particular structural conformation.

5 | SPLICING-DEPENDENT REGULATION OF INTRONIC CLUSTERS

Like unique miRNAs, miRNA clusters can be intergenic, when their transcription is driven by their own promoter, but they can also be included into other transcriptional units. Although there are cases where miRNAs are harbored within exons or UTRs, it is more common to find them within introns of both coding and noncoding host genes (Chang et al., 2015; Kim & Kim, 2007; Rodriguez et al., 2004). Splicing is a major event in the processing of RNA Pol II transcripts and constitutes the first level of post-transcriptional regulation occurring upstream (or concomitantly) of pre-miRNA cleavage by the Microprocessor. If one host gene harbors several miRNAs, and they are not located within the same intronic or exonic unit, they cannot be considered as clusters. This is because splicing is thought to be completed during pre-mRNA synthesis and its fast dynamics makes it unlikely to find miRNAs from distant introns or exons physically linked on a single unprocessed RNA molecule. On the contrary, they are rapidly separated and follow distinct fates within their respective introns or exons. Even though long-distance interactions cannot be excluded, this does not leave much room for further regulatory mechanisms related to polycistronic organization.

However, splicing, and more particularly alternative splicing patterns can constitute another layer of miRNA regulation, if they are located within the same or in adjacent introns/exons. A detailed study of splicing patterns of the BamHI A Rightward Transcript (BART), which contains most of the miRNAs expressed by the EBV, revealed the importance of intron/exon retention for processing of the various miRNA precursors located on the long RNA molecule (Edwards et al., 2008). The 21 BART miRNA hairpins form two groups usually considered as 2 distinct clusters, lying 4.5 kb apart, but starting as one single transcript. Analysis of splice isoforms derived from this region in different EBV-infected cell lines showed that the BART miRNAs are grouped on four distinct introns (Figure 5a). Differential intron/exon retention between different cell lines correlates with variations in relative miRNA expression. This led to the hypothesis that distinct splicing patterns may account for the differential miRNA accumulation, by either increasing the efficiency of processing of some precursors or impairing the processing of others. This might be related to the assembly of spliceosomes and the splicing process itself at different splice sites. Indeed, it has been shown that the splicing machinery can interact with and have both stimulatory or inhibitory/competitive functions on the Microprocessor activity (Agranat-Tamir et al., 2014; Janas et al., 2011; Kataoka et al., 2009; Kim & Kim, 2007; Mattioli et al., 2014; Morlando et al., 2008).

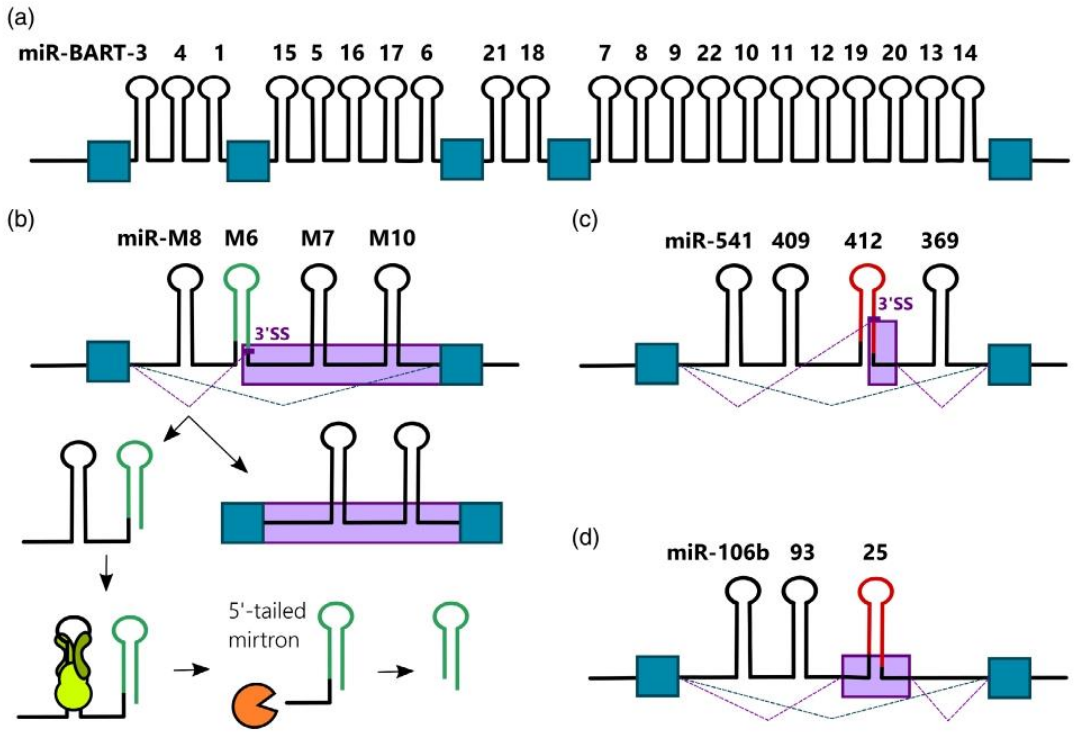

FIGURE 5 Modulation of miRNA processing by splicing. (a) Schematic view of Epstein–Barr virus transcript containing BamHI A Rightward Transcript miRNAs. Blue rectangles represent exons that may or may not be included into the spliced variants. (b) Processing of MDV-1 transcript through alternative splicing. An alternative 3′ splice site divides the cluster in two parts and induces alternative processing of pri-miR-M6 as a 5′-tailed mirtron (shown in green). Purple rectangles represent alternative exons. (c,d) Alternative splicing patterns of murine miR-451~369 and human miR-106b~25 clusters, respectively. Negatively impacted miRNAs are shown in red

Another interesting insight into the interplay between alternative splicing and pre-miRNA processing was revealed by the study of another viral miRNA cluster, mir-M8/M10 encoded by the Marek's disease herpesvirus (MDV-1). This cluster contains four miRNA precursors located within the first intron of the LAT noncoding RNA. Rasschaert et al. have discovered an alternative 3′ splice site, which splits the cluster in two parts (Rasschaert et al., 2016). While the first two miR-M8 and -M6 remain intronic, the two other miR-M7 and -M10 become a part of the following exon. Interestingly, upon this splicing event, miR-M6, which is located just upstream of the novel splice site is not processed anymore by the Microprocessor. Instead, it is processed as a 5′ tailed mirtron, whose 3′ end is directly defined by splicing and 5′ end is released by Drosha cleavage of the flanking pre-miRNA and the action of an unknown exoribonuclease. Mutation of the alternative splice site negatively impacts miR-M6 expression indicating that this event is required for its optimal expression. On the contrary, the alternative splicing itself is limited by the presence of miR-M7 stem-loop, which may help to balance the switch between the two processing pathways (Figure 5b). Regarding the alternative exon that is generated, it could serve two purposes. It can either cleave by the Microprocessor to give rise to pre-miR-M7 and -M10 or it can serve as a functional noncoding gene product and increase viral transcriptome diversity.

Mammalian genes also undergo alternative splicing at loci containing miRNA clusters. One interesting example is the mouse *Mirg* gene, which gives rise to a long noncoding RNA enriched in brain tissue. Melamed et al. found that the splicing pattern of this RNA varies during development, leading to progressive inclusion of an alternative exon in adult brain, as compared to embryonic brain tissue (Melamed et al., 2013). This exon is found inside an intron containing four miRNA precursors, miR-541, miR-409, miR-412, and miR-369. Interestingly, miR-412 hairpin overlaps with the alternative 3′ splice site so that the alternative splicing and miR-412 processing are mutually exclusive. Therefore, this particular splicing event not only splits the miRNA cluster apart (which does not seem to impact the expression of the remaining miRNAs), but it specifically hinders the processing of miR-412 (Melamed et al., 2013) (Figure 5c).

TABLE 1 Experimentally validated post-transcriptional regulatory mechanisms in miRNA polycistrons

Cluster	Regulatory mechanism	References
Hsa-mir-15a~16-1	Cluster assistance, miR-16-1 assists miR-15a processing, with the involvement of SAFB2 and ERH proteins.	Hutter et al. (2020)
Hsa/mmu-mir-17~92a	Intramolecular contacts and higher order inhibitory structure allow hierarchical differential processing of individual pri-miRNAs, various *trans*-acting factors are involved, that is, hnRNP A1 regulating pre-miR-18a cleavage	Chaulk et al. (2011), Chaulk et al. (2014), Chakraborty and Krishnan (2017), Donayo et al. (2019), Du et al. (2015), Guil and Cáceres (2007), Han et al. (2020), Kooshapur et al. (2018), Michlewski et al. (2008)
Hsa-mir-23~24–1	Uncoupling of miR-24-1 from the rest of the cluster through alternative splicing	Ramalingam et al. (2014)
Hsa-mir-106b~25	Uncoupling of miR-25 from the rest of the cluster through alternative splicing.	Ramalingam et al. (2014), Agranat-Tamir et al. (2014)
Hsa-mir-144~451	Cluster assistance, miR-144 assists miR-451 processing, with the involvement of ERH protein.	Fang and Bartel (2020), Kwon et al. (2020), and Shang et al. (2020)
Hsa-mir-181a-1~181b-1	Cluster assistance, miR-181a-1 assists miR-181b-1 processing, with the involvement of SAFB2 protein.	Fang and Bartel (2020) and Hutter et al. (2020)
Hsa-mir-191~425	Cluster assistance, miR-191 assists miR-425 processing, with the involvement of SAFB2 protein.	Hutter et al. (2020)
Hsa-mir-374a~545	Cluster assistance, miR-545 assists miR-374a processing	Shang et al. (2020)
Hsa-mir-374b~421	Cluster assistance, miR-421 assists miR-374b processing	Fang and Bartel (2020) and Shang et al. (2020)
Hsa-mir-376c~376a-1	*Trans*-acting noncoding RNA	Soler et al. (2021)
Hsa/mmu-mir-497~195a	Cluster assistance, miR-497 assists miR-195a processing. *Trans*-acting noncoding RNAs	Guo et al. (2018), Lataniotis et al. (2017), Liz et al. (2014) Xiao et al. (2018)
Mmu-mir-541~369	alternative splicing abolishes expression of miR-412 overlapping an alternative splice site	Melamed et al. (2013)
Dme-mir-11~988	Cluster assistance, expression of miR-11 is fine-tuned by the presence of miR-988	Truscott et al. (2016)
Dme-mir-100~125	Cluster assistance, miR-125 requires the presence of at least one of the remaining pri-miRNAs	Truscott et al. (2016)
Ebv-mir-BARTs	Distinct splicing patterns between miRNA containing introns	Edwards et al. (2008)
Ebv-mir-BHRF1-2~BHRF1-3	Cluster assistance, expression of miR-BHRF1-3 is fine-tuned by the proximity of miR-BHRF1-2 and a poly-A site	Haar et al. (2016)
Kshv-mir-K10/12	miR-K1 and miR-K3 required for the processing of the remaining pre-miRNAs	Vilimova et al. (2021)
Mdv1-mir-M8~M10	Alternative splicing splits the cluster in two parts, M-6 becomes a 5′ tailed mirtron	Rasschaert et al. (2016)

A slightly different situation has been described in the case of two other intronic clusters, mir-23b~24-1 and mir-106b~25. Both clusters contain three miRNAs each and alternative splice sites in between them can give rise to bicistronic subclusters, while the remaining miRNA finds itself into an exon (Agranat-Tamir et al., 2014; Ramalingam et al., 2014). At least in the case of miR-25, its exonic location negatively impacts its expression (Agranat-Tamir

et al., 2014) (Figure 5d). In addition, these unique splice variants were observed in response to particular environmental stimuli such as hypoxia and they seem to originate from distinct transcripts, shorter than the entire host-gene and starting from an independent transcription start site (Ramalingam et al., 2014). The fact that these splice isoforms do not belong to the host gene isoforms shows that they may be produced exclusively to uncouple the cluster miRNA expression. This would therefore allow to keep the expression of some miRNAs, while preventing the expression of others.

Thus, alternative splicing patterns can differentially affect the expression of polycistronic miRNAs, either by intron/exon selection or crosstalk between the splicing and the Microprocessor machineries.

6 | CONCLUSION

The propensity of miRNA genes for clustering in operon-like structures has been known since the first miRNAs were discovered in animal genomes. However, we only start to understand that the functional significance of such polycistrons not only rely in their synchronized expression, but also in the emergence of previously unrecognized regulation modes. As we have seen above, various post-transcriptional molecular mechanisms can explain the differential accumulation of polycistronic miRNAs. Both *cis*-acting sequences or structural elements and *trans*-acting factors, such as RNA-binding proteins and noncoding RNAs, can recruit the Microprocessor complex, regulate the access of the miRNA processing machinery or optimize the processing of some pre-miRNAs. In addition, particular splicing patterns may be utilized to uncouple the processing of the precursors, thus adapting to conditions requiring changes in abundance of the different mature miRNAs. Table 1 provides a summary of miRNA clusters for which the mechanism at play in their post-transcriptional regulation was experimentally validated. Post-transcriptional regulation of miRNA clusters presents new challenges and avenues of research concerning miRNAs and indicates that we should consider them as important entities that are not only here to carry individual regulatory units. We also need to reconsider what defines a miRNA cluster per se. They are frequently defined based on genomic proximity and occasionally inferred from expression data clustering together miRNAs from a genomic region being co-expressed under certain conditions (Chaulk et al., 2016). However, as we have just seen, co-expression is not necessarily an imperative. What is more, identification of genomic miRNA clusters strongly depends on the selected threshold distance separating two miRNA loci. As an illustration, estimating the number of miRNA clusters within the human genome resulted in 99 distinct clusters encompassing 352 miRNA genes if the threshold used was 10 kb (Wang, Luo, et al., 2016), and 153 clusters containing 468 miRNAs when the cut-off was shifted to 50 kb (Kabekkodu et al., 2018). This criterium is quite arbitrary and does not provide information about the true polycistronic nature of the presumed miRNA clusters. It is indeed more challenging to evaluate the exact number of genuine polycistrons unless their primary transcripts are thoroughly characterized. Some efforts have been made to predict the promoter regions and transcription start sites (TSSs) or poly(A) signals for individual miRNA genes, which can be helpful for defining whether neighboring miRNA loci are or are not transcribed together as polycistrons (Chang et al., 2015; Chien et al., 2011; de Rie et al., 2017; Perdikopanis et al., 2021; Rodriguez et al., 2004; Saini et al., 2007; Yu et al., 2006). However, experimental validation is still rather scarce and concerns only a handful of well-expressed and studied clusters (Chaulk et al., 2016). The task may prove even more challenging if we take into account the cell-type and context-dependent expression and the fact that independent TSSs can give rise to subclusters under certain circumstances (Nayak et al., 2018; Ozsolak et al., 2008). Needless to say, future studies should explicitly differentiate between the two notions of genomic and polycistronic miRNA clusters, and deal with them carefully with respect to the scope of their research. Along the same line, a large majority of studies focusing on miRNA function and biogenesis still focus on single isolated miRNAs or their precursors, which brings about questions regarding the fate of their neighbors if they are located within a cluster. Knock-out experiments aiming to delineate the function of a particular miRNA may lead to skewed conclusions if the context of cluster is not properly addressed. Given the novel paradigms, some of the published results may need to be revised accordingly.

On another note, understanding the regulation at play within miRNA clusters opens new possibilities for manipulation and adaptation of novel genetic tools. As such, the design of new strategies for therapeutic intervention is particularly appealing. For example, one may take advantage of a well-designed single molecule targeting the primary transcript to impact the expression of several miRNAs at once. An RNA aptamer and an antisense oligonucleotide were proposed to inhibit the expression of the entire mir-17~92a and KSHV mir-K10/12 clusters, both involved in pathology (Lünse et al., 2010; Subramanian et al., 2015; Vilimova et al., 2021). Alternatively, the construction of artificial clusters expressing several therapeutical or synthetic miRNAs, each at a different level, could help to broadly modulate

physiological or pathological signaling (Wang, Xie, et al., 2016; Yang et al., 2013). However, these approaches are still in their infancy and will require more work before they can be applied to real-life settings. Our knowledge of miRNA cluster regulation is still incomplete and fragmented. It is therefore problematic to define common or distinct features for each type of regulation and it is impossible to extrapolate the already available information to other clusters. To further complicate the picture, the regulation might be not only cluster- but also context-dependent, intertwined with the activity of specific factors and regulatory networks, that might be cell line/tissue-specific or rewired in diseased or stress conditions. In addition, while it is determinant, processing of the primary transcript is not the only level at which miRNAs are regulated. Indeed, the mature miRNA abundance results from a balance between efficient production and pre- and mature miRNA decay. Ultimately, all these aspects will have to be considered, if we want our knowledge of the regulation mechanisms during the processing of natural miRNA clusters to be translated one day into successful applications.

AUTHOR CONTRIBUTIONS

Monika Vilimova: Conceptualization (equal); funding acquisition (supporting); writing – original draft (lead); writing – review and editing (equal). **Sébastien Pfeffer:** Conceptualization (supporting); funding acquisition (lead); project administration (lead); supervision (lead); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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5. miRNAs and viruses

Viruses are known to take advantage of any cellular mechanisms which can enhance their survival and replication cycle. They not only hijack the fundamental gene expression machinery, but also tap into other metabolic and regulatory functions. They can make use of specific viral regulators, mimic the ones produced by the cell or simply steel cellular genes. The older the co-evolution between the host and the virus, the more sophisticated are these viral maneuvers. Given the fundamental function of miRNAs in cell homeostasis and gene regulation, it is not surprising that viruses have evolved to exploit them as well.

One way a virus can takes advantage of the miRNA machinery is by diverting the function of miRNAs present within the cell or induce the expression of others because they can directly or indirectly optimize infection conditions. On the contrary, some viruses specifically eliminate some miRNAs or inhibit the entire miRNA pathway if this is deleterious to their replication (Cullen, 2011; Girardi et al., 2018; Piedade and Azevedo-Pereira, 2016). But the most striking adaptation to miRNA machinery is by viruses that have evolved to encode their own set of miRNAs. The advantages of viral miRNAs are multifold. They represent a powerful tool to establish favorable cellular environment by reshaping the pattern of viral and host gene expression. At the same time, they are non-immunogenic and do not occupy much space in the crowded viral genomes.

First identified in Epstein Barr virus (EBV) (Pfeffer et al., 2004), numerous viral miRNAs have been listed so far. Starting from one single miRNA encoded by certain polyomaviruses (Seo et al., 2008; Sullivan et al., 2005), the larger genomes of herpesviruses allow to accommodate dozens of them. For example, EBV encodes 25 miRNA precursors which give rise to 44 different miRNAs (Pfeffer et al., 2004; Walz et al., 2010). It is generally agreed that DNA viruses have a higher propensity to express viral miRNAs. Since they essentially replicate in the nucleus and make use of the host transcription, viral pri-miRNAs are naturally accessible and recognized by the cellular miRNA biogenesis machinery. Consequently, the expression of these viral miRNAs is entirely performed by the host apparatus and usually follows the canonical biogenesis pathway. On the contrary, it is generally believed that RNA viruses are devoid of miRNA genes. To date, one retrovirus, the bovine leukemia virus (BLV), was shown to express *bona fide* miRNAs (Kincaid et al., 2012) and several other

RNA viruses, such as SARS-Cov-2 (Pawlica et al., 2021), were reported to encode putative miRNAs. Yet most of such reports rely on deep sequencing and computational prediction, missing further experimental evidence (reviewed by (Nanbo et al., 2021)). The controversy around RNA virus-expressed miRNAs is also based in the RNA virus life cycle, which is usually accomplished within the cytoplasm, thereby exposing genomic RNA to RISC complexes. Thus, perfectly complementary mature viral miRNAs would risk to target the RNA they originate from. In addition, excision of a miRNA precursor from the viral genome or antigenome would lead to their destabilization (Cullen, 2011; Grundhoff and Sullivan, 2011).

5.1 Herpesviruses and miRNAs

Herpesviruses can be considered as champions in miRNA utilization. The long-term co-evolution with their host allowed them to master even subtle regulatory mechanisms present within the cell and manipulate them in a highly refined fashion (Pellett and Roizman, 2013). Because miRNAs provide access to the host gene expression networks, but also control viral gene expression, they are well tailored to modulate host-pathogen interactions, especially for a virus aiming at lifelong persistence. Indeed, miRNAs seem to be particularly useful for the hallmark of herpesvirus infection, the latent cycle (Grundhoff and Sullivan, 2011). With the exception of Varicella-zoster-virus (VZV) and the roseolovirus HHV-7, all human herpesviruses encode miRNAs. However, there is no evolutionary conservation between their sequences (Walz et al., 2010). This indicates that each member of this family has evolved its own miRNAs by adaptation to its specific infectious context and viral life cycle.

6. Herpesviruses

To date, there are about 200 different viruses in the *Herpesviridae* family. They are enveloped double-stranded DNA viruses. It was estimated that they arose 180-220 million years ago (McGeoch et al., 1995) and they belong to the most complex viruses in terms of gene diversity. Their large genomes (125-240 kbp) code for approximately 70-170 viral proteins (Davison, 2007). Moreover, their genomic output is increased by the presence of alternative promoters and splicing events as well as expression of various non-coding RNAs.

6.1 Classification

The viruses of the family *Herpesviridae* infect three classes of vertebrates: birds, reptiles and mammals (Davison et al., 2009). However, they usually present a restricted host range, limited to one single species and nine of them infect humans (Pellett and Roizman, 2013). These are herpes simplex type 1 (HSV-1), herpes simplex type 2 (HSV-2), varicella zoster virus, cytomegalovirus (CMV), three roseoloviruses (HHV-6A, HHV-6B and HHV-7), Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV).

Herpesviridae have been further classified into three subfamilies, based on genomic data and their biological properties among which the most evident is the preference for each subfamily to establish a long-term latency in a certain cell lineage (Davison, 2007; Pellett and Roizman, 2013). HSV-1, HSV-2, VZV belong to *Alphaherpesvirinae* and are characterized by latent residency in neurons. The other two subfamilies prefer cells of haematopoietic origin. *Betaherpesvirinae* (CMV, HHV-6A, HHV-6B and HHV-7) typically target monocytes, macrophages and T lymphocytes, while *Gammaherpesvirinae* (EBV, KSHV) tend to reside in B lymphocytes. However, it should be noted that most of the viruses can infect various cell types and probably persist also in secondary target cell reservoirs. Moreover, cells of epithelial lineages are also highly susceptible to herpesvirus infection. In some cases, such as for HSV or EBV, massive replication in these cells is required for efficient host-to-host dissemination. As to the other herpesviruses, it is plausible that at some point, they all replicate in epithelia, especially because oral and genital mucosa constitute the main transmission routes for all of them (Grinde, 2013).

6.2 Clinical importance in humans

Altogether, the nine human herpesviruses achieve outstanding global infection rate with virtually 100% of adult individuals carrying at least one of them (Grinde, 2013). Several reasons may explain this exceptional success in host occupancy. First, the vast majority of infections occur during early childhood and are asymptomatic, or accompanied by only mild clinical manifestations, included during high viral activity (Grinde, 2013). This reflects the perfect adaptation to the host cell exploitation without eliciting strong immune response (Griffin et al., 2010; Pellett and Roizman, 2013). Secondly, the virus can persist within host cells for extended periods without being deleterious to host cells and with almost no harm to the carrier. Consequently, the virus can colonize specific body compartments where it remains for the rest

of the host's life. These reservoirs are characterized by low viral activity restricted to the expression of only a handful of viral latent genes. A "dormant" state is thus maintained in balance with the host immune system that becomes unable to completely eradicate the virus (Griffin et al., 2010). Third, this particular survival strategy relies on the ability to switch back to active phase and cycling between latency and lytic replication (Pellett and Roizman, 2013; Yan et al., 2019). Following specific environmental and intracellular cues, it can be induced into periodic "awakening" resulting in progeny virus production. Yet the reactivation is also usually completely indolent since many of the herpesviruses have been shown to asymptomatically shed through oropharyngeal or genital mucosa. Finally, relatively high frequency of such reactivations is the main factor for human-to-human dissemination (Damania and Cesarman, 2013; Grinde, 2013).

However, this view of a benign parasite maintaining itself within the host organism does not correlate with the actual clinical importance of herpesviruses. This is because the uncorrupted immune status is veritably crucial for such smoothly running host-pathogen relationship. Hence any hurdle to the capacity of the immune system to react appropriately is susceptible to lead to a failure in terms of control of viral replication or latency (Della Chiesa et al., 2019; Grinde, 2013; Münz, 2019). In reality, the quality of immune surveillance is often submitted to various internal or external stresses that may lead to clinical complications and disease. Among such stress factors, there are innate and acquired immunosuppression, but also hormonal changes, physiologic and emotional stress, exposure to sunlight and ageing (Yan et al., 2019). These triggers promote reactivation and uncontrolled replication accompanied by a spectrum of clinical symptoms, from cutaneous lesions to severe neurologic sequelae and post-transplantation complications such as host-to-graft disease. Not only the lytic, but also latent infection bears a risk of pathologic outcomes, in particular oncogenesis, which can result from long-term infection with EBV and KSHV (Hatano et al., 2021; Münz, 2019).

7. Kaposi's Sarcoma Associated Herpesvirus

Kaposi Sarcoma Associated Herpesvirus (KSHV) known also as human herpesvirus 8 (HHV-8) was the last human herpesvirus to be discovered in 1994 by Yuang Chang and Patrick Moore (Chang et al., 1994). It infects mostly endothelial and B-cells, where it can establish latency and causes pathology. Sporadically, it can be found in monocytes, lymphoid organs,

bone marrow and epithelial cells. The inter-individual transmission occurs through saliva, most frequently in early childhood and from mother-to-child. In non-endemic regions, it is frequently transmitted sexually and through organ transplantation (Damania and Cesarman, 2013; Yan et al., 2019).

Compared to other human herpesviruses, KSHV is not ubiquitous and high prevalence rates are geographically limited to Sub-Saharan Africa where the virus is endemic. In this area, 50% of the adult population carry KSHV. In contrast, the virus was originally rare in Asia, Europe and North America. The only exception are Mediterranean regions which are also considered endemic, reaching an infection rate of 35%. However, the spread of KSHV was greatly enhanced by the outbreak and expansion of the HIV pandemic since HIV co-infection is a risk factor for infection as well as development of KSHV-related pathology. Consequently, current prevalence in North America and the rest of Europe has been estimated to ~10% (Wong and Damania, 2017; Yan et al., 2019).

7.1 KSHV-associated diseases

Whereas primo-infection with KSHV is completely asymptomatic, at least four pathologic conditions are associated to its long-term latent persistence and reactivation from latency. KSHV belongs to human oncoviruses with etiology well recognized in the Kaposi's sarcoma and the Primary Effusion Lymphoma (PEL). KSHV is also involved in another lymphoproliferative disorder called Multicentric Castelman's disease (MCD) and more recently, it has been linked to the KSHV inflammatory cytokine syndrome (KICS) (Goncalves et al., 2017).

7.1.1 Kaposi's sarcoma (KS)

A viral etiology of the skin cancer described by Moritz Kaposi (1872) was first suggested in the 1970s when herpes-like viral particles were observed in cell cultures derived from diseased tissues (Giraldo et al., 1972). It was however only in 1994 that this novel human herpesvirus was isolated from KS lesions and characterized (Chang et al., 1994). The transforming potential in KSHV is now an established fact since almost 100% of KS cells contain KSHV genome. The malignancy starts in cells of lymphatic endothelial origin. A typical mark of the transformed cells observed in histologic preparations of KS lesions is their elongated shape, which earned them the name spindle cells. Spindle cells contain latent KSHV episomes (Dupin et al., 1999; Zhong et al., 1996). A small percentage of cells (1-5%) however

undergo spontaneous lytic reactivation, which has been shown to be important for initiation and progression of KS. Secretion of pro-inflammatory and pro-angiogenic factors by lytic cells is indeed paramount for KS development since KS tumors require sustained paracrine stimulation. In addition, the tumors are maintained through continuous infection and transformation of neighboring cells. This makes KS different from classical tumors formed by clonal proliferation. Another characteristic feature is the highly heterogenous character of the cell populations forming the tumor mass. Next to the KSHV-infected spindle cells, the lesions are richly vascularized and infiltrated with immune cells (Damania and Cesarman, 2013; Dupin et al., 1999).

Initial sites of cancer proliferation are located within the dermis and in proximal lymphatic nodes. In more advanced stages, visceral lesions appear in the respiratory and gastrointestinal tract. There are several clinical sub-types of KS depending on variable patient populations (in terms of age or immune status) and varying in severity of clinical manifestations (Antman and Chang, 2000). The "classic" variant affects mostly elderly male patients in the Mediterranean region and can remain relatively innocuous and limited to slowly progressive skin lesions appearing mostly on limbs. African areas with high KSHV circulation struggle with the "endemic" variant of KS, which occurs in adults and children, is more aggressive and represents the most common cancer in males (Cesarman et al., 2019). What is more, with the onset and alarming advance of HIV epidemic, pediatric Kaposi Sarcoma starts to become a matter of great concern in several African countries where it became the most common childhood cancer (El-Mallawany et al., 2018). In fact, co-infection with other viruses such as HIV (and recently SARS-Cov2 (Chen et al., 2021)) favors KSHV reactivation thereby increasing the risk of KS development. With the progression of the HIV pandemic, a category on its own has been associated to HIV-immunocompromised cases. This so-called "epidemic" variant is indeed the most frequent cancer in HIV patients and represents one of the main manifestations accompanying the onset of AIDS. Indeed, 50% of KSHV infected AIDS patients develop KS if not treated with HAART (highly active antiretroviral therapy) (Martin et al., 1998). The last clinical category of KS is the "iatrogenic" variant, which occurs in immunodeficient patients and can be induced by immunosuppressive post-transplantation treatments. With the exception of HIV seropositive and therapeutically immunosuppressed patients, the development of KS in KSHV carriers remains rather low (<1/100 000 person-years in the US and >1 /1 000 person-years in endemic zones) (Cesarman et al., 2019). Similarly to other cancers induced by oncoviruses, KSHV infection is not sufficient for KS tumorigenesis.

This is a multifactorial process, which requires an accumulation of multiple cell-disturbing events leading to transformation.

Current treatments of KS involve mostly chemotherapy with the use of cytostatics and cytotoxic molecules (vinblastin, bleomycin, doxorubicin) as well as anti-angiogenics (paclitaxel). Since KSHV replication maintains the cancerous proliferation, drugs blocking viral replication such as ganciclovir and foscarnet have been successfully used. In HIV patients, HAART therapy has been shown to be efficient in both prevention and treatment (Cesarman et al., 2019). In post-transplant patients, withdrawal or reduction of the immunosuppressive drugs also leads to KS regression, however at the risk of loss of the graft (Antman and Chang, 2000; Goncalves et al., 2017). However, despite the treatment options available to date, the disease tends to recur. In one study involving 129 patients, only 30% were disease-free 10 years post primary treatment (Brenner et al., 1999). The fact remains that a specific and efficient treatment for KS is still missing (Cesarman et al., 2019).

7.1.2 Primary Effusion Lymphoma (PEL)

PEL is a rare and aggressive form of non-Hodgkin lymphoma with a poor outcome (Goncalves et al., 2017). It derives from transformation and clonal proliferation of post-germinal B-cells, blocked at a late stage of differentiation towards antibody-secreting plasma cells. All malignant cells are KSHV positive and retain numerous copies of KSHV genome (20-200 episomes/cell). For the most part, KSHV remains latent and undergo lytic replication only in small number of infected cells (1-2%) (Miller et al., 2007).

PEL usually develops in the pleural space in form of an effusion in body cavities (pleural, peritoneal, pericardial) without a solid tumor mass. In some cases, diseased cells can also spread to lymph nodes, lungs and gastro-intestinal tract where limited solid-tumor formation has been demonstrated (Dupin et al., 1999).

Most PEL cases arise in elderly and immunocompromised patients, i.e. HIV seropositive individuals. About ~80% of PEL tumors are also EBV positive and EBV co-infection has been shown to enhance KSHV-induced tumorigenesis, indicating that EBV co-infection is an important risk factor for PEL development (Cesarman et al., 1995; McHugh et al., 2017). The disease is rapidly fatal and current chemotherapeutic regimes are not specific and not efficient, yielding only 30-40% 2-year survival rates (Andrei and Snoeck, 2015; Goncalves et al., 2017).

7.1.3 Multicentric Castelman Disease (MCD) and KICS

MCD and KICS are inflammatory conditions directly or indirectly caused by high viral reactivation and in the case of MCD linked with neoplastic proliferation. Both diseases are associated with deregulated production of inflammatory cytokines IL-6 and IL-10, in addition to KSHV-encoded vIL-6. Aberrant increase in these cytokines leads to a range of non-specific symptoms such as fever, night sweats, weight loss, nausea, rash and fatigue and they are accompanied with increased viral loads as a result of actively replicating KSHV (Polizzotto et al., 2013; Uldrick et al., 2010). MCD is also characterized by expansion of germinal center B-lymphocytes stimulated by the cytokine excess, which affect lymph nodes, spleen and liver. While the clinical manifestations are mild in some people, they can be life-threatening in others, especially in patients with PEL or KS. In these cases, MCD and KICS flares contribute to poor prognosis and higher mortality rates. Similarly to KS and PEL, the prominent risk factor for both MCD and KICS is immunosuppression and the overall prevalence is higher in HIV-positive patients (Goncalves et al., 2017).

7.2 KSHV architecture and genomic structure

Infectious KSHV virions measure approximately 150 nm and structurally, they are composed of a lipid bilayer envelope, amorphous tegument layer and a capsid protecting the genome. The capsid exhibits an icosahedric architecture and is formed essentially by the major capsid protein (MCP) (Damania and Cesarman, 2013; Renne et al., 1996). KSHV genome consists of a double-stranded linear DNA molecule of about 165 kbp in length. The coding region of the genome encompases approximately 145 kbp and is flanked by 30-45 tandemly arranged CG-rich terminal repeats allowing its circularization in infected nucleus. There are 87 KSHV open reading frames (ORFs) (Russo et al., 1996). Most of them are annotated according to homology with genes previously identified in close homologs such as the monkey Herpesvirus saimiri (HVS). In addition to these conserved genes, the virus encodes a unique set of genes specific for KSHV, which are designated with a prefix K (K1-K15). Another group of genes represent viral homologs of host genes, for example v-IL6 (viral interleukin 6) or v-Cyc (viral cyclin). In addition, several regulatory RNAs such as long-non-coding RNAs, circular RNAs (circRNAs) and miRNAs complete KSHV gene repertoire (Borah et al., 2011; Damania and Cesarman, 2013; Russo et al., 1996; Tagawa et al., 2018).

7.3 KSHV life cycle

Infection is initiated upon interaction between the glycoproteins on viral envelope and heparan sulfates at the surface of target cells (Figure 5). The involvement of other receptors such as integrins, DC-SIGN and CD98 has been also reported (Chandran, 2010). The entry into host-cell occurs through endocytosis. Upon fusion of the viral envelope with the endosomal membrane, the tegument layer and the nucleocapsid are released into the cytoplasm. The tegument contains proteins and several mRNAs which prime cell environment and facilitate the early events of infection. The nucleocapsid is transported along microtubule network towards the nucleus where it docks on a nuclear pore. The viral genome is then released into the nucleoplasm where it circularizes. Immediately, the circular viral episome is assembled into nucleosomes and behaves like host chromosomal DNA, recruiting host transcriptional machinery and regulatory epigenetic marks. It does not integrate into host genome, but remains independent, though attached to host chromosomes. As already mentioned above, KHSV can adopt two different infectious modes, lytic replication and latency, which both require distinct molecular behavior. (Damania and Cesarman, 2013). Upon primo-infection, the default gene expression program leads towards lytic replication, which allows an initial KSHV establishment, increase of genome copy number and spread within the target cell populations (Dittmer et al., 1999). However, most of the infected cells quickly exhibit also latent gene expression indicating that the decision to enter lytic or latent cycle is made during the early stages of infection. Finally, latent expression program remains predominant in nearly all infected cells (Damania and Cesarman, 2013; Yan et al., 2019).

7.3.1 Lytic phase

The course of lytic replication follows a particular gene expression pattern, which consists of the progressive expression of 3 subsets of viral genes: immediate early, early and late. Immediate early genes are transcription factors and regulators of viral gene expression, i.e. the replication transactivator RTA (ORF50). Expression of RTA is the only pre-requisite for lytic program initiation, whether it is during primo-infection or latent-to-lytic switch. RTA acts as transcription factor binding to the promoters of further immediate early and early genes thus triggering the temporal gene expression cascade. RTA (and some other early genes) is also present in mature virions so that the replicative cycle can be initiated directly upon entry. Among the early gene products are proteins involved in viral DNA replication and nucleic acid metabolism to provide the necessary nucleotide pools for intensive genome synthesis. There

are also modulators of cellular functions such as host transcription and immune evasion. Finally, late genes complete the cycle by producing structural proteins. The encapsidation takes place within the nucleus and the capsid then migrates through nuclear envelope and endoplasmic reticulum progressively acquiring tegument and envelope layers. Mature viral particles are then released through exocytosis. Massive viral egress ultimately leads to cell destruction (Aneja and Yuan, 2017; Damania and Cesarman, 2013; Yan et al., 2019).

7.3.2 Latent phase

In contrast to the lytic replication, which mobilizes the entire viral gene expression, during latency, viral gene activity is extremely limited (Renne et al., 1996). Hence it can be considered as the ultimate form of immune evasion avoiding exposure of immunogenic viral proteins. Most of the viral episome is heavily methylated and is enriched with repressive epigenetic marks, i.e. H3K27-me3 (Fröhlich and Grundhoff, 2020). The transcriptional activity focuses on the major latency locus encoding a handful of specific latent genes (Dittmer et al., 1998). Their function is centered on what allows the virus to hide within the cell. This involves counteracting immune surveillance, extending the host cell life span and finally, maintaining the latent state by modulating cell signaling pathways or directly repressing viral gene expression. For instance the multifunctional Large nuclear antigen (LANA/ORF73) acts through several mechanisms to silence lytic genes and maintain the latency (Uppal et al., 2014). First, it recruits cellular epigenetic regulators, such as DNMT3a and the polycomb repressive complex 2 (PRC2), to the promoters of lytic genes. Second, it acts as a transcriptional repressor directly binding to the promoter of RTA in order to avoid inappropriate lytic reactivation. LANA's other functions involve inhibition of pro-apoptotic cues, and increase of the infected cell life span through binding p53 and Rb proteins. During the latency phase, the virus replicates its genome simultaneously to the host cell using host DNA polymerase. LANA also plays a role in episome replication through binding to latent origin of replication. Finally, LANA anchors viral genomes to host chromosomes thereby helping their effective segregation between daughter cells during mitosis. In addition to LANA, other latent genes include two viral homologs of apoptosis and cell cycle modulators, vFLIP (FLICE inhibitory protein) and viral cyclin (v-cyc). Finally, latent gene expression involves at least three oncogenic and anti-differentiation Kaposin polypeptides as well as viral miRNAs (Damania and Cesarman, 2013; Yan et al., 2019).

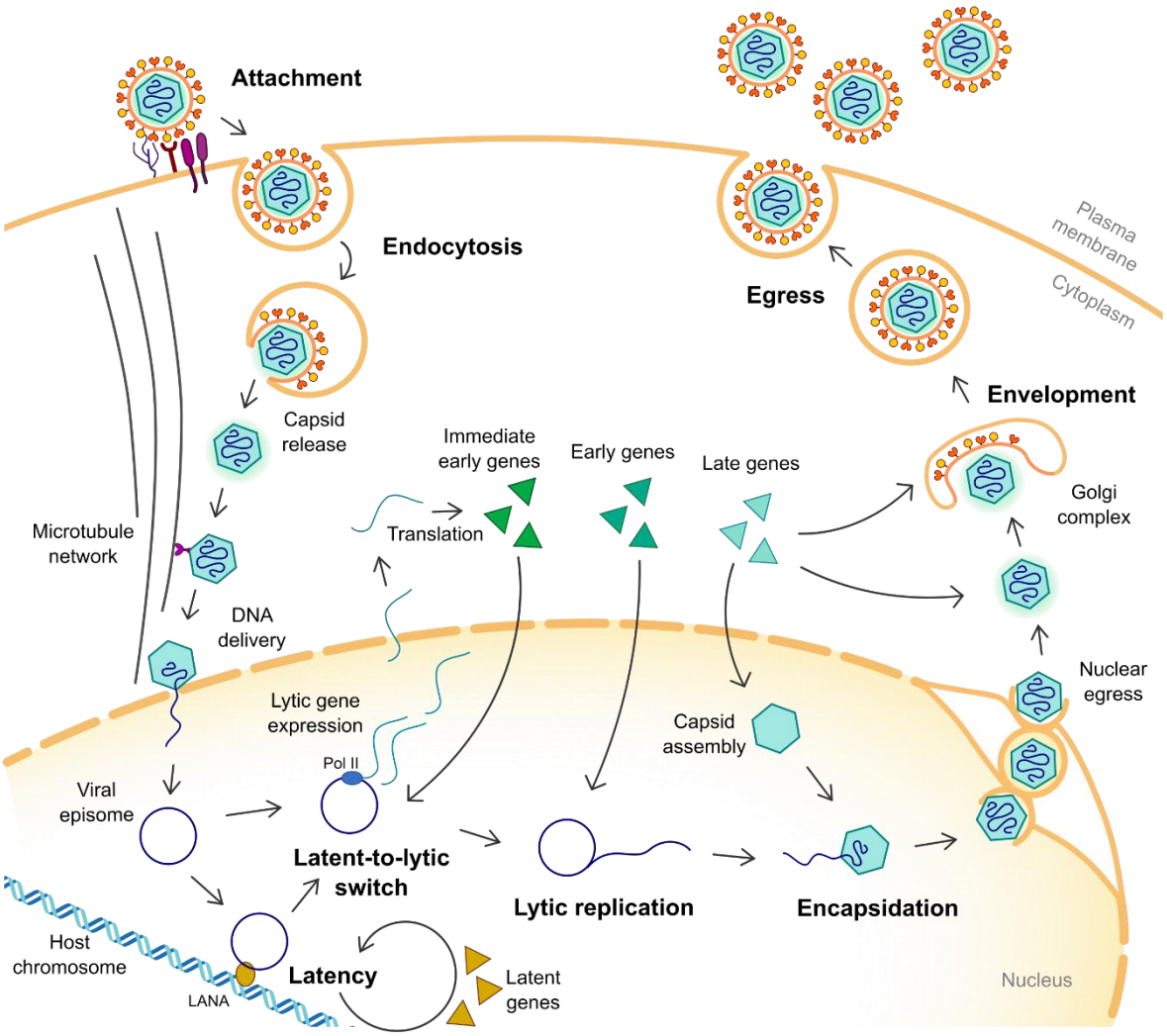

Figure 5: KSHV life cycle. Virion attachment to cellular receptors and heparan sulfates triggers the endocytosis allowing virus entry into the host cell. Following the fusion of the endosome and viral envelope, the capsid is released and can interact with dynein motor proteins that carry it towards the nucleus. Viral DNA is injected through the nuclear pore and circularizes in the nucleoplasm. If lytic phase is initiated, viral lytic genes are expressed in a temporal cascade involving immediate early genes, early genes and late genes. These allow to initiate and regulate viral DNA replication (rolling circle mechanism) and the generation of progeny virions. Nucleocapsids are assembled in the nucleoplasm, then migrate through the nuclear envelope, which is accompanied with a primary envelopment and de-envelopment. In the cytoplasm, the nucleocapsid acquires the tegument layer and the viral envelope by budding through Golgi vesicles. Finally, new virions are released after fusion of the vesicle and plasma membrane of the cell. During the latent phase, viral gene expression limits itself to genes primarily allowing latency maintenance (see the main text). Viral episomes remain attached to the host chromosome via LANA and replicate simultaneously with the host cell. During latency, the virus keeps the capacity to re-enter the lytic cycle.

8. KSHV miRNAs

KSHV encodes 12 miRNA genes (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005). Given their genomic proximity to the K12 ORF (Kaposin A), they were named with the label K12 and a number, i.e. miR-K12-1 to miR-K12-12. For the sake of simplicity, they are often referred to as miR-K1, miR-K12 etc. Altogether, they give rise to at least 25 miRNAs, 24 produced from each strand of the pre-miRNAs and an abundant isoform generated via A-to-I conversion of miR-K10-3p by ADAR1 (miR-K10a and miR-K10b) (Gottwein et al., 2011; Lei et al., 2012; Pfeffer et al., 2005).

With the exception of miR-K5 and miR-K9 whose precursors present polymorphisms leading to their decreased processing in some cell lines (Gottwein et al., 2006; Umbach and Cullen, 2010), there is a high overall conservation of miRNA sequence between different KSHV isolates and between tumors of KS and PEL. This indicates that miRNAs play important functions in KSHV life cycle and related pathology (Gottwein, 2012; Marshall et al., 2007).

8.1 KSHV miRNA expression

KSHV miRNAs are all encoded in a single genomic region, the major latency locus, which indicates their preferential and high expression during latency. Indeed, miRNA profiling of RISC occupancy in latently infected cells has shown that the proportion of viral miRNAs can reach up to 70% of the total miRNA population (Haecker et al., 2012). Other studies using small RNA sequencing also support the elevated expression of KSHV miRNAs in different cell lines and infection models, ranging from <10% to >80% of total miRNA reads. (Contrant et al., 2014; Dölken et al., 2010; Gottwein et al., 2011; Umbach and Cullen, 2010)

The same genomic locus contains also latent protein-coding genes LANA, v-Cyclin, v-FLIP and Kaposins (Figure 6). Several latent and lytic promoters in the locus allow to generate alternative polycistronic transcripts thereby expressing all or a subset of the latent proteins and the miRNAs (Li et al., 2002; Pearce et al., 2005; Sadler et al., 1999). miR-K1 to miR-K-9 and miR-K-11, are clustered together within a large intron spanning over ~4 kb. miR-K10 and miR-K12 are localized within the Kaposin A ORF and its 3'UTR respectively. While the intronic cluster is dependent on a transcription by 2 latent promoters, the Kaposin locus, and thus miR-K10 and -12, is also under the control of a lytic promoter leading to increased expression of these miRNAs during lytic cycle (Cai and Cullen, 2006).

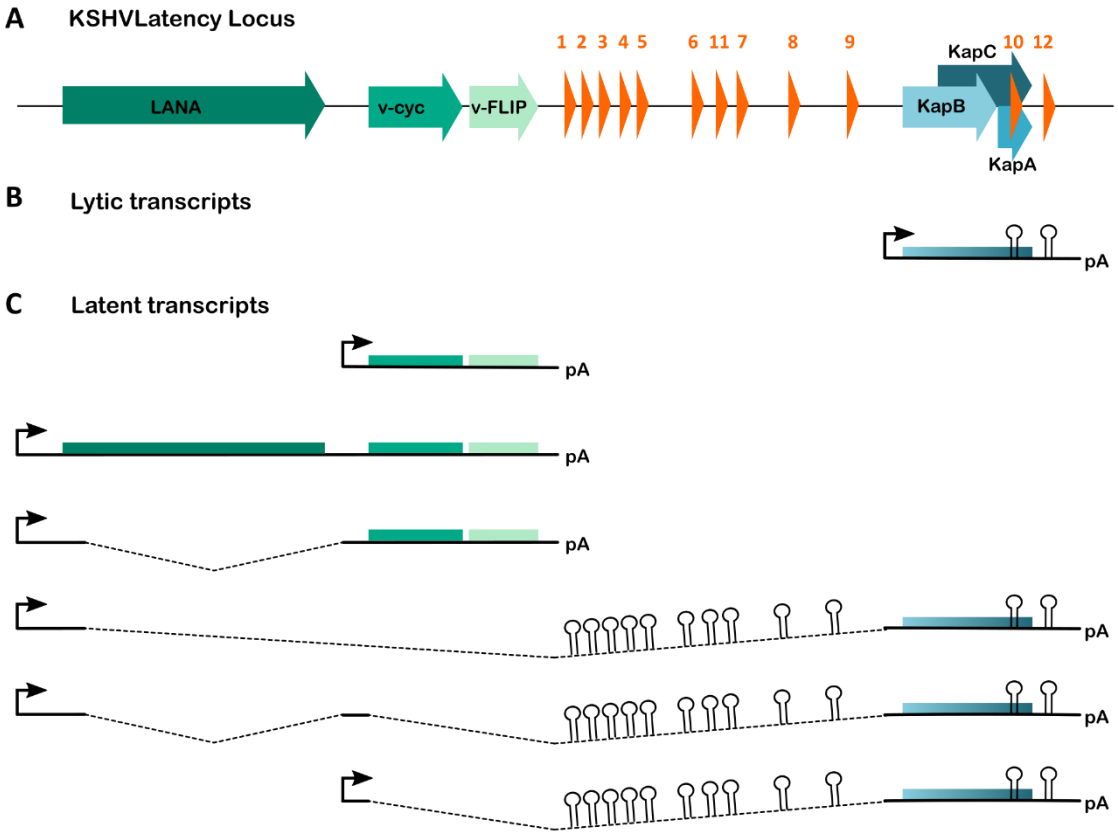

Figure 6: KSHV major latency locus architecture and transcription products. A) Schematic representation of the genomic region showing ORFs as arrows and miRNA genes as numbered orange arrowheads. Kaposin locus gives rise to at least three overlapping polypeptides, Kaposin A, B and C, owing to differential translation initiation. B and C) Lytic and latent promoters generate a complex transcription pattern with numerous alternatively spliced and polyadenylated mRNAs. The most abundant transcripts are represented. Black arrows represent transcription start sites, thick lines exons and dashed lines introns. According to (Cullen, 2011; Gottwein, 2012).

8.2 KSHV miRNA functions

Owing to their non-immunogenic nature, miRNAs are a tool of choice for the virus to modulate the intracellular environment and maintain itself over long periods of time. miRNA-dependent regulation is now well appreciated as essential for KSHV biology, allowing it to avoid immune clearance and to control viral and host life cycle.

Numerous groups set to identify KSHV mi RNA targets and study the impacts of miRNA activity at the cellular level. By investigating the phenotypes upon depletion or overexpression of a given miRNA, relationships to targeted genes could be drawn and functional validations performed. Other studies aimed at bringing a broader insight into KSHV targetome. By using microarray- and sequencing-based high-throughput techniques, such as

RIP-CHIP (immunoprecipitation of RISCs followed by microarray analysis (Dölken et al., 2010)), PAR CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (Gottwein et al., 2011)), HITS CLIP (High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (Haecker et al., 2012)) and CLASH (cross-linking ligation and sequencing of hybrids (Gay et al., 2018)) brought to light dozens of candidate targets based on increased association with AGO, many of which could be further validated. Finally, the actual target repertoire is still growing and a non-exhaustive list of the experimentally validated targets is presented in Table 2.

Table 2: Experimentally validated KSHV miRNA targets

Target	miRNA(s)	Functional consequence	Referece(s)
BACH1	miR-K11	Increased viability under oxidative stress	(Gottwein et al., 2007; Qin et al., 2010; Skalsky et al., 2007)
BCLAF1	miR-K5, miR-K9-3p, miR-K10a	Inhibition of caspase activity	(Ziegelbauer et al., 2009)
CASP3	miR-K1, miR-K3, miR-K4-3p	Inhibition of apoptosis	(Suffert et al., 2011)
CASTOR1	miR-K1, miR-K4-5p	Increased proliferation through activation of mTOR1 pathway	(Li et al., 2019)
C/EBPβ	miR-K11	B-cell proliferation induced by increased IL-6 and IL-10 production	(Boss et al., 2011)
CDKN1A / p21	miR-K1	Inhibition of p21-dependent cell-cycle arrest	(Gottwein and Cullen, 2010)
FOS	miR-K11	Regulation of cell cycle	(Gottwein et al., 2007)
GRK2	miR-K3	Pro-angiogenic and pro-migratory function	(Hu et al., 2015; Li et al., 2016a)
IKBKE / KKε	miR-K11	Attenuation of interferon signaling	(Gottwein et al., 2007; Liang et al., 2011)
IRAK1	miR-K9	Immune evasion through reduction of inflammatory cytokines	(Abend et al., 2012)
MAF	miR-K1, miR-K6-5p, miR-K11	Endothelial cell de-differentiation	(Hansen et al., 2010)
MCPIP1	miR-K4-5p, miR-K6-3p, miR-K10a	Stabilization of viral miRNAs and IL-6 mRNA	(Happel et al., 2016)
MICB	miR-K7	Inhibition of stress-induced NK cell recognition and activation	(Nachmani et al., 2009)
MYD88	miR-K5	Immune evasion through reduction of inflammatory cytokines	(Abend et al., 2012)
NFIB	miR-K3	Latency maintenance through inhibition of RTA transactivation	(Lu et al., 2010a)
IκBα	miR-K1	Latency maintenance through constitutive NFκB activation	(Lei et al., 2010; Moody et al., 2013)

Jarid2	miR-K11	Increased survival through epigenetic remodelling	(Dahlke et al., 2012)
RBL2	miR-K4-5p	Latency maintenance through de-repression of DNA methyl transferases	(Lu et al., 2010b)
SH3BGR	miR-K6-3p	Increased angiogenesis and migration by activation of STAT3 pathway	(Li et al., 2016b)
SMAD5	miR-K11	Cell growth strimulation through attenuation of TGFβ signaling	(Liu et al., 2012b)
TGFBRII	miR-K10	Cell growth strimulation through attenuation of TGFβ signaling	(Lei et al., 2012)
THBS1	miR-K1, miR-K3 miR-K6, miR-K11	Increased angiogenesis and proliferation	(Samols et al., 2007)
TWEAKR	miR-K10	Reduced apoptosis and inflammatory cytokine production	(Abend et al., 2010)
XAF1	miR-K11	Inhibition of apoptosis	(Gottwein et al., 2007)
KSHV RTA	miR-K9-5p, miR-K5, miR-K7	Inhibiton of KSHV lytic reactivation	(Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b)

KSHV miRNAs are also known to impact cell signaling pathways involved in metabolism and cell growth, such as AKT, JAK-STAT, TGF-β, mTor and NFκB (Lei et al., 2010; Li et al., 2019, 2016a, 2016b; Moody et al., 2013). This leads to an increased life span of infected cells and re-programming of differentiation pathways. De-regulation of cell cycle and inactivation of control check points are the hallmarks of malignant transformation. Thus, KSHV miRNAs directly favor progression towards cancer. Indeed, several groups have shown that mutant viruses lacking all or a subset of miRNAs exhibit decreased potential to yield cancerous phenotypes, such as resistance to stress and apoptosis, increased proliferation, angiogenesis and migration (Gay et al., 2021; Moody et al., 2013; Zhu et al., 2016). In addition several miRNAs were also shown to target or deregulate long non-coding RNAs, such as ANRIL and MEG3 which were both associated to cancer (Sethuraman et al., 2017).

The KSHV miRNA targetome is diverse and includes viral and cellular transcripts, thus optimizing infection conditions at various levels. One of the crucial functions of KSHV miRNAs is the establishment and maintenance of latency. For example, at least three miRNAs (miR-K5, miR-K7, miR-K9-5p) were shown to directly target the latent-to-lytic switch protein RTA in order to prevent inappropriate lytic reactivation (Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b). What is more, miR-K3 decreases the levels of the transcription factor NFIB able to activate RTA promoter (Lu et al., 2010a). Epigenetic silencing of RTA expression

occurs through miR-K4-3p-mediated repression of RBL2 that in turn represses several DNA-methyltransferases, thus increasing the overall methylation status of viral episomes (Lu et al., 2010b).

Virus survival within the cell also depends on efficient immune evasion. This is achieved through deregulated cytokine secretion, inhibition of antigen presentation and preventing host cytotoxic response. Among the miRNAs impairing host immune surveillance, miR-K7 targets MICB which is a stress-induced ligand for NK (natural killer) cells, thus avoiding destruction of infected cells (Nachmani et al., 2009).

Regarding the oncogenic function of KSHV miRNAs, most of attention has been drawn to miR-K11. Interestingly, miR-K11 presents the same seed sequence as the cellular miR-155. miR-155 is a known regulator of B-cell development and it has been coined an "oncomiR" owing to its aberrant overexpression measured in several cancers (Jiang et al., 2010; Rodriguez et al., 2007; Rufino-Palomares et al., 2013; Thai et al., 2007). It has been shown that miR-K11 can phenocopy miR-155 since it regulates the same set of target genes, among them BACH1, IKBKE, FOS and XAF1, which have functions in cell cycle, differentiation and cell survival (Gottwein et al., 2007; Skalsky et al., 2007). In addition, when overexpressed in humanized mouse models, both miRNAs induce B-cell hyperplasia and hyperproliferation (Boss et al., 2011; Costinean et al., 2006; Dahlke et al., 2012). Given the high expression of miR-K11 in infected cells, it is conceivable that it takes an active part in KSHV-induced oncogenesis.

However, miR-K11 is not the only KSHV miRNA mimicking a cellular ortholog. In fact, miR-K3, miR-K6 and miR-K10 also present seed homology to miR-23, miR-16 family and miR-142 respectively (Gottwein et al., 2011; Morrison et al., 2019; Skalsky et al., 2007). This kind of evolutionary convergence seems to be a convenient way for the virus to integrate and benefit from pre-existing host regulatory networks that can foster its survival. It is also an illustration of the very long co-evolution of the virus with its host.

Next to the canonical target-related function, two other KSHV miRNAs were shown to exert an additional regulatory activity in *cis*. Since pre-miR-K10 and pre-miR-K12 are integral parts of Kaposin transcripts (Figure 6), their excision by the Microprocessor destabilizes Kaposin mRNAs. Thus the fate of these transcripts relies on a competition between miR-K10 and miR-K12 processing and Kaposin mRNA translation (Lin and Sullivan, 2011).

9. Model systems for the study of KSHV

In vivo, the exclusive human tropism has prevented establishment of efficient animal models to follow KSHV transmission and pathogenesis. While successful infection was reported in SCID mice engrafted with human hematopoietic tissues, these animals did not develop pathologic conditions similar to humans (Damania and Cesarman, 2013; Dittmer et al., 1999). Therefore, our knowledge of KSHV infectious cycle is either inferred from closely related herpesviruses (such as MHV68), or based on cell culture experiments.

Infection of numerous adherent cell lines is possible *in vitro*. Cells of endothelial origin, HeLa, HEK293, primary keratinocytes and fibroblasts, as well as non-human-derived cell lines CHO (chinese hamster ovary) and Vero (African green monkey kidney) can be infected with variable efficiency. On the other hand, the primary target cells, B-lymphocytes, are refractory to infection *in vitro*, which supports the idea that not yet identified factors are necessary for their physiological infection *in vivo* (Bechtel et al., 2003; Myoung and Ganem, 2011; Zhou et al., 2002).

In cell culture, latency is established almost immediately. However, most of the cells do not undergo transformation and tend to lose viral episomes over passages, with only a small fraction being able to maintain latent viral genomes over time. A possible explanation may lie in the epigenetic status of these cells or in uneven episome partitioning during cell division (Chiu et al., 2017; Grundhoff and Ganem, 2004). Interestingly, the loss of viral episomes has been observed also in cells derived from primary KS spindle cells, further demonstrating the importance of the complex local environment in KS lesions.

On the contrary, numerous cell lines could be established from patients with PEL. They usually harbor high number (40-80) of viral episomes per cell (Zhou et al., 2002) and are helpful to study KSHV in a physiologically relevant B-cell environment.

Given that only 1-5% of cells in culture undergo spontaneous lytic reactivation, in order to study events related to lytic cycle, they need to be induced by chemical agents, such as phorbol esters (TPA) or histone deacetylase inhibitors (sodium butyrate, valproic acid) (Miller et al., 1997, 2007; Renne et al., 1996). Such reactivation is however only moderately efficient with at most 15-30% of cells expressing lytic genes. Ectopic expression of RTA has also been

used to induce lytic cycle, usually reaching higher reactivation rates (Lukac et al., 1998; Myoung and Ganem, 2011; Pearce et al., 2005).

Several stable cell lines were established using infections with recombinant viruses, which reproduce latent phenotype, are readily reactivable, and maintain viral DNA through antibiotic selection (Myoung and Ganem, 2011). These can be based on recombination *in cellulo* by transfecting a linearised cassette into PEL cells and then collecting viral particles upon reactivation of pre-selected cells (Vieira and O'Hearn, 2004). Another interesting approach relies on the bacmid technology, producing a bacterial artificial chromosome containing the entire KSHV genome. This allows easier genetic manipulation and generation of mutant variants, given that viral DNA can be recombined and propagated in bacterial cultures (Brulois et al., 2012; Jain et al., 2016; Zhou et al., 2002). Finally, insertions of fluorescent markers downstream of lytic and latent promoters has allowed to track latent and lytic infections directly in cultured cells (Vieira and O'Hearn, 2004).

10. Thesis objectives and hypotheses

The importance of miRNA-mediated regulation in KSHV infection and pathogenesis is well established. However, much less is known about the mechanisms that in turn regulate their biogenesis and accumulation. The aim of this work was to decipher the molecular mechanisms that modulate KSHV miRNA processing at the post-transcriptional level. We focused on the intronic cluster containing ten out of the twelve KSHV miRNAs, which are arrayed in tandem on one single primary transcript. Recent evidence shows that such polycistronic organization may provide background to peculiar regulatory mechanisms orchestrating the processing of individual cluster members. In addition, we and others have noticed distinct expression patterns leading to differential accumulation of the ten miRNAs, even though they are processed from one single precursor molecule (Contrant et al., 2014; Gottwein et al., 2011; Haecker et al., 2012; Umbach and Cullen, 2010). One conceivable explanation would be that the processing of each miRNA precursor depends on their structural (and sequence) features required for efficient Microprocessor activity. Thus, the most optimally folded precursors would correspond to the highest expressed mature miRNAs. According to the results obtained previously in the laboratory, the optimality of pre-miRNA substrates frequently, but not always correlate with the steady-state levels of mature miRNAs within infected cells (Contrant et al., 2014). This strongly points toward the existence of additional post-transcriptional mechanisms defining the fate of individual pre-miRNAs along their biogenesis. Furthermore, expressing the miRNAs from constructs containing either the entire cluster, or only one pre-miRNA leads to dramatically different expression levels between the two conditions. We also observed that swapping the positions of some pre-miRNAs within the cluster affects the relative accumulation of the corresponding mature miRNAs (Contrant et al., 2014). This indicates that the context of the cluster is highly relevant for individual miRNA expression.

Recognition of the pri-miRNA and its cleavage by the Microprocessor is the first and the most important step of miRNA processing. As such, it plays a decisive role in final miRNA accumulation (Conrad et al., 2014; Feng et al., 2011; Louloupi et al., 2017). In addition, Microprocessor can exhibit variable kinetics within polycistronic miRNA transcripts (Louloupi et al., 2017). Therefore, we decided to focus on this first biogenesis step, in order to further understand the regulation within the KSHV miRNA cluster. More particularly, we set to investigate two aspects potentially influencing Microprocessor dynamics within the cluster: (1) regulation by elements in *cis* and (2) regulation by co-factors in *trans*.

First, we hypothesized that the polycistronic organization *per se* may play a pivotal role in the accumulation of individual KSHV miRNA. We have indeed shown that the kinetic of the Microprocessor is influenced by the context of the cluster. In addition, we have shown that the maturation of miRNAs within the cluster is interdependent and relies on the presence of certain pre-miRNAs serving as regulatory elements in *cis*.

As RNA molecules, including miRNA processing intermediates, are rarely devoid of protein partners, in the second part, we were interested in potential co-factors whose binding may participate in KSHV miRNA processing events. Such co-factors may have different effects with respect to Microprocessor activity:

1. Co-factor binding to structure/sequence motifs of pre-miRNAs may result in preferential recruitment of the Microprocessor complex.
2. Co-factor binding may prevent efficient pre-miRNA processing by impeding Microprocessor recognition/cleavage.
3. Co-factor binding may alter pre-miRNA secondary structure, so that it becomes more or less favorable for Microprocessor recognition/cleavage.
4. Co-factor binding in the context of a miRNA polycistron can participate to the higher order organization of pre-miRNA processing, by exerting one of the above activities or through another molecular mechanisms.

We have benefited from data obtained previously in the laboratory, that have identified candidate proteins binding to individual KSHV pre-miRNAs. The aim was to validate their function in miRNA biogenesis and characterize their mode of action.

III. Results

1. *Cis*-regulation within a cluster of viral miRNAs

Given the importance of Microprocessor cleavage efficiency for mature miRNA expression, we set to determine the kinetic of the cleavage events within the primary transcript containing the KSHV miRNA cluster (pri-miR-K10/12). We used an *in vitro* processing assay of the pri-miR-K10/12 incubated in cell extracts overexpressing Drosha/DGCR8 followed by quantitative northern blot analysis. We were able to demonstrate that the ten miRNA precursors on the cluster possess highly variable kinetic properties, as seen by differential pre-miRNA accumulation rates. This helped us to rank the pre-miRNAs according to their propensity to be efficiently cleaved. Interestingly, we noticed that the variation between cleavage efficiencies of individual pre-miRNAs did not always correlate with the respective expression of their mature forms. In particular pre-miR-K1 and -K3 are very efficient Microprocessor substrates, whereas corresponding miR-K1 and miR-K3 do not accumulate to high levels in infected cells. This incoherence led us to further investigate the function of these pre-miRNAs within the cluster, hypothesizing that they may be involved in a post-transcriptional regulatory mechanism. First, deletion of both pre-miR-K1 and -K3 led to a striking decrease in the levels of the remaining miRNAs within the cluster. In addition, replacement of pre-miR-K1 by an unrelated pre-miRNA, pre-let-7a-1, could rescue the cluster expression. We therefore concluded that it is not the presence or the downstream function of pre-miR-K1 itself that is important, but the presence of a pre-miRNA at this particular position within the cluster. Further investigation of the phenomenon indicated that pre-miR-K1 and -K3 may be required as integral parts of the primary transcript to exert their regulatory function, hence they act as *cis* regulatory elements. Based on these findings, we developed an approach to inhibit the expression of the entire cluster by using one single antisense oligonucleotide targeting pre-miR-K1 processing. These results are presented in the publication included afterward.

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Cis regulation within a cluster of viral microRNAs

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ABSTRACT

MicroRNAs (miRNAs) are small regulatory RNAs involved in virtually all biological processes. Although many of them are co-expressed from clusters, little is known regarding the impact of this organization on the regulation of their accumulation. In this study, we set to decipher a regulatory mechanism controlling the expression of the ten clustered pre-miRNAs from Kaposi's sarcoma associated herpesvirus (KSHV). We measured *in vitro* the efficiency of cleavage of each individual pre-miRNA by the Microprocessor and found that pre-miR-K1 and -K3 were the most efficiently cleaved pre-miRNAs. A mutational analysis showed that, in addition to producing mature miRNAs, they are also important for the optimal expression of the whole set of miRNAs. We showed that this feature depends on the presence of a canonical pre-miRNA at this location since we could functionally replace pre-miR-K1 by a heterologous pre-miRNA. Further *in vitro* processing analysis suggests that the two stem-loops act in *cis* and that the cluster is cleaved in a sequential manner. Finally, we exploited this characteristic of the cluster to inhibit the expression of the whole set of miRNAs by targeting the pre-miR-K1 with LNA-based antisense oligonucleotides in cells either expressing a synthetic construct or latently infected with KSHV.

INTRODUCTION

Kaposi's sarcoma herpes virus (KSHV) or Human herpes virus 8 is a gammaherpesvirus associated with cancers such as Kaposi's sarcoma, B-lymphomas or the proliferative disorder Castelman disease. Its genome is a ∼165 kb dsDNA molecule that encodes >90 open reading frames (ORFs) as well as 25 mature microRNAs (miRNAs) (1). KSHV establishes lifelong persistent infection with a restricted expression of viral genes. However, a small percentage (<3%) of cells support lytic replication and under certain conditions KSHV can reactivate from latency to lytic replication. A dynamic balance between the latent and lytic phases of KSHV replication is critical to establish a successful virus infection, maintain latency, and is involved in pathogenic effects such as tumorigenesis (reviewed in (2)).

Interestingly, all KSHV precursor (pre-)miRNAs are expressed on the same polycistronic transcript, which is associated with latency (3–5). Ten of them (pre-miR-K1 to -K9 and miR-K11) are clustered within an intron of ∼4 kb between ORF71 (v-FLIP) and the kaposin genes, and are expressed under the control of a latent promoter (6). Pre-miR-K10 and pre-miR-K12 localize within the ORF and the 3′UTR of the kaposin gene, respectively, and are controlled by both latent and lytic promoters (6,7).

KSHV miRNAs are able to regulate the expression of both viral and cellular genes that are essential to virus infection and associated diseases. Abundantly expressed during the latent phase, they directly participate in its maintenance, for instance by repressing directly, or indirectly through targeting of NF-kB pathway, the replication and transcription activator (RTA), which is crucial for viral reactivation (8–10). They also promote tumorigenesis by modulating apoptosis, angiogenesis or cell cycle (e.g. (11–14)). Finally, KSHV miRNAs also enhance immune evasion and viral pathogenesis by regulating host immune responses (e.g. (15–18). See also (19) for a recent review on KSHV miRNA functions.

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Although we now know of numerous functions of KSHV miRNAs due to active research in the field, we still do not have a precise understanding of the regulation of expression of these key viral factors. In animals, miRNA biogenesis is a multi-step process including two maturations by RNase III enzymes. MiRNA genes are generally transcribed by RNA polymerase II as a long primary transcript (pri-miRNA) of several kilobases that can contain one or several miRNA precursor hairpins (pre-miRNA). First, the pri-miRNA is processed in the nucleus by the Microprocessor, comprising the RNAse III type enzyme Drosha and its cofactor DGCR8. After export into the cytoplasm, the pre-miRNA is further processed by another RNase III enzyme, Dicer, associated with TRBP. The final result is a duplex of miRNAs (5p and 3p) from which one of the strands is preferentially incorporated into an Argonaute protein to form the RISC complex, which can then be directed toward target mRNAs (reviewed in (20)). Alternative pathways of miRNA biogenesis exist such as Drosha-independent processing of mirtrons or Dicer-independent Ago2-dependent miR-451 cleavage (21–24).

About 25–40% of human miRNAs are found in clusters (25,26). There are usually two to three miRNAs in a cluster. However, a few larger clusters were also described such as the conserved mammalian pri-miR-17–92 that contains 6 members, or the imprinted C19MC that contains 46 tandemly-repeated pre-miRNA genes (27–29). Co-expression may be essential as it was shown that many clustered miRNAs regulate common biological processes as is the case for KSHV miRNAs (e.g. (18)). Even though clustered miRNAs are co-transcribed, the resulting mature miRNAs are found at different levels in the cell (e.g. (30,31)). This suggests that complex regulation events occur downstream of the transcription. Two independent studies from Zeng and Orom laboratories revealed the key importance of maturation by the Microprocessor to explain the global level of cellular miRNAs (32,33). Maturation of pri-miRNAs by the Microprocessor is controlled by sequence and structural features of miRNA hairpin, defining the basal level of pre-miRNAs excised. In addition, protein cofactors may interact with specific motifs or structure of the stem-loop and thus modulate the Microprocessor activity (see (34–36). Recently, several studies demonstrated interdependent processing in the context of bicistronic pri-miRNA where an optimal miRNA hairpin assists the processing of a neighboring suboptimal one (37–42). Such interdependency has not yet been documented in the case of larger miRNA clusters.

Previously, we demonstrated the importance of RNA secondary structure of the long primary transcript containing the ten intronic miRNAs from KSHV (pri-miR-K10/12) for the accumulation of mature miRNAs (31). Here, we show *cis* regulation within this large viral miRNA cluster. We observed that the ten miRNA hairpins from the KSHV intronic cluster are processed *in vitro* by the Microprocessor with different efficiencies. Intriguingly, high processing levels of miR-K1 and miR-K3 hairpins were not consistent with the low level of accumulation of their mature miRNAs in infected cells (31), suggesting that these miRNA hairpins could serve other purposes than solely producing mature miRNAs. Indeed, specific deletion of pre-miR-K1 or pre-miR-K3 within the cluster significantly reduce the expression of the remaining miRNAs in the cell. Moreover, only the pre-miRNA feature is sufficient to support such regulatory mechanism since replacement of pre-miR-K1 by the heterologous pre-Let-7a-1 restores expression of clustered miRNAs to the wt level. Further experiments of *in vitro* processing assays using pri-miRNA fragments (mimicking cleavage of pre-miR-K1 or pre-miR-K3) suggest that regulation may occur before pre-miR-K1 and -K3 are cut by the Microprocessor or that processing of the cluster is sequential. Finally, we developed an antisense strategy based on Locked Nucleic Acid (LNA) oligonucleotides to post-transcriptionally downregulate the expression of the whole KSHV miRNA cluster. Using an LNA targeting either the 5p or 3p of the pre-miR-K1 sequence, we managed to significantly reduce the levels of clustered KSHV miRNAs in cells transfected with a synthetic construct. We also showed that in KSHV-infected cells, the levels of neosynthesized miRNAs derived from the cluster dropped significantly upon LNA targeting of pre-miR-K1, indicating that this could be a useful strategy to block the entire cluster in infected cells.

MATERIALS AND METHODS

Cells and media

HEK293FT-rKSHV cells were generated by infecting HEK293FT cells with concentrated rKSHV.219 virus (43) in the presence of 8 μg/ml polybrene (Sigma) and by spinoculation (800 g for 30 min at room temperature). Puromycin selection was applied to select for rKSHV.219 infected cells. The virus stock used to infect HEK293FT cells was generated by treating iSLK.219 cells (44) with 1 μg/ml doxycycline and 1.35 mM sodium butyrate and collecting virus particles 48 h post-reactivation by ultracentrifugation.

Adherent HEK293Grip and HEK293FT-rKSHV cell-lines were cultured in a humidified 5% CO_2 atmosphere at 37°C in DMEM medium containing 10% fetal calf serum (FCS). In addition, HEK293FT-rKSHV were grown with 2,5 μg/ml puromycin (for viral genome maintenance).

RNA preparation

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific).

Pre-miRNAs, wt and mutants of pri-miR-K10/12, derived from BCBL-1 cell line, were transcribed from PCR-generated DNA templates carrying a T7 promoter (see Supplementary Table S1 for primers). *In vitro* RNA synthesis was done by T7 RNA polymerase (Ambion). Pre-miRNAs were purified on denaturant polyacrylamide gel and long pri-miR-K10/12 derived transcripts (up to ∼3 kb) were salt purified using Monarch® PCR and DNA cleanup kit (New England BioLabs). After acidic phenol extraction and ethanol precipitation, the RNAs were pelleted and recovered in MilliQ water.

Northern blot analysis

RNAs were resolved on a 8% urea-acrylamide gel, transferred on a nylon membrane (Amersham Hybond-NX,

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GE-Healthcare Life Sciences), crosslinked to the membrane by chemical treatment at 60°C using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Sigma) for 1 h 30 min. MiRNAs and pre-miRNAs were detected with specific 5′-^{32}P labeled oligonucleotides (Supplementary Table S1). The signals were quantified using a Fuji Bioimager FLA5100. miR-16 was probed as a loading control.

In vitro Drosha miRNA processing assays

Drosha and DGCR8 were overexpressed in 10-cm Petri Dish Hek293Grip cells using pCK-Drosha-Flag and pCK-Flag-DGCR8. After 48 h, cells were washed with ice-cold PBS, centrifuged and pellet was resuspended in 120 μl ice-cold lysis buffer (20 mM Tris–HCl pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol and mini-complete EDTA-free protease inhibitor (Roche)). The cell suspension was sonicated during 5 min at high amplitude, 30 s on and 30 s off using Bioruptor™ UCD-200 (Diagenode), centrifuged for 10 min at 10 000 g, 4°C, and the supernatant was used for *in vitro* processing assays.

500 or 1000 fmol of *in vitro* transcribed wt or mutant pri-miR-K10/12 RNAs were denatured 3 min at 95°C, cooled on ice 3 min and folded in 1× structure buffer provided by Ambion during 30 min at 37°C. Processing assays were performed in 30 μl containing 15 μl of total protein extract (10 μg/μl) or 15 μl of lysis buffer, 6.4 mM $MgCl_2$, 30 U Ribolock (Thermo Scientific™, Thermo Fisher Scientific). Just after addition of 170 μl elution buffer (2% SDS, 0.3 M sodium acetate), reaction was terminated by acidic phenol extraction followed by ethanol precipitation with 5 μg glycogen. After resuspension in formamide loading buffer, cleavage products were analyzed by northern blot. For quantification, *in vitro* transcribed and gel purified pre-miRNAs and synthetic miRNA oligonucleotides (IDT) were loaded at increasing concentration (from 1.5 to 25 fmol). A standard curve was generated by plotting the signal intensity against the amount of pre-miRNAs loaded and was used to calculate the absolute amount of pre-miRNAs produced by *in vitro* Drosha processing.

Kinetic analysis

The experimental cleavage curves show different rates and different fractions of cleavage of each pre-miR. In addition, the experimental cleavage curves most often show a maximum followed by a slight decrease of the amount of pre-miR, which requires to introduce a secondary cleavage event of the newly formed pre-miR (either from a contaminant RNase, or from Dicer). The model in use is thus:

$$\text{pri}-\text{miR} \xrightarrow{k_{Drosha}} \text{pri}-\text{miR}(i) \xrightarrow{k_{RNAse}} \text{miR}(i)? \quad (1)$$

At first, it was attempted to model the rates of enzymatic cleavage (k_{Drosha} and k_{RNAse}) according to Michaelis–Menten kinetics, but it turned out to be inefficient due to a very large correlation of the parameters K_M and V_{max}. We finally used the following simplest possible mathematical model:

$$\frac{dK_i}{dt} = k_i^+(f_i R_0 - K_i - K_i^*) - k_i^- K_i;\ \frac{dK_i^*}{dt} = k_i^- K_i \quad (2)$$

with R_0 the total concentration of the pri-miRNA, K_i the time-dependent concentration of the *i*th pre-miR, f_i the fraction of R_0 used by Drosha to produce K_i , and K_i^* the time-dependent concentration of the secondary-cleavage product (miR(*i*) in Equation (1)). Since Drosha act differently on the pri-miR to produce each pre-miR, it is necessary to replace k_{Drosha} with a particular cleavage rate K_i^+ for each pre-miR and, similarly, it is necessary to replace k_{RNASe} with k_i^- for each secondary-cleavage rate. This simple model, therefore, does not try in any way to differentiate situations wherein a particular pre-miR would be obtained either from the first cleavage of the full pri-miR, or from subsequent cleavage of a fragment of it. The previous linear differential Equation (2) are readily integrated, which gives the cleaved fraction $Y_i = K_i(t)/R_0$ necessary to fit the *i*th experimental curve:

$$Y_i = \frac{K_i(t)}{R_0} = f_i k_i^+ \frac{e^{-k_i^- t} - e^{-k_i^+ t}}{k_i^+ - k_i^-} \quad (\text{if } k_i^+ \neq k_i^-) \quad (3)$$

$$Y_i = \frac{K_i(t)}{R_0} = f_i k_i^+ t\, e^{-k_i^+ t} \quad (\text{if } k_i^+ = k_i^-) \quad (3')$$

The results obtained with this model are in Figure 1 and Supplementary Figure S2.

Mutagenesis and miRNA expression in human cells

Plasmid pcDNA-K10/12, derived from pcDNA5 (Invitrogen) and containing the wild type (wt) pri-miR-K10/12 (14) was mutated using the Phusion site-directed mutagenesis kit (Thermo Scientific) and transformation of *Escherichia coli* DH5alpha strain. Positive clones were identified by sequencing (GATC Biotech, France). Four deletion mutants were designed: ΔK1, ΔK3, ΔK7 and ΔK9 where the respective pre-miRNA sequence, was deleted. ΔK1-Let7 corresponds to ΔK1 mutant where the pre-Let7a-1 was inserted in place of pre-miR-K1 (see Figure 2). Expression tests were conducted as follow: 2 μg of plasmids (wt or mutated) were used to transfect HEK293Grip cells in 6-well/plate. Total RNA was collected after 48 h and miRNA expression was analyzed by northern blot, using 9 μg of total RNA and standard protocol. miR-16 was probed as a loading control and used for signal normalization.

Antisense LNA treatments

HEK293Grip cells in 6-well/plate were transfected with 1 μg of wt pcDNA-K10/12 in combination with 20 nM LNA oligonucleotide (see Supplementary Table S1 for sequence). Total RNA was collected after 48 h and miRNA expression was analyzed by northern blot, using 10 μg of total RNA and standard protocol. miR-16 was probed as a loading control and used for signal normalization.

HEK293FT-rKSHV were seeded in 12-well/plates. When approximately 50–60% confluent, they were transfected with 20 nM LNA by using Lipofectamine 2000 reagent (Invitrogen). On the next day, they were detached by 50 μl of 0,05% Trypsin-EDTA (Gibco), resuspended in fresh medium and half of the cells were transferred into a new well and allowed to seed. On the next day they were

transfected again with 20 nM LNA. They were collected by direct lysis in Trizol reagent (Ambion) one day after the second transfection.

4sU metabolic labeling, neosynthesized RNA pull-down and RT-qPCR analysis

The experimental procedure was adapted from the protocol developed by the Nicassio laboratory (45). One day prior to LNA transfection, 5 million HEK293FT-rKSHV cells were seeded in a 10 cm culture dish. 50nM LNA were transfected using Lipofectamine 2000 (Invitrogen) in total culture volume of 10 ml. After 24 h, culture medium was collected, filtered (45 μm) and 4sU was added to reach final concentration of 300 μM. The medium was then transferred back to the cells which were allowed to incorporate the 4sU during 3 h (incubation at 37°C, 5% CO_2, in dark). After that, cells were detached in ice-cold PBS and collected by centrifugation. RNA was extracted by TRIzol reagent (Invitrogen) by using 3 ml of the reagent per dish.

70 μg of total RNA were biotinylated by incubation with 160 μl of EZ-Link HPDP-Biotin (Thermo Scientific, 1 mg/ml in DMF), and biotinylation buffer (final concentration 10 mM Tris pH 7.4, 1 mM EDTA) in total volume of 490 μl during 2 h at 25°C. Following PCI (Roth) extraction and isopropanol precipitation, the RNA was washed with EtOH 75% and dissolved in 80 μl of RNase-free water. Biotinylated RNA was then pulled-down on Dynabeads MyOne Streptavidin T1 (Invitrogen) by using 80 μl of beads per condition. The beads were first washed twice in buffer A (80 μl of 100 mM NaOH, 50 mM NaCl), then once in buffer B (100 mM NaCl) and finally resuspended in 160 μl of buffer C (2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.1% Tween-20). The volume of RNA was increased to 160 μl and added to the beads. After 15 min of rotation on a wheel at room temperature, the beads with captured RNA were washed three times in 320 μl of buffer D (1 M NaCl, 5 mM Tris pH 7.5, 0.5 mM EDTA, 0.05% Tween-20). The RNA was then eluted with 160 μl of elution buffer (10 mM EDTA in 95% formamide) by heating at 65°C for 10 min. Trizol-LS (Invitrogen) and chloroform were used for eluted RNA extraction and after addition of 1.5 V of EtOH 100% to the aqueous phase, RNA was recovered on miRNeasy columns (Qiagen) in final volume of 30 μl.

3 μl of purified RNA were reverse-transcribed by using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems) and a pool of eight specific stem-loop primers (miR-K1, miR-K3, miR-K4, miR-K11, miR-16, let-7a1, miR-92, U48, 0.5 μl each). RT reaction was then diluted twice and 1 μl used to perform qPCR in total volume of 10 μl, by using TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems) and 0.5 μl of individual TaqMan miRNA assays (Applied Biosystem). qPCR was realized on CFX96 Touch Real-Time PCR Detection System (Biorad). Analysis of input RNA was performed in the same way on 100 ng of total RNA. In order to determine the amount of neosynthesized miRNAs relative to the input, we first calculated the enrichment of miRNA levels in the pull-down relative to the input after normalizing the data to Let-7. This ratio was then compared between the specific treatment (LNA @K1*) and the control treatment (LNA Ctrl), which was arbitrarily set to 1.

Primary transcript pri-miR-K10/12 was analyzed after prior treatment with DNase I (Invitrogen) or TURBO™ DNase (Invitrogen). 5 μl of purified or 1 μg of input RNA was treated and subsequently reverse-transcribed (using $\frac{1}{4}$ of reaction volume for non-reverse-transcribed control) with Superscript IV (ThermoFisher Scientific) according to the manufacturer protocol. cDNA was diluted twice before using 1 μl for quantitative PCR Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The same approach than for mature miRNAs was used to determine the amount of neosynthesized pri-miRNAs except that the data were normalized to the CYC1 mRNA instead of Let-7.

RT-qPCR analysis of the primary transcript in HEK293Grip cells transfected with LNA was performed according to the same procedure, however by diluting the cDNA 10 times.

miRNA expression in HEK293FT-rKSHV cells transfected with LNAs without metabolic labeling was measured similarly to the 4sU-samples, except for the reverse transcription step, which was performed individually for each RT stem-loop primer (0.5 μl) and without diluting the resulting cDNA. 100 ng of total RNA was used for each RT reaction.

Primer sequences are indicated in the Supplementary Table S1.

RESULTS

Clustered KSHV pre-miRNAs are processed *in vitro* by the Microprocessor with different efficiencies

In this work, we focused on the polycistronic feature of the KSHV intronic pri-miRNA containing ten miRNA hairpins (miR-K1 to miR-K9, and miR-K11), that we referred to as pri-miR-K10/12 (Supplementary Figure S1). Our previous results showed that pri-miR-K10/12 adopts a well-organized 2D structure, composed of multiple hairpins with all miRNA sequences found in stem-loops. Interestingly, the secondary structural features of miRNA stem-loops correlate to some extent with the cellular abundance of mature miRNAs. Indeed, optimally folded stem-loops tend to lead to more abundant miRNAs. Moreover, we demonstrated that the structural context of miRNA hairpins within the primary transcript is important since swapping miRNA stem-loops or expressing miRNAs individually results in differential miRNA accumulation in cells (31).

To further understand the mechanism behind the regulation of expression of polycistronic KSHV miRNAs, we assessed the *in vitro* processing efficiency of the different pre-miRNAs within the cluster. To do so, we took advantage of *in vitro* processing assays using total extracts obtained from cells over-expressing Drosha and DGCR8 and *in vitro* transcribed pri-miR-K10/12 (~3.2 kb) (Figure 1A). Accumulation levels of all pre-miRNAs from the cluster were analyzed at different time points using quantitative northern blot analysis (Figure 1B). Figure 1 and Supplementary Figure S2 show the results obtained from two independent experiments (Exp#1 and Exp#2).

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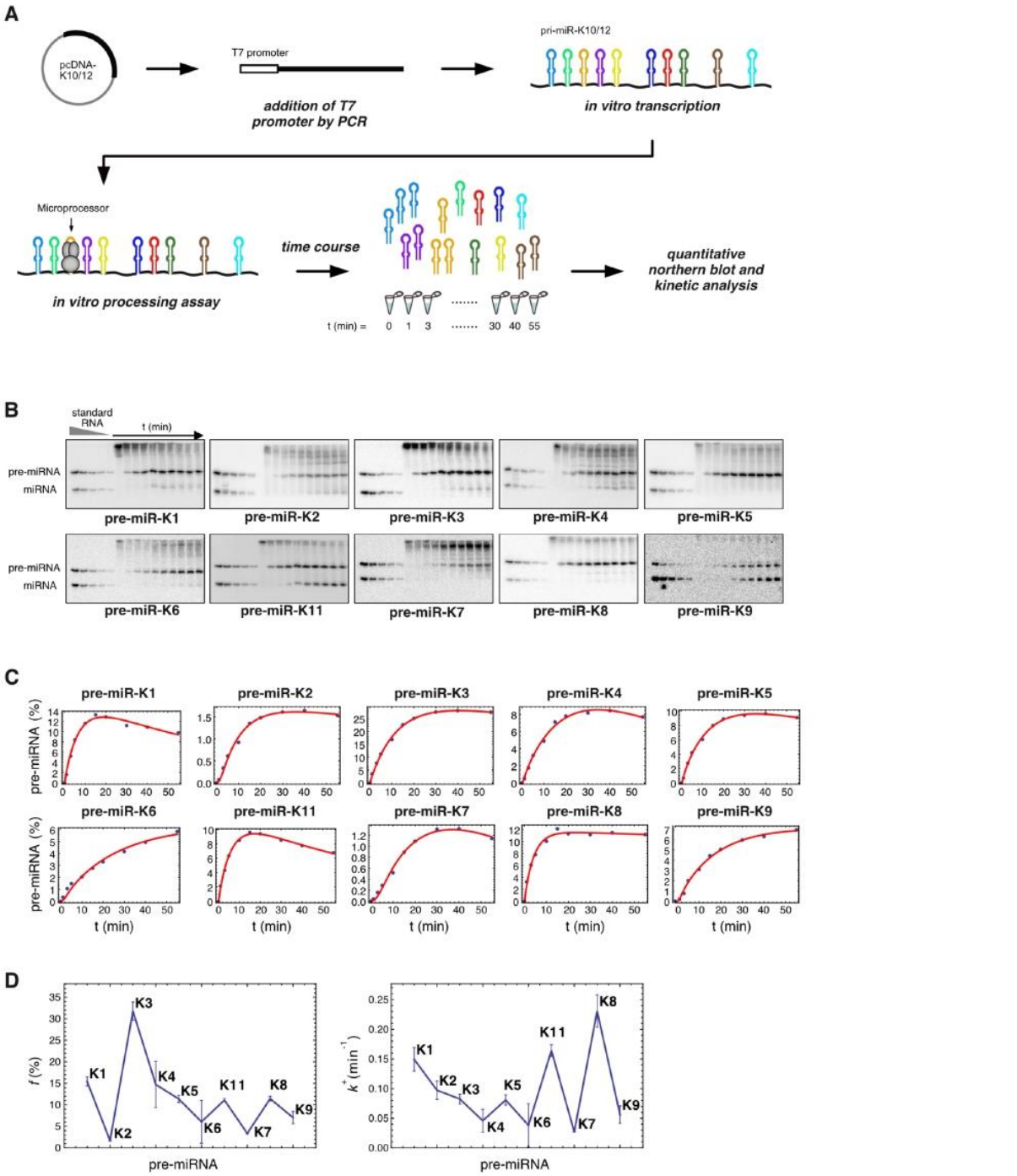

Figure 1. Kinetic analysis of KSHV clustered pre-miRNAs maturation *in vitro* by the Microprocessor. (**A**) Overview of *in vitro* processing assays starting with synthesis of DNA template containing a T7 promoter by PCR from the pcDNA-K10/12 plasmid and *in vitro* transcription to generate pri-miR-K10/12 containing the 10 KSHV clustered pre-miRNAs. *In vitro* processing assays was performed by incubating pri-miR-K10/12 with total protein extract of HEK293Grip cells overproducing Drosha and DGCR8. Pre-miRNAs production was monitored along the time and quantified by northern blot analysis. (**B**) Northern blot analysis of the time course of *in vitro* processing assays using 500 fmol of *in vitro* transcribed pri-miR-K10/12 and HEK293Grip cells total protein extract where Drosha and DGCR8 were overexpressed. *In vitro* transcribed pre-miRNAs and synthetic RNA oligonucleotides were loaded at increasing concentration as standards. (**C**) Cleavage curves were obtained after plotting pre-miRNA product, in percentage of initial pri-miR-K10/12 substrate, according to time. The fits were obtained with the model involving three free parameters per curve (compare with Supplementary Figure S3 for the more stringent model with two free parameters per curve). (**D**) Processing efficiencies (left panel) and cleavage rate (right panel) were plotted in respect to miRNA hairpins showing variation among the clustered pre-miRNAs. The error bars come from standard procedures used to fit the experimental curves by minimizing the residuals between the experimental points and their theoretical estimates. Data are from Exp#1 (see Supplementary Figure S2 for Exp#2).

In the conditions used, all pre-miRNAs were produced from the unique pri-miR-K10/12 substrate. Pre-miRNAs were the major products, and, in some cases, we also observed accumulation of mature miRNAs due to residual Dicer activity in the total protein extract. Experimental cleavage curves were fitted using a simple kinetic model with three free parameters per curve (Figure 1C). This led to excellent agreement compared to a more stringent model with only two free parameters per curve (see Materials and Methods and Supplemental Method). The numerical results are shown in Supplementary Table S2. Interestingly, we noticed that the sum of all pre-miRNAs is (114 ± 8) % and (110 ± 17) % for Exp#1 and Exp#2, respectively, which is 10-fold lower that the maximum possible value of 1000 % if pri-miRNA gave rise to all ten possible pre-miRNAs. Since these two sums are not significantly different from 100 %, this suggests that each pri-miR-K10/12 would be cleaved only once and would produce only one particular pre-miRNA. However, we set our experimental conditions such that the initial concentration of substrate will be high enough to ensure that we measured the maximum rate of cleavage for each miRNA stem-loop. In that respect, the Microprocessor was probably saturated by the full-length pri-miR-K10/12 at the disadvantage of cleaved RNA fragments. As a consequence, we overlook the possibility to observe several rounds of cleavage.

Comparison of processing efficiencies f_i and of the cleavage rate constant k_i^+ for each KSHV pre-miRNA showed important differences between pre-miRNAs as illustrated in Figure 1C and Supplementary Figure S2C. Indeed, average accumulation levels vary from 1.4 % for pre-miR-K2 up to 36 % for pre-miR-K3 products (Table 1); cleavage rate constants ranged from 0.027 min^{-1} for pre-miR-K7 to 0.23 min^{-1} for pre-miR-K8 in Exp#1 (Figure 1 and Supplementary Table S2). Kinetic parameters were not averaged due to a higher activity of the Microprocessor in Exp#1 versus Exp#2. Drosha/DGCR8 concentration was estimated to be about three-fold greater in Exp#2 based on the relative values of cleavage rates in the two experiments. The agreement between the two experiments is rather good for the processing efficiencies f_i (correlation coefficient = 0.95) and lower for the kinetic constants of cleavage k_i^+ (correlation coefficient = 0.52) (Supplementary Figure S4). We can nevertheless conclude that there are significant differences of processing efficiencies between the different KSHV pre-miRNAs. To exclude that the observed differences might have arisen from various stability of the different pre-miRNAs and not their processing efficiencies, we assessed the stability of two pre-miRNAs that appeared well-processed in our experiment (pre-miR-K1 and -K8) and two pre-miRNAs that are less well-processed (pre-miR-K11 and -K7) (Supplementary Figure S5). Briefly, *in vitro* transcribed pre-miRNAs were incubated in total cellular extract from HEK293Grip cells and pre-miRNAs stability was measured at various time points by northern blot analysis. Overall, we did not observe any striking difference in the decay rate between them, which could account for the variation measured in our *in vitro* cleavage assays.

We previously determined hairpin optimality features for each KSHV pre-miRNA (31), which we decided to update in order to take into account novel features that have been published since (46,47). In particular, we calculated the Shannon entropy for each miRNA hairpin as highlighted in Supplementary Figure S6. The data obtained indicated that overall a lower Shannon entropy could be observed along the stem in well-processed miRNA hairpins, with the exception of stem-loop miR-K11 and miR-K9, as described previously (47). Supplementary Table S3 summarizes the updated optimality features observed for KSHV pre-miRNAs. This allowed us to compare processing efficiencies f_i of miRNA hairpins with their hairpin optimality feature and their corresponding miRNA abundance in infected BCBL-1 cells (as previously determined (31)) (Table 1). MiRNA hairpins were ranked from the best to the worst substrates, which allowed us to define two groups, *i.e.* the well or moderately processed ($f_i \geq 10$ %, miR-K1, -K3, -K4, -K8 and -K11) and the less efficiently processed (miR-K2, -K6, -K7 and -K9). The miR-K5 hairpin was not ranked since the results were too discordant between the two experiments. Overall, well processed miRNA hairpins corresponded to optimally folded stem-loops. However, with the exception of miR-K4, this did not correlate with the level of accumulation of mature miRNAs in infected cells (31). Thus, miR-K1 and -K3 are embedded within hairpins that are optimal and the best processed by the Microprocessor *in vitro* (16 and 36% respectively), whereas the level of mature miRNAs is quite low (~3 and ~8%, respectively). Accordingly, they were defined as over-processed. On the opposite, miR-K11

Table 1. Correlation between KSHV miRNA hairpin processing efficiency with their hairpin optimality and cellular abundance of their corresponding mature miRNAs

KSHV miRNAs	Hairpin optimality[a]	Processing efficiencies f_i (%)	Cellular abundance in BCBL-1 (%)[b]	Correlation/comment
K3	+	**36.0 ± 5.7**	8.15	No/over-processed
K1	+	**16.0 ± 1.4**	3.03	No/over-processed
K4alt	+[c]	**13.5 ± 2.1**	**18.64**	Yes
K8	+	**12.0 ± 1.4**	2.10	No/over-processed
K11	+	**10.3 ± 1.0**	**23.14**	No/sub-processed
K9	-	7.4 ± 0.5	6.70	Yes
K6	+	4.5 ± 2.3	**14.25**	No/sub-processed
K7	+	3.6 ± 0.2	**22.03**	No/sub-processed
K2	-	1.4 ± 0.5	1.15	Yes
K5	-	2.8 / 11.0	0.80	No/no conclusion

The miRNAs are ranked according to their processing efficiencies ($n = 2$). We defined well or moderately processed pre-miRNAs as accumulating above 10% (percentage are in bold). MiR-K5 hairpin was not ranked since processing efficiencies were too distant in the two experiments.
In grey are emphasized the values of f_i parameter that correlate with one or the two other parameters, allowing to define miRNA hairpin as over-processed, processed accordingly to hairpin optimality and miRNA level or sub-processed. In the case of miR-K5, no conclusion could be drawn due to variation of data between experiments.
[a]Hairpin optimality takes into account presence of primary and secondary features required for optimal processing by the Microprocessor as described previously (31) and in Supplementary Table S3.
[b]Relative cellular abundance of viral miRNAs in BCBL-1 is expressed in percentage and abundant miRNAs were defined as above 10% (in bold) (from (31)). For miR-K4, -K6 and -K9 hairpins, percentage were obtained after addition of miRNA abundance of 5p and 3p arms.
[c]In the case of miR-K4 hairpin, whereas experimentally determined structure was not optimal, it could be manually folded into an alternative and more optimal structure (see Supplementary Table S3).

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hairpin is only processed at ∼10% although it is the most abundant miRNA in cells, representing ∼23% of viral clustered miRNAs. This hairpin was thus defined as being subprocessed. The same held true for miR-K6 and -K7 hairpins. Of the remaining hairpins, only miR-K2, -K4 and -K9 showed a good correlation between their optimality feature, processing efficiencies and cellular abundance.

Altogether, our kinetic analysis therefore shows different processing efficiencies of KSHV miRNA hairpins within the polycistronic pri-miR-K10/12, which did not fully correlate with the fact that the miRNA hairpin was optimal or not or with the accumulation level of the respective mature miRNAs in infected cells. These discrepancies may reflect complex regulation of their biogenesis. Indeed, subprocessing of miRNA hairpins may be explained by the requirement of additional elements such as protein cofactors that may be absent or present at low levels in our *in vitro* assay. In addition, we also observed cases of over-processed miRNAs, such as miR-K1 and miR-K3, which suggests that processing by the Microprocessor may serve here another purpose than solely producing mature miRNAs.

Deletion of pre-miR-K1 or pre-miR-K3 globally impairs the expression of the remaining miRNAs from the cluster

To further study the processing of the KSHV miRNA cluster, we used a plasmid allowing expression of the pri-miR-K10/12 sequence driven by a CMV promoter (14). Although it seems that there is a better accumulation of miRNAs at the 5′ extremity of the cluster, the wild type construct gives rise to all ten miRNAs and their relative expression level is close to what can be measured in latently infected BCBL1 cells (Supplementary Figure S7).

To investigate the potential other role of miR-K1 and -K3 processing we generated mutant constructs in which we deleted individually pre-miR-K1 or pre-miR-K3 sequences within the polycistronic pri-miR-K10/12. Other miRNA sequences from the cluster were unchanged. We then assessed the impact of these deletions on the expression of the remaining clustered miRNAs in the cell. As negative controls, we deleted pre-miR-K7 and pre-miR-K9, that are located in the middle and at the 3′ end of the cluster, respectively, and are not well processed *in vitro* by the Microprocessor. The resulting mutants were named ΔK1, ΔK3, ΔK7 and ΔK9 (Figure 2A). These were expressed in HEK293Grip cells and the accumulation levels of all mature miRNAs from the cluster were assessed by northern blot analysis (Figure 2B and C).

Interestingly, the expression of all miRNAs in the cluster was globally and drastically decreased compared to the wt construct in the ΔK1 and ΔK3 mutants, whereas it was only moderately or mostly unaffected in the ΔK7 and ΔK9 mutants. ΔK1 construct led to miRNA levels significantly reduced down to ∼28% (3.6-fold) and ∼52% (1.9-fold) for miR-K3 and miR-K7, respectively, when compared to the wt plasmid. All the miRNAs from the cluster were negatively affected, whatever the distance between pre-miR-K1 and the impacted miRNAs. For example, the farthest miR-K9 was even more impacted than the closest miR-K2 (∼38% (2.6-fold) versus ∼49 % (2-fold), respectively) (Figure 2C). Deletion of pre-miR-K3 within the cluster was even more deleterious for the expression of the rest of the clustered miRNAs. Indeed, miRNA levels were decreased down to ∼6% (16.7-fold) (miR-K5) or ∼18% (5.6-fold) (miR-K2). In the case of ΔK7 mutant, a moderately negative impact was observed for some miRNAs with the most affected being miR-K11, which level was reduced down to ∼54% (1.9-fold). In the case of miR-K11, this may be due to a local effect of pre-miR-K7 deletion on the folding and/or processing of the adjacent miR-K11 hairpin.

Altogether, our results suggest that deletion of pre-miR-K1 or -K3 within pri-miR-K10/12 globally and drastically impacts the expression of the remaining miRNAs from the cluster in cells. This global and severe impact is specific to pre-miR-K1 and pre-miR-K3 since it was not observed for two other pre-miRNAs in the cluster.

Expression of clustered miRNAs can be rescued by replacing pre-miR-K1 by a heterologous pre-miRNA

The analysis of deletion mutants indicates that pre-miR-K1 and -K3 appear to be required for the optimal expression of KSHV clustered miRNAs. This may be due either to (i) their ability to recruit the microprocessor, as stem-loop structures, or (ii) specific sequences that establish tertiary contacts within the cluster or that are recognized by protein cofactors.

To investigate which hypothesis should be favored, we inserted a heterologous pre-miRNA sequence in lieu of pre-miR-K1 into the ΔK1 mutant and measured the expression of the rest of the clustered miRNAs. We chose the pre-Let-7a-1 sequence since both its primary sequence and its secondary structure, especially in its apical loop, is very different from those of pre-miR-K1, therefore lowering the possibility to form the same tertiary contacts or to recruit the same cofactors. The resulting mutant ΔK1-Let7 construct was expressed in HEK293Grip cells and the expression of miRNAs was assessed by northern blot analysis (Figure 2B, C). Whereas pre-miR-K1 was as expected not produced from the mutant construct, Let-7a expression was increased ∼2-fold when compared to the endogenous expression, showing that Let-7a within the context of the cluster was fully recognized and processed by the Microprocessor. The level of accumulation of all the KSHV miRNAs was measured and compared to the wt construct. Interestingly, expression of all of them was restored almost to the wt level, showing that replacing pre-miR-K1 with a heterologous pre-miRNA is sufficient for optimal production of the other miRNAs in the cluster. In conclusion, our results suggest that a pre-miRNA structure at this position within the cluster is sufficient to optimize the expression of KSHV miRNAs from this construct.

In vitro, mimicking initial cleavage of miR-K1 or miR-K3 hairpins impacts the processing of only few miRNA hairpins from the cluster

Pre-miR-K1 or pre-miR-K3 are necessary for the optimal expression of the other miRNAs from the cluster, and at least for pre-miR-K1, this is independent of primary sequence but rather relies on the presence of a miRNA stem-loop structure. According to that observation, we hypothesized that cleavage of pre-miR-K1 or pre-miR-K3 by the

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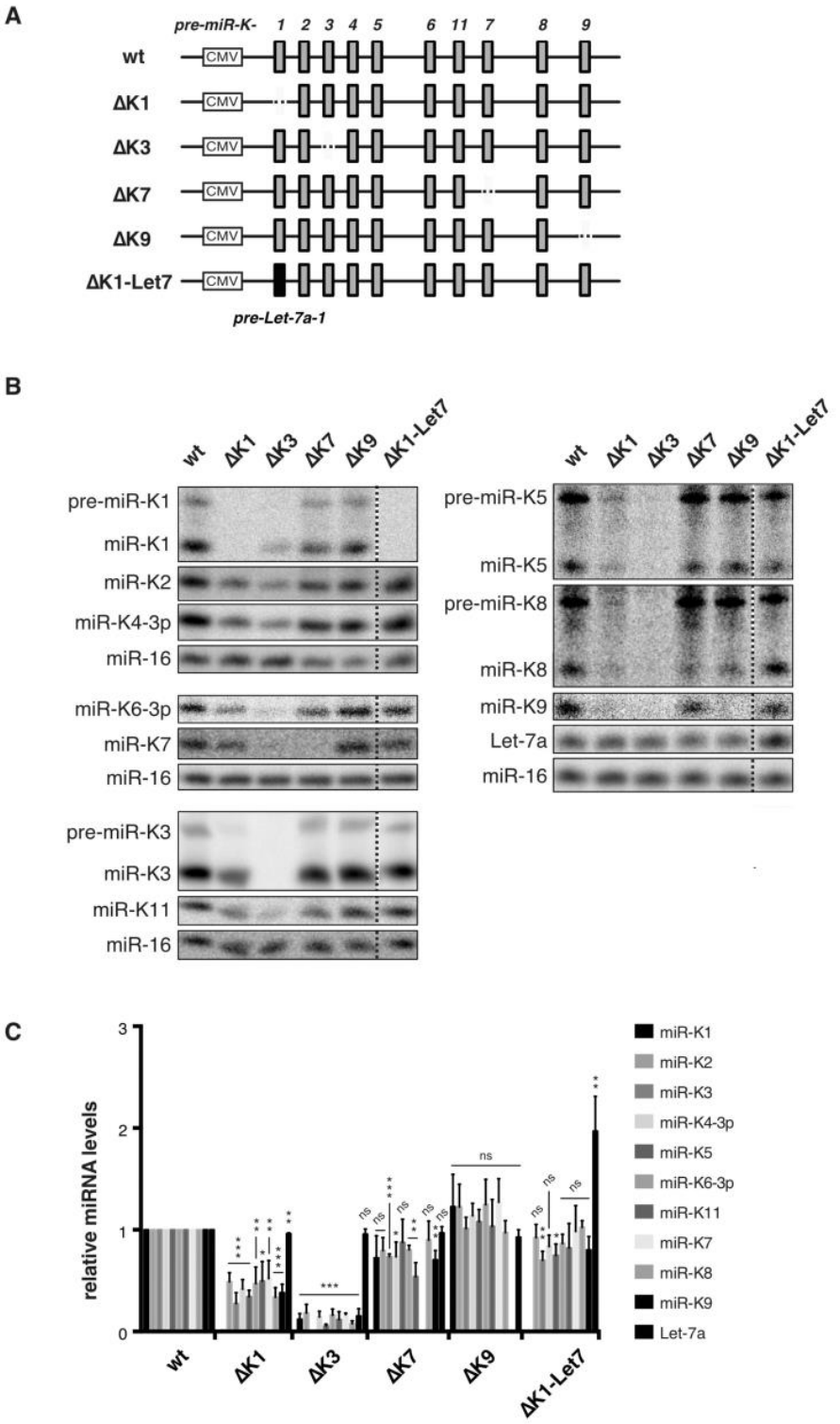

Figure 2. Mutational analysis reveals *cis* regulation within KSHV miRNA cluster. (**A**) Schematic view of pri-miR-K10/12 wt or mutant constructs used in the study. KSHV or hsa Let-7a-1 miRNA hairpins are represented by grey and black bars, respectively. Cytomegalovirus (CMV) promoter is shown. (**B**) Northern blot analysis of the accumulation of mature miRNAs, after overexpression of wt and mutant constructs in Hek293Grip cells ($n = 3$). MiR-16 was probed as a loading control. Dotted lines indicate where the blot was cut. (**C**) Histogram showing the relative expression of the clustered miRNAs from the mutated pri-miR-K10/12 constructs compared to the wt. Error bars were obtained from three independent experiments and *P*-values were obtained using unpaired *t* tests comparing wt versus mutant for each miRNA. ns: non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Microprocessor may help to favor the processing of the other miRNAs from the cluster. If true, an RNA mimicking the initial cut of one or the other of these two pre-miRNAs would lead to a better processing of the remaining pre-miRNAs.

To test this, we performed *in vitro* processing of two different *in vitro* transcribed RNA mimics, namely cut-K1 and cut-K3. Figure 3A gives a schematic view of the RNA molecules. Briefly, cut-K1 is composed of an RNA fragment containing pre-miR-K2 to pre-miR-K9 and including pre-miR-K11. Its 5′ end starts just downstream of the 3′ end of pre-miR-K1 3p arm, as it would be after Microprocessor cleavage. Cut-K3 RNA is the combination of two independent RNA fragments. One comprises pre-miR-K1 and pre-miR-K2 and its 3′ end finishes just upstream of the 5′ end of pre-miR-K3 5p arm. The second embeds pre-miR-K4 to pre-miR-K9, including pre-miR-K11, and its 5′ end starts just downstream of the 3′ end of pre-miR-K3 3p arm. These constructs were incubated with total protein extracts from cells over-expressing Drosha and DGCR8 and pre-miRNA products were analyzed by northern blot analysis (Figure 3B). We analyzed pre-miRNAs based on their proximity (pre-miR-K1, -K3 and -K4) or not (pre-miR-K6, -K7, -K11 and -K8) to the deleted pre-miRNAs and the fact that their levels obtained in our kinetic analysis were low compared to the corresponding mature miRNA levels measured in infected cells (miR-K6, -K7 and -K11). Pre-miR-K4 and -K8 were chosen as controls since they were both efficiently processed.

Cut-K1 RNA gave rise to all the tested pre-miRNAs from the cluster, with the exception of pre-miR-K1 as expected. Whereas most of them are produced to similar levels as from the wt transcript, pre-miR-K3 and to a minor extent pre-miR-K4 accumulates significantly ∼1.6- and ∼1.3-fold more respectively when compared to wt condition.

RNA mimicking the cleavage of pre-miR-K3 led to significantly more pre-miR-K11 (∼1.5-fold) and to a milder extent more pre-miR-K8 (∼1.3-fold) whereas pre-miR-K1, -K4 and -K6 levels were unchanged. Pre-miR-K7 showed a small increase (∼1.25-fold) but this was not statistically significant. Whereas cut-K1 showed rather local effect, cut-K3 increased the processing levels of pre-miRNAs at long distances.

Altogether, our results show that initial processing of pre-miR-K1 or pre-miR-K3 does not dramatically improve *in vitro* the overall maturation by the Microprocessor of the other miRNAs within the cluster. On the contrary, it affects the processing of only few pre-miRNAs. These results may emphasize the necessity of miR-K1 or miR-K3 hairpins to be an integral part of the cluster to exert a *cis*-regulatory function and/or a sequential processing of the different pre-miRNAs.

Blocking pre-miR-K1 cleavage by an antisense LNA oligonucleotide phenocopies its deletion

The use of antisense oligonucleotides has been described as an efficient approach to suppress miRNA function by sponging the mature miRNA (48). Interestingly, Hall et al. described that antisense LNA can also inhibit miRNA maturation steps (49). Indeed, an LNA oligonucleotide targeting the liver specific miR-122 also binds to pri-miR-122 and pre-miR-122, invading the stem-loop structure and hindering recognition by the Microprocessor and Dicer. This may account for ∼30% of the total inhibition of miR-122 activity. We therefore decided to use a similar strategy to block the processing of miR-K1 hairpin in order to downregulate the whole cluster. The LNA oligonucleotide that we used consists of 20 nt fully complementary to mature miR-K1-5p arm and contains 8 LNA residues in the middle part (from nt 8 to nt 15) (Supplementary Table S1). Using a similar oligonucleotide, Gao and colleagues managed to efficiently suppress miR-K1 activity (9). In their study, they did not assess whether this was solely due to the sponging effect of mature miRNA, or whether this also decreased miRNA biogenesis.

The previously described construct containing the KSHV miRNAs cluster was transfected in HEK293Grip cells together with an LNA targeting miR-K1, namely LNA@K1, or a control LNA (Supplementary Table S1). We then measured the levels of mature miRNAs from the entire cluster by northern blot analysis (Figure 4A, B). As expected, miR-K1 accumulation was strongly decreased (∼3.7-fold) upon treatment with LNA@K1 compared to treatment with the control LNA. Interestingly, the levels of all the other miRNAs within the cluster were also negatively affected (∼2.2- to almost 6-fold decrease) (Figure 4B). As a control, endogenous Let-7a was not affected, since its level was unchanged whatever the LNA treatment. Thus, inhibiting processing by antisense LNA targeting miR-K1 phenocopies the impact of ΔK1 mutant on the expression of the clustered miRNAs.

Since LNAs also have the capacity to bind DNA, there is a possibility that LNA@K1 could interfere with transcription of the miRNA cluster and thus explain such a global effect. We therefore performed RT-qPCR to evaluate the levels of pri-miR-K10/12 in control LNA and LNA@K1 conditions. Figure 4C shows that pri-miR-K10/12 level was not affected by LNA@K1 treatment, ruling out a possible inhibition at the transcription step.

In conclusion, we were able to negatively affect the expression of the 10 clustered miRNAs of KSHV solely by targeting miR-K1 sequence. Since it does not interfere with transcription, this downregulation most likely occurs at the post-transcriptional level, probably by interfering with the Microprocessor recognition and/or cleavage of pre-miR-K1.

Targeting miR-K1 inhibits the expression of the cluster in infected cells

So far, we have demonstrated that the whole cluster can be downregulated by using one single molecule targeting miR-K1 sequence. However, our experimental settings did not mirror natural conditions of KSHV infection, since the cluster was expressed from a plasmid. In order to test whether this *cis* regulation exists in a context closer to physiological infection, we decided to apply our antisense LNA strategy in HEK293FT cells carrying recombinant KSHV genomes (HEK293FT-rKSHV) (43). Similar to physiological conditions, rKSHV in cells remains mostly in latent state and it produces all viral miRNAs, even though their global

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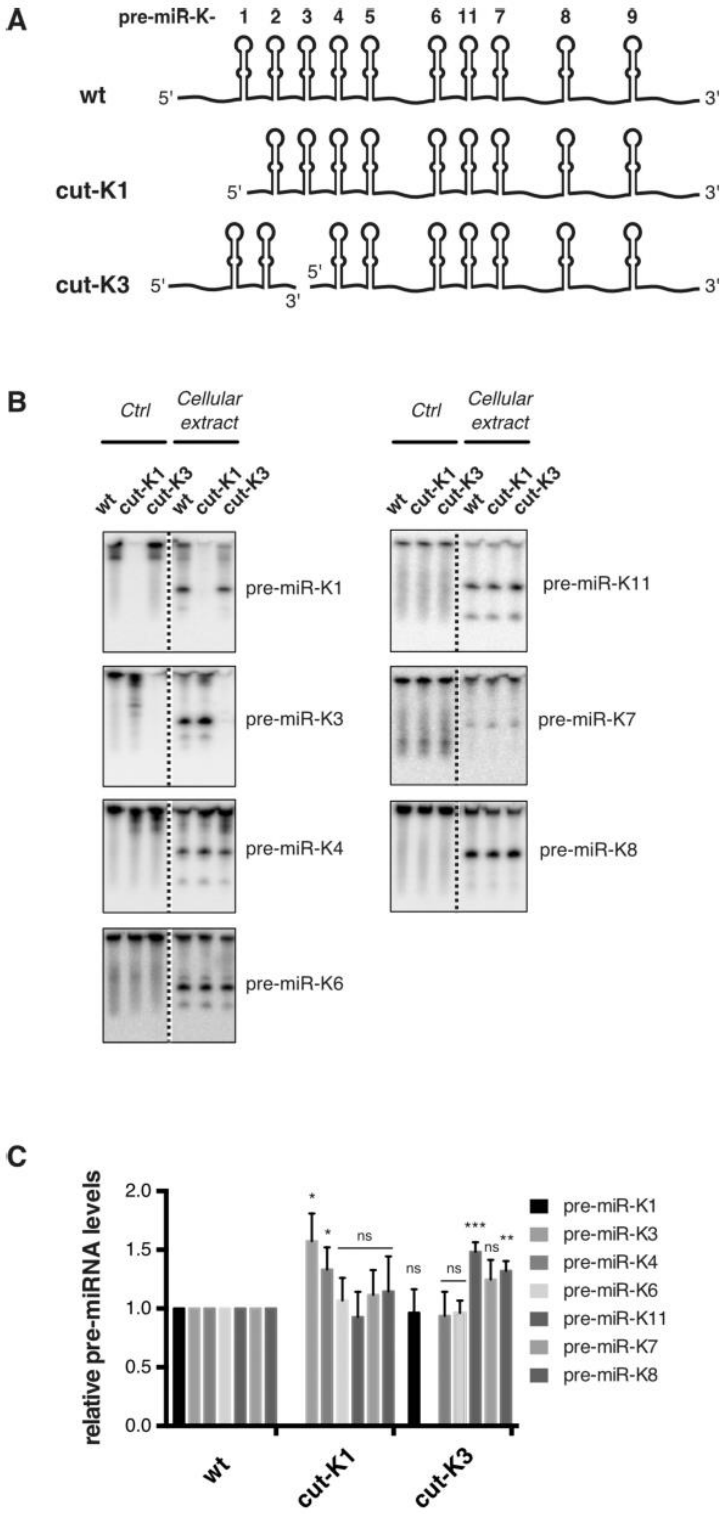

Figure 3. *In vitro* maturation assays using RNA mimicking miR-K1 and miR-K3 hairpins cleavage. (**A**) Schematic view of *in vitro* transcribed RNAs used in the study. Cut-K1 and cut-K3 RNAs mimic cleavage products by Drosha/DGCR8 of pre-miR-K1 and pre-miR-K3, respectively. As a result, cut-K3 is composed of 2 RNA fragments. (**B**) Northern blot analysis of pre-miRNAs produced after 45 min incubation of 1000 fmol of *in vitro* transcribed RNAs with HEK293Grip cells total protein extract where Drosha and DGCR8 were overexpressed (right part of blot) or lysis buffer (left part of blot). Dotted lines indicate where the blot was cut. (**C**) Histogram showing the relative level of pre-miRNAs compared to the wt. Error bars were obtained from three independent experiments and p-values were obtained using unpaired t tests comparing wt versus mutant for each pre-miRNA. ns: non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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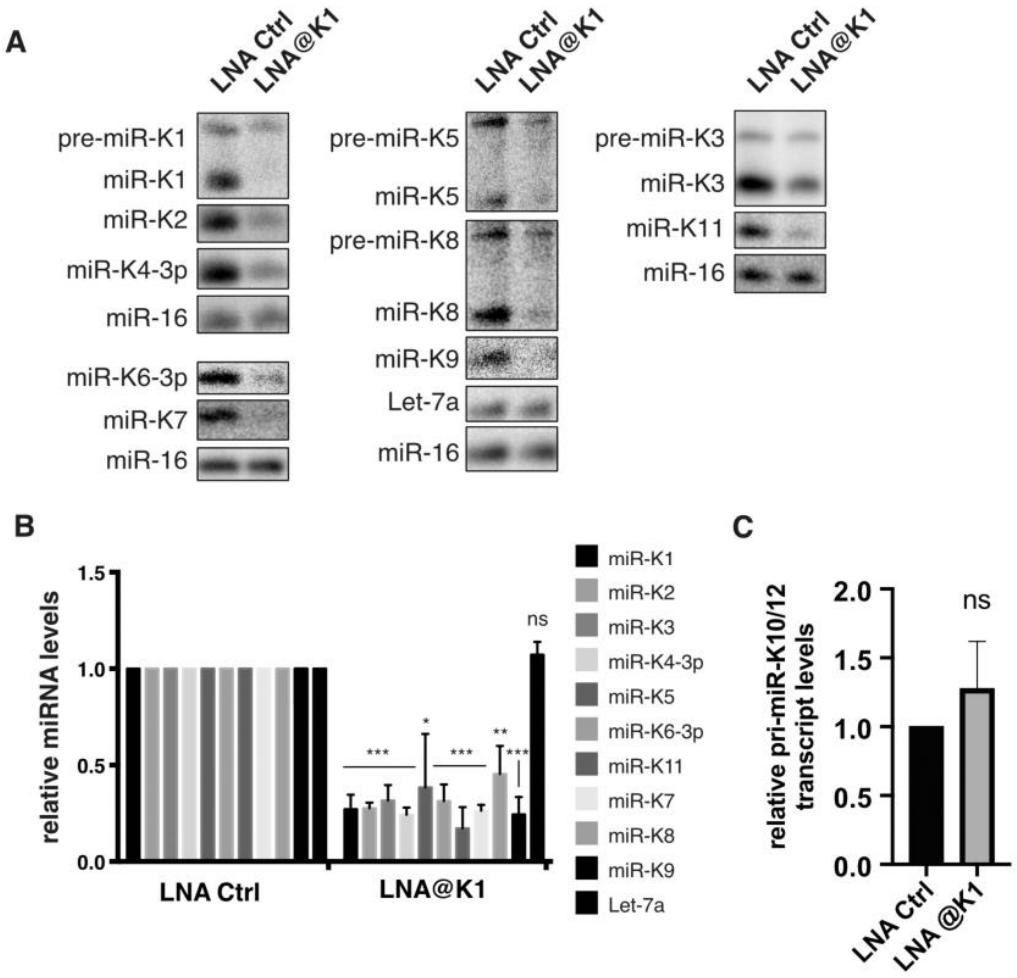

Figure 4. Antisense LNA targeting miR-K1 inhibits expression of other miRNAs from the cluster. (**A**) Northern blot analysis of the accumulation of mature miRNAs, after overexpression of wt plasmid in HEK293Grip cells during 48h ($n = 3$), with 20 nM control LNA (Ctrl LNA) or antisense LNA to miR-K1 (LNA@K1) treatment. Let-7a and miR-16 were probed as a control of miRNA expression and as a loading control, respectively. (**B**) Histogram showing the relative expression of the different miRNAs upon treatment with LNA@K1 compared to control LNA. Error bars were obtained from 3 independent experiments and p-values were obtained using unpaired t test with ns: non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**C**) The expression of the KSHV miRNA primary transcript pri-miR-K10/12 was measured by RT-qPCR in total RNA samples from (B) and normalized to GAPDH.

expression is much lower, probably due to the number of viral episomes per cell.

By using the same antisense oligonucleotide as previously described (LNA@K1) in these cells, we were not able to see any global downregulation of the cluster, with the exception of miR-K1(data not shown). We hypothesized that most of the LNAs transfected into the cells were probably sponged by the mature miR-K1 molecules already abundantly present in the cell, as opposed to the situation where miRNAs are expressed from a plasmid concomitantly with the LNA treatment. Thus, only a limited amount of the LNA would be available for the microprocessor inhibition and the potential impact on other miRNAs is too low to be measured.

To cope with this situation, we designed a new LNA complementary to the opposite strand of pre-miR-K1 stem loop (LNA@K1*). Given that this new molecule will not be sequestrated by the mature miR-K1, more oligonucleotides can reach the nucleus and interfere with pre-miR-K1 processing. In addition, this would also confirm that the effect we observe on the expression of the cluster is dependent on the processing event of pre-miR-K1 and not on a downstream function of the mature miR-K1.

First, to show that LNA@K1* can indeed interfere with processing of miRNA from the cluster, we performed the experiment in HEK293Grip cells by co-transfecting the pri-miR-K10/12 construct with either LNA@K1* or a control LNA and assessed the expression of mature miR-K1, -K4 and -K11 by RT-qPCR. Upon treatment with LNA@K1*, the accumulation of all three miRNAs dropped substantially compared to the treatment with the control LNA, while the level of Let-7a remained unchanged (Figure 5A). As previously, a potential impact of LNA treatment on cluster transcription was verified and only a mild decrease (30%) of pri-miR-K10/12 could be observed between control and LNA@K1* conditions (Figure 5B). We thus

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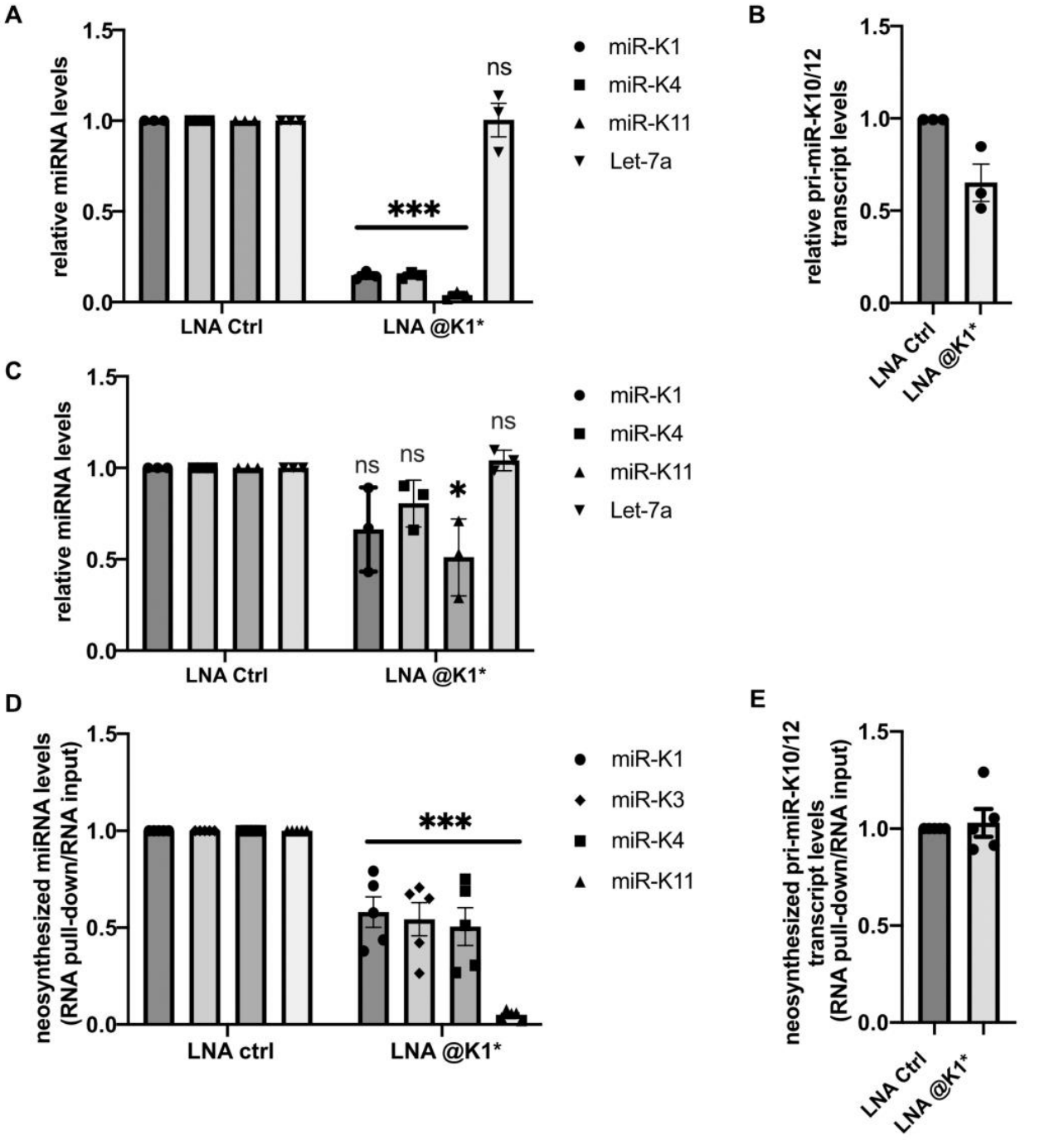

Figure 5. Inhibition of pre-miR-K1 processing impacts the expression of other viral miRNAs in infected cells. (**A**) Levels of mature miRNAs in HEK293Grip cells co-transfected with pri-miR-K10/12 expression plasmid and LNAs complementary to miR-K1* or control LNA. The analysis was performed on total RNA extracted 48 h post-transfection and miRNA levels were normalized to U48. (**B**) Measure of pri-miR-K10/12 expression in samples from (A), GAPDH was used as a normalizer. (**C**) Levels of mature miRNAs in HEK293FT-rKSHVcells transfected twice with 20nM of LNAs complementary to miR-K1* or control LNA. U48 was used as a normalizer. (**D**) Accumulation of neosynthesized miRNAs in HEK293FT-rKSHV cells transfected with either LNA complementary to miR-K1* or control LNA. 3 h of metabolic labelling with 300 μM 4sU was performed 24 h after LNA transfection and levels of mature miRNAs were measured in total RNA (input) and in isolated newly synthesized fraction (pull-down). To account for variation in pull-down efficiencies, enrichment of miRNA levels in pull-down over input were determined after normalizing to Let-7 levels. (**E**) Accumulation of neosynthesized pri-miR-K10/12 measured in the samples from (**D**). The same approach was used to determine the enrichment of pri-miRNA levels in the pull-down over input except that CYC1 was used to normalize the data instead of Let-7. Mature miRNAs and pri-miR-K10/12 in all experiments were quantified by RT-qPCR. All results are displayed relative to control samples set to 1. Bars represent mean ± s.e.m of three (A, B, C) or five (D, E) independent experiments. Statistical significance was verified by unpaired t test with ns: non-significant, $*P < 0.05$, $***P < 0.001$.

confirmed the LNA@K1*-mediated post-transcriptional inhibition occurring during miRNA processing. However, transfection of this LNA oligonucleotide into HEK293FT-rKSHV cells resulted only in a mild impact on KSHV miRNA expression, as demonstrated for miR-K1, -K4 and -K11. While only miR-K11 levels were significantly reduced, miR-K1 and -K4 did not decrease in a statistically significant manner (Figure 5C). This might be explained by a differential stability of the different viral miRNAs in infected cells. In contrast to ectopically expressed cluster, infected cells already contain a certain amount of mature viral miRNAs and their differential turnover would directly influence the sensitivity of our assay. If the half-lives of miR-K1 and -K4 are longer than the half-life of miR-K11, then it might prove difficult to assess the impact of inhibiting their processing. As a solution, we set to measure the accumulation of newly synthesized miRNAs. LNA-transfected cells were therefore incubated with 4-thiouridine (4sU), which allowed the isolation of novel transcripts via selective biotinylation and pull-down on streptavidin beads. Due to the variation between the efficiency of individual pull-downs, viral miRNAs were analyzed in total RNA (input) as well as in isolated pull-down fraction and their enrichment in pull-down over input was expressed relative to Let-7a. Following two different experimental protocols, we observed a consistent and significant reduction in neosynthesized miR-K1, -K3, -K4 and -K11 upon treatment with LNA@K1* (Figure 5D and Supplementary Figure S8). In addition, we verified by RT-qPCR that the LNA had no impact on the levels of neosynthesized pri-miRNA transcript (Figure 5E). Thus, we have shown that the expression of the KSHV miRNA cluster can be inhibited by using one single oligonucleotide targeting pre-miR-K1 and that this phenomenon indeed takes place also within KSHV-infected cells. Although at this stage, we cannot formally conclude that what is important for the *cis*-regulation is the processing event or the presence of a stem-loop, our results clearly point toward the importance of pre-miR-K1.

DISCUSSION

In this study, we explored a complex layer for miRNA biogenesis regulation in which the expression of miRNA hairpins within a large miRNA cluster is interdependent. We showed that two miRNA hairpins, namely miR-K1 and -K3 hairpins, within the intronic KSHV miRNA cluster were required for the optimal expression of the remaining miRNAs. Indeed, their deletion within an expression plasmid drastically diminished clustered miRNAs expression in cell. Similarly, antisense LNAs that bind to the pre-miR-K1 hairpin and inhibit its processing by impeding the recognition and/or cleavage by the Microprocessor led to global downregulation of the cluster. This strategy also allowed to decrease viral miRNAs in the more natural context of infected cells. Furthermore, our data showed that the pre-miRNA feature *per se*, at least for pre-miR-K1, is responsible for this regulation since pre-miR-K1 could be replaced by a heterologous pre-miRNA. Altogether, these results indicate that miR-K1 and miR-K3 hairpins are important *cis*-regulatory elements for the expression of the KSHV clustered miRNAs. Previously, the Krueger laboratory produced bacmid constructions containing KSHV genome deleted from individual KSHV miRNAs and they also noticed a decrease in expression of other viral miRNAs in mutants deleted of miR-K1 and miR-K3 (50). Here, we explain these observations through the *cis*-regulatory function of these two pre-miRNAs.

Interdependency of clustered miRNA hairpins was documented previously for different miRNAs and in diverse species (37–40,40,41). However, it has so far only been studied for small clusters of two or three miRNA hairpins where a helper hairpin assists the processing of a neighboring suboptimal hairpin. Here, we show *cis* regulation for the first time within a large cluster of 10 miRNA hairpins. Thus, 'assisted' miRNA hairpins are not all proximal to the helper hairpins. In addition, they are not necessarily suboptimal as demonstrated by our 2D structure probing data published previously (31). So, in the case of KSHV miRNAs cluster, *cis* regulation might result from a more complex mechanism. One possibility could rely on the recruitment of the Microprocessor by miR-K1 and miR-K3 hairpins, inducing its local concentration to re-initiate further maturation events on the same polycistronic pri-miRNA. From a conceptual point of view, this might be compared to ribosome re-initiating translation on a same mRNA. However, from a mechanistic point of view, Microprocessor would not scan the pri-miRNA but rather cycle from the cleaved miRNA hairpin and relocate on promiscuous miRNA hairpin. Two alternate but not mutually exclusive models may help this repositioning. One model would be that the globular and compact 3D structure of pri-miRNA may *per se* help the Microprocessor to relocate. Indeed, structural study performed on pri-miR-17–92 shows such organization (30). We previously determined the 2D structure of KSHV miRNA cluster using SHAPE method (31). Although we could not conclude on a compact arrangement of the viral pri-miRNA, we did observe numerous stem–loops, containing or not miRNAs, that could participate to long-distance interaction to maintain such compact 3D structure. A second model would involve protein cofactors able to assist in the recruitment of the Microprocessor on the neighboring miRNA hairpin. Very recently, it was shown that ERH and SAFB2 can interact with the Microprocessor and their ability to dimerize may even mediate multimerization of the Microprocessor and allow its simultaneous binding to several hairpins (37,40,42,51). However, this model remains to be clearly established. For example, the role of ERH and SAFB2 proteins may be specific to only certain miRNA clusters and not all clustered miRNAs may depend on such assistance. Indeed, using CRISPR/Cas9 editing, Lataniotis et al showed that editing of miR-195 led to a decrease of its neighboring miR-497, whereas no such interdependency was observed for miR-106–25 or miR-17–92 clusters (39). Another study, based on genetically engineered mice, also showed that deletion of any miRNA from the cluster miR-17–92 did not alter the expression of the other miRNAs (52). In the case of the KSHV cluster, pre-miR-K1 and pre-miR-K3 may interact with high affinity with a protein cofactor, potentializing further interaction with the other pre-miRNAs. This protein cofactor might then recruit or improve the Microprocessor activity.

Another mechanism may rely on specific structural constrains that could be resolved after cleavage of miR-K1 and miR-K3 hairpins, rendering the other miRNA hairpins more accessible to the Microprocessor. Indeed, previous studies reported that the globular fold of the pri-miR-17–92 may autoregulate its processing (30,53,54). Chaulk et al. demonstrated that the compact fold of the cluster is adopted through a specific tertiary contact between pre-miR-19b and a non-miRNA hairpin resulting in reduced miR-92a expression whereas this inhibitory effect could be abolished by disrupting this contact (54). However, our data obtained from our *in vitro* processing assays of RNA mimicking initial cleavage of miR-K1 or miR-K3 hairpins show neither global nor drastic improvement of other miRNA hairpins maturation. This suggests that miR-K1- and miR-K3-dependent regulation may rather occur *in cis*, when the two hairpins are still present in the cluster. Cleavage of the cluster may also happen in a hierarchical way similarly to pri-miR-17–92 (55). In that case, the restricted impact of pre-miR-K1 or pre-miR-K3 cleavage may reflect that processing of the KSHV cluster occurs in different steps having each downstream additive positive effect. Unfortunately, the experimental design of our *in vitro* processing assays did not allow to follow complete processing of the cluster. Thus, we probably observed only the first cleavage events.

Another intriguing aspect of this work is the fact that despite their efficient processing by the Microprocessor, the levels of mature miR-K1 and -K3 are low in infected cells. It would be interesting to know if these two pre-miRNAs are as well efficiently exported into the cytoplasm and/or processed by Dicer and how the excess of pre-miRNAs is eliminated from the cell. It was shown previously that MCP-1-induced protein-1 (MCPIP1), a suppressor of miRNA biogenesis and involved in immunity, could directly cleave KSHV pre-miRNAs through its RNase domain (56,57). However, while their results show that pre-miR-K1 and -K3 can be bound and cleaved by MCPIP1, almost all remaining KSHV pre-miRNAs are prone to be degraded as well, suggesting that another protein or mechanism might be involved in selective decay of excessive pre-miR-K1 and –K3.

KSHV miRNAs are involved in a multitude of functions related to the viral life cycle, immune escape, and pathogenesis (reviewed by e.g. (58,59)). Latent infection is one of the biggest hurdles in the treatment of KSHV-related pathologies. Therefore, the role of viral miRNAs in latency maintenance is of great importance. Several groups have shown that artificial reduction of levels of particular KSHV miRNAs can lead to higher viral reactivation. For example, the main transactivator of lytic cycle RTA is directly regulated by at least two miRNAs, miR-K7-5p and miR-K5 (10,60). In addition, miR-K1 indirectly controls latency maintenance by downregulation of the inhibitor of NF-kB pathway, IkBa (9). Furthermore, many of their protein targets validated to date are involved in pathways important in oncogenesis (61). Interestingly, miR-K11 presents the same seed sequence as a well-known oncomiR miR-155 and both miRNAs target a common subset of genes (62,63). The Gao's and Renne's groups have shown that several KSHV miRNAs participate to the transforming potential of KHSV by targeting cell growth and survival pathways (64,65). Targeting several, if not all the KSHV miRNAs can therefore represent a valuable therapeutic option. Recently, Ju et al. have proposed a therapeutic strategy based on LNA-modified oligonucleotides complementary to miR-K1, -K4 and -K11 coupled to carbon dots for better intracellular delivery (66). While they use a combination of three different molecules, our results suggest that blocking the processing of one single miRNA might lead to a global decrease of the entire miRNA cluster. Given that about 25 to 40% of all human miRNAs are embedded in clusters (25,26), and given the growing body of evidence that they are implicated in disease, the ability to suppress their expression as a whole might be of importance for future therapies.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article (and its supplementary file). Requests for material should be made to the corresponding authors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: A.F., S.P., M.C. and M.V. conceived the project. M.C., M.V., S.P. and A.F. designed the work. M.C., M.V., R.R. and A.F. performed the experiments and analyzed the results. P.D. performed the kinetic analysis. E.E. and P.M.O. generated the HEK293FT-rKSHV cells. A.F. and S.P. coordinated the work and S.P. assured funding. M.V., P.D., S.P. and A.F. wrote the manuscript with input from the other authors. All authors reviewed and approved the final manuscript.

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SUPPLEMENTAL MATERIAL

Cis regulation within a cluster of viral microRNAs

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SUPPLEMENTAL METHOD

Kinetic analysis

Practical problems and fitting method. There is a significant upward curvature of the low-amplitude cleavage curves around t = 0 (see for example pre-mir-K2 and pre-miR-K7 for 20130530). This was interpreted as a linearity problem of the IP response since this is only visible for the low-amplitude curves (accordingly, it is almost invisible with the Exp#1 data having higher amplitudes). Such a feature cannot be accounted for by equations (3) and (3') (see main manuscript) imposing a downward curvature around t = 0. In order to nevertheless use these equations, a simple correction was devised to mimic this lack of linearity for the lowest values of $K_i(t)/R_0$. For this, instead of using directly $Y_i = K_i(t)/R_0$ to fit the experimental curves, a modified value of Y_i was used according to the response function:

$$Y_i \rightarrow \mathcal{R}(Y_i) = \frac{(Y_i/\varepsilon)^2}{1+(Y_i/\varepsilon)^2}\, Y_i + \frac{1}{1+(Y_i/\varepsilon)^2}\, {Y_i}^2 \qquad (4)$$

with ε a small value. This response function gives $\mathcal{R}(Y_i) \approx {Y_i}^2$ for Y_i of order ϵ, which provides the upward curvature close to $Y_i = 0$, and it is transformed smoothly into $\mathcal{R}(Y_i) = Y_i$ for increasing values of Y_i above ϵ. This *ad-hoc* procedure was quite effective to improve the quality of results, particularly for the Exp#2 data. The value of ε was tuned to 0.5 % to obtain the best fit of all curves with a minimum value of the global sum of the errors on f_i and k_i^+ for both data sets. Such a low value indicates that the correction is indeed a minor one. Practically, the fit was done with the function NonlinearModelFit (with Method → "ConjugateGradient") in *Mathematica* V.11 from Wolfram Research.

Using a more stringent kinetic model of pri-miRNA cleavage. The simple model used in the study allowed us to obtain excellent fits (Figure 1 and Figure S1), but with three free parameters (f_i , k_i^+ and k_i^-) per experimental curve. In order to use a more stringent test, we imposed two restrictions for a better representation of reality. First, we imposed that the variations of the rates of cleavage by Drosha from one experiment to another one should only result from the amount of Drosha in each experiment. For this, we imposed a strict proportionality of the two sets of k_i^+. Second, we imposed that the cleaved fraction f_i of R_0 yielding the pre-miR K_i was the same for the two experiments (Figure S2). This more stringent method, therefore, involves only two adjustable parameters per curve, which represents quite a significant reduction of the degrees of freedom.

The fitting of the experimental cleavage curves with the kinetic model with three free parameters (see Material and Methods) per curve led to excellent agreement (Figure 1B and Figure S1B). Note that the slight correction for non-linearity (see Supplemental Material) was important to obtain this result. As expected, the results with a more stringent model with only two free parameters per curve were less good but mostly for the low-amplitude curves with the lowest signal-to-noise ratio (Figure S2). This indicates that the excellent agreement with three parameters was not simply the result of a meaningless numerical fit, which is in good support for the simple kinetic model in use. The numerical results are shown in Table S1.

***In vitro* pre-miRNA stability assays**

Measure of stability of *in vitro* transcribed pre-miRNAs was performed in the same conditions as the in vitro Drosha miRNA processing assays (see Materials and Methods) except that whole cell lysate was used without overexpressing Drosha and DGCR8. Briefly, 500 fmol of each of the four tested pre-miRNAs were pooled together, denatured, let to refold and incubated in total HEK293Grip cells extract for increasing times at 37°C. 1/10 of the phenol-extracted and ethanol-precipitated RNAs was used for northern blot analysis. A standard curve was generated by loading decreasing amounts (50 to 3.125 fmol) of corresponding *in vitro* transcribed pre-miRNAs.

Supplemental bibliography

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SUPPLEMENTAL TABLES

Table S1. Sequences of oligonucleotides used in this study.

Usage	Sequence 5' to 3'	Name
RNA preparation	GAAATTAATACGACTCACTATAGAATGCGTGCTTCTGTTTGAAGG	Pri-miR-K10/12 T7 forward primer
	TTTACCGAAACCACCCAGAGGC	Pri-miR-K10/12 Reverse primer
	GAAATTAATACGACTCACTATAGATTACAGGAAACTGGGTGTAAG	Pre-miR-K1 T7 forward primer
	GGTTGCAGGAAACAGGTGCTG	Pre-miR-K1 reverse primer
	GAAATTAATACGACTCACTATAGAACTGTAGTCCGGGTCGATC	Pre-miR-K2 T7 forward primer
	CAGCTCTAGCCCTGGAAGATC	Pre-miR-K2 reverse primer
	GAAATTAATACGACTCACTATAGTCACATTCTGAGGACGGCAG	Pre-miR-K3 T7 forward primer
	TGTCACATTCTGTGACCGCGA	Pre-miR-K3 reverse primer
	GAAATTAATACGACTCACTATAGAGCTAAACCGCAGTACTCTAGGG	Pre-miR-K4 T7 forward primer
	TCAGCTAGGCCTCAGTATTCTA	Pre-miR-K4 reverse primer
	GAAATTAATACGACTCACTATAGAGGTAGTCCCTAGTGCCCTAAG	Pre-miR-K5 T7 forward primer
	CCGGCAAGTTCCAGGCATCCTA	Pre-miR-K5 reverse primer
	GAAATTAATACGACTCACTATAGCCAGCAGCACCTAATCCATCG	Pre-miR-K6 T7 forward primer
	CTCAACAGCCCGAAAACCATCA	Pre-miR-K6 reverse primer
	GAAATTAATACGACTCACTATAGAGCGCCACCGGACGGGGATTTATG	Pre-miR-K7 T7 forward primer
	AGCGCCAGCAACATGGGATCA	Pre-miR-K7 reverse primer
	GAAATTAATACGACTCACTATAGACTCCCTCACTAACGCCCCG	Pre-miR-K8 T7 forward primer
	CGTGCTCTCTCAGTCGCGCCTA	Pre-miR-K8 reverse primer

	GAAATTAATACGACTCACTATAGACCCAGCTGCGTAAACCCCG	Pre-miR-K9 T7 forward primer
	TTACGCAGCTGCGTATACCCAG	Pre-miR-K9 reverse primer
	GAAATTAATACGACTCACTATAGGTCACAGCTTAAACATTTCTAG	Pre-miR-K11 T7 forward primer
	TCGGACACAGGCTAAGCATTAA	Pre-miR-K11 reverse primer
	GAAATTAATACGACTCACTATAGGCTCGTGTTTGGGCAAAACACATC	Cut-K1 T7 forward primer
	Same as pri-miR-K10/12 reverse primer	Cut-K1 reverse primer
	Same as pri-miR-K10/12 T7 forward primer	Cut-K3 5' fragment T7 forward primer
	TAGCCCATTACAGGCATTGTAG	Cut-K3 5' fragment reverse primer
	GAAATTAATACGACTCACTATAGGCCCCTCCAGGTCCAAGCGACG	Cut-K3 3' fragment T7 forward primer
	Same as pri-miR-K10/12 reverse primer	Cut-K3 3' fragment reverse primer
Northern blot	GCTTACACCCAGTTTCCTGTAAT	Probe for miR-K1
	CAGATCGACCCGGACTACAGTT	Probe for miR-K2
	TCGCTGCCGTCCTCAGAATGTGA	Probe for miR-K3
	TCAGCTAGGCCTCAGTATTCTA	Probe for miR-K4-3p
	CCGGCAAGTTCCAGGCATCCTA	Probe for miR-K5
	CTCAACAGCCCGAAAACCATCA	Probe for miR-K6-3p
	AGCGCCAGCAACATGGGATCA	Probe for miR-K7
	CGTGCTCTCTCAGTCGCGCCTA	Probe for miR-K8
	AGCGGGGTTTACGCAGCTGGGT	Probe for miR-K9
	TCGGACACAGGCTAAGCATTAA	Probe for miR-K11
	CGCCAATATTTACGTGCTGCTA	Probe for hsa miR-16

	AACTATACAACCTACTACCTCA	Probe for hsa Let-7a
Mutagenesis	GGTGCTGCCCAGGACGGCCGGATGCGGGCGCTCGTGTTTGGGCAAAACACATCCGCTGCC	Forward primer to delete pre-miR-K1
	GGCAGCGGATGTGTTTTGCCCAAACACGAGCGCCCGCATCCGGCCGTCCTGGGCAGCACC	Reverse primer to delete pre-miR-K1
	CGCAACAGCTACAATGCCTGTAATGGGCTACCCCTCCAGGTCCAAGCGACGAACCGCCCG	Forward primer to delete pre-miR-K3
	CGGGCGGTTCGTCGCTTGGACCTGGAGGGGTAGCCCATTACAGGCATTGTAGCTGTTGCG	Reverse primer to delete pre-miR-K3
	GATACCACGCAGCCGCGCATATTGGCGTTGTCACGGCCCGTGTGCCAGCCGCCTGGACG	Forward primer to delete pre-miR-K7
	CGTCCAGGCGGCTGGCACACGGGCCGTGACAACGCCAATATGCGCGGCTGCGTGGTATC	Reverse primer to delete pre-miR-K7
	CTATTCCAGTAGGTATACCCAGCTGGGTCTACCCGGCTGGGTAAATCCAGCTGTAATTC	Forward primer to delete pre-miR-K9
	GAATTACAGCTGGATTTACCCAGCCGGGTAGACCCAGCTGGGTATACCTACTGGAATAG	Reverse primer to delete pre-miR-K9
	TGCTGCCCAGGACGGCCGGATGCGGGCGTGAGGTAGTAGGTTGTATAGTTTTAGGG	Forward primer to insert pre-Let-7a-1
	CAGCGGATGTGTTTTGCCCAAACACGAGGAAAGACAGTAGATTGTATAGTTATCTC	Reverse primer to insert pre-Let-7a-1
Antisense LNAs	ATTGAATCAAACAGCCGACCAA	Control LNA
	GCTTACACCCAGTTTCCTGTAAT	LNA targeting miR-K1
	GGTTGCAGGAAACAGGTGCTGCC	LNA targeting miR-K1*
qPCR	CTTTGGTATCGTGGAAGGACT	*GAPDH* fw
	CCAGTGAGCTTCCCGTTCAG	*GAPDH* rev
	CCAGGGAAGCTGTTCGACTATTTC	*CYC1* fw
	CCAGGGAAGCTGTTCGACTATTTC	*CYC1* fw
	AAAACAGGAAGCGGGTTGGAC	*Pri-miR-K10/12* fw
	CCGCACCCTGCGTAAACAACC	*Pri-miR-K10/12* rev

T7 promoter sequence and LNA residues are underlined.

Table S2. Kinetic parameters for cleavage of KSHV miRNA hairpins within pri-miR-K10/12 by the Microprocessor *in vitro*.

	Exp#1			Exp#2		
pre-miR	***f* (%)**	**10 x k^+ (min^{-1})**	**100 x k^- (min^{-1})**	***f* (%)**	**10 x k^+ (min^{-1})**	**100 x k^- (min^{-1})**
K1	15.0 ± 1.0	1.50 ± 0.20	1.10 ± 0.20	17.0 ± 0.6	0.30 ± 0.01	3.00 ± 0.20
K2	1.7 ± 0.2	0.97 ± 0.20	0.32 ± 0.30	1.0 ± 0.1	0.53 ± 0.08	0.80 ± 0.30
K3	32.0 ± 2.0	0.82 ± 0.08	0.34 ± 0.20	40.0 ± 0.9	0.22 ± 0.005	2.20 ± 0.10
K4	15.0 ± 5.0	0.46 ± 0.20	1.70 ± 1.00	12.0 ± 12.0*	0.18 ± 0.18*	1.80 ± 1.80*
K5	11.0 ± 0.8	0.81 ± 0.09	0.57 ± 0.20	2.8 ± 0.6	0.58 ± 0.20	0.15 ± 0.50
K6	6.1 ± 5.0	0.38 ± 0.40	0.00 ± 2.00	2.8 ± 2.0	0.41 ± 0.30	0.00 ± 1.00
K11	11.0 ± 0.3	1.60 ± 0.10	1.10 ± 0.10	9.6 ± 9.6*	0.26 ± 0.26*	2.60 ± 2.60*
K7	3.4 ± 0.06	0.27 ± 0.005	2.70 ± 0.10	3.7 ± 0.1	0.14 ± 0.004	1.40 ± 0.10
K8	11.0 ± 0.5	2.30 ± 0.30	0.10 ± 0.10	13.0 ± 3.0	0.71 ± 0.20	1.80 ± 0.90
K9	7.0 ± 1.0	0.56 ± 0.10	0.05 ± 0.50	7.7 ± 6.0	0.32 ± 0.30	0.78 ± 2.00

Pre-miRNA accumulation levels *f* are in percentage of cleaved pri-miRNA in the assay (initial concentration = 16.7 nM, see Material and Methods). Cleavage rate constants k^+ and k^- are associated to cleavage by the Microprocessor or to residual Dicer or another RNase activity, respectively.

* when errors were extremely higher than the determined values, they were arbitrarily set at 100 %.

Table S3. Primary sequence and structural features determinants of miRNA stem-loops from KSHV pri-miR-K10/12.

Region or motif	**Terminal loop**	**Basal stem**		**Flanking single-stranded segments**	**$U_{-14}G_{-13}$ from the 5' cleavage**	**GUG/UGU in apical loop**	**'mismatched GHG' at 7-9 nt from the basal junction**	**Shannon entropy along the miRNA stem**	**No of positive criteria**
Criterion for optimal structure	([10-14] nt long)	~11 nt (±2)	Stable platforms	(at least one, ≥9 nt long)				low	
miRNA stem loop									
K3	Y (13)	Y (11)	Y	Y	Y	Y	N	Y	**7**
K1	Y (11)	Y (11)	Y	N	N	N	Y	Y	**5**
K11	Y (11)	Y (10)	Y	N	Y	Y	N	N	**5**
K8	Y (11)	N (7)	+/-	Y	N	Y	N	Y	**4.5**
K4alt	N (6)	Y (11)	Y	Y	N	N	N	Y	**4**
K6	N (9)	Y (11)	Y	Y	N	Y	N	N	**4**
K7	Y (14)	Y (13)	Y	N	N	Y	N	N	**4**
K9	N (8)	Y (11)	Y	N	N	N	N	Y	**3**
K4	N (6)	N (7)	+/-	N	N	N	N	Y	**1.5**
K2	N (16)	N (0)	N	N	Y	N	N	N	**1**
K5	N (9)	N (3)	+/-	N	N	N	N	N	**0.5**

H=any nt but G
In grey, expression is over 10%

SUPPLEMENTAL FIGURES AND LEGENDS

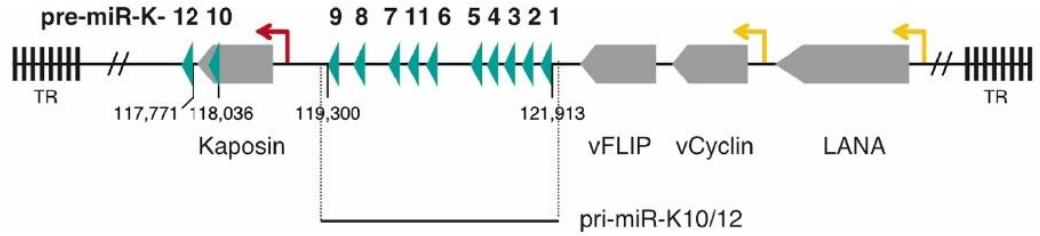

Figure S1. Genomic organization of KSHV miRNAs and location of pri-miR-K10/12. The twelve KSHV pre-miRNAs are localized in the latency locus and are indicated by green arrow heads. Ten of them are clustered in an intron (pre-miR-K1 to -K9 and pre-miR-K11) from which the sequence referred to as pri-miR-K10/12 derives, whereas pre-miR-K10 and -K12 are in the coding region and in the 3'UTR of Kaposin mRNA, respectively. Sequence coordinates were derived from reference sequence NC_009333.1. Open reading frames are in grey. Lytic promoter is represented by a red arrow and latent promoters by yellow arrows. TR, Terminal Repeats.

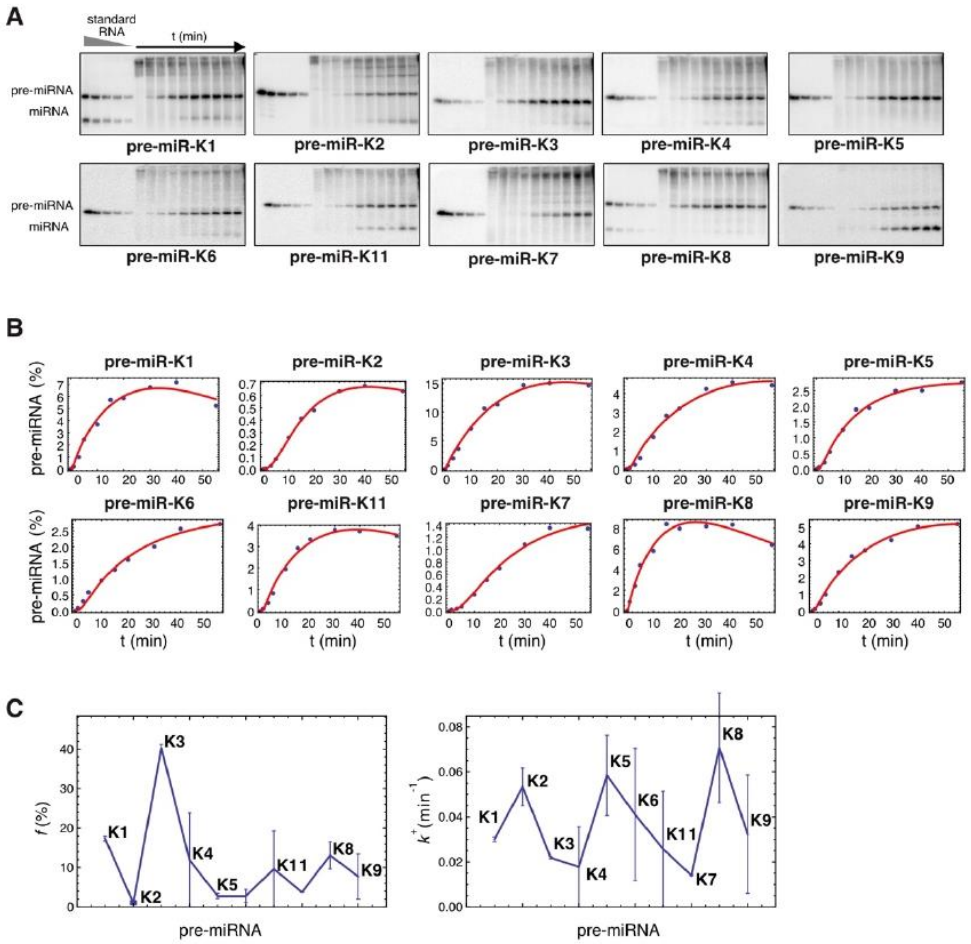

Figure S2. Kinetic analysis of KSHV clustered pre-miRNAs maturation *in vitro* by the Microprocessor (Exp#2).

(A) Northern blot analysis of the time course of *in vitro* processing assays using *in vitro* transcribed pri-miR-K10/12 and Hek293Grip cells total protein extract where Drosha and DGCR8 were overexpressed. *In vitro* transcribed pre-miRNAs and synthetic RNA oligonucleotides were loaded at decreasing concentrations as standards.

(B) Cleavage curves were obtained after plotting pre-miRNA product, in percentage of initial pri-miR-K10/12 substrate, according to time. The fits were obtained with the model involving three free parameters per curve (compare with Figure S3 for the more stringent model with two free parameters per curve).

(C) Processing efficiencies (left panel) and cleavage rate (right panel) were plotted in respect to miRNA hairpins showing variation among the clustered pre-miRNAs. The error bars come from standard procedures used to fit the experimental curves by minimizing the residuals between the experimental points and their theoretical estimates.

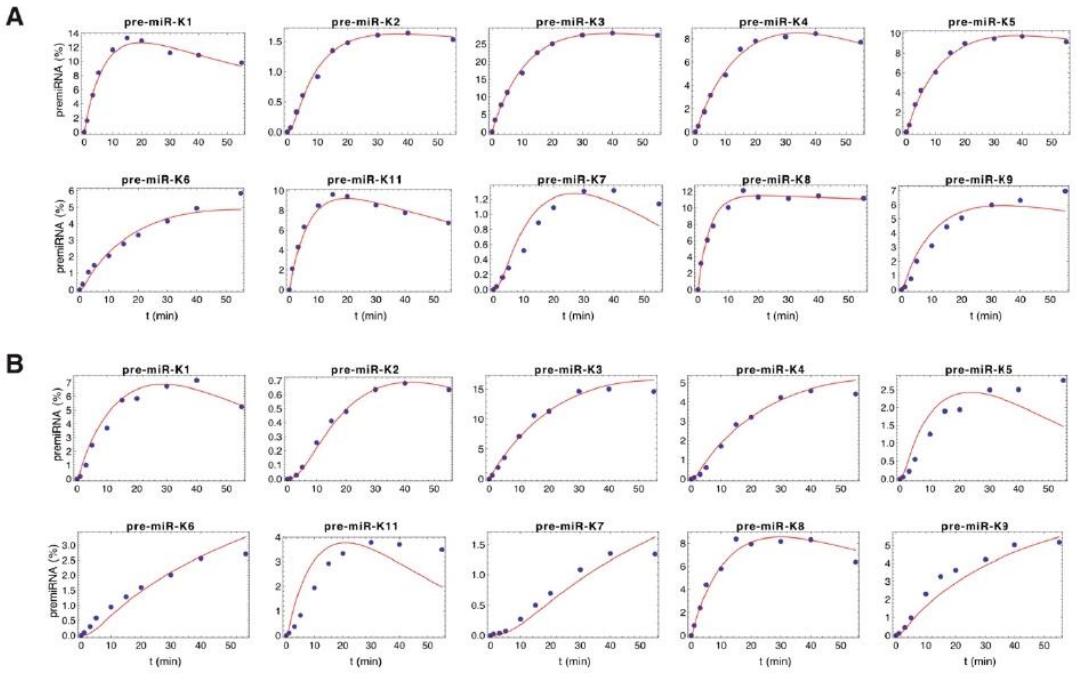

Figure S3. Joint fitting of the experimental curves for Exp#1 (A) and Exp#2 (B) with two free parameters per curve.

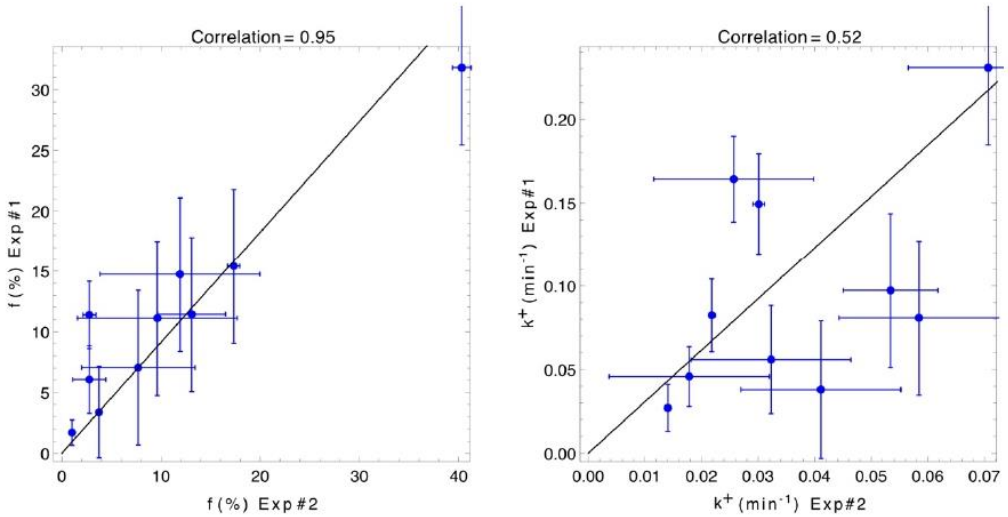

Figure S4. Correlation among experiments of *in vitro* processing assays.
Processing efficiencies (f in percentage, left panel) and cleavage rate constants (k^+ in min^{-1}, right panel) were compared between the two experiments analyzed in this study, namely Exp#1 and Exp#2.

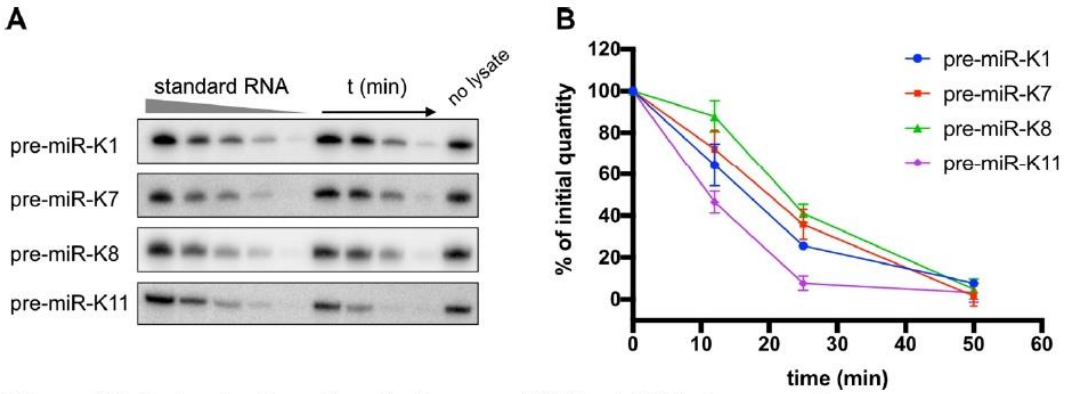

Figure S5. Determination of synthetic pre-miRNAs stability in processing assays. *In vitro* transcribed pre-miRNAs were incubated in whole cell lysate from HEK293Grip cells and submitted to conditions used for *in vitro* processing assays. Their decay was followed over time. Northern blots (A) were quantified by using standard pre-miRNAs and results from three replicates were plotted (B) relative to pre-miRNA quantity at 0 min.

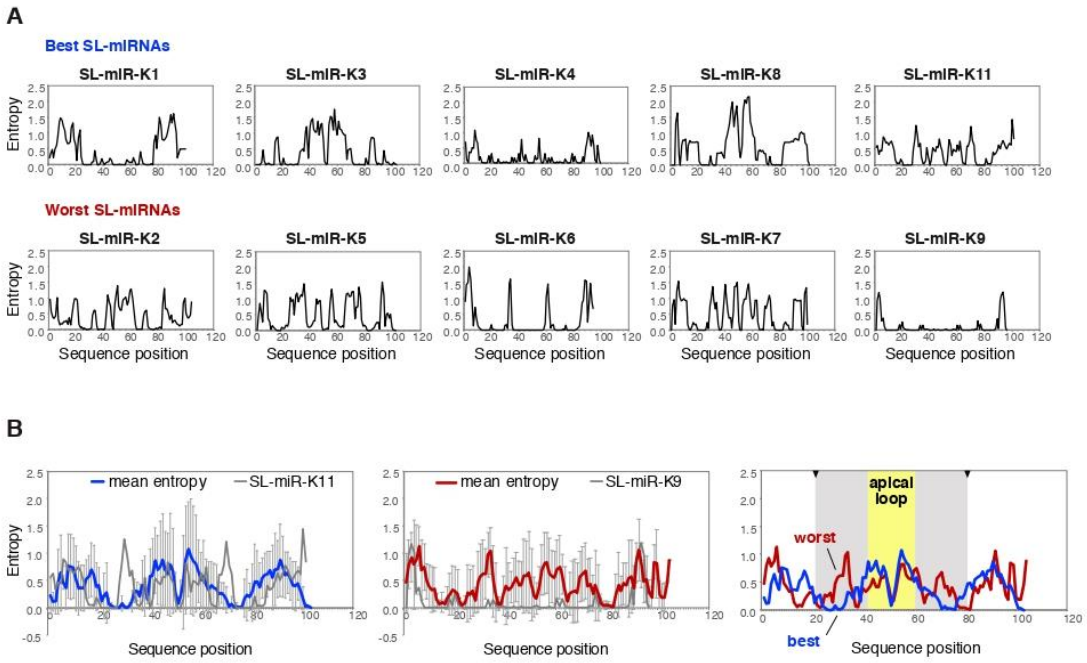

Figure S6. Positional entropy of KSHV miRNA hairpins.

(A) Shannon entropy was plotted across the sequence of individual miRNA hairpins, namely stem-loop (SL)-miRNAs, including the pre-miRNA plus 20 nucleotides on both sides, using RNAfold from ViennaRNA Web Services (Institute for Theoretical Chemistry, University of Vienna) (1, 2).

(B) Mean entropies of the best substrates (processed over 10%, blue curve, left panel) and of the worst substrates (processed below 10%, red curve, middle panel) were plotted across the sequence. Comparison of the two curves (right panel) show that the best substrates are enriched for low entropy along the stem in contrast to the worst substrates, in agreement with data published in Rice et al (3). However, in the two groups, exceptions come with SL-miR-K11 (processed over 10% but showing high entropy along the stem, grey curve, left panel) and SL-miR-K9 (processed below 10% but showing low entropy, grey curve, middle panel). The approximate location of apical loop and the stem is highlighted in yellow and grey, respectively, and cleavage sites are indicated by arrow heads.

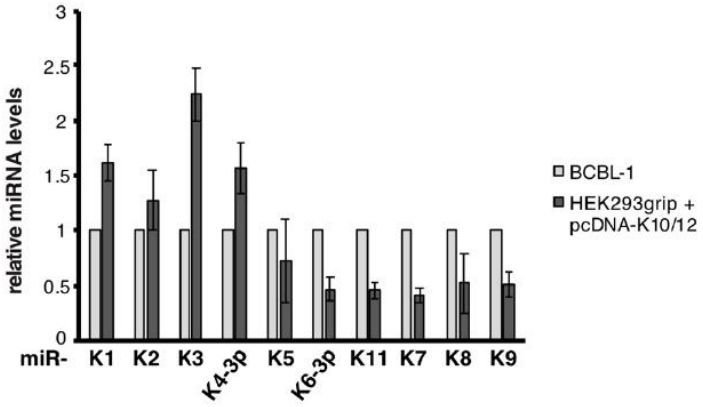

Figure S7. Relative expression of KSHV miRNAs from HEK293grip cells transiently transfected with pcDNA-K10/12 compared to expression in BCBL-1 infected cells. Values were obtained by quantifying signals from northern blot analysis. Error bars derive from three independent experiments except for miR-K1, -K2, -K4-3p and -K6-3p where n=2.

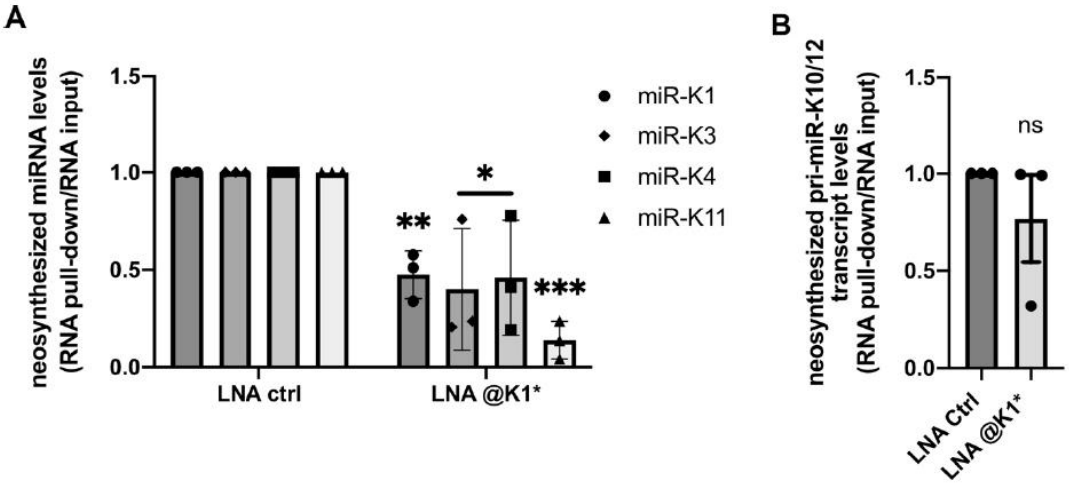

Figure S8. Quantification of neosynthesized miRNAs upon treatment with 20 nM of LNA oligonucleotides.

HEK293FT-rKSHV cells were transfected with 20 nM LNA complementary to miR-K1* or control LNA. 24 hours after transfection, they were incubated with 100µM 4sU for another 16 hours. Neosynthesized transcripts having incorporated 4sU were isolated and levels of mature miRNAs (A) and primary transcript (B) were measured by RT-qPCR. Histograms show ratios of enrichment in pull-down over input RNA relative to Let-7 levels which were set to 1 in control samples. Enrichment of primary transcript was determined relative to CYC1. Bars represent mean ± s.e.m of three experiments. Statistical significance was verified by unpaired t test with ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

2. Identification and characterization of co-factors involved in KSHV miRNA biogenesis

Protein co-factors binding to miRNA precursors are well known modulators of miRNA biogenesis (Michlewski and Cáceres, 2019; Treiber et al., 2019). What is more, some co-factors have been specifically involved in clustered miRNA regulation, enabling for example the phenomenon of cluster assistance (Hutter et al., 2020; Kwon et al., 2020). Therefore, involvement of such co-factors might help to understand the molecular mechanisms regulating the biogenesis of the KSHV miRNA cluster. In this second part of the PhD project, we sought to identify potential co-factors and characterize their mode of action with respect to KSHV miRNA regulation.

2.1 Identification of candidate co-factors

In order to identify putative co-factors involved in the biogenesis of KSHV miRNAs, we took advantage of data generated previously in the laboratory aiming at detecting proteins bound to individual pre-miRNAs within the KSHV miRNA cluster (Creugny, 2019). Briefly, each pre-miRNA hairpin including 20 nt upstream and downstream sequences was *in vitro* transcribed and ligated to a biotinylated DNA oligonucleotide adaptor. Once coupled to streptavidin-coated beads, the bait was incubated with nuclear extract from BC-3 cells (KSHV infected PEL cell line). Following the RNA-pulldown, elution of proteins bound to the baits was performed by DNase I treatment (Figure 7).

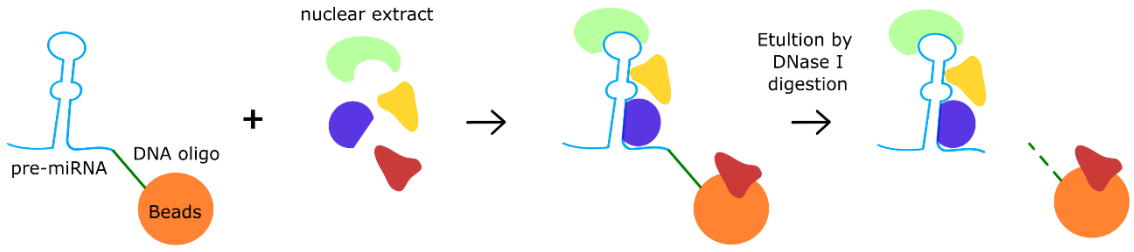

Figure 7: Scheme of RNA pull-down approach used for identification of proteins binding individual KSHV pre-miRNAs. Proteins from BC-3 nuclear fraction enriched on pre-miRNA baits were analyzed by mass spectrometry.

Proteomic analysis of the eluted samples allowed to identify altogether 137 proteins potentially interacting with one or a subset of KSHV pre-miRNAs. These were mostly RNA-binding proteins and some of them have been already identified as miRNA biogenesis co-factors of cellular miRNAs, such as MSI2, HUR and FUS (Choudhury et al., 2013; Morlando et al., 2012). As a positive control for validation of the approach, let-7a-1 bait was used in parallel and as expected, hnRNP A1 (Guil and Caceres, 2007; Michlewski et al., 2008) and LIN28B (Piskounova et al., 2011; Viswanathan et al., 2008) were identified among its binding partners.

2.2 Selection of candidate proteins

Starting with the large set of proteins identified in the pulldown assays, we decided to narrow down the list of putative co-factor candidates, so that we could proceed to their functional validation. Several criteria were considered in the choice of the candidates. For example, all of them are known RBPs and present nuclear localization making their involvement in Microprocessor-mediated maturation more plausible. A summary of the selected candidates is presented in Table 3.

Table 3: Candidate co-factors selected for further validation

Candidate co-factor	Enriched on bait	Known molecular function
SUGP1 / SF4	Pre-miR-K1	mRNA splicing
hnRNPL	Pre-miR-K1	Alternative mRNA splicing, transcription regulation
hnRNPK	Pre-miR-K1 and -K3	mRNA splicing, transcriptional regulation, DNA damage response
RBM45	Pre-miR-K1 and -K3	Alternative mRNA splicing, DNA damage
HNRL1 / hnRNPUL1	Pre-miR-K1, -K2, -K3, -K7, -K11, pre-miR-155, pre-let-7	mRNA splicing and nuclear export, DNA damage response, transcriptional activation

Considering our finding that pre-miR-K1 acts as a regulatory element in *cis*, allowing optimal expression of the entire cluster, it seemed interesting to explore a potential co-factor function in this regulation. Therefore, we were looking for proteins specifically enriched on the pre-miR-K1 bait. Among them, SUGP1 (SURP and G-patch domain-containing protein 1) known also as Splicing Factor 4 (SF4) showed specific and exclusive binding to pre-miR-K1. SUGP1 is a component of the spliceosome (Baltz et al., 2012; Rappsilber et al., 2002) and plays important role in mRNA splicing, i.e. in 3’ splice site recognition (Alsafadi et al., 2021; Kim et al., 2016a; Zhang et al., 2019). Another protein binding specifically to pre-miR-K1 was

hnRNPL (heterogenous nuclear ribonucleoprotein L). This is another RBP involved in mRNA metabolism acting as a regulator of alternative splicing (Liu et al., 2012a; Loh et al., 2015; Rossbach et al., 2009). An additional role for this protein was suggested in transcriptional regulation (Hollensen et al., 2020; Kuninger et al., 2002).

Since pre-miR-K3 is also required for optimal expression of the remaining clustered miRNAs, this was another interesting path to explore. However, we did not retrieve any protein specifically and significantly enriched on pre-miR-K3 baits. Yet we found two proteins, hnRNPK (heterogenous nuclear ribonucleoprotein K) and RBM45 (RNA-binding protein 45), that were identified in parallel on pre-miR-K1 and pre-miR-K3. Since both precursors act as *cis* regulators of the cluster, they might also bind to the same proteins to achieve their regulatory function. HnRNPK is an mRNA splicing factor and a transcriptional regulator in response to apoptotic stimuli and DNA damage (Hollensen et al., 2020; Moumen et al., 2005; Pelisch et al., 2012). RBM45 is a spliceosome-interacting protein, recently shown to play a key function in the alternative splicing of a viral mRNA (Li et al., 2016c; Wang et al., 2020). It was also related to DNA damage response and brain development (Gong et al., 2017; Tamada et al., 2002).

Finally, one protein was included following a different rationale. The heterogeneous nuclear ribonucleoprotein U-like protein (hnRNPUL1) or HNRL1 was retrieved with almost all baits used in the RNA pulldown, including the two cellular pre-miRNAs used in the analysis, pre-miR-155 and pre-let-7a-1. This indicates that HNRL1 might function as a more general co-factor, potentially regulating numerous viral and cellular miRNAs. In addition, while none of the other proteins were previously related to the miRNA biogenesis, there are clues in the literature pointing towards its potential implication in miRNA maturation. The protein has been identified in interaction with the Microprocessor (Gregory et al., 2004) and it can bind to cellular pri-miRNAs, as shown by Van Nostrand *et al*. in a large-scale eClip experiment (Van Nostrand et al., 2020). However, the specific activity of HNRL1 in miRNA biogenesis was never confirmed and further explored. HNRL1 known functions include transcriptional regulation, mRNA transport and processing and it also plays important roles during DNA damage response and embryonic development (Blackwell et al., 2022; Gabler et al., 1998; Kzhyshkowska et al., 2003; Polo et al., 2012; Zhang et al., 2022).

Interestingly, involvement in mRNA metabolism and association with splicing is a common feature of all the selected candidates. This could make sense since the KSHV miRNA

cluster is itself located within an intron. Thus, proteins brought to the processing sites by the spliceosome, might be also available to participate in miRNA maturation.

2.3 Validation and functional analysis of candidate co-factors

In order to confirm the function of selected candidates in the process of KSHV miRNA biogenesis, we first decided to assess the impact of their depletion on mature miRNA accumulation in cells. We hypothesized that if a given candidate is a genuine co-factor, its depletion should impact at least the processing of the pre-miRNA(s) it could bind to, if not the levels of all the KSHV miRNAs (in line with the importance of pre-miR-K1 and -K3 in the entire cluster expression). In addition, the observed increase or decrease in miRNA levels would provide information regarding the stimulatory or inhibitory function of the co-factor of interest.

2.3.1 Knockdown of candidate proteins

Even though we have greatly limited the number of selected candidates, validating five of them still represents a considerable amount of work. For the sake of time and simplicity, we therefore opted for transient knockdown (KD) of each protein in order to rapidly distinguish the most interesting candidate(s). This was to be performed in HEK293FT cells containing recombinant rKSHV.219 (HEK293FT-rKSHV) or a KSHV bacmid (HEK293FT-Bac16), which are used as a model for KSHV infection (Brulois et al., 2012; Vieira and O'Hearn, 2004). These cells reproduce the latent phase of KSHV life cycle and express all KSHV miRNAs from their native genomic context, thus providing conditions close to natural infection. First, we set to determine a protocol for efficient transfection of small interfering RNAs (siRNA) into HEK293FT-rKSHV and/or HEK293FT-Bac16. To be sure to compare a well observable effect, the optimization was performed with siRNAs targeting Drosha, which should have an important negative effect on miRNA accumulation. Several protocols were tested. Results of the best transfection test are shown in Figure 8. Even though efficient downregulation of Drosha was observed, this did not lead to an important decrease in the levels of all the tested miRNAs, as measured by RT-qPCR.

Even though a ~30-40% decrease was observed for miR-K4 and -K11 and the cellular let-7a, the level of miR-K1 was not impacted. Another problem was that KD of a co-factor modulating Drosha's activity would probably yield less pronounced effects that the KD of the main cropping enzyme itself. Therefore, we were not sure whether this approach is sensitive

enough to truly report on co-factor activity. What is more, owing to their high stability and potentially differential half-life, studying phenomena related to miRNA biogenesis might require prolonged KD, to compensate for slow miRNA turnover and observe effects at the level of newly produced miRNAs. Taken together, we concluded that transient transfection is not suitable to study miRNA co-factors in our settings.

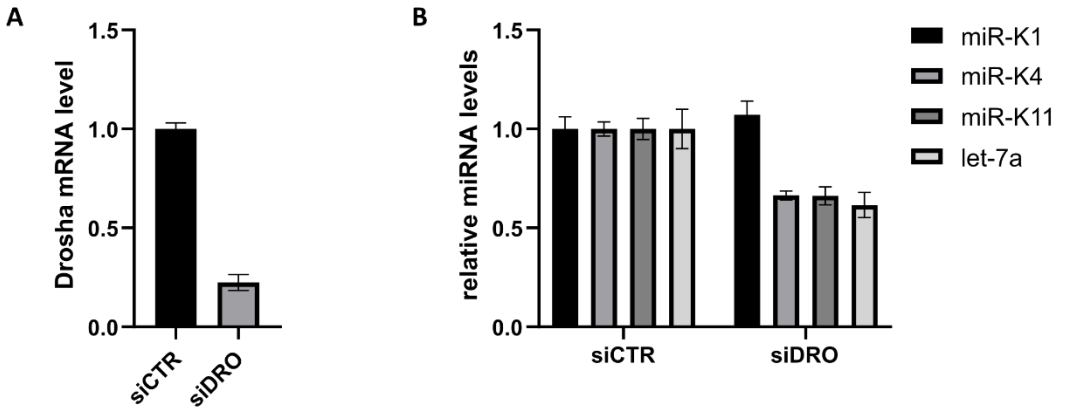

Figure 8: Optimization of transient KD protocol in HEK293FT-Bac16 cells using siRNAs. HEK293FT-Bac16 cells were transfected three times with 20nM of control siRNAs (siCTR) or siRNAs targeting Drosha (siDRO). Cells were collected for analysis 24h after the last transfection. A) Efficiency of Drosha downregulation and B) levels of selected miRNAs were measured by RT-qPCR and normalized to GAPDH or U48. Error bars represent standard deviation of 2 technical replicates.

To overcome this problem, we chose to perform lentiviral transductions using short hairpin RNAs (shRNAs) targeting the proteins of interest. This allows to generate stable cell lines repressing the target gene over long periods of time, due to the transgene integration and sustained shRNA expression from a Pol III promoter. In addition, lentiviral delivery also brings the possibility to transduce naturally infected B lymphocytes, which are otherwise hard-to-transfect cells. We decided to use the BCBL1 cell line (KSHV induced body-cavity based lymphoma) (Renne et al., 1996), which is a good study model, because the cells express high amounts of all KSHV miRNAs and represent physiological KSHV infection.

Two shRNAs were designed for each candidate protein. However, we were not able to obtain shRNA constructs targeting hnRNPK. Thus, the transductions were performed only with the remaining constructs, i.e. shSUGP1, shHNRNPL, shRBM45 and shHNRL1, as well as with the non-targeting control shScramble (shSCR). Stable cell lines were generated in two

individual transduction experiments followed by antibiotic selection. However, at the time of writing, only the first set of samples (one shRNA for SUPG1, RBM45 and HNRL1) could be analyzed, from which the results will be presented below. It should be noted that not all transductions allowed to obtain growing cell lines. In fact, none of the shHNRNPL-transduced conditions survived the selection, as well as one of the two shRBM45. This might be related to the essential character of hnRNPL protein suggested previously (Bertomeu et al., 2018; Manzano et al., 2018) or to sequence-specific toxicity of the given shRNAs.

2.3.2 Analysis of phenotypes in BCBL1-shRNA cell lines

After transducing BCBL1 cells with constructions bearing shRNAs, the cells were under antibiotic selection for more than two weeks before they were collected for RNA and protein analysis. First, efficiency of shRNA-mediated KD was verified by measuring the levels of mRNAs by RT-qPCR and for HNRL1, protein levels were analyzed also by western blot (Figure 9 A-C). Both shSUGP1 and shHNRL1 cell lines display satisfying knockdown efficiency with ~20% remaining mRNAs. However, only ~50% decrease was observed for RBM45 mRNA, which raises the question whether this limited downregulation can lead to measurable output at the level of miRNAs. Indeed, there was no difference in the accumulation of mature KSHV miRNAs (miR-K1, -K3, -K4 and -K11) in shRBM45 samples compared to the shSCR (Figure 9 D). This might simply result from the inefficient downregulation and another (more efficient) shRNA should be tested before this possibility is excluded. Interestingly, we observed a 1.9-fold upregulation in the level of let-7a (Figure 9 G). This might also indicate that RBM45 is not involved in KSHV miRNA biogenesis (at least in the case of the four miRNAs tested), while it can have a direct or undirect impact on the regulation of let-7a. In contrast, HNRL1 and SUGP1 silencing leads to a marked 3 to 4.5-fold increase in all the KSHV miRNAs tested (Figure 9 E, F). In parallel, we have also measured a more modest increase (~1.5 to 3-fold) in the levels of three cellular miRNAs, let-7a, miR-16 and miR-92a (Figure 9 H, I). This indicates that both proteins might act as more general co-factors, impacting global miRNA accumulation, even though KSHV miRNAs seem to be more sensitive to such regulation. In the case of HNRL1, this would not be surprising given its broad binding in the pulldown assays. However, at this point, we cannot draw any definitive conclusion, since these observations will have to be reproduced with at least one other shRNA cell line for each protein.

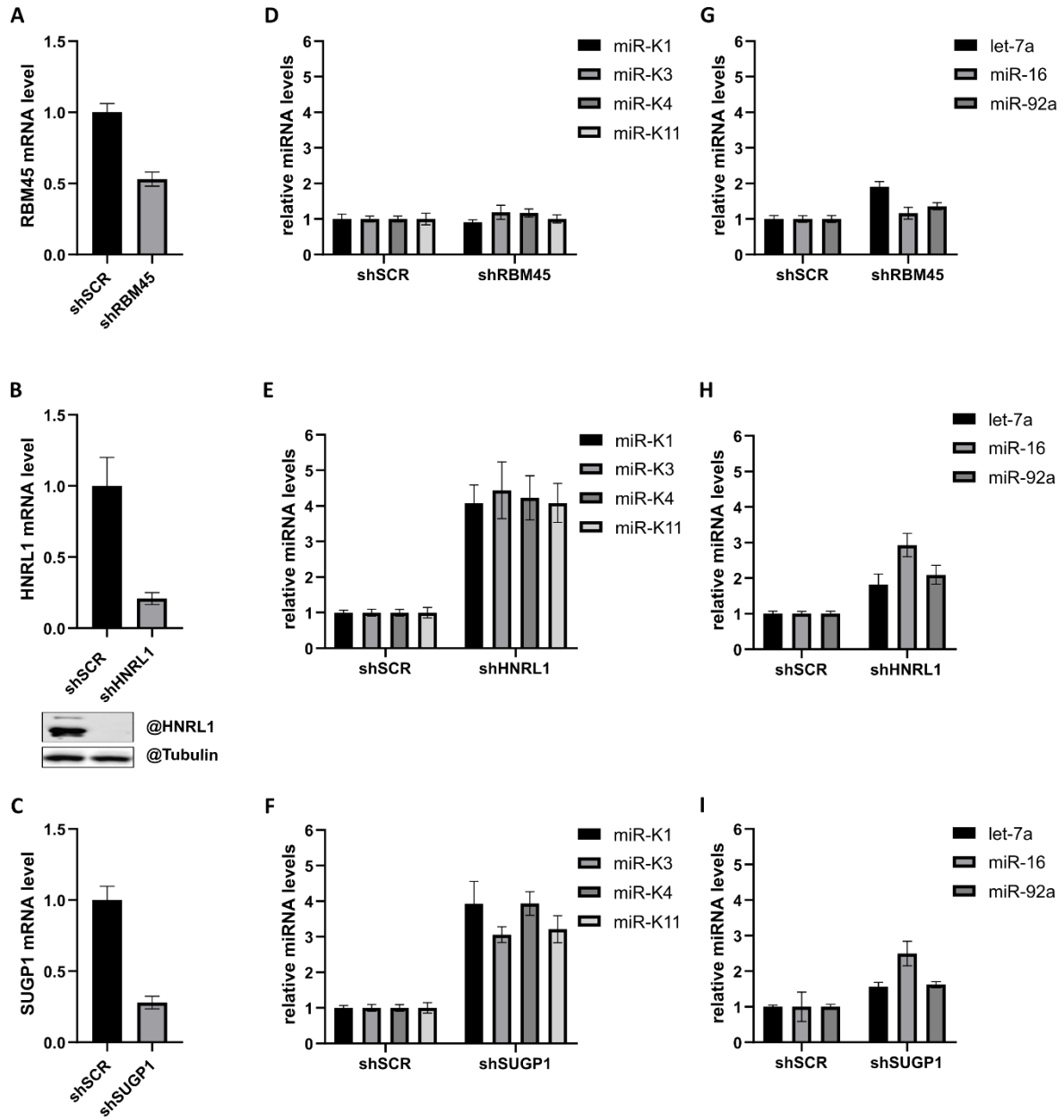

Figure 9: Phenotypic analysis of BCBL1 transduced with shRNAs targeting RBM45, HNRL1 and SUGP1. A, B, C) KD induced by each construct was verified by RT-qPCR and for shHNRL1 also by WB. Expression of viral (D, E, F) and cellular (G, H, I) miRNAs was measured by Taq-Man RT-qPCR and normalized to GAPDH or U48. Error bars represent standard deviation of 2 or 3 technical replicates.

Another possibility to explain increased miRNA levels in BCBL1-shHNRL1 and shSUGP1 cell lines could be an effect on the expression of components of the miRNA pathway. Therefore, we verified the expression of Drosha and Dicer mRNAs. As presented in Figure 10 A, B, neither of the two genes is upregulated arguing against a potential stimulatory effect of HNRL1 and SUGP1 KD on the general miRNA machinery. On the contrary, we observe a decrease of both Drosha and Dicer mRNAs. This may be a consequence of the overall miRNA

overexpression in the cells, since the processing enzymes can be in turn targeted by some cellular miRNAs (Martello et al., 2010; Tokumaru et al., 2008).

To exclude that the increase in KSHV miRNAs result from enhanced transcription of the KSHV miRNA locus, we measured the levels of the primary transcript (pri-miR-K10/12) containing the KSHV miRNA cluster. No increase of pri-miR-K10/12 in neither of the cell lines was observed, as compared to the shSCR (Figure 10 C, D). This further supports the hypothesis that HRNL1- and SUGP1-mediated miRNA regulation occurs at the post-transcriptional level.

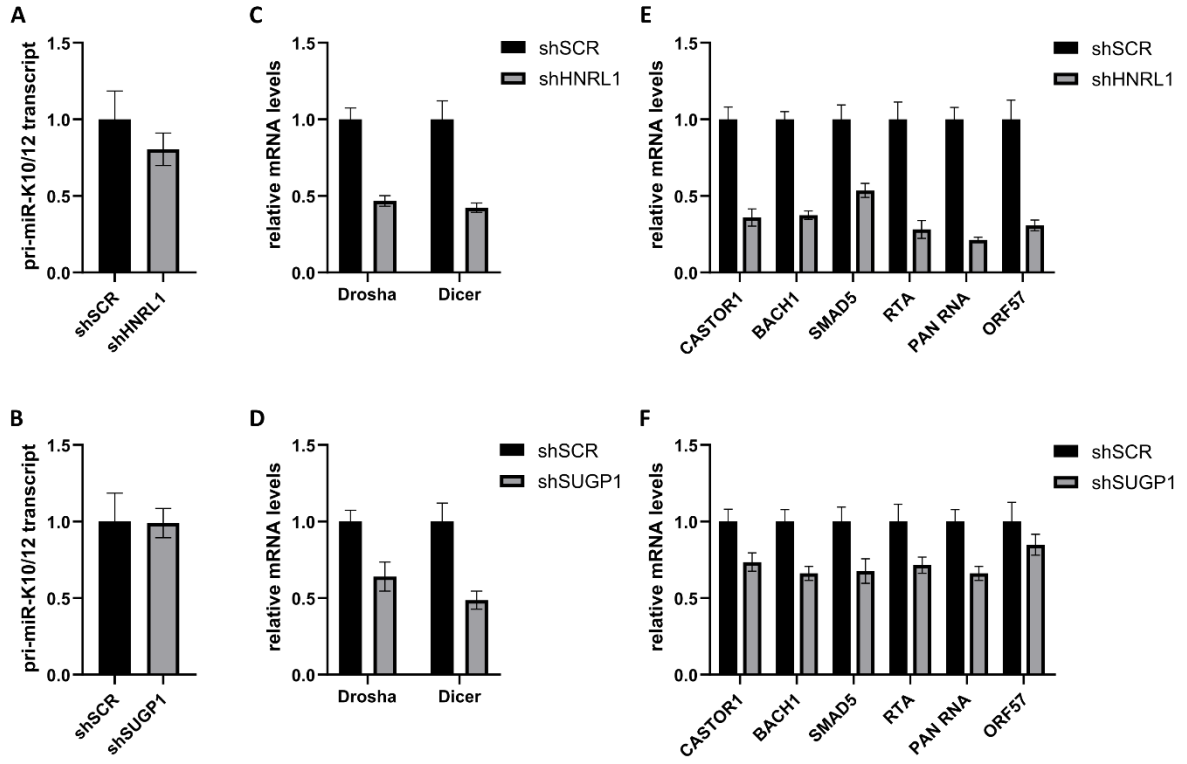

Figure 10: Further phenotypic analysis of BCBL1-shHNRL1 and shSUGP1. Expression of the main miRNA pathway enzymes (A, B), of the primary miRNA transcript (C, D), of KSHVmiRNA target genes, as well as lytic genes (E, F) was measured by RT-qPCR and normalized to GAPDH or U48. Error bars represent standard deviation of 3 technical replicates.

To further explore the phenotype of BCBL1-shHNRL1 and -shSUGP1, we were interested in the impacts of the KSHV miRNA upregulation on their known cellular and viral targets. CASTOR1 has been validated as a target for miR-K1 and also suggested as potential target of miR-K4 (Li et al., 2019). BACH1 and SMAD5 are targeted by miR-K11 (Gottwein et al., 2007; Liu et al., 2012b; Skalsky et al., 2007). At least three viral miRNAs, miR-K5, -K7 and -K9-5p, were shown to directly target the KSHV transactivator RTA, thereby inhibiting

lytic reactivation (Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b). We also tested the expression of two other lytic genes, PAN RNA and ORF57, whose transcription is activated downstream of RTA, hence should be indirectly impacted by miRNA activity (Lukac et al., 1998; Song et al., 2001). Consistent with the increase in KSHV miRNA levels, we observed a reduction in almost all direct and indirect target mRNAs. However, these effects were more pronounced in BCBL1-shHNRL1 and rather modest in BCBL1-shSUGP1.

To conclude this part, HNRL1 and SUGP1 are interesting co-factor candidates. Since their repression in BCBL1 induces increased expression of KSHV miRNAs, they seem to function as negative regulators of KSHV miRNA biogenesis acting at the post-transcriptional level. Their function in cellular miRNA control is also a possibility.

2.4 Generation of a knock-out mutant of HNRL1 in HCT116 cell line

In our pulldown experiments, HNRL1 was able to bind numerous pre-miRNA baits, leading to the hypothesis that it may function as a global miRNA regulator. To test whether cellular miRNAs are regulated by this protein, we decided to assess its function outside of the context of KSHV infection. To this end, we turned to the colon cancer cell line HCT116, which has the advantage of being near diploid and is frequently used to generate CRISPR-Cas9 mutants (Golden et al., 2017; Jallepalli et al., 2001). First, we assessed the impact of HNRL1 knock-down in this cell line. Following a well-established protocol, we performed siRNA transfection and assessed the expression of let-7a. Despite being identified on pre-let-7a-1 baits, HNRL1 depletion did not lead to any variation in mature let-7a-1 in HCT116 cells (Figure 11).

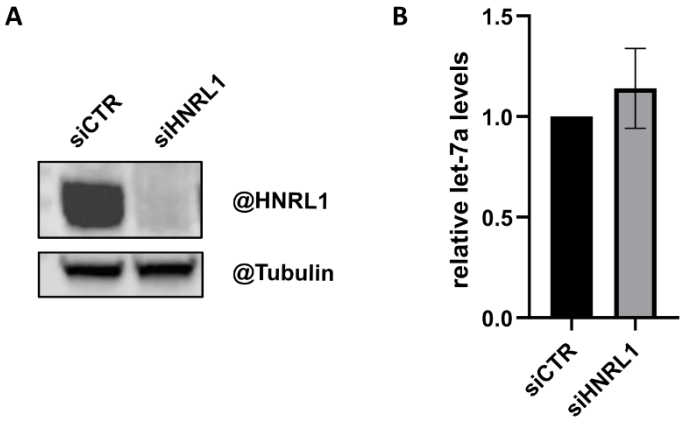

Figure 11: Transient KD of HNRL1 in HCT116 cells. The cells were transfected three times with 20nM siCTR or siHNRL1 and harvested 48h after the last transfection. A) Efficiency of HNRL1 KD was verified by western blot. B) Expression of let-7a-1 was measured by TaqMan RT-qPCR and normalized to U48 levels. The graph shows the mean of two independent experiments and error bars represent standard deviation.

As previously described, elevated miRNA stability may be a hurdle to approaches using transient transfections. Therefore, we decided to generate a knock-out (KO) cell line. This would be useful not only for the analysis of HNRL1 depletion on miRNA expression, but also for further functional analysis that may require HNRL1-free background.

2.4.1 Experimental approach

In order to generate a loss-of-function mutation in the HNRL1 gene (HNRL1 KO), we took advantage of the CRISPR-Cas9 system (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). We designed two pairs of guides supposed to induce Cas9-mediated DNA cleavage simultaneously in the second exon and in the following intron of the HNRL1 gene, thereby generating a deletion of 633 and 387 nt respectively (Figure 12 A). Upon co-transfection of the two guides and the Cas9 nuclease into HCT116, transfected cells were selected through antibiotic treatment and grown as single clones until they were screened by PCR (genomic DNA) and by western blot. From the 10 (guide pair 1) and 12 (guide pair 2) initially growing clones, only one (clone 2.2) showed no expression of HNRL1 protein (Figure 12 B).

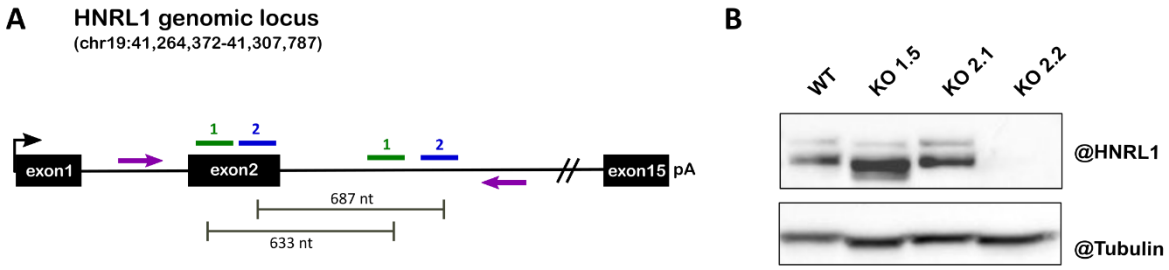

Figure 12: CRISPR-Cas9 strategy for HNRL1 KO generation. A) Schematic representation of HNRL1 genomic locus and RNA guide design. Two pairs of guides (green and blue lines) were used simultaneously to generate indicated deletions. PCR primers used for DNA screening and sequencing are also indicated (purple arrows). B) Western blot analysis of selected clones. Numbers represent the pair of guides used for transfection (1 or 2) and the number of the respective clone (5, 1 or 2).

2.4.2 HNRL1 KO mutant characterization and phenotype analysis

To confirm that the clone 2.2 is indeed KO for HNRL1, a genomic fragment containing the deletion locus was amplified by PCR and sequenced. Sequencing results showed that while there is a deletion of 330 nt within the intron targeted by one of the guides, it did not occur at the expected cleavage site and it would not induce protein depletion. However, the KO phenotype was still successfully generated by a 5 nt deletion within the 2nd exon, at the site

targeted by the other guide RNA. This induced a frameshift and abolished HNRL1 expression. (Figure 13 A). Most likely no cleavage occurred at the other site targeted by the second guide RNA, since no alteration of the genomic sequence was observed.

Next, we were interested in the impact HNRL1 KO would have on cellular miRNA expression. Accumulation of three miRNAs, let-7a, miR-16 and miR-92a was measured and compared to the maternal cell line. Unexpectedly, none of the miRNAs presented increased expression, as observed in the shHNRL1-transduced BCBL1 (Figure 13 B, Figure 9 H). On the contrary, miR-92a seems to be rather slightly decreased. This discrepancy might be due to a context-specific activity of HNRL1, which could act as co-factor in infected B lymphocytes and not in HCT116. Alternatively, it could be also an issue of this particular KO clone.

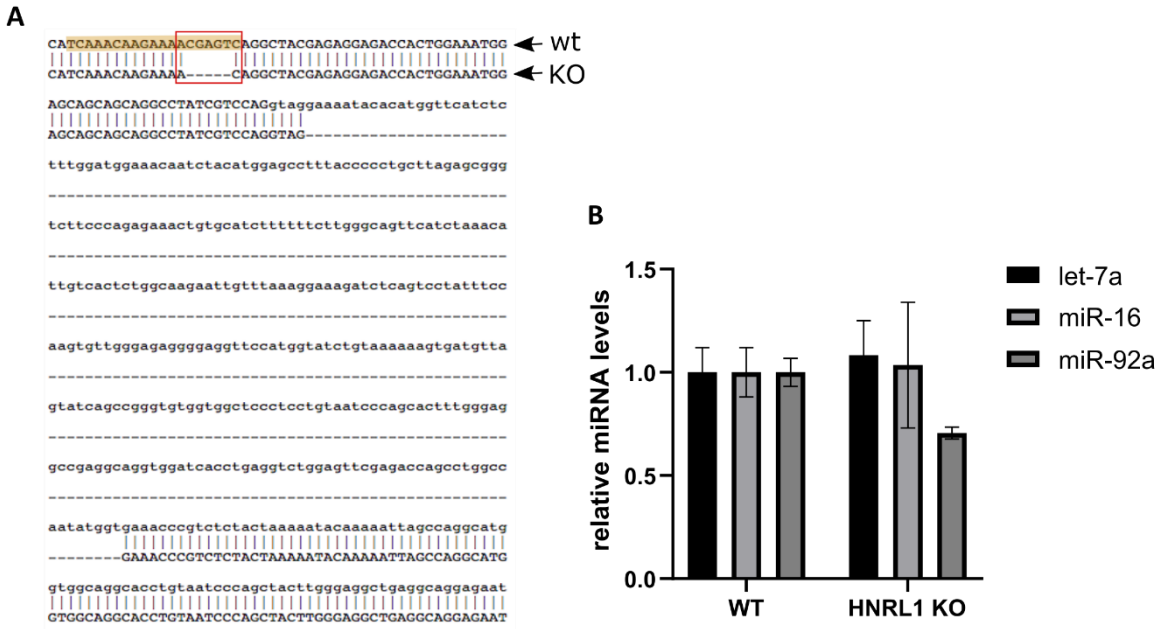

Figure 13:Analysis of generated HCT116-HNRL1 KO cell line. A) Sequence analysis of PCR-amplified genomic region encompassing expected deletion site. Exonic and intronic sequences are in upper and lower case respectively. The site targeted by the RNA guide, as well as the resulting deletion are highlighted. C) TaqMan RT-qPCR analysis of miRNA expression normalized to U48 in HCT116-HNRL1 KO cells. Error bars represent standard deviation of 2 or 3 technical replicates.

Taken together, these experiments allowed to gain a first insight into the function of two potential co-factors involved in the biogenesis of viral and cellular miRNAs, i.e. HNRL1 and SUGP1. Our first results indicate that they both negatively impact the production of KSHV-expressed miRNAs at a post-transcriptional level. Depletion of either of the candidates in BCBL1 cell leads to increased miRNA expression concomitant with increased target gene repression indicating that this regulation is relevant also for cellular phenotype. However, these results will have to be further confirmed. Concerning the potential regulation of cellular miRNAs by HNRL1, we did not observe the same expression profiles in the two experimental systems used and further investigation is required to explain this discrepancy.

IV. Discussion and perspectives

Kaposi's sarcoma associated herpesvirus (KSHV) is a human oncovirus, also responsible for primary effusion lymphoma and multicentric Castleman's disease. To date, KSHV still represents a therapeutic challenge, since no specific anticancer therapy, nor efficient antiviral molecules are available (Wong and Damania, 2017). At the origin of KSHV-related pathogenesis is the lifelong viral latency, an infection mode the virus enters in order to escape immune recognition. The latency is characterized by a minimal expression of viral proteins while the host cell environment is continuously remodelled through viral non-coding RNAs such as miRNAs. Non-immunogenic, they represent a valuable tool for the virus to control itself and its host cell without eliciting an immune reaction. KSHV miRNAs are expressed at high levels and directly participate to cell transformation by targeting genes involved in cell cycle, apoptosis or immune response (Piedade and Azevedo-Pereira, 2016; Ramalingam et al., 2012). With regard to their propensity to trigger severe pathologic outcomes including cancer, it is of great importance to fully understand processes that underly the production of these pathogenic molecules.

At the heart of this work, the regulation of the biogenesis of the KSHV miRNA cluster is essential for the virus to produce sufficient and appropriate levels of each of the miRNAs. While the cluster is transcribed as one primary transcript, the corresponding miRNAs accumulate to strikingly different levels (Contrant et al., 2014; Gottwein et al., 2011; Haecker et al., 2012; Umbach and Cullen, 2010). This clearly indicates that their respective accumulation is decisive for their function and must be tightly fine-tuned to meet the needs of the virus. Recent evidence has shown that the concept of a miRNA as an individual regulatory unit becomes less applicable for miRNA clusters. Reports focusing on other, usually smaller, miRNA polycistrons make us realize that numerous and complex post-transcriptional mechanisms come into play, that does not necessarily exist for individual miRNAs. By taking advantage of the host machinery, KSHV miRNAs follow the canonical miRNA biogenesis like most of their cellular counterparts. However, all along the maturation process, distinct regulatory mechanisms, which are only poorly understood, take place. Our work demonstrates that the regulation is multifaceted and diverse molecular aspects intertwined, making the task of characterizing them even more challenging.

We decided to focus on the first step of miRNA biogenesis by the Microprocessor complex, since it is considered as the key step determining final accumulation of mature miRNAs (Conrad et al., 2014; Feng et al., 2011; Louloupi et al., 2017). What is more, the polycistronic nature of the cluster is most likely to exert inherent regulation at this biogenesis stage. Our investigation was concentrated on the function of some pre-miRNAs acting as regulatory elements in *cis* and on the discovery of protein co-factors modulating the cluster processing in *trans*. Both facets likely contribute to the complex landscape of KSHV miRNA processing.

1. KSHV cluster regulation in *cis*

Studying the activity of the Microprocessor within the KSHV cluster by *in vitro* processing assays, we were able to determine the efficiency of Microprocessor cleavage of each individual pre-miRNA on the KSHV. Striking incoherence between the highly productive cleavage of pre-miR-K1 and pre-miR-K3 and the low accumulation of their respective mature miRNAs in infected cells indicated that these precursors might have another function than the sole miRNA production. Indeed, deletion of each of the two pre-miRNAs led to a significant decrease in the expression of all the miRNAs within the cluster. This led to the conclusion that pre-miR-K1 and pre-miR-K3 act as *cis* regulatory elements that are required for optimal cluster expression. Interdependence between the processing of KSHV miRNAs provided evidence of a higher order regulation operating within the polycistron. What is more, we developed an approach to exploit this regulation in order to inhibit the expression of the whole cluster. We demonstrated that a global downregulation of KSHV miRNAs can be achieved by using a single LNA-based antisense oligonucleotide interfering with the processing of pre-miR-K1.

1.1 Molecular mechanism?

Even though we have demonstrated the importance of *cis* regulatory elements pre-miR-K1 and pre-miR-K3 for the cluster expression, the molecular mechanism at play remains unknown. Replacement of pre-miR-K1 by a cellular pre-miRNA, pre-let-7a-1, could rescue the deletion phenotype and restore the expression of the cluster. This indicates that at least for pre-miR-K1, the mode of action is unrelated to its primary sequence or to the downstream activity of the corresponding mature miR-K1. At the same time, deletion of the pre-miR-K3 showed a

more pronounced effect on the expression of the cluster than the deletion of pre-miR-K1. Although we did not test the effect of its replacement by a cellular miRNA, it seems that the mechanism involved might be distinct that for pre-miR-K1. Indeed, using LNA oligonucleotides targeting pre-miR-K3 led to only modest downregulation of the cluster when tested in our plasmid-based system (data not shown). This could be explained either by inefficient LNA design, which could be further optimized, or there is also a possibility that pre-miR-K1 and -K3 cannot be targeted in the same way because they act through distinct mechanisms. Interestingly, in previously published SHAPE analysis, pre-miR-K3 apical loop did not present highly reactive residues, indicating that it may be involved in tertiary interactions with other parts of the cluster (Contrant et al., 2014). It has been shown that pre-miRNA stem loops within polycistrons can mediate regulatory functions through the interaction with other structural elements (Chaulk et al., 2014). Hence structural constraints could not only impede the accessibility of the hairpin to the LNA oligonucleotides, but could also be the basis for a regulation mechanism specific to pre-miR-K3.

1.2 Further perspectives

Regarding the observed *cis* regulatory function of pre-miR-K1 and/or -K3, two main models (not mutually exclusive) could be proposed. First, after efficient cleavage of pre-miR-K1 or -K3, the rest of the transcript might be primed for further sequential processing of the remaining pre-miRNAs. This would reflect a hierarchy of events when a first cleavage is required in order to overcome a structural constraint. However, our *in vitro* processing assays using fragmented pri-miR-K10/12 mimicking the molecules resulting from pre-miR-K1 or -K3 cleavage did not improve overall cluster processing. This indicates that the two pre-miRNAs do probably not maintain an inhibitory structure within the transcript, which would be released after their cleavage. On the contrary, they seem to be required in *cis* in order to accomplish their regulatory function.

The second model relies in the efficient recruitment of the Microprocessor to the polycistron, that would be promoted by pre-miR-K1 and -K3. Given that they are both very efficient Microprocessor substrates, they might attract the complex to the primary transcript in a mechanism analogous to the cluster assistance, where a helper hairpin is required for neighbour pre-miRNA processing. To verify this possibility, additional mutant constructs could be tested, further determining the specific features of the *cis* regulatory elements required for the regulation to take place. For example, replacing pre-miR-K1 (and -K3) by another pre-

miRNA, which is a poor Microprocessor substrate (such as miR-541 (Fang and Bartel, 2020; Kwon et al., 2020; Shang et al., 2020)) or by a non-pre-miRNA would confirm whether the regulatory element needs to be a well-structured pre-miRNA. Moreover, forced recruitment of the Microprocessor to the primary transcript could be tested in order to recapitulate the effect of pre-miR-K1 (and -K3). In this experiment, the regulatory pre-miRNA would be replaced by a B-box allowing to tether the Microprocessor fused with phage Lambda N-peptide and mimic its recruitment without subsequent cleavage of the pre-miRNA (Shang et al., 2020).

Finally, replacement of the regulatory pre-miRNA by a heterologous pre-miRNA was performed only for pre-miR-K1 leaving the possibility, that pre-miR-K3 regulation is sequence-dependent. Therefore, analogous experiment could be carried out. If the pre-miR-K3 is specifically required, additional variants, such as the substitution of apical loop or the stem might help to localize potentially important structural/sequence motifs.

2. miRNA processing regulation by co-factors

Given that KSHV miRNAs are processed exclusively using the host miRNA machinery, it would not be surprising if additional cellular factors participated to the fine-tuning of viral miRNA levels. We were therefore also interested in another layer of KSHV miRNA regulation relying on protein co-factors. We hypothesized that implication of co-factors might either explain the variable relative abundance of the different miRNAs, by selective enhancement or inhibition of a particular miRNA processing, or help to decipher mechanistic implications of the regulation in *cis*. We established a list of potential co-factors based on the previously analyzed interactome of selected KSHV miRNA precursors, namely pre-miR-K1 and pre-miR-K3. While we have noted interesting phenotypes related to some of the putative co-factors, it should be noted, that these are fairly preliminary results and further experimentation need to be carried out before any definitive conclusions can be drawn.

2.1 Loss-of-function study of selected candidates

Loss-of-function studies are commonly used to delineate the function of all types of gene products. Starting with a higher number of potential candidates, using RNAi based methods, rather than genomic deletion, is a more practical and faster way to identify the most interesting candidates. In our case, transient siRNA-mediated knockdown proved to be

inefficient, presumably due to the long half-life of mature miRNAs (Figure 8). Therefore, we opted for an approach allowing long-term repression of putative co-factors by stably expressed shRNAs, so that the downstream impact on miRNA accumulation can be measured. Another advantage of the knockdown experiments is the possibility to study essential proteins, which would not be feasible if they are knocked out. Indeed, most of the proteins on our list (HNRL1, SUGP1, hnRNPK, hnRNPL) were previously identified as essential genes or at least as cell fitness-impacting genes by large scale screens performed by the Tyers' and Gottwein's laboratories (Bertomeu et al., 2018; Manzano et al., 2018). Therefore, KO cells might not be viable, while KD leads to residual protein levels that could support cell survival. Interestingly, we succeeded at generating HNRL1 KO in HCT16 cells and even though we did not precisely measure cell growth in culture, we did not notice markedly impaired growth indicating that at least in HCT116 cells, this protein is likely not essential. Similarly, no particular growth issues were noticed in the BCBL1 cell lines transduced with shHNRL1 and shSUGP1.

At the same time, even by using shRNA-mediated knock-down, we did not manage to generate cell lines to study all the selected candidates. None of the shHNRNPL-transduced cells survived the selection, despite repeated attempts. This could indicate that residual hnRNP L proteins levels are perhaps not sufficient to maintain cell viability. In the case of shRBM45, only one of the two shRNA constructs allowed us to generate viable cell lines. This might result from some sequence-specific toxicity of the given shRNA, e.g. due to off-target effects. What is more, the successful construct only yielded a knockdown efficiency below 50%, which leaves also open the possibility that the cells cannot support massive loss of RBM45, thus explaining the non-viability of the first construct. We did not find in literature any report suggesting the essential nature of this protein, but cell type-specific requirement cannot be formerly excluded.

Another interesting idea that could help to explain our issues with shRNA cell line generation is that long-term shRNA expression was shown to be toxic *in vivo* (Grimm et al., 2006; McBride et al., 2008). In these reports, mice treated with Pol III driven shRNA constructs (similar to ours) presented cell death and organ failure several weeks post-shRNA transduction, presumably due to the oversaturation of the miRNA pathway and/or toxic accumulation of unprocessed precursors and antisense stands. This might indicate that this type of shRNA approach is not suitable for long-lasting experimental settings, since a long-term shRNA expression could have deleterious effects for cell survival. Yet, we succeeded at generating some of the cell lines, even though some of them survived the selection period only after a

second transduction attempt. This might be explained also by the principle of lentiviral transduction that relies on random insertion of the transgene. The resulting heterogenous polyclonal cell populations present highly variable transgene expression, as a consequence of variable epigenetic status at the integration locus. We might speculate that the cell line survival depends on a balance in transgene expression level, which must not be too strong, yet sufficient for survival in the selective environment. Consequently, cells would counter-select for weak expressors over time, while strong expressors would progressively die out due to the toxic effects of accumulating shRNAs. In favor of this hypothesis, we observed in our cultures a progressive decrease of the mCherry signal carried by the lentiviral construct (data not shown). In the shRNA lentiviral plasmids we used, mCherry is fused to the blasticidin resistance gene and included into the transgene cassette in order to follow up transduction efficiency. While 24-48h post-transduction, we observed a strong fluorescence in a large proportion of transduced cells, fluorescence faded out with time, even though cells remained viable (i.e. blasticidin resistant). On the other hand, cell lines that did not survive the selection, were also efficiently transduced (as seen by mCherry signal 24-48h post-transduction). Their progressive death might therefore reflect the fact that the well-balanced expressors were not present in those transduced cell pools.

If other functional studies of the same or additional co-factors were to be planned in the future, these technical considerations would have to be taken into account. To cope with potential shRNA toxicity, analogous systems based on miRNA-like molecules were successfully used (Boudreau et al., 2009; McBride et al., 2008). For example, the Zuber lab has optimized the native miR-30 backbone termed “miR-E”, which allows production of artificial miRNAs transcribed by RNA Pol II (Fellmann et al., 2013). These molecules achieve highly efficient target downregulation, yet their expression levels are not higher than endogenous miRNAs, thus avoiding oversaturation of miRNA machinery.

2.2 Candidate co-factors: HNRL1 and SUGP1

Analysis of the BCBL1 cell lines we obtained with shHNRL1 and shSUGP1 showed a potential involvement of both proteins in the regulation of KSHV miRNA biogenesis. Depletion of either protein seems to positively impact the expression of KSHV miRNA cluster (and potentially also cellular miRNAs). However, these findings will have to be confirmed using at least one other shRNA construct because at this moment, we cannot formally rule out the possibility of off-target effects. Overexpression of the proteins would be also helpful to validate

HNRL1 and SUGP1 involvement by inducing opposite effects to their depletion. What is more, the question of the molecular mechanism remains open since at this point, we cannot say at what level of the miRNA biogenesis this regulation would occur. We will therefore only speculate about the possible result interpretation, as well as suggest scenarios and additional experimental approaches to elucidate the function of HNRL1 and SUGP1 in more details.

2.2.1 Mode of action

The increase of KSHV miRNAs upon HNRL1 and SUGP1 depletion indicates a repressive function for both proteins (Figure 9). This could happen at various steps during miRNA biogenesis. We did not measure an increase in the transcription of the cluster, which points towards one of the post-transcriptional steps (Figure 10). Regulation of the Drosha/DGCR8 activity at the level of the primary transcript represents the most plausible scenario, since both co-factors are predominantly nuclear and in the case of HNRL1, interaction with the Microprocessor was already demonstrated (Gregory et al., 2004). Yet we cannot exclude the possibility that the co-factors bind to pre-miRNAs rather than pri-miRNAs. We started with the assumption that the proteins interact with miRNA hairpins used as baits in pulldown experiments, thus cannot distinguish between pri- and pre-miRNA interactomes. Therefore, the co-factors could also interact with the precursors downstream of the Microprocessor and recruit or stimulate the activity of decay factors such as MCPIP1 (Suzuki et al., 2011). However, SUGP1 was identified only on pre-miR-K1 bait. Given that other KSHV miRNAs are coordinately upregulated upon SUGP1 depletion, it is more easily conceivable that this protein acts at the level of pri-miRNA when all the precursors are still on the same primary transcript. In addition, this would support our finding that pre-miR-K1 act as a *cis* regulatory element controlling the fate of the remaining miRNAs within the cluster. SUGP1 could therefore inhibit the expression of the entire cluster through interaction with pri-miR-K1 only. To confirm this hypothesis, the binding of co-factors to the pri-miRNAs and/or to the Microprocessor complex should be verified (Co-IP, RIP). In addition, *in vitro* processing assays in cell lysates overexpressing or depleted of the given co-factor would validate the co-factor involvement in Microprocessor cleavage. Similar to the B-box-based tethering of the Microprocessor proposed above, SUGP1 and HNRL1 could be tethered to the primary transcript to see whether or not they can represses its processing in cells.

Concerning the interaction of our co-factor candidates with their putative pri-miRNA substrates, no consensus RNA binding sequence was identified either for HNRL1 or SUGP1 to

the best of our knowledge. However, the proteins possess the B30.2/SPRY and SURP domains respectively, that could confer them RNA-binding capacity (Bohnsack et al., 2021; Choudhury et al., 2017). In addition, HNRL1 binding to human pri-miRNA transcripts was shown in large scale eCLIP experiments (Van Nostrand et al., 2020) and both proteins are involved in mRNA metabolism as spliceosome-associated factors. However, direct binding of the protein to pri-miRNAs should be first verified (RIP, CLIP, EMSA), before we could study further the characteristics of their binding. For example, determination of protein domains involved in the interaction as well as RNA sequence/motif pre-requisites would be of interest. Previously, pri-miRNA terminal loops were proposed to confer binding specificity and serve as landing platforms for *trans*-acting factors (Michlewski et al., 2008; Treiber et al., 2017). Hence it is possible that terminal loops are responsible for SUGP1 and/or HNRL1 recruitment. This might be verified by pre-miRNA mutagenesis. To go even further, biochemical and structural approaches (crystallography, SHAPE, RNase footprinting) were previously used for deeper characterization of interactions between pri-miRNAs and their respective co-factors (Chen et al., 2016; Choudhury et al., 2013; Kooshapur et al., 2018; Michlewski and Cáceres, 2010).

Intriguingly, depletion of HNRL1 and SUGP1 leads to a similar phenotype, the upregulation of the KSHV miRNA cluster. First, the two proteins could act through two distinct repressive mechanisms, HNRL1 by non-specific binding to all pre-miRNAs within the cluster and SUGP1 by antagonizing pre-miR-K1 stimulatory activity. Alternatively, similar miRNA expression may also indicate that the two proteins act through a convergent pathway, coordinately repressing KSHV miRNAs in a single process, perhaps as one effector complex. One could imagine a scenario, in which SUGP1 binds to pre-miR-K1 and recruits HNRL1 that in turn interacts with the remaining precursors, thereby mediating the blockade of their processing. This question could be addressed by knocking down both factors where two unrelated mechanisms could be distinguished by additive effect of the simultaneous depletion of the two proteins. It would be also interesting to test whether HNRL1 and SUGP1 physically interact.

The repressive activity of our putative co-factors could result from direct interaction and alteration of Microprocessor activity on the KSHV cluster. Alternatively, they could also mask pre-miRNAs and prevent their recognition, or recruit additional factors exhibiting repressive activity. Interestingly, SUGP1 contains one G-patch domain, which is known to interact with and activate RNA helicases for ATP hydrolysis (Bohnsack et al., 2021). RNA helicases exert

numerous activities on their RNA substrates, such as unwinding dsRNA strands, untangling complex secondary structures and dislocating other RBPs. Several RNA helicases have been shown to participate in cellular miRNA biogenesis (DDX5, DDX17, DDX1) (Han et al., 2014; Ngo et al., 2019; Wang et al., 2012) by inducing structural rearrangements of pri-miRNAs, thereby modulating Microprocessor activity. An interesting example is the DDX3X, that was shown to display opposite regulatory functions in distinct miRNA biogenesis. Whereas it can promote the expression of a subset of individual miRNAs, it was also shown to inhibit the processing of the miR-183~182 cluster during retina development (Krol et al., 2015; Zhao et al., 2016). A number of RNA helicases were identified in the RNA pull-down experiment. Notably, among them was DDX15, which was already identified as binding partner of SUGP1 (Hegele et al., 2012). Similar to HNRL1, DDX15 was retrieved with almost all pre-miRNA baits, thus being another interesting candidate in KSHV miRNA biogenesis. DDX15 could be recruited by SUGP1 and exert the repression, perhaps through alteration of the pre-miRNA or the entire primary transcript structure. Along this line, further investigation of SUGP1 and HNRL1 interactome, crossed with pull-down data, could help to understand the mechanism of KSHV miRNA repression.

Next to the direct interference with the Microprocessor activity, there is also a possibility that HNRL1 and SUGP1 take part on mechanisms that modulate miRNA maturation less directly, such as splicing. Processing of intronic miRNAs is intimately related to splicing and there is a regulatory crosstalk between the Microprocessor and the splicing machinery (Agranat-Tamir et al., 2014; Janas et al., 2011; Mattioli et al., 2014). This can have context-dependent outcomes, leading to enhancement or inhibition of both phenomena. HNRL1 and SUGP1 are both involved in alternative splicing (Alsafadi et al., 2021; Blackwell et al., 2022; Zhang et al., 2019) and HNRL1 has been shown to play a role also in the back-splicing event leading to production of the circRNA circMAN1A2 in response to DNA damage (Li et al., 2021). In addition, another recent report by Nguyen et al. has shown that the protein hnRNPU, closely related to HNRL1, forms a complex with DDX3X. This complex is required for increased splicing of the miR-155 host gene and concomitant decrease in the miRNA processing. In some leukemia cell lines, formation of the hnRNPU/DDX3X complex is impaired, thus leading to increased expression of the pro-proliferative miR-155 (Nguyen et al., 2021). KSHV latency locus undergoes a complex set of transcription patterns, whose regulation is not completely understood. This includes termination read-through events and alternative splicing and the KSHV miRNA cluster itself is expressed from an alternative intron (Figure 6).

Hence, interaction of splice factors with intronic pri-miRNAs might help or prevent the assembly of the spliceosome or change the dynamics of splicing events. This could in turn lead to a global impact on KSHV miRNAs, such as seen in HNRL1 and SUGP1 KD. Involvement of splicing in the repression by HNRL1 and SUGP1 could be investigated by monitoring splicing events (spliced or unspliced RNA) in infected cells or by using artificial minigene constructs.

To sum up, regarding the molecular mechanism underlying the repressive role of HNRL1 and SUGP1 in KSHV miRNA biogenesis, there are plenty of open questions and there are many ways to address them. This will be one of the tasks for future research in the laboratory.

2.2.2 Impact on cellular miRNAs

Although our principal focus was directed towards KSHV miRNA regulation, some of our preliminary results provide hints that cellular miRNAs could be also concerned. Not only HNRL1 can bind in vitro to pre-let-7a-1 and pre-miR-155, but also levels of miR-16 (and more modestly miR-92a and let-7a) are impacted in HNRL1 knock-down BCBL1 cell line (Figure 9). Regarding SUGP1 and RBM45, since they were not retrieved with pre-let-7a-1 bait, the regulation of this miRNA was not expected. The possibility remains that this regulation could be undirect. Given the similar profiles of cellular miRNA expression between shHNRL1 and shSUGP1 cells, there might also be coordinated action of both co-factors, as discussed above. Even though KD of HNRL1 and SUGP1 impact viral miRNAs to a much greater extent (~3-4.5-fold), the more moderate increase (~1.5-3-fold) of the three cellular miRNAs measured, is perhaps also of significance (Figure 9). Indeed, other groups that studied the involvement of repressive co-factors in human miRNA biogenesis, often measured no more than two-fold miRNA upregulation upon the co-factor depletion (Chen et al., 2016; Choudhury et al., 2013; Suzuki et al., 2011; Wu et al., 2015). Therefore, the possibility that HNRL1 and SUGP1 also regulate cellular miRNAs should not be discarded.

However, arguing against this hypothesis is the fact that none of the cellular miRNAs investigated was increased by the KO of HNRL1 in HCT116 cells (Figure 13). One possibility to explain such a discrepancy between the two experimental systems is that the variation in miRNA levels in BCBL1 cells results from an off-target effect of the shRNA (since it was not yet verified by another shRNA) and may not be reproducible. The binding of HNRL1 to pre-

let-7a-1 observed in the pulldown assays may also represent a false positive. This can be expected in large scale screens identifying numerous potential binding candidates by mass spectrometry. In addition, RNA pulldown represents an *in vitro* approach potentially forcing interactions, which may not occur in cells.

However, we might also speculate, that the regulation requires a cell-type-specific context, thus occurring only in lymphocytes and not in epithelial HCT116 cells. This might be due to the expression of a particular factor or even to the KSHV infection. To address these questions, we could use a non-infected lymphoblastoid cell line such as BJAB (Burkitt lymphoma) to reproduce repression experiments and study the cellular miRNA expression. Upregulated, or not, levels of let-7a, miR-16 and miR-92a (as well as potentially other miRNAs) would provide clues regarding to cell-type specificity or infectious context.

Along with the hypothesis that HNRL1 and SUGP1 can have a broader impact on miRNA biogenesis, we might as well expand our investigation to the entire cellular miRNA population. Small RNA sequencing after depletion of the co-factors would allow to get a global picture of the pool of regulated miRNAs, as well provide deeper insight into their molecular signatures. For example, it might be interesting to determine, whether the regulated miRNAs tend to reside within a particular genomic context, such as clusters or introns.

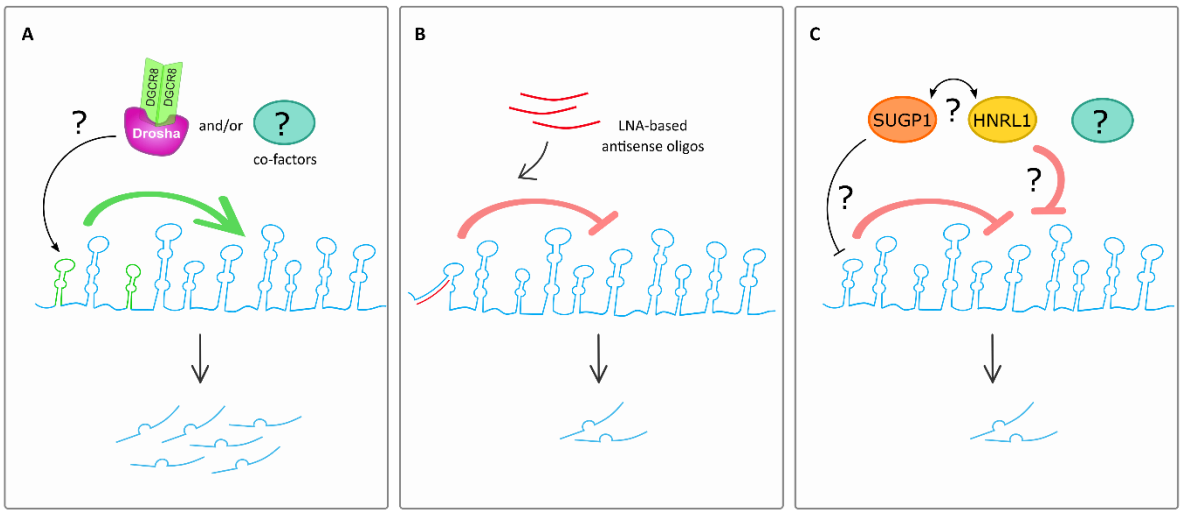

Figure 14: Regulation of KSHV miRNA cluster. Cis-regulatory elements pre-miR-K1 and -K3 allow optimal expression of the entire KSHV miRNA cluster (A). By using one single antisense oligonucleotide, pre-miR-K1-mediated regulation is inhibited, thus leading to decreased expression of all the miRNAs within the cluster (B). The cluster is also subject to the repressive activity of protein co-factors, such as SUGP1 and HNRL1 (C). Molecular mechanisms and potential additional factors underlying the regulation remain to be determined (indicated by question marks).

V. Conclusion

Nowadays, a multitude of diagnostic and therapeutical approaches seeks to implement miRNA-based techniques. These small regulatory molecules have been proposed not only as reliable biomarkers for disease stage and prognostics, but also as drug targets. What is more, there is a future for therapeutic molecules mimicking miRNAs with beneficial disease-reverting activity (Rupaimoole and Slack, 2017). Several innovative techniques experiment with the use of clustered miRNA mimics, especially owing to their simultaneous expression (Amen et al., 2022; Choi et al., 2015; Wang et al., 2016a; Yang et al., 2013). However, a growing body of evidence points to the fact that the control of polycistronic miRNA expression requires diverse post-transcriptional regulation. This new concept is highly relevant to miRNA biology and becomes a field of study on its own. Even though the mechanisms underlying the post-transcriptional regulation of clustered miRNAs hold great potential, the limits of our current knowledge hinder their real-life application. After all, before the regulation of clustered miRNAs is not completely understood, these approaches cannot be fully mastered and translated into new therapies.

In this work, we have joined the efforts to elucidate post-transcriptional polycistronic miRNA regulation by focusing on the pathogenic KSHV miRNA cluster. First, we have demonstrated the requirement of *cis* regulatory elements for an optimal expression of the cluster. Based on these findings, we provided a proof of principle for the use of antisense oligonucleotides able to inhibit the processing of the primary transcript. Finally, we might have uncovered another regulatory phenomenon. This would be related to the presence of co-factors antagonizing the *cis*-mediated regulation or involved in an independent repressive process (Figure 14). Unveiling the mechanistic bases of these co-factor activity will be the next research challenge and the first experiments are underway in the laboratory.

To our knowledge, this is the first extensive and detailed study of a large viral miRNA cluster, considering it as an interdependent unit. This approach helped us to highlight the complexity of KSHV miRNA regulation and to move closer towards its understanding.

VI. Materials and methods

1. Cell culture

1.1 Cell culture conditions

Adherent HCT116 and HEK293FT-Bac16 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % fetal calf serum (FCS, Dominique Dutscher). The medium for HEK293FT-Bac16 growth was also supplemented with 100 µg/ml Hygromycin (InvivoGen) to maintain KSHV genomes. BCBL-1 lymphocytes were grown in a Roswell Park Memorial Institute medium (RPMI 1640, Gibco) supplemented with 10 % FCS. All cell lines were cultured at 37°C and 5 % CO_2 atmosphere.

1.2 Transfections

Detached 110 000 HEK293FT-Bac16 cells were transfected in a 48-well format in final volume 200 µL. 20nM siRNAs (ON-TARGET plus smart pool, Dharmacon) were reverse-transfected into the cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. On the next day, the same amount of siRNAs was transfected again into attached cells. 24 hours later, the cells were split and again reverse-transfected with 20 nM siRNAs. 24 hours after the last transfection, they were washed with cold PBS and lysed in 200 µL Tri-reagent (Trizol, Sigma). HCT116 were reverse-transfected in 6-well plates using 600 000 cells/well and 2 wells per condition. Three transfections were performed with 20nM similarly to the procedure applied to HEK293FT-Bac16. The main difference was that the cells were harvested 48 hours after the last transfection. One well was collected for RNA and the second for protein analysis.

2. Lentiviral transductions

2.1 shRNA constructions

All the vectors (pLV-shRNA) used for lentiviral transductions were purchased from VectorBuilder. Two shRNAs sequences were designed for each target gene, as well as one non-

targeting control (shScramble). Vectors were received as glycerol stocks and amplified by being cultured at 30°C on LB-agar plates, then in liquid LB medium supplemented with 100 mg/mL Carbenicillin. After plasmid purification (NucleoBond Xtra Macherery Nagel), correct sequences were verified by sequencing (Eurofins Genomics). Further stocks were produced by heat-shock transformation of E.coli (NEB® Stable Competent E. coli) using the protocol recommended by the supplier.

Table 4: Sequences of shRNAs designed for lentiviral transductions. Target sequence is underlined.

Target	ShRNA sequence	Targeted region	Vector ID
Scramble	CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG	No target	VB010000-0015hpw
HNRL1 #1	CCTCATGCAGTTGGTTGTAAACTCGAGTTTACAACCAACTGCATGAGG	3'UTR	VB210920-1042qye
HNRL1 #2	CCGTGTATGCTTCGAGATGAACTCGAGTTCATCTCGAAGCATACACGG	CDS	VB210920-1050fcu
hnRNPL #1	TAGAGGCTTACTTAACCTTAACTCGAGTTAAGGTTAAGTAAGCCTCTA	3'UTR	VB210920-1064kdb
hnRNPL #2	GCCGACAACCAAATATACATTCTCGAGAATGTATATTTGGTTGTCGGC	CDS	VB210920-1066wup
hnRNPK #1	AGATTTGGCTGGATCTATTATCTCGAGATAATAGATCCAGCCAAATCT	CDS	VB210920-1053quc
hnRNPK #2	CGTTATTGTTGGTGGTTTAAACTCGAGTTTAAACCACCAACAATAACG	3'UTR	VB210920-1056vrz
RBM45 #1	TAATGTAGCATCAGCTATTTACTCGAGTAAATAGCTGATGCTACATTA	CDS	VB210920-1067pxf
RBM45 #2	CGAGTATTGCAGCATTATTAACTCGAGTTAATAATGCTGCAATACTCG	CDS	VB210920-1068zad
SUGP1 #1	CATCCTGGCGAAATCACAAATCTCGAGATTTGTGATTTCGCCAGGATG	CDS	VB210920-1070yha
SUGP1 #2	CTCGGAAAGTGATAGAGAAATCTCGAGATTTCTCTATCACTTTCCGAG	CDS	VB210920-1071esn

2.2 Lentivirus production

Lentiviral particles were produced using the 2nd generation plasmids, psPAX2 carrying HIV-1 gag as well as HIV-1 pol and pVSV-G plasmid bearing the VSV envelope protein G, to accompany pLV-shRNA. One day prior transfection, 700 000 HEK293T cells were seeded into 6-well plates (2 wells per condition). Using standard Lipofectamine 2000 protocol (Invitrogen), 1.3 µg of psPAX2, 0.33 µg pVSV-G and 1.7 µg pLV-shRNA were transfected in total volume

of 2 mL. After 72h, culture supernatants were filtered (0.45 µm, PES filters), aliquoted and viral stocks were frozen until further use at -80°C.

2.3 Lentivirus transduction

One day before transduction, BCBL1 cells were diluted to the concentration of 0.5 M cells/mL. Transduction was performed by incubation of 500 µL of viral stock and 8 µg/mL polybrene with 500 000 cells resuspended in fresh RPMI medium supplemented with 10% FCS. To enhance infection efficiency, the cell/virus suspension was centrifuged at 800 x g during 2 hours at 32 °C. Then, supernatants were discarded and transduced cells resuspended in fresh medium.

2.4 Stable cell line selection

24 hours post-transduction, Blasticidin (InvivoGen) was added to the cell cultures reaching final concentration of 7.5µg/mL. After 16 days of selection, RNA and protein samples were harvested and remaining cultures frozen. Given the high mortality of cells leading to loss of most of the transduced cell lines during the first trial, the second attempt was performed by using 5 µg/mL Blasticidin during the first two weeks. Then antibiotic concentration was increased to 7.5 µg/ml and the cells were cultured for another week before they were collected for analysis or frozen.

3. HNRL1 KO generation

3.1 Design and cloning of CRISPR guideRNAs (gRNA)

For the CRISPR-Cas9-mediated knock-out of HNRL1 generation we designed 2 pairs of RNA guides (gRNAs) targeting HNRL1 gene within its third exon and third intron (see Results 2.4.1). We used the plasmid px459 V2.0 (Addgene, #62988) that carries CMV-driven Cas9 gene together with one guide RNA (gRNA) under the control of U6 promoter. The gRNAs were designed on http://crispor.tefor.net/ and their cloning was performed based on the Zhang lab protocol (Ran et al., 2013). Briefly, 10µM sense and antisense DNA oligonucleotides corresponding to a gRNA were denatured at 95°C for 5 min and annealed by slow cooling down to the room temperature (RT). Next, 0.1 µM of annealed oligos were added to 30 ng of the vector previously linearized by BbsI during 4h at 37°C and purified by phenol-chloroform

extraction. The DNA fragments were ligated by T4 DNA ligase (Thermofisher) during one hour at RT in total reaction volume of 20 µL. One half of the reaction was used to transform XL-1 blue competent cells and plasmids purified from bacteria grown with ampicillin (100 mg/mL) were verified by sequencing

Table 5: DNA sequences of used for gRNA cloning. gRNA sequences are underlined and bases at extremities correspond to restriction sites required for cloning into px495 V2.0.

gRNA	sense	antisense
Pair 1 guide 1	CACCGGACTTGGGTATCGTAGAAT	AAACATTCTACGATACCCAAGTCC
Pair 1 guide 2	CACCGGATGTTAGTATAAGCACCC	AAACGGGTGCTTATACTAACATCC
Pair 2 guide 1	CACCGTCAAACAAGAAAACGAGTC	AAACGACTCGTTTTCTTGTTTGAC
Pair 2 guide 2	CACCGGAGCCTTCTAAGTCTAATC	AAACGATTAGACTTAGAAGGCTCC

.

3.2 Transfection of HCT116 and clone selection

One day prior to transfection, 600 000 HCT116 cells were plated into 6-well plates. px459 V2.0 plasmids carrying two distinct gRNAs were co-transfected at 1 µg each using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. On the next day, Puromycine (InvivoGen) was added to the culture medium to reach final concentration 1.5 µg/mL. 24h after the beginning of the selection, the cells were passed into new wells and new selective medium was added. After 72h, most of cells were dead and surviving cells were individualized and placed into 96-well plates using the technique of limiting dilution (0.5 cell/well). Individual clones were grown in non-selective medium for 18 days before they were passed and analyzed.

3.3 Clone screening and validation

Monoclonal colonies growing in 96-well plates were detached and half of the cells were used for DNA analysis, while the other half was passed into a bigger plate format. Cells harvested for analysis were collected by centrifugation at 500 x g during 10 min at 4°C, then resuspended in 300 µL lysis buffer containing 50 mM Tris-HCl pH8, 100 mM EDTA pH8, 100 mM NaCl, 1% SDS, 0.1 mg/mL Proteinase K (Roche). After 4h incubation at 55°C under shaking, one volume of PCI (Roth) was used to extract genomic DNA, which was then EtOH-precipitated. 100 ng genomic DNA was used as template for PCR analysis performed by GoTaq Master Mix (Promega) and primers Fw: 5'TTCTTCATGAACCGCCCCTC3' and Rev: 5'TGAGGCCTGACACACACTTT3'. Samples were analyzed by electrophoresis on 2%

agarose gel and clones giving rise to smaller amplicons, as compared to wt cell line, were allowed to grow, while the wt clones were discarded. When the cultures expanded enough, protein samples were collected, as well as cells for a second DNA analysis. This time, DNA was extracted by using the Monarch® Genomic DNA Purification Kit (NEB) to obtain better quality genomic DNA preparations. 1 µg template DNA was amplified by Phusion High-Fidelity DNA polymerase (Thermo Scientific) and gel-purified with the Monarch® DNA Gel Extraction Kit (NEB). PCR amplicons were sequenced (Eurofins Genomics).

4. Protein analysis

All protein samples were resuspended in the RIPA lysis buffer (150 mM NaCl; 0.1 % TritonX-100; 0.5 % sodium deoxycholate; 0.1 % sodium dodecyl sulphate; 50 mM Tris HCL pH 8; protease inhibitor (cOmplete™ Protease Inhibitor Cocktail EDTA-free tablets ,Roche). After 30 min on ice, the cell debris were eliminated by centrifugation at 13 000 x g during 10 min at 4°C. Proteins were quantified using the Bradford method (Bio-Rad Protein Assay).

4.1 Western blot

25 µg proteins were prepared in 2X Laemmli buffer (126 mM Tris-HCL pH 6.8; 0.2 % 2-mercaptoethanol; 20 % glycerol; 4 % SDS; 0.004 % bromophenol blue) and denatured for 5 min at 95°C. They were resolved on SDS-PAGE (10% PA) and transferred on a nitrocellulose membrane (Hybond nitrocellulose, GE Healthcare). Prior to blocking (5% milk, 0.2% tween-20 in 1X PBS), the quality of transfer was verified by Ponceau staining (0.1% red Ponceau in 5% acetic acid). Blocking was performed at room temperature for 1 hour. Primary antibodies against HNRL1 (Santa Cruz, sc-393975) and Gamma-Tubulin (Sigma, T6557) were diluted 1/500 and 1/10 000 respectively in the blocking solution and incubated with the membranes over night at 4°C. After three washing steps (0.2% tween-20 in 1X PBS), HRP-conjugated secondary antibodies were allowed to bind during 1h at RT. Next, the membranes were washed again three times in 0.2% tween-20 in 1X PBS and once in 1X PBS only. Detection was performed after incubation with the ECL Western blot detection reagents (GE Healthcare) on Fusion FX (Vilber Smart Imaging).

5. RNA analysis

All samples assigned to RNA analysis were extracted using the Tri-reagent (Trizol, Sigma) according the manufacturers instructions. After one freeze/thaw cycle, quantification of RNA concentrations was performed by the optical density measure on Nanodrop2000 spectrophotometer.

5.1 RT-qPCR analysis of coding genes and pri-miR-K10/12

Prior to RT-qPCR analysis, 1 µg total RNA was treated with DNase TURBO^{TM} (Invitrogen) during 30 min at 37°C. Then, after addition of 15 mM EDTA (Invitrogen), the enzyme was inactivated by heating to 75°C for 10 min. ¾ of the RNA were used for reverse transcription using SuperScript IV reverse transcriptase kit (Invitrogen) and random 9-mer primers, whereas the rest was treated as non-reverse-transcribed control (NRT). The cDNA was diluted 1/10 and 1 µL was used per qPCR reaction performed with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) using CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Generated data were analyzed with the help of the software CFX manager (Bio-Rad) using the $2^{-\Delta\Delta Ct}$ method.

Table 6: List of primers used for qPCR analysis.

Target	Forward primer	Reverse primer
BACH1	ATTCATGCTTCTGTTCAGCCAA	GGCACTGAGAAGCAGGATCTTT
CASTOR1	AACTCCACATCCTGGAGCAC	GGAATCCTTCCTCATCGACA
DICER	GTACGACTACCACAAGTACTTC	ATAGTACACCTGCCAGACTGT
DROSHA	TAGGCTGTGGGAAAGGACCAAG	GTTCGATGAACCGCTTCTGATG
GAPDH	CTTTGGTATCGTGGAAGGACT	CCAGTGAGCTTCCCGTTCAG
HNRL1	TTGAGCACCGAGAGGATAGG	GGTATCATCAAAGTCATCTTCATCC
ORF57	TGGCGAGGTCAAGCTTAACTTC	CCCCTGGCCTGTAGTATTCCA
PAN RNA	GCCGCTTCTGGTTTTCATTG	TTGCCAAAAGCGACGCA
RBM45	TCAGCAAGTACACACCTGAGT	AGATGATCGGGACTGAGCAAT
RTA	CCCAAACGAAAGCAGAGAAG	GGTGCAGCTGGTACAGTGTG
SMAD5	CCAGCAGCTGCAGCCTCAAAAT	TGCCGGTGATATTCTGCTCCCCAA
SUGP1	GATGTTGCAGGAAAGGCTAACC	TTCCCGTTTCTTCTGAGCGAT
pri-miR-K10/12	AAAACAGGAAGCGGGTTGGAC	CCGCACCCTGCGTAAACAACC

5.2 miRNA analysis by TaqMan RT-qPCR

100 ng of total RNA were used for stem-loop reverse transcription by TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) by using a pool of eight specific primers (miR-K1, miR-K2, miR-K3, miR-K4, miR-K11, let-7a, miR-16, miR-92a, U48) that were supplied in the individual TaqMan miRNA Assays (Applied Biosystems). 0.5 µL of each primer were used in a total reaction volume of 15 µL. If non-infected cells were analyzed, only 4 primers (let-7a, miR-16, miR-92a, U48) were used for the RT step. RT reaction was performed according to the manufacturer's instructions. Resulting cDNAs were diluted twice and 1µl was used to assay the expression of individual miRNAs in separate qPCR reactions containing specific primers and probes (TaqMan miRNA Assays, Applied Biosystems) and TaqMan Universal Master Mix II, no UNG (Applied Biosystems), in total reaction volume of 10 µL. CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was used to perform the PCR and record fluorescent signal and data were analyzed using the $2^{-\Delta\Delta Ct}$ method and CFX manager software (Bio-Rad).

Table 7: TaqMan Assays used in the study.

Target	Reference (ID)
Kshv-miR-K12-1-5p	197204_mat
Kshv-miR-K12-3-5p	008316
Kshv-miR-K12-4-3p	197240_mat
Kshv-miR-K12-11-3p	008562
Hsa-let-7a-5p	000377
Hsa-miR-16-5p	000391
Has-miR-92a-3p	000431
RNU48	001006

VII. References

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Appendix

I. Résumé de la thèse

1. Introduction

Parmi les ARN non codants les mieux étudiés chez les animaux figurent les microARN (miARN). Il s'agit de petits ARN de 20-22 nt qui agissent comme de puissants régulateurs post-transcriptionnels de l'expression des gènes et contrôlent pratiquement tous les processus biologiques (Bartel, 2018). L'importance des miARN pour le développement, la prolifération, le métabolisme ou la réponse au stress est bien décrite et l'altération de leur expression ou leur activité a été liée à des maladies telles que le cancer (Rufino-Palomares et al., 2013).

La biogenèse des miARN consiste premièrement en la reconnaissance et le clivage des structures en tige-boucle au sein d'un transcrit primaire (pri-miARN) par le Microprocesseur constitué d'une RNase de type III, Drosha et de son cofacteur DGCR8. Les pré-miARN ainsi générés sont ensuite exportés vers le cytoplasme où ils subissent un second clivage par Dicer. Les miARN matures qui en résultent sont incorporés au sein d'une protéine Argonaute (AGO) pour constituer le complexe RISC (RNA-induced silencing complex). Lors de l'appariement des bases entre la séquence « seed » du miARN (c'est-à-dire les nt 2 à 7 ou 8) et une séquence complémentaire de l'ARNm cible situé habituellement dans la région 3' non traduite (3'UTR), RISC recrute d'autres facteurs pour bloquer la traduction et déstabiliser l'ARNm cible.

Le rôle fondamental des miARN au sein d'une cellule est de réguler finement son protéome en l'adaptant à différents contextes et en répondant à divers signaux internes et externes. Cependant, cela signifie également que l'expression des miARN elle-même doit être étroitement contrôlée dans l'espace et dans le temps. Outre les mécanismes de contrôle de l'activité transcriptionnelle au niveau des promoteurs des miARN, une régulation post-transcriptionnelle a lieu à chaque étape de la voie de biogenèse des miARN (Creugny et al., 2018; Treiber et al., 2019). Par exemple, l'efficacité de la reconnaissance et le clivage des précurseurs par la machinerie cellulaire dépend à la fois de leurs caractéristiques intrinsèques (motifs de séquence, structure secondaire) qui en font des substrats plus ou moins efficaces,

mais aussi des cofacteurs modulant l'activité des principaux acteurs de leur biogenèse comme le Microprocesseur ou Dicer. Ainsi de nombreux facteurs et des processus accessoires affinent les niveaux des miARN matures qui sont nécessaires pour leur fonction biologique. Ces mécanismes de régulation post-transcriptionnelle peuvent agir de manière spécialisée, en modulant la biogénèse d'un seul miARN, ou ils peuvent avoir un impact sur des sous-ensembles plus larges, voire sur l'ensemble des miARN exprimés à un moment donné.

Dans ce travail, nous nous sommes intéressés à la régulation de la biogenèse des miRNAs lors du premier clivage par le Microprocesseur. La reconnaissance du pri-miARN et son clivage par le Microprocesseur est la première étape de la maturation des miARN. En tant que telle, elle joue un rôle décisif dans l'accumulation finale des miARN (Conrad et al., 2014; Feng et al., 2011; Louloupi et al., 2017). Alors que Drosha et DGCR8 forment le noyau enzymatique nécessaire et suffisant à la maturation des pri-miRNA (Denli et al., 2004), il a été démontré que dans les cellules, la majeure partie du Microprocesseur réside au sein de complexes plus importants contenant de nombreuses protéines accessoires (Gregory et al., 2004). Ces dernières appartiennent pour la plupart à un grand groupe de protéines désignées comme des protéines de liaison à l'ARN (RBP). Les RBP sont impliquées dans tous les aspects du métabolisme de l'ARN, dont également la biogenèse de miARN. A ce jour, l'implication de plusieurs RBP a été démontrée dans la régulation du clivage par le Microprocesseur. Leur activité peut affecter spécifiquement un miARN ou plus globalement un ensemble plus grand de miARN et leur effet peut être soit positif/stimulateur, soit négatif/inhibiteur vis-à-vis de la génération du ou des miARN en question (Creugny et al., 2018; Michlewski & Cáceres, 2019).

Un aspect important de la biologie des miRNA est le fait qu'un grand nombre d'entre eux sont regroupés en clusters génomiques et co-exprimés sur des pri-miARN polycistroniques Outre l'avantage sélectif en termes d'évolution (Marco et al., 2013), l'expression en cluster permet une activité simultanée et coopérative des miRNA en régulant plusieurs gènes cibles à la fois (Kim et al., 2009; Lataniotis et al., 2017; Li et al., 2021; O'Donnell et al., 2005). Ceci permet de réguler par exemple plusieurs facteurs d'une même voie de signalisation et peut induire une réponse plus robuste au niveau cellulaire. Cependant, nous commençons seulement à comprendre que l'importance fonctionnelle de ces polycistrons ne repose pas seulement sur leur expression synchronisée, mais aussi sur l'émergence de modes de régulation précédemment inconnus. Divers mécanismes moléculaires post-transcriptionnels contrôlent la maturation des précurseurs individuels et peuvent expliquer l'accumulation différentielle des miRNA

polycistroniques. Des séquences ou des éléments structurels agissant en *cis* et des facteurs agissant en *trans*, tels que des protéines de liaison à l'ARN et des ARN non codants, peuvent recruter le Microprocesseur, réguler son accès au pri-miRNA et optimiser la maturation de certains pré-miRNA au détriment des autres. De plus, la structure tri-dimentionnelle qu'adopte le pri-miRNA peut impliquer des conformations autorégulatrices. Tout cela fait des clusters des systèmes dynamiques au sein desquels les miARN fonctionnent de manière interdépendante. Sur cet aspect de régulation post-transcriptionnelle de miARN polycistroniques, nous avons publié une une revue dans le journal WIREs RNA (Vilimova & Pfeffer, 2022).

Dans le cadre de ce projet de thèse, nous avons étudié les méchanismes de régulation post-transcriptionnelle qui régulent l'expression d'un cluster de miARN exprimé par un oncovirus humain, l'herpèsvirus associé au sarcome de Kaposi (KSHV). En plus du sarcome de Kaposi d'où il a été isolé pour la première fois, le KSHV est l'agent étiologique de plusieurs maladies lymphoprolifératives telles que le lymphome primitif des séreuses et la maladie de Castleman (Goncalves et al., 2017). La pathogenèse est liée notamment à la capacité du virus de persister tout au long de la vie de l'individu infecté appelée la latence. Au cours de la phase latente, le KSHV échappe au système immunitaire, notamment en exprimant un nombre réduit de protéines. Il maintient par contre une expression élevée d'ARN non-codants, tels que les microARN (miARN).

Le génome du KSHV contient 12 gènes de miRNA (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005). Dix de ces miARN, miR-K1 à miR-K9 et miR-K11, sont regroupés dans un grand cluster intronique s'étendant sur ~4 kb. miR-K10 et miR-K12 sont localisés dans la séquence codante (ORF) et dans la 3'UTR de la Kaposine A respectivement. Les différents précurseurs viraux empruntent la même voie de biogenèse que les miARN cellulaires en se servant de la machinerie de l'hôte. Ceci mène à une expression élevée des miARN viraux, notamment pendant la phase latente du cycle viral. En effet, la proportion de miRNA viraux peut atteindre jusqu'à 70 % de miRNA associés aux complexes RISC dans les cellules infectées (Haecker et al., 2012).

Leur caractère non-immunogène fait de ces molécules un outil de choix pour le virus afin de contrôler son propre cycle réplicatif, mais également l'environnement cellulaire, ce qui lui permet de se maintenir sur de longues périodes. Les miARN du KSHV sont également connus pour avoir un impact sur les voies de signalisation cellulaire impliquées dans le métabolisme et la croissance, l'apoptose et la réponse au stress. Cela conduit à une

augmentation de la durée de vie des cellules infectées et à une reprogrammation des voies de différenciation. De plus, afin d'éviter l'élimination par le système immunitaire, les miARN ciblent également des facteurs responsables de la surveillance immunitaire. Ainsi, les miARN du KSHV participent directement à la transformation cancéreuse (Gottwein, 2012; Ramalingam et al., 2012).

L'importance de la régulation médiée par les miARN dans l'infection et la pathogenèse du KSHV est donc bien établie. Cependant, on en sait beaucoup moins sur les mécanismes qui régulent leur biogenèse et leur accumulation. L'objectif de ce travail était de déchiffrer les mécanismes moléculaires qui modulent la biogenèse des miRNA du KSHV au niveau post-transcriptionnel. Nous nous sommes intéressés au cluster intronique contenant dix pré-miARN du KSHV dont l'organisation polycistronique pourrait permettre l'apparition de méchanismes de régulation spécifiques. Notre laboratoire a précédemment étudié et quantifié l'expression de ces miARN dans des cellules infectées. Les résultats obtenus ont montré que les miARN matures s'accumulent à des niveaux très différents dans les cellules infectées bien qu'ils soient exprimés sur le même transcrit primaire (Contrant et al., 2014). Selon les résultats obtenus précédemment au laboratoire, l'optimalité des substrats de pré-miARN, estimée sur la base de séquence et de structure secondaire, est fréquemment, mais pas toujours, en corrélation avec les niveaux de miARN matures dans les cellules infectées (Contrant et al., 2014). Cela indique l'existence de mécanismes post-transcriptionnels supplémentaires qui modulent la maturation des pré-miARN individuels tout au long de leur biogenèse. De plus, l'expression des miARN à partir de constructions contenant soit le cluster entier, soit un seul pré-miARN conduit à des niveaux d'expression très différents entre les deux conditions. Nous avons également observé que le changement des positions de certains pré-miARN au sein du cluster affecte l'accumulation relative des miARN matures correspondants (Contrant et al., 2014). Le contexte du cluster serait donc important pour l'expression des miRNA individuels.

Afin d'expliquer ces observations, nous avons décidé de déchifrer les mécanismes de régulation post-transcriptionnelle qui s'appliquent lors de la maturation des pré-miARN par le Microprocesseur. Cette étape de biogenèse est en effet considérée comme une étape clé qui détermine l'abondance des miARN matures. Pour cela, nous nous sommes focalisés sur deux aspects pouvant influencer la dynamique des microprocesseurs au sein du cluster : (1) la régulation par des éléments en cis et (2) la régulation par des cofacteurs en trans.

2. Résultats

2.1 Régulation en *cis*

Tout d'abord, nous avons émis l'hypothèse que l'organisation polycistronique du cluster de miARN du KSHV en elle-même pouvait jouer un rôle dans la régulation de la maturation par le Microprocesseur et par conséquent dans l'accumulation des miRNA individuels. Afin de vérifier cette hypothèse, nous avons décidé de suivre l'activité enzymatique du Microprocesseur sur le cluster du KSHV. Pour cela, nous avons effectué des expériences de maturation in vitro qui consistent à incuber le transcrit primaire contenant le cluster de miRNA du KSHV (pri-miR-K10/12) dans des extraits cellulaires surexprimant Drosha et DGCR8. L'accumulation des pré-miARN clivés au cours du temps a été analysée par northern blot quantitatif. Ceci nous a permis de déterminer l'efficacité de maturation des différents pré-miARN au sein du long transcrit primaire. Nous avons pu démontrer que les dix précurseurs de miARN du cluster possèdent des propriétés cinétiques très variables, comme le montrent les taux d'accumulation différentiels des pré-miARN. Ainsi, nous avons pu classer les pré-miARN selon leur capacité à être efficacement clivés. Nous avons également défini l'optimalité des pré-miARN en tant que substrats pour le Microprocesseur basée sur des critères issues de la littérature. De manière intéressante, l'efficacité de clivage ne correspondait pas toujours à l'optimalité des tiges-boucles individuelles ce qui indique que la cinétique du Microprocesseur est en effet influencée par le contexte du cluster. De plus, nous avons constaté que la variation entre les efficacités de clivage des pré-miARN individuels n'était pas toujours en corrélation avec l'expression respective de leurs formes matures. Nous avons notamment remarqué des incohérences importantes entre le clivage très productif de certains pré-miARN (pré-miR-K1 et -K3) et une faible accumulation des miARN correspondants dans les cellules infectées. Ceci suggère donc que ces deux pré-miARN jouent un rôle supplémentaire que donner naissance aux miARN correspondants. Les deux pré-miARN pourraient par exemple assurer une fonction régulatrice au sein du cluster.

Afin de tester cette hypothèse, nous avons délété le pré-miR-K1 ou le pré-miR-K3. Dans les deux cas, ceci a fortement réduit l'expression de tous les autres miARN du cluster ce qui renforce l'hypothèse d'une fonction régulatrice des deux pré-miARN au sein du polycistron. Au contraire, leur expression a été restaurée en insérant un pré-miARN hétérologue, le pré-Let-7a-1, à la place du pré-miR-K1 délété. Ainsi, la fonction régulatrice ne semble pas dépendre de

la séquence du pré-miR-K1, ni de la fonction du miR-K1 mature, mais plutôt de la structure en tige-boucle du précurseur. Une étude plus approfondie du phénomène en utilisant des transcrits tronqués immitant le clivage du pré-miR-K1 et -K3 a permis d'écarter la possibilité que leur clivage est requis afin de libérer des contraintes structurales au sein du transcrit primaire. Au contraire, ils semblent être nécessaires en tant que parties intégrantes du pri-miARN pour exercer leur fonction régulatrice. Ainsi nous avons déduit qu'ils agissent comme des éléments régulateurs en *cis*. Ces éléments sont essentiels à l'expression optimale du cluster du KSHV et prouvent que la maturation des miRNA au sein du cluster est interdépendante.

Par la suite, la connaissance de ces éléments régulateurs nous a permis de développer une nouvelle stratégie d'inhibition de l'expression de tout le cluster. Nous avons ciblé le pré-miR-K1 et empêché sa maturation en utilisant un oligonucléotide antisens (ASO) ce qui a entraîné la diminution globale de tous les miARN du cluster. Nous avons apporté la preuve de principe en exprimant le cluster de manière ectopique dans des cellules HEK293Grip.

Afin de confirmer que cette régulation en *cis* existe dans un contexte plus physiologique, nous avons décidé d'appliquer la même stratégie également dans les cellules infectées par le KSHV (HEK293T-rKSHV). Cependant, la transfection des ASO dans ce modèle cellulaire n'a pas permis d'observer des changements significatifs des niveaux de miARN viraux, même si une tendance à la baisse était visible. Ceci pourrait s'expliquer par l'abondance de miARN dans ces cellules, la variabilité de demi-vie entre les différents miARN et surtout leur grande stabilité en général, ce qui entraîne des difficultées techniques dans les expériences de transfection transitoire. Afin de résoudre ce problème, nous avons mis en place une méthode de marquage métabolique à la thiouridine (4SU) qui est incorporée dans les transcrits d'ARN nouvellement sunthétisés. Ceci nous a permis de mesurer les niveaux de miARN produit ultérieurement à la transfection des ASO et d'observer une baise substantielle de plusieurs miARN du KSHV suite à l'inhibition de la maturation du pré-miR-K1. Nous avons donc confirmé l'existence de la régulation en cis dans un contexte physiologique. Ces résultats ont été publiés au cours de la troisième année de thèse dans le journal Nucleic Acids Research (Vilimova et al., 2021).

2.2 Régulation en *trans*

Comme les molécules d'ARN, y compris les précurseurs des miARN, sont rarement dépourvues de partenaires protéiques, dans un second volet de ce projet de thèse, nous nous sommes intéressés aux cofacteurs potentiels qui pourrait participer à la biogenèse des miARN

du KSHV. Il s'agirait là de protéines qui modulent l'activité du Microprocesseur en interagissant à la fois avec ce dernier et avec les pré-miARN régulateurs. Les cofacteurs se liant aux précurseurs des miARN sont des modulateurs bien connus de la biogenèse des miRNA cellulaires (Michlewski & Cáceres, 2019; Treiber et al., 2019). De plus, certains cofacteurs ont été spécifiquement impliqués dans la régulation des miRNA en cluster, permettant par exemple le phénomène d'assistance qui stimule la maturation des précurseurs suboptimaux grâce à la proximité d'un précurseur plus optimal (Hutter et al., 2020; Kwon et al., 2020). Dans le cadre de notre projet, ces cofacteurs peuvent avoir des effets différents sur la biogenèse des miARN du KSHV : (1) La liaison des cofacteurs aux motifs de structure/séquence des pré-miARN peut entraîner un recrutement préférentiel du complexe de Microprocesseur. (2) La liaison du cofacteur peut inhiber la maturation efficace du pré-miARN en empêchant la reconnaissance et/ou le clivage par le Microprocesseur. (3) La liaison des cofacteurs peut modifier la structure secondaire du pré-miARN, de sorte qu'elle devienne plus ou moins favorable à la reconnaissance et/ou au clivage par le Microprocesseur. (4) La liaison des cofacteurs dans le contexte d'un polycistron peut participer à l'organisation hiérarchique de la maturation des pré-miARN, en exerçant l'une des activités ci-dessus ou via d'autres mécanismes moléculaires. Ainsi, l'activité de ces protéines dans la biogenèse du cluster permettrait d'expliquer l'accumulation différentielle des miARN individuels. De plus, en continuité avec le phénomène de l'interdépendance de la maturation au sein du cluster développé dans la première partie, le recrutement des cofacteurs en *trans* serait une possibilité pour expliquer l'action des pré-miARN en tant que régulateurs en *cis*. Par conséquent, l'implication de tels cofacteurs pourrait ajouter une autre dimension à notre compréhension des mécanismes moléculaires régulant la biogenèse du cluster de miARN du KSHV.

Afin de mettre en évidence ces facteurs, nous avons étudié l'interactome des différents pré-miARN du cluster. Pour cela, nous avons utilisé les résultats obtenus au laboratoire précédemment grâce à une approche de « RNA pulldown » suivie d'une analyse par spectrométrie de masse (Creugny, 2019). Brièvement, les tige-boucles comprenant un pré-miARN et 20 nt en amont et en aval ont été transcrites in vitro, couplées à des billes magnétiques et incubées avec un extrait nucléaire de cellules BC-3 issues d'un lymphome induit par le KSHV. L'analyse des protéines retenues sur les pré-miARN a permis d'identifier au total 137 protéines interagissant potentiellement avec un ou avec un sous-ensemble de pré-miARN du KSHV. Il s'agissait principalement de protéines de liaison à l'ARN et certaines d'entre elles ont déjà été identifiées comme des cofacteurs de la biogenèse des miARN cellulaires, telles que

MSI2, HUR et FUS (Choudhury et al., 2013; Morlando et al., 2012). Comme contrôle positif pour la validation de l'approche, pré-let-7a-1 a été utilisé en parallèle et a retenu des protéines hnRNP A1 et LIN28B, connues pour intervenir dans sa biogenèse (Guil & Caceres, 2007; Michlewski et al., 2008; Piskounova et al., 2011; Viswanathan et al., 2008).

En partant du large ensemble de protéines identifiées dans les essais de « RNA pulldown », nous avons décidé de réduire la liste des candidats cofacteurs putatifs, afin de pouvoir procéder à leur validation fonctionnelle. Plusieurs critères ont été pris en compte dans le choix des candidats. Par exemple, tous sont des RBP connus et présentent une localisation nucléaire rendant leur implication dans la maturation médiée par Microprocessor plus plausible. Considérant notre découverte que le pré-miR-K1 agit comme un élément régulateur en *cis*, permettant une expression optimale de l'ensemble du cluster, il semblait intéressant d'explorer une fonction potentielle de cofacteurs dans cette régulation. Nous avons donc recherché des protéines enrichies sur pré-miR-K1. Parmi elles, SUGP1 (SURP and G-patch domain-containing protein 1) a montré une liaison spécifique et exclusive au pré-miR-K1. Une autre protéine se liant spécifiquement à pré-miR-K1 était hnRNPL (heterogenous nuclear ribonucleoprotein L). Puisque le pré-miR-K3 est également requis pour l'expression optimale des miRNAs sur le cluster, nous avons également cherché parmi ses interactants potentiels. Cependant, nous n'avons pas retrouvé de protéine spécifiquement et significativement enrichie sur pré-miR-K3. Par contre, nous avons trouvé deux protéines, hnRNPK (heterogenous nuclear ribonucleoprotein K) et RBM45 (RNA-binding protein 45), qui ont été identifiées en parallèle sur les pré-miR-K1 et pré-miR-K3. Étant donné que les deux précurseurs possèdent une fonction régulatrice analogue au sein du cluster, ils pourraient également interagir avec les mêmes protéines. Finalement, HNRL1 ou hnRNPUL1 (heterogenous nuclear ribonucleoprotein U-like 1) a été identifiée avec presque tous les pré-miARN utilisés dans les « RNA pulldown », y compris les deux pré-miRNA cellulaires utilisés dans l'analyse, pre-miR-155 et pre-let-7a-1. Cela indique que HNRL1 pourrait fonctionner comme un cofacteur plus général, régulant potentiellement de nombreux miARN viraux et cellulaires. En outre, alors qu'aucune des autres protéines n'était précédemment liée à la biogenèse des miARN, nous avons trouvé dans la littérature des indices en faveur de l'implication potentielle de HNRL1 dans la maturation des miARN. Par exemple, la protéine a été identifiée en interaction avec le Microprocesseur (Gregory et al., 2004) et elle peut se lier aux pri-miARN cellulaires (Van Nostrand et al., 2020). Cependant, l'activité spécifique de HNRL1 dans la biogenèse des miARN n'a jamais été confirmée et explorée plus en détail.

Afin de vérifier l'implication des candidats sélectionnés dans le processus de biogenèse des miARN du KSHV, nous avons d'abord décidé d'évaluer l'impact de leur déplétion sur l'accumulation des miARN matures dans les cellules. Selon notre hypothèse, si un candidat donné est un véritable cofacteur, sa déplétion devrait avoir un impact sur la biogenèse des pré-miARN auxquels il se lie. De plus, considérant l'importance des pré-miR-K1 et -K3 dans l'expression de l'ensemble du cluster, un cofacteur pourrait moduler les niveaux de tous les miARN du cluster en régulant un de ces deux pré-miARN. En outre, l'augmentation ou la diminution observée des niveaux de miARN fournirait des informations sur la fonction stimulante ou inhibitrice du cofacteur en question.

Premièrement, les expériences de répression (knockdown) transitoire par des siARN (small interfering RNA) n'ont pas permis de mesurer un impact suffisamment marqué sur les niveaux de miARN matures pour pouvoir distinguer la réelle implication d'un cofacteur dans leur maturation. Ceci était probablement dû à la stabilité élevée des miARN dans les cellules. Pour surmonter ce problème, nous avons choisi d'effectuer des transductions lentivirales en utilisant des shRNA (short hairpin RNA) ciblant les protéines d'intérêt. Cela permet de générer des lignées cellulaires stables réprimant le gène cible sur de longues périodes, grâce à l'intégration du transgène et à l'expression continue du shRNA. En outre, cette méthode nous a permis de transduire des lymphocytes B infectés naturellement, qui sont des cellules difficiles à transfecter. Nous avons décidé d'utiliser la lignée cellulaire BCBL1 issue d'un lymphome induit par le KSHV, qui constitue un bon modèle d'étude, car les cellules expriment des quantités élevées de tous les miARN du KSHV et représentent l'infection physiologique par le KSHV. Cependant nous n'avons pas réussi à générer des lignées déplétées de toutes les protéines d'intérêt ce qui pourrait être lié à leur caractère essentiel ou à une toxicité des shRNA.

Parmi les trois lignées qui ont pu être caractérisées, shRBM45, shHNRL1 et shSUGP1, aucune variation de miARN viraux n'a été observé dans les shRBM45. En revanche, la répression de HNRL1 et SUGP1 a eu pour l'effet une augmentation marqué de tous les miARN du KSHV testés, en plus d'une augmentation plus modeste de trois miARN cellulaires, miR-16, miR-92a et let-7a. Ces résultats indiquent que les deux protéines pourraient inhiber la production de la totalité du cluster du KSHV, mais également avoir un impacte plus global sur l'expression des miARN cellulaires. En vérifiant l'expression du transcrit primaire pri-miR-K10/12 et des ARNm de Drosha et de Dicer, nous avons démontré que cette augmentation de miARN ne provient pas d'une stimulation transcriptionnelle du cluster, ni d'une dérégulation

de la machinerie de biogenèse. Cela confirme l'hypothèse selon laquelle la régulation des miRNA médiée par HRNL1 et SUGP1 aurait lieu au niveau post-transcriptionnel et les deux protéines seraient impliquées en tant que cofacteurs. Pour explorer davantage le phénotype de lignées shHNRL1 et shSUGP1, nous nous sommes intéressés également à l'impact de la régulation des miARN du KSHV sur leurs cibles cellulaires et virales connues. Cohérent à l'augmentation des niveaux de miARN du KSHV, nous avons observé une réduction de presque tous les ARNm cibles testés. Finalement, ces résultats nous ont permis d'identifier des cofacteurs intéressant ayant une activité inhibitrice sur la production des miARN étudiés et ayant des effets phénotypiques sur l'expression des cibles des miARN régulés.

Dans nos expériences de pulldown, HNRL1 a été capable de lier de nombreux pré-miARN, y inclus des pré-miARN cellulaires. Ceci nous a motivés à l'étudier également en dehors du contexte de l'infection par le KSHV. Pour vérifier si les miRNA cellulaires sont régulés par cette protéine, nous avons généré une délétion génomique (KO) de la protéine dans la lignée cellulaire de cancer du côlon HCT116. Ensuite, nous nous sommes intéressés à l'impact que le KO de HNRL1 aurait sur l'expression des miARN cellulaires. L'accumulation de trois miARN, let-7a, miR-16 et miR-92a a été mesurée et comparée à celle de la lignée cellulaire d'origine. De manière inattendue, aucun des miARN n'a présenté une expression augmentée, comme cela a été observé dans la lignée BCBL1 transduite par shHNRL1. Cette divergence dans les deux systèmes expérimentaux utilisés pourrait être due à une activité contexte-dépendante de HNRL1, qui pourrait agir comme cofacteur dans les lymphocytes B infectés et non dans la lignée épithéliale HCT116. D'autres expériences seront nécessaires afin de vérifier cette possibilité.

Pour conclure, ces expériences ont permis d'obtenir un premier aperçu de la fonction de deux cofacteurs potentiels impliqués dans la biogénèse des miARN viraux et cellulaires, à savoir HNRL1 et SUGP1. Nos premiers résultats indiquent qu'ils ont tous les deux un impact négatif sur la production des miARN exprimés par le KSHV au niveau post-transcriptionnel. La déplétion de l'un ou l'autre des candidats dans les cellules infectées par le KSHV conduit à une augmentation de l'expression des miARN viraux accompagnée d'une augmentation de la répression de leurs gènes cibles. La régulation des miARN cellulaires par ces cofacteurs reste également une possibilité. Cependant, à l'heure actuelle, nous ne pouvons pas conclure de manière définitive quant au rôle de ces protéines dans la biogenèse des miARN, car nous ne disposons que de résultats préliminaires. D'autres expériences seront nécessaires pour

confirmer ces résultats et pour caractériser le mode d'action HNRL1 et SUGP1 dans la biogenèse des miARN.

3. Conclusion

Les travaux effectués dans le cadre de ce projet de thèse ont permis de révéler de nouveaux mécanismes de régulation post-transcriptionnelle impliqués dans l'expression des miARN du KSHV. En effet, nos résultats démontrent que l'expression de ce cluster de miARN viraux subit une régulation complexe et interdépendante à plusieurs niveaux. Tout d'abord, nos recherches se sont concentrées sur la fonction de certains pré-miARN agissant comme éléments régulateurs en *cis* qui permettent l'expression optimale de tous les miARN du cluster du KSHV (Vilimova et al., 2021). Nous avons fourni la preuve d'une régulation d'ordre supérieur opérant au sein de ce polycistron. À notre connaissance, il s'agit de la première étude approfondie et détaillée d'un grand cluster de miRNA viraux, en le considérant comme une unité interdépendante. De plus, ces résultats s'inscrivent dans un nouveau domaine de recherche, encore peu exploré, de la régulation des miARN exprimés en cluster.

Un autre aspect qui contribue à la complexité de la biogenèse des miARN du KSHV est le recrutement de facteurs accessoires dont l'activité en *trans* module la maturation des miARN individuels ou du cluster dans son ensemble. Nous avons donc cherché à identifier des cofacteurs potentiels et à caractériser leur mode d'action en ce qui concerne la régulation des miARN du KSHV. Ces travaux ont permis de mettre en évidence des protéines candidates potentiellement impliquées dans la régulation du cluster en se liant à des pré-miARN du KSHV. L'analyse phénotypique des lignées stables déplétées des deux protéines d'intérêt, HNRL1 et SUGP1, indique que leur activité inhiberait la maturation de la totalité du cluster. Ceci impliquerait donc un mécanisme antagoniste à la régulation médiée par les éléments en *cis* qui pourrait s'appuyer sur ces derniers ou agir de manière indépendante. Ces résultats prometteurs devront cependant être confirmés par d'autres approches complémentaires. En perspective à ce projet, une étude plus approfondie de la fonction et du mode d'action de ces protéines pourrait améliorer notre compréhension de la maturation des miARN du KSHV.

Finalement, d'un point de vue méthodologique, nous avons démontré la possibilité de cibler les éléments régulateurs en *cis* avec des oligonucléotides antisens afin de moduler l'expression l'intégrité du cluster du KSHV. Etant donné qu'il s'agit d'un cluster à activité

oncogène, cette approche pourrait s'avérer intéressante en vue d'un futur développement thérapeutique. Alors que d'autres groupes de recherches ont développé des méthodes d'élimination des miARN individuels dans leurs formes matures (Ju et al., 2020; Rupaimoole & Slack, 2017), notre méthode est novatrice puisqu'elle vise l'étape de maturation en amont. Ainsi, elle présente l'avantage d'inhiber tout un ensemble de miARN pathogènes avec une seule molécule thérapeutique.

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Monika VILÍMOVÁ

Étude des mécanismes de régulation de biogenèse des microARN exprimés par l'Herpèsvirus associé au sarcome de Kaposi

Study of mechanisms regulating the biogenesis of microRNAs expressed by Kaposi's sarcoma-associated herpesvirus

Résumé

Les microARN (miARN) sont d'importants régulateurs de l'expression génique exprimés par presque tous les eucaryotes, ainsi que certains de leurs virus associés. Dans le cadre de ce projet, nous avons étudié la biogenèse d'un cluster de miARN exprimé par un oncovirus humain, l'Herpèsvirus associé au sarcome de Kaposi (KSHV). L'objectif était de caractériser les mécanismes de régulation post-transcriptionnelle qui régissent la maturation ce de cluster. Nous avons démontré que certains des miARN précurseurs (pré-miARN) servent d'éléments régulateurs en *cis* et sont nécessaires pour l'expression de la totalité du cluster. En se basant sur ces résultats, nous avons développé une méthode d'inhibition globale de l'expression du cluster. Finalement, nous avons également étudié l'implication de potentiels co-facteurs HNRL1 et SUGP1 qui pourraient réguler la biogenèse des miARN en *trans*.

Mots-clés : miARN, polycistron, Microprocesseur, co-factor, régulation post-transcriptionnelle, KSHV

Abstract

MicroRNAs (miRNAs) are important regulators of gene expression expressed by almost all eukaryotes and also some of their associated viruses. In this project, we have focused on the biogenesis of a miRNA cluster expressed by the human oncovirus, Kaposi's sarcoma associated herpesvirus (KSHV), with the aim to decipher the post-transcriptional molecular mechanisms controlling their processing. We have shown that some of the viral precursor miRNAs (pre-miRNAs) can act as *cis* regulatory elements and are required for the optimal processing of the remaining pre-miRNAs within the cluster. An approach to inhibit the global expression of the cluster was developed based on these findings. In addition, we were interested in potential implication of protein co-factors HNRL1 and SUGP1 that could regulate the miRNA expression in *trans*.

Keywords: miRNA, polycistron, Microprocessor, co-factor, post-transcriptional regulation, KSHV