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Human Cytomegalovirus Reprograms the Expression of Host Micro-RNAs whose Target Networks are Required for Viral Replication: A Dissertation

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HUMAN CYTOMEGALOVIRUS REPROGRAMS THE EXPRESSION OF HOST MICRO-RNAS WHOSE TARGET NETWORKS ARE REQUIRED FOR VIRAL REPLICATION

A Dissertation Presented
By

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Microbiology and Physiological Systems

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Dedication

I dedicate my doctoral dissertation to my family. I am truly blessed to have such a commending group of caring and compassionate people in my life. I have learned to be the person I am today from your actions and ideals. I wish that all members of my family were still with us so that I could thank them personally, but I know that they all are aware of my appreciation. I love you all more than you know, and I know that I don't say it enough. This dissertation is a testament to what a strong family can help someone accomplish, and I will be forever grateful for having you in my life.

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List of Symbols

HCMV	human cytomegalovirus
HSV	herpes simplex virus
EBV	epstein barr virus
KSHV	kaposi's sarcoma associated virus
VZV	varicella zoster virus
WNV	west nile virus
RNAi	RNA interference
siRNA	short-interfering RNA
miRNA	micro-RNA
ncRNA	non-coding RNA
PTGS	post-transcriptional gene silencing
MRE	miRNA recognition element
TFBS	transcription factor binding site
UTR	untranslated region
gB	glycoprotein b
pp65	phosphoprotein 65 kDa
IE	immediate early
E/DE	early/delayed early
L	late
MIEP	major IE promoter
DDR	DNA damage response
HTS	high throughput sequencing
MAPK	mitogen activated kinase
CREB	cyclic-amp response element binding protein
CRE	CREB response element
SP1	specificity protein 1
KRAS	Kirsten rat sarcoma viral oncogene homolog

Abstract

The parasitic nature of viruses requires that they adapt to their host environment in order to persist. Herpesviruses are among the largest and most genetically complex human viruses and they have evolved mechanisms that manipulate a variety of cellular pathways and processes required to replicate and persist within their hosts. Human cytomegalovirus (HCMV), a member of the β-herpesvirus sub-family, has the capacity to influence the expression of many host genes in an effort to create an optimal environment for infection. One mechanism utilized by HCMV to alter gene expression is the host RNA interference (RNAi) pathway. This is evidenced by a requirement of host factors to process viral micro-RNAs (miRNAs) and by the dynamic expression of host miRNAs during infection.

The work presented in this dissertation demonstrates that productive HCMV infection reprograms host miRNA expression in order to positively influence infection. I was able to identify a cohort of infection-associated host miRNAs whose change in expression during infection was highly significant. Using the enhancer-promoter sequences of this panel of host miRNAs, I statistically enriched for the presence of functional transcription factor binding sites that regulated the expression of two highly conserved clusters of host miRNAs: miR132/212 and miR143/145. Given that inhibiting their infection-

associated change in expression during infection was detrimental to viral replication, it suggests that HCMV mechanistically influences the expression of these miRNA clusters. In order to determine the functional relevance of these miRNAs, I assembled a cohort of potential miRNA target genes using gene expression profiles from primary fibroblasts. By statistically enriching for miRNA recognition elements (MRE) in the respective 3'-UTR sequences, I generated a miRNA target network that includes thousands of host genes. I evaluated the efficacy of our novel miRNA target prediction algorithm by confirming the functionality of enriched MREs present in the 3'-UTR of KRas and by confirming anecdotal miRNA targets from published studies. Gene ontology terms enriched from infection-associated host miRNA target networks suggest that the utility of host miRNAs may extend to multiple host pathways that are required for viral replication. The targeting of multiple miRNAs to shared genes increased the statistical likelihood of target site enrichment. I propose that identifying cooperative miRNA networks is essential to establishing the functional relevance of miRNAs in any context. By combining contextual data on the relative miRNA/mRNA abundance with statistical MRE enrichments, one will be able to more accurately characterize the biological role of miRNAs.

Chapter I:

Introduction

A. The Herpesvirales Order and Human Cytomegalovirus

Herpesviruses

The first identified herpesvirus, Pseudorabies virus, was isolated in 1902 by Aladar Aujeszky (14, 240). Since then, at least one herpesvirus has been identified in almost all animal species (Table 1.1) (99). Herpesviruses were historically classified based on the architecture of the virion. The identification of numerous novel herpesvirus species and the characterization of their replication and pathogenesis eventually led the establishment of the *Herpesviridae* family. The more recent availability of extensive nucleotide sequence data resulted in the establishment of the higher taxonomic order *Herpesvirales*, which is divided into three distinct families of herpesviruses based on their species restrictions and sequence conservation (Table 1.1) (75). The *Herpesviridae* family represents one of the largest and most genetically complex families of viruses. It is speculated that the family is between 200 and 300 million years old, implying that they have been co-evolving with animals throughout their evolutionary divergence (155, 220). The extensive host range of the *Herpesviridae* highlights the longevity of this family, and its ability to adapt to and persist within such discrete environments has allowed these viruses to survive multiple mass extinction events (270, 271). Co-evolution with their hosts has allowed them to

persist within the respective hosts for their entire lifetime without commonly causing debilitating or malignant disease.

Family	Sub-Family	Genus	Hosts
Alloherpesviridae	None	Batrachovirus Cyprinivirus Ictalurivirus Salmonivirus	Frog, Eel & Fish
Herpesviridae	Alpha (α)	Simplexvirus Varicellovirus Iltovirus Mardivirus Scutavirus Unassigned	Human, Bird, Turtle, Cow, Marsupial, Primate, Rabbit, Antelope, Cat, Deer, Dog & Horse
	Beta (β)	Cytomegalovirus Roseolovirus Muromegalovirus Proboscivirus Unassigned	Human, Primate, Mouse, Elephant, Guinea Pig, Pig & Tree Shrew
	Gamma (γ)	Lymphocryptovirus Rhadinovirus Macavirus Percavirus Unassigned	Human, Primate, Antelope, Cow, Hippopotamus, Pig, Sheep, Horse, Weasel, Mouse, Hamster, Seal
	Unassigned	Unassigned	Iguana
Malacoherpesviridae	None	Ostreavirus Aurivirus	Oyster Abalone

Table 1.1: *The Herpesvirales order.* Listed in this table are all known members of the *Herpesvirales* order. They are organized into family, sub-family and genus. The far right column lists the host-range for the respective family in order to illustrate the ubiquitous nature of the herpesviruses. Each respective genus can harbor up to 17 individual species with both unique and similar hosts. There are currently 96 documented herpesviruses listed by the International Committee on Taxonomy of Viruses.

The distinct biological properties of the *Herpesviridae* family delineate it into three separate sub-families: alpha (α), beta (β) and gamma (γ) (Table 1.1 and 1.2). These sub-families are based on the host range, the duration of the

replication cycle, cytopathology and specific characteristics of latency (Table 1.2) (99, 281). The sub-families are more definitively classified on the basis of genome arrangement and sequence variability, but these distinctions are generally accurately defined by the classifications that are based on biological properties. The mapping of phylogenetic relationships through DNA sequencing has, however, helped to establish viral ancestry and determinants of species specificity (155, 221).

Sub-Family	Human Herpesvirus Species	Biological Properties
Alpha (α)	Herpes Simplex Virus 1 (HSV1)	- Variable host range - Short reproductive cycle - Rapid spread in culture - Efficient destruction of infected cells - Establishment of latency primarily in sensory ganglia
	Herpes Simplex Virus 2 (HSV2)	
	Varicella Zoster Virus (VZV)	
Beta (β)	Human Cytomegalovirus (HCMV)	- Restricted host range - Long reproductive cycle - Slow spread in culture - Infected cells frequently become enlarged (cytomegalia) - Latency can be established in secretory glands, lymphoreticular cells, kidneys and other tissues
	Human Herpesvirus 6 (HHV6)	
	Human Herpesvirus 7 (HHV7)	
Gamma (γ)	Epstein-Barr Virus (EBV)	- Restricted host range - In vitro replication in lymphoblastoid cells, and lytic infections can occur in some types of epithelioid and fibroblastic cells - Specificity to either T- or B-lymphocytes - Establishment of latency primarily in lymphoid tissue - Associated with cellular transformation
	Kaposi's Sarcoma Associated Herpesvirus (KSHV)	

Table 1.2: *The human herpesviruses.* The human herpesviruses are all members of the *Herpesviridae* family. Their sub-family and species classifications are illustrated in this table along with the biological properties that distinguish the respective sub-families.

The enveloped herpesvirus virion ranges in size from 120 – 200 nm, and its surface is studded with an array of glycoproteins. The electron dense dsDNA genome is enclosed within an icosahedral capsid structure (12, 110, 301). Between the capsid and the envelope is a distinct, protein-rich region known as the tegument. This ordered structure contains viral proteins that are required for regulating a multitude of processes involved in activation of viral gene expression, immune evasion and virion assembly (227, 230). The herpesvirus genome ranges in size from 120 kbp – 230 kbp, and it has the unique capacity to code for a large array of enzymes involved in nucleic acid metabolism, protein modification/processing and DNA replication. A distinct set of core genes involved in nucleotide metabolism, DNA replication and virion structure are conserved within the *Herpesviridae* family, but not all are essential for viral replication (6, 229). Herpesviruses characteristically replicate and encapsidate their DNA genomes in the nucleus and production of infectious progeny inevitably kills the host cell (Figure 1.1) (226, 227). Herpesviruses can also establish a latent reservoir where the viral genome is maintained with minimal viral gene expression. This life-long latent infection can be reactivated under various circumstances, and this can subsequently cause various symptomatic disorders (Figure 1.1) (99). Human cytomegalovirus (HCMV) represents the largest and most genetically complex member of the *Herpesviridae* family. Its presence among the human population is ubiquitous, and the virus has

developed elaborate strategies that allow it to persist for the lifetime of the host with little to no apparent pathology.

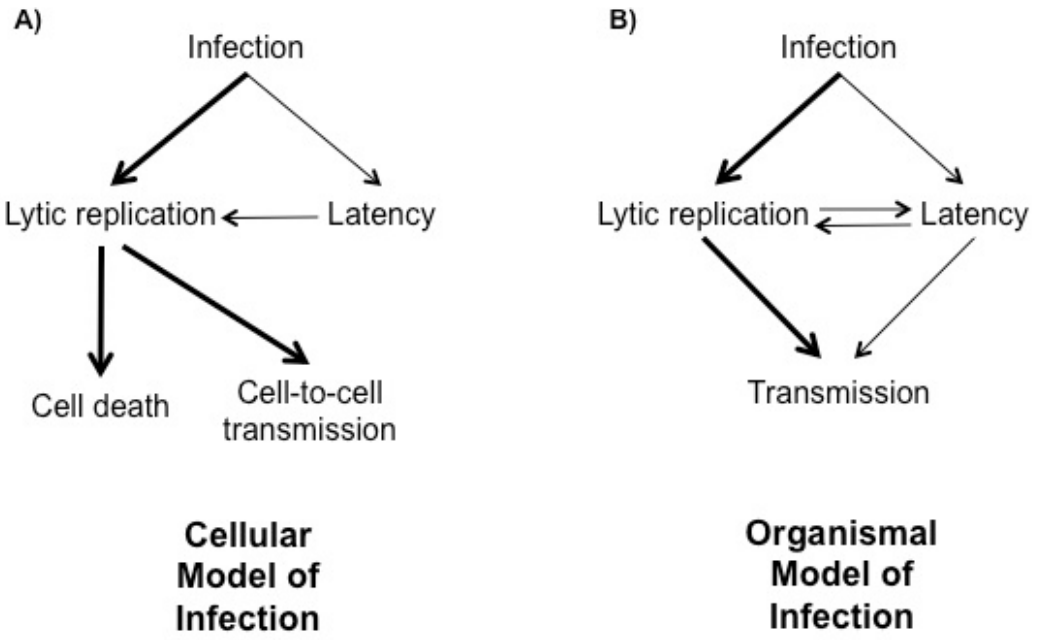


Figure 1.1: *Progression of herpesvirus infections.* This figure illustrates the progression of herpesvirus infections in cell culture and *in-vivo*. Following primary infection, lytic replication is initiated and latent infections are established in order to allow for dissemination of the infection and for the retention of latent virus. The thickness of each arrow indicates the relative frequency at which each event occurs. *This figure was adapted from Fields Virology* (99).

Human cytomegalovirus

Human cytomegalovirus is a member of the β-herpesvirus subfamily. It is a ubiquitous, species-restricted pathogen with seropositivity rates ranging from 50% to greater than 95% depending on the demographic being tested (214). HCMV is spread horizontally by close or intimate contact with bodily fluids, so the rates of seroprevalence increase with age and are linked with socioeconomic standards of living (45, 147, 290). HCMV exhibits three characteristic types of infection: productive, persistent and latent (99). Each is characterized by the extent of viral gene expression and viral genomic replication occurring in the infected cell, and by the relative amount of infectious progeny virus being produced. Primary productive infections are typically asymptomatic and virus can be shed for a period of months to years in urine, feces, saliva, tears, semen and cervical secretions (99, 141). Productive replication of HCMV is believed to inevitably kill the host cell, and infection can be propagated either through cell-to-cell spread or through primary infection of neighboring cells by newly generated mature virions (99, 212). Although HCMV is a species-restricted human virus, it exhibits a promiscuous tropism pattern within its host. Not every cell-type is capable of supporting a complete productive replication cycle, but the majority of human cells and organs can be infected (32, 299). This phenomenon contributes to the unique patterns of virus shedding, dissemination and pathogenesis. Within the host, mature virus is thought to be shed from fully permissive cells such as

fibroblasts, epithelial cells and smooth muscle cells. This theory is supported by the established cell culture model systems with these particular cell types (333, 334). Dissemination of HCMV infection *in-vivo* is thought to be mediated by immature myelomonocytic leukocytes and dendritic cells (DC). Although leukocytes are not fully permissive to productive replication, it is speculated that they passively transfer the virus to other organs within the host (99, 299). The surveillance functions of these cells are ideal for transporting the virus to other tissues and organs. Differences in cell tropism among the various strains of HCMV *in vitro* are a result of the loss of tropism-specific genes (42, 53, 165). These factors mediate similar effects *in-vivo,* but the availability of host factors required for viral replication also contribute to cell tropism. Host factors also contribute to the establishment and maintenance of latent HCMV infection. Episomal DNA is latently maintained in CD34+ monocyte progenitors with minimal viral gene expression. These cells do not support productive replication but they allow for maintenance of latent genomes and reactivation of viral DNA (224, 326). In instances of immunosuppression, viral gene expression can be activated and productive replication can commence again. The exit and entrance into the latent state is partially mediated through epigenetic silencing of viral gene expression through chromatin modification (272, 304). It has also been proposed that alterations in host miRNA expression profiles contribute to maintenance of the latent infection (263). The requirement of host factors is a hallmark of HCMV replication and it is necessary for precise control of almost all aspects of infection.

HCMV can also be spread vertically from mother to child via trans-placental congenital infection, intrapartum transfer or through breast milk (150, 275, 324). Given the ability of the virus to cross the placental barrier, congenital HCMV infection is the leading cause of birth defects associated with an infectious agent.

Congenital infection with HCMV can result in the formation of various neurological sequelae, some of which can lead to serious developmental disorders (4, 182). Approximately 1% of all live births present active HCMV infection, and roughly 10% of those births exhibit symptomatic HCMV disease (213). Historically, it was presumed that primary infections during gestation were responsible for the majority of the cases of congenital HCMV disease. However, more recent studies have shown that pre-existing maternal infections and primary infections of infants have similar incidences of disease (106, 361). Congenital HCMV disease can be diagnosed by identifying intrauterine developmental abnormalities such as organ enlargement or brain malformations, but the infectious nature of the disease can only be inferred through maternal HCMV antibody seropositivity since there is considerable overlap with the pathological sequelae of other infectious and genetic disorders (35, 274). HCMV infection also presents a significant risk to immuno-compromised hosts, as weakened immune surveillance can allow for extensive viral replication. Patients undergoing allogenic stem cell transplants, solid organ transplants, AIDS patients or patients with autoimmune diseases are all at significant risk of either acquiring new infections or for reactivating latent virus (62, 84, 331). Cases of HCMV

disease in immuno-competent hosts have also been described (57, 327). It is speculated that the virus is the cause of up to 8% of all cases of mononucleosis (169). HCMV infection is also associated with the manifestation of numerous diseases including atherosclerosis, hypertension, glioblastoma multiforme (GBM) and Alzheimer's disease (20, 86, 210, 264, 314). The role that the virus plays in disease causality or progression has not been accurately defined. However, the presence of the virus in the relevant cell types and tissues is a strong positive correlation of disease development. Regardless of the type of infection, the severity of HCMV disease is dependent on the efficiency of host immune surveillance and the degree of viral replication that occurs.

The HCMV virion has the characteristic enveloped structure and the viral membrane is studded with a complex network of glycoproteins required for cell binding, fusion, entry and cell-to-cell spread (39). The virion itself is pleomorphic and is larger than that of the other human herpesviruses (200 - 300 nm). Its dsDNA genome encased within a ~125 nm icosahedral capsid and this is supported by the viral tegument structure, which contains a multitude of host and viral proteins (Figure 1.2 A) (99, 340). The most abundant of these proteins are pp65, a viral phosphoprotein required for inhibiting the cellular interferon response and pp71, a viral transactivator (329, 340). The functions of tegument proteins are quite diverse. They support the structure of the virion, regulate fusion and uncoating, dampen the host responses to infection and transactivate the expression of viral genes. The ~235 kbp viral genome is the largest of the

Herpesviridae family, and it has the capacity to code for ~170 viral genes and 15 viral miRNAs (Figure 1.2 B & C) (118, 225, 316). It is organized into unique-long (U_L) and unique-short (U_S) regions, which are flanked by terminal (TR_L and TR_S) and internal (IR_L and IR_S) repeat sequences (Figure 1.2 C) (99). HCMV represents one of the largest and most genetically complex human viruses. The requirement of cellular factors is essential to the progression of infection, and a hallmark of HCMV infection is its characteristic ability to reprogram host gene expression in order to create an optimal environment for infection (373). The impact that infection has on host gene expression can be observed even at the initial events of virion binding (37).

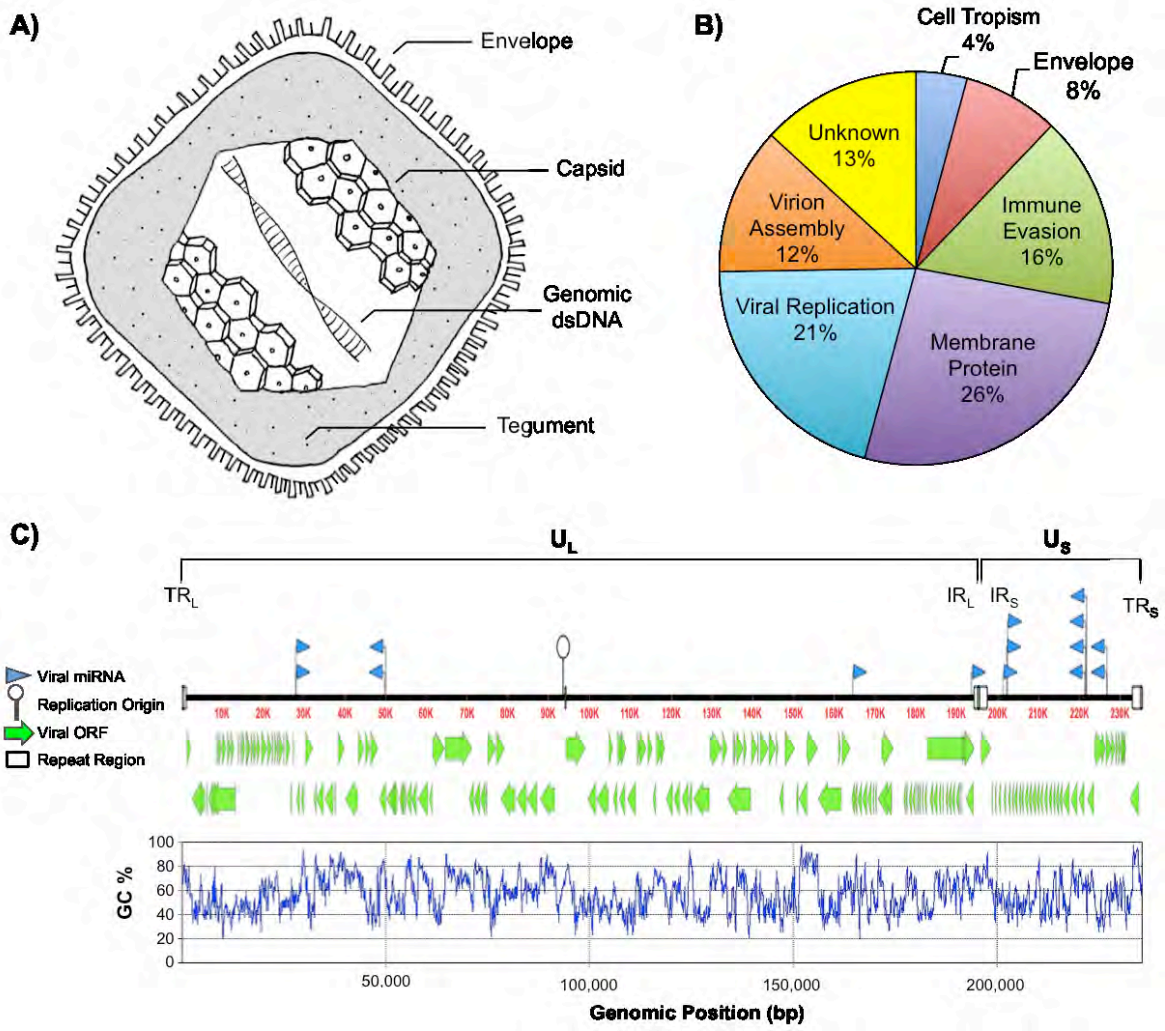


Figure 1.2: *HCMV virion structure and genomic coding capacity.* (A) The HCMV virion is illustrated with the characteristic structural and genomic components. (B) The coding capacity of the dsDNA genome is broken down according to the gene functions. The chart illustrates the documented function of all the characterized viral genes. (C) The HCMV genome is represented to show the coding regions of the viral open reading frames and their orientation, the repeat regions, the viral miRNAs and the replication origin. The legend next to the genome illustration relates how each respective segment is labeled. Below the genome annotation is a plot showing the GC-content of the viral genome based on a calculation made using a sliding window of 50 bp.

HCMV infection begins at the plasma membrane where viral glycoproteins mediate binding and entry through interactions with host proteoglycans and membrane proteins (Figure 1.2). The glycoprotein gB, one of the most highly conserved herpesvirus core proteins, regulates heparan sulfate binding along with other complexes of glycoproteins such as gH:gL, gM:gN and gO (99). Although these complexes do not represent all of the viral glycoproteins, they are thought to be the most important given that they are required for viral replication (133). Other cell membrane components such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor alpha (PDGFRα) and integrins have also shown to influence virion binding and fusion. Regulating the coordinated interaction of each of these molecules is thought to be essential for infection (97, 313, 348, 349). Membrane fusion in fibroblasts occurs at the plasma membrane, but in other relevant cell types entry and fusion occurs within endocytic vesicles (99, 284).

Virion fusion at the plasma membrane releases the nucleocapsid into the cell cytoplasm along with viral tegument proteins, which immediately begin to counteract host antiviral defenses (Figure 1.3) (2, 40, 284). The capsid is transported along microtubules to the nucleus within minutes of infection, and the linear viral genomic DNA is deposited into the nucleus allowing for immediate transactivation of viral gene expression (Figure 1.3) (2). Shortly after the linear viral DNA is deposited into the nucleus, the parental genome is circularized (222). This will later allow for the formation of concatameric genomes via rolling circle

replication. Viral gene transcription is mediated by host RNA polymerase II and follows an ordered cascade: immediate early (IE) → early/delayed early (E/DE) → late (L).

Expression of IE genes occurs shortly after the viral genome is deposited into the nucleus and does not depend on the prior expression or function of other viral genes. IE genes are typically involved in trans-activating gene expression, inhibiting antiviral responses and altering the cellular environment in preparation for DNA replication (40, 51, 69). The expression of IE genes is also dependent on the availability of host transcription factors, which regulate activity of the major immediate early promoter (MIEP) (146, 181, 288). The immediate early proteins IE1 and IE2 are critical mediators of infection. They influence the expression and function of host transcription factors required for regulating viral gene expression and viral DNA replication (48, 127, 262, 366, 367). Immediate early genes also activate the expression of E/DE genes, which initiates by 6 hpi and continues through 18 – 24 hpi. These genes are required for viral DNA replication, nucleotide synthesis and capsid maturation. Expression of E/DE genes is dependent on the prior expression of IE genes because they are critical trans-activators of viral gene expression and because they regulate the accumulation of host factors required for viral gene expression (56). HCMV infection requires the contribution of host factors for mediating viral DNA replication, gene expression and protein translation. Infected cells appear to be in a G_1/S- or M-like phase of the cell cycle, but replication of host cell DNA is blocked. This

environment generates host factors required for viral DNA replication (48, 49, 99, 131). Temporal accumulation of E/DE gene products allows for the initiation of viral DNA replication, which occurs between 18 – 24 hpi (Figure 1.3) (2). The episomal genome is the substrate for viral DNA replication. It allows for the formation of concatameric genomes via rolling circle replication. Viral genomic replication occurs in distinct compartments within the nucleus and host cell cycle and DNA damage response (DDR) factors aid in the process (79, 87, 256). Accumulation of nascent viral genomic DNA subsequently leads to expression of late (L) genes, which is dependent on the initiation of viral replication and the prior expression of IE and E/DE genes. Late genes regulate genomic encapsidation, virion assembly and egress. As viral DNA is synthesized, encapsidation proteins recognize the concatameric template through *cis*-acting sequences and insert unit-length DNA into the procapsid structure (2, 339). A nucleolytic cleavage of the concatameric DNA generates the mature nucleocapsid. This process is reminiscent of the “head full” assembly of dsDNA bacteriophages. Although the mechanistic aspects of this process are best understood in Herpes simplex virus (HSV) models, functional packaging proteins have been identified in HCMV and they are assumed to share similar roles in the process (291, 353). Virion assembly and envelopment occurs in a two-step process (227). The assembled nucleocapsid is first transported out of the nucleus through dissolution of the nuclear lamina (176, 226, 238). Following nuclear egress, the capsid is then enveloped in the cytoplasm by large inclusions

of the golgi body and the endoplasmic reticulum (2, 13, 303). This process is guided by the interaction of tegument proteins with the capsid and envelope glycoproteins (171, 354). The enveloped virion is then transported to the cell membrane and fusion results in the egress from the infected cell. Progeny virus can be detected in infected cell supernatants as early as 48 hpi. The ability of HCMV to reprogram host gene expression and to commandeer host signaling pathways is essential to viral replication, dissemination and persistence.

The necessity of host genes for regulating infection is a hallmark of HCMV infection. These factors are involved in almost every aspect of viral replication and they contribute to the establishment of each distinct phase of infection. One particular pathway that has recently been shown to contribute to HCMV replication is the host RNA interference pathway (RNAi). Viral encoded miRNAs depend on the host RNAi pathway for processing and functional implementation, and infection impacts the expression of host miRNAs whose proper function is required for efficient viral replication (116, 316, 347).

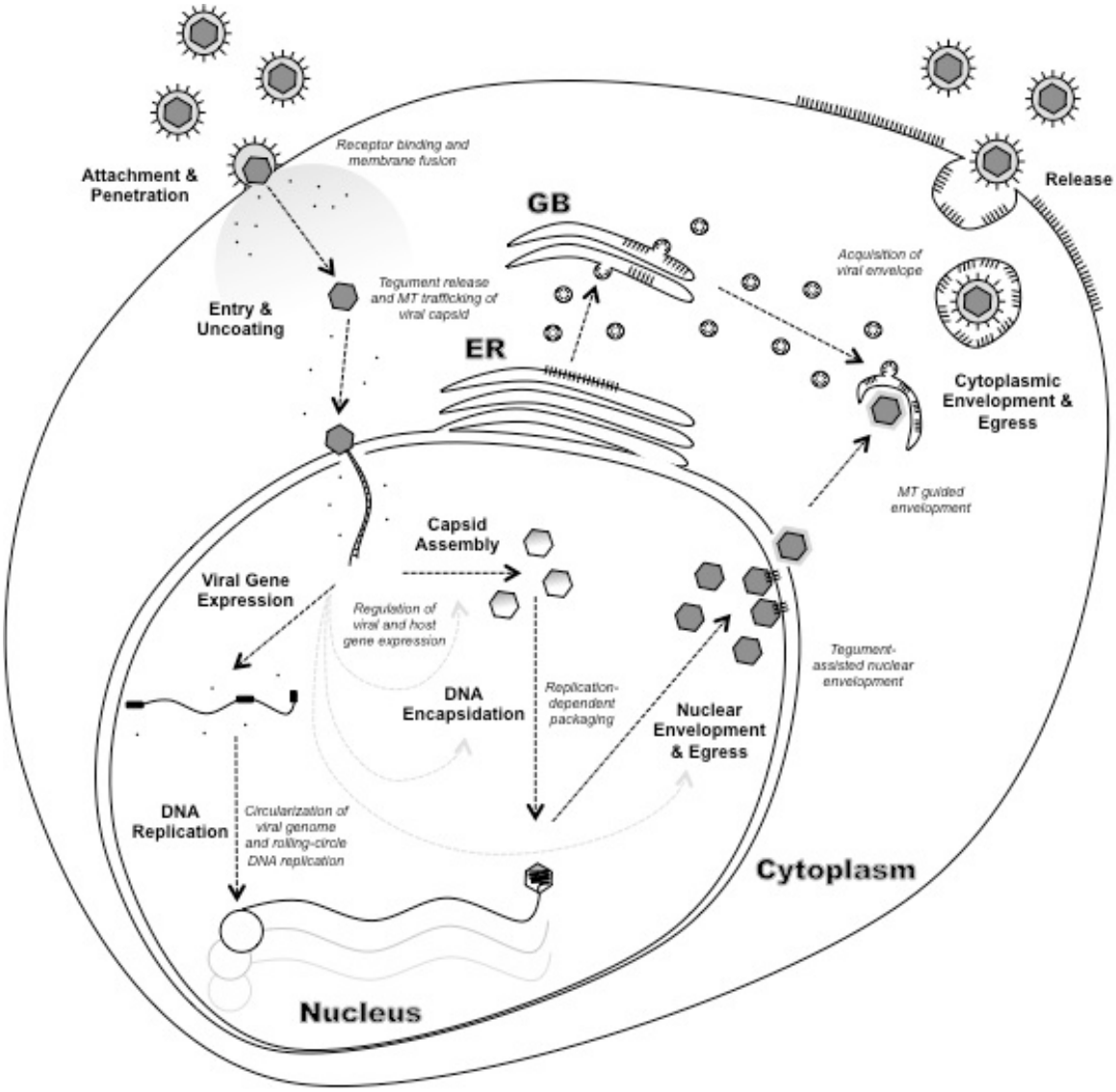


Figure 1.3: *Human cytomegalovirus replication.* The life cycle of HCMV replication is illustrated in this figure. Upon attachment at the plasma membrane, the viral capsid and tegument proteins are released into the cell cytoplasm. The capsid is trafficked to the nucleus and viral gene expression begins almost immediately after the genome has been deposited into the nucleus. Viral genomic replication occurs via a rolling circle mechanism, and immature capsids are packaged and assembled in the nucleus. After budding from the nucleus, the capsids acquire the tegument layer and are enveloped in the cytoplasm. After acquiring the viral envelope, the assembled virions bud from the cytoplasmic membrane. Virion maturation continues after the particle is released as envelope glycoproteins and tegument proteins undergo conformation changes.

B. RNA Silencing

Discovery of RNA-mediated post-transcriptional gene silencing

The fortuitous discovery of RNA interference began with the observation that transient transduction of a chimeric expression vector into plants actually resulted in repressed expression of a chalacone synthase transgene (245). Around the same time, other groups working in plant and fungal models observed similar phenomena wherein transient over-expression lead to concomitant repression of transgene expression (76, 282, 338). Terms such as post-transcriptional gene silencing (PTGS), quelling and co-suppression were adopted to describe the apparent dissidence of these observations, but the general consensus in the field was that nucleic acid was guiding antisense-mediated repression of gene expression. Even before the characterization of the biogenesis and targeting of short interfering RNAs (siRNAs) or microRNAs (miRNAs), various groups had adopted effective antisense-mediated transduction strategies for disrupting gene expression (100, 123). These initial observations were later addressed by the finding that a heterochronic, non-coding *C. elegans* gene, lin-4, was essential for creating a temporal decrease in LIN-14 protein levels during development (185). Here it was suggested that a small *lin-4* transcript, which harbored complementarity to the LIN-14 3'-UTR, was regulating LIN-14 protein translation through a sequence-specific RNA-RNA

interaction. It was proposed that dsRNA acted as a “trigger” for inducing an antisense-mediated repression of gene expression. Subsequent work validated that the transient introduction of dsRNA with complementarity to a target gene of interest was sufficient to “interfere” with target gene expression. From these observations, the term “RNA interference” was born (101,158, 251). Further characterization of the lin-4 locus and other similar forward genetic studies showed that short interfering RNA duplexes, which are expressed from inherited genetic loci, are capable of negatively regulating gene expression through sequence-specific interactions (88, 89, 232, 273, 307). The establishment that dsRNA was the substrate mediating RNAi catalyzed the biochemical characterization of miRNA processing and helped identify miRNA coding genes.

The pioneering work that identified the biochemical components required for RNAi-mediated regulation of gene expression was carried out in plant, fungus, fly and nematode model systems. Subsequent exploratory comparisons later identified orthologous genes in vertebrates. There is conservation of core RNAi pathway components among eukaryotes, but the distinctions between species lie mainly at the molecular level of miRNA processing and targeting. In line with this theme was the identification of an RNA-dependent RNA polymerase (RdRP) as the first essential gene required for mediating RNAi in plants and fungus (66, 74). These genes were identified through the use of “quelling-defective” strains of *Neurospora crassa* (which identified *qde*-1) and random mutagenesis screens of *Arabidopsis thaliana* (which identified *sgs-2* and *sgs-3*) (67, 90, 233).

Subsequent studies also identified an RdRP orthologue in *C. elegans* (302, 309). It was proposed that the role of the RdRP was to amplify the aberrant dsRNA intermediates required to initiate PTGS, and later this theory would be substantiated by the biochemical characterization of the anti-viral PTGS response in plants (91, 196, 233). The necessity of an RdRP for propagating PTGS is one of the main determinants distinguishing RNAi in plants, nematodes and fungi from that in flies and vertebrates (113). Soon after, a flurry of similar studies identified other functional components of the RNAi pathway including proteins with homology to a RecQ DNA helicase (*qde-3*) and RNAseD (*mut-7*) (68, 162). These findings supported the idea that dsRNA directed the enzymatic degradation of target RNAs, which resulted in PTGS. The initial division of the field between different animal and plant model systems helped establish sets of conserved genes that were required for mediated PTGS. This was critical for identifying RNAi pathway components in other species through homology comparisons, and it also helped establish the idea that RNAi was derived from a common ancestral mechanism (93). The identification of these first sets of essential genes gave way to an enormous expansion of the field of RNAi and led to the precise biochemical characterization of miRNA biogenesis and targeting in mammals.

miRNA biogenesis and targeting

miRNAs represent a specific class of small non-coding RNAs (ncRNA). The characterization of ncRNAs in eukaryotes is continually expanding, but the biogenesis and function of mammalian miRNAs is well understood (reviewed in 113, 164). miRNA genomic coding loci can be located either within the intronic sequences of other genes (intragenic) or they can be found within the vacant flanking sequences between coding genes (intergenic) (Figure 1.4). miRNA coding loci have also been identified in exonic coding sequences, but the occurrence of these loci is relatively rare (279, 322). Intragenic miRNA transcription is presumably coincident with that of the respective coding gene, but evidence suggesting that these miRNAs can also be independently expressed has yet to be formally discredited (279). miRNA coding loci can consist of a single miRNA, or they can be clustered with other miRNAs that will be expressed in the same poly-cistronic transcript (188) (Figure 1.4 and 1.5).

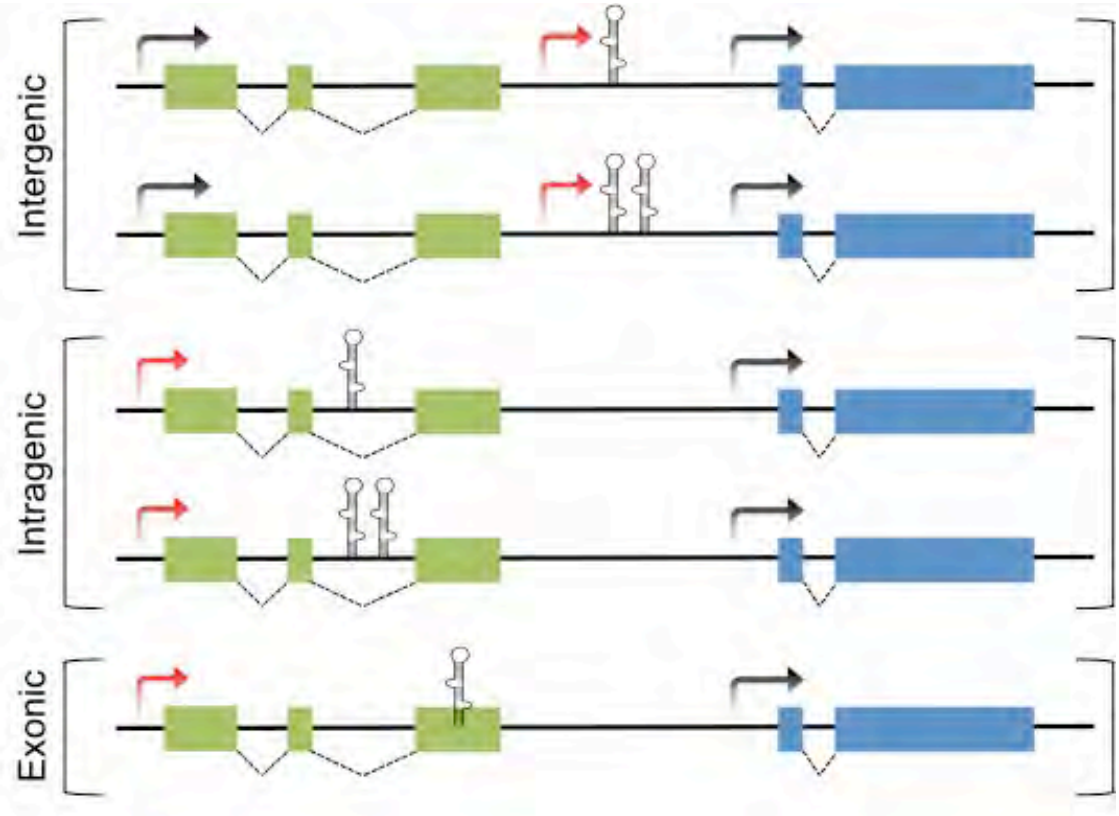


Figure 1.4: *miRNA genomic coding loci*. The various miRNA genomic coding arrangements and clustering patterns are illustrated in this figure. The green and blue boxes represent the exonic coding sequences of two arbitrary genes, and the genomic miRNA coding loci (hairpin structures) are represented alongside those genes in order to illustrate intergenic, intragenic and exonic miRNA coding loci.

Similar to other classically defined genes, miRNA expression is mediated by RNA polymerase II (RNAPII), and miRNA transcripts are capped and poly-adenylated (43, 189). RNAPIII has been shown to be able to direct transcription of miRNAs as well, but it is thought that the presence of repetitive Alu elements near miRNA coding genes may be largely responsible for this phenomenon (36). Primary miRNA (pri-miRNA) transcripts form a characteristic hairpin structure that is recognized in the nucleus and cleaved into a pre-miRNA hairpin by a

micro-processor complex containing the RNAse III enzyme *Drosha* (Figure 1.5) (77, 115, 128, 179). The pre-miRNA hairpin is actively transported out of the nucleus through Exportin 5, where it is bound and processed in the cytoplasm by the RNAse III enzyme *Dicer* (Figure 1.5) (34, 142, 161, 170, 363). The resulting dsRNA duplex is then unwound and the thermodynamic stability of the 5' end of the duplex dictates which strand is utilized as the "guide" to regulate mRNA translation (Figure 1.5) (247, 279, 295). The mature miRNA, which is ~22 nt in length, is incorporated into a miRNA-containing ribonucleoprotein complex (miRNP) also known as the miRNA-induced silencing complex (miRISC), and the *Argonaut* protein AGO2 directs binding to the target mRNA (Figure 1.5) (201, 223).

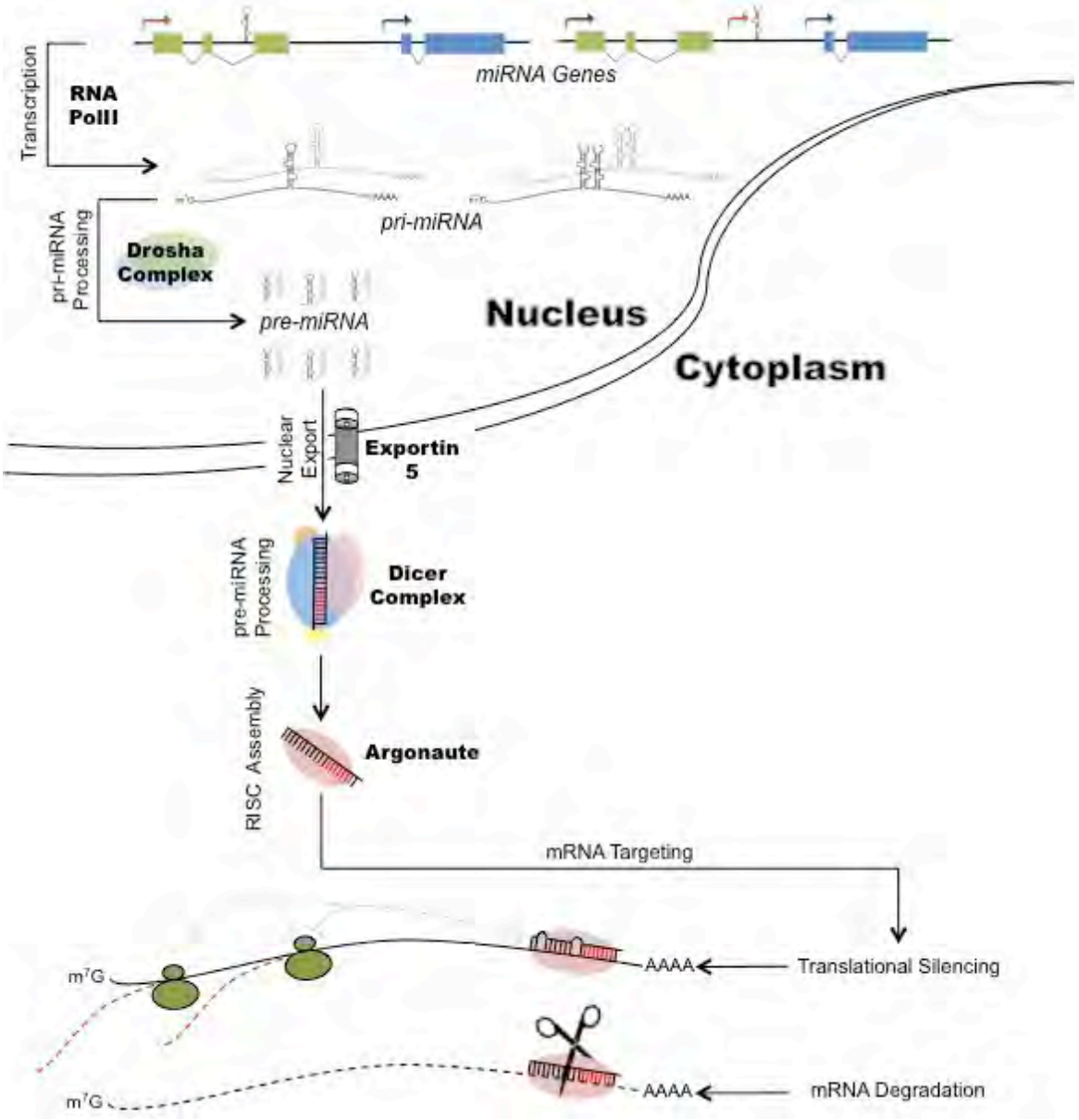


Figure 1.5: *Mammalian miRNA biogenesis*. The biochemical mechanism of mammalian miRNA biogenesis is illustrated in this figure. miRNA transcription is typically mediated by RNA polymerase II, and pri-miRNA transcripts are enzymatically processed in the nucleus by the Drosha complex. Pre-miRNA hairpins are actively transported into the cytoplasm, where they are processed into dsRNA duplexes by the RNAse III enzyme Dicer. The mature miRNAs are loaded into RISC miRNP complexes, and they post-transcriptionally mediate gene expression through mRNA translational inhibition or transcript degradation.

Micro-RNA recognition elements (MRE) guide miRISC to the target mRNAs. Typically, MREs are located in the 3'-UTR of mRNA messages and they exhibit complementarity to nucleotides 2-7 at the 5' region of the mature miRNA known as the "seed" region (119, 192, 193, 246). Although the majority of mammalian MREs have been shown to be located in 3'-UTR sequences, functional MREs in the 5'-UTR and in exonic coding sequences have also been described (211, 276). The extent of seed complementarity and the composition of flanking sequences around the MRE have also been shown to influence the efficacy of the miRNA in inhibiting mRNA translation (Figure 1.6) (23). siRNAs exhibit extensive complementarity to their target message and typically induce mRNA cleavage upon binding (Figure 1.6) (143, 218). This effect is also seen with the majority of plant miRNAs as they also harbor extensive target complementarity (reviewed in 344). miRNA binding in vertebrates typically results in translational repression, but the exact mechanisms governing translational repression are still being defined (reviewed in 254). The observation that miRNAs can be associated with polysomes suggests that this effect occurs after translation initiation (216, 257). Subsequent studies indicated that the mechanism of translational repression involves de-adenylation of the target transcript and inhibition of translation initiation through interference with cap binding (26, 140, 357). Even though the majority of mammalian miRNAs induce translational inhibition and the majority of plant miRNAs induce mRNA cleavage, exceptions to these dogmas have been observed in both systems (362,

364). In rare cases, miRNA targeting has even been shown to increase protein translation (70, 341). Taken together, the available literature suggests that there are aspects of miRNA biogenesis and targeting that have yet to be fully appreciated and characterized. Regardless of this assumption, the characterization of miRNA-mediated translational inhibition through MRE recognition in a target's 3'-UTR is very well established.

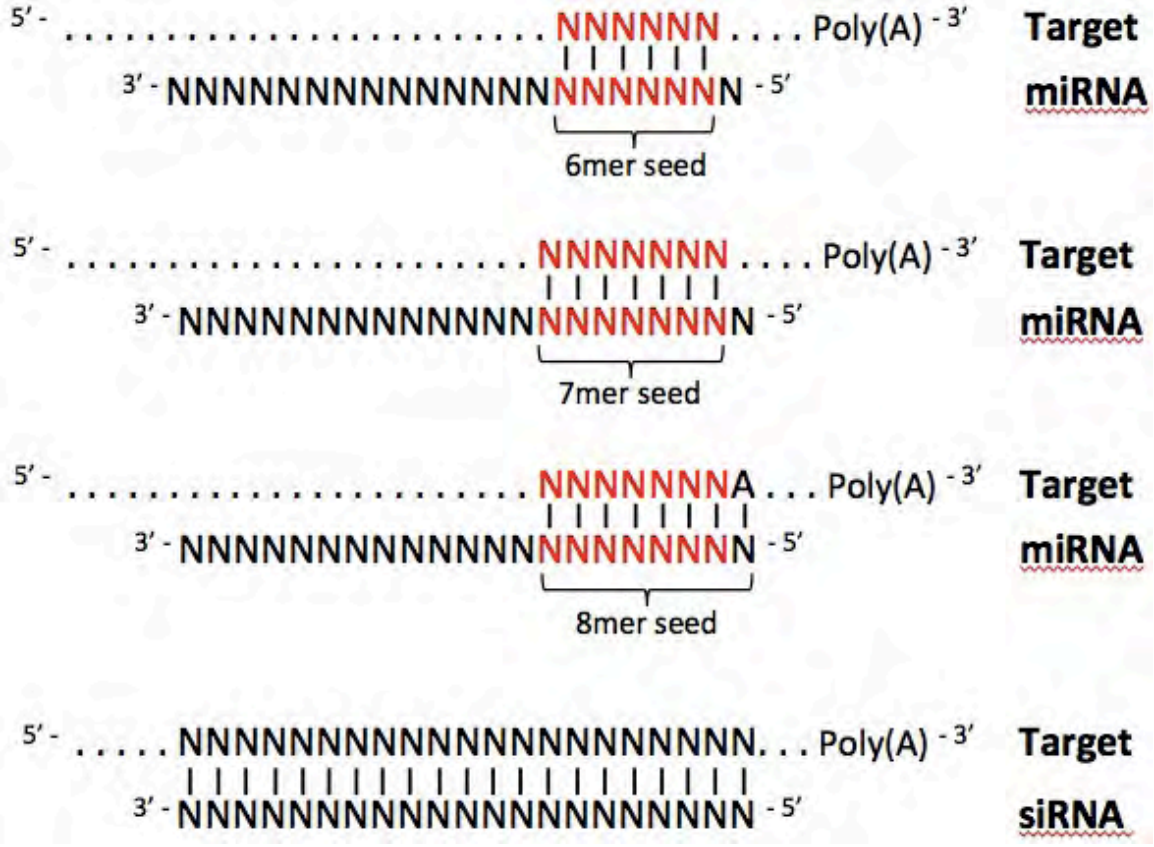


Figure 1.6: *miRNA seed sequences are targeting determinants*. Nucleotides 2-7, near the 5'-end of a mature miRNA are known as the "seed sequence". This region dictates the targeting repertoire of the miRNA based on complementarity to a target mRNA. Different seed types have been described based on the extent of seed complementarity and, 5'-flanking sequences. These respective seed sequences are designated based on the number of nucleotides contributing to MRE recognition (6mer, 7mer and 8mer). As a reference for comparison, the complementarity of an siRNA is shown alongside the different miRNA seed sequences.

Using these targeting criteria, various groups have developed mathematical algorithms that identify functional MREs in the 3'-UTRs of predicted target genes (27, 73). Various factors beyond complementarity to the miRNA seed have been shown to influence the efficacy of miRNA binding, and the respective prediction protocols each use different criteria such as site conservation, UTR architecture, site spacing, site number and strength of the various seed types to score the predictions (38, 81, 134, 166, 175, 205, 287). Concordance among each prediction algorithm can be seen in their establishment that mammalian MREs are over represented in 3'-UTRs and that extensive site complementarity and site number are strong predictive measures of MRE functionality. Currently, the Sanger miRBase suggests that the human genome codes for over 2000 functional miRNAs. Given the small sequence requirement for miRNA targeting, it is predicted that every protein coding gene is potentially regulated by a miRNA and that over half of these sites are evolutionarily conserved (107). Combinatorial approaches that joined transcriptomic and proteomic analyses indicated that not only are miRNAs capable of simultaneously controlling the expression of many different genes, but that there is a significant amount of variability in the degree translational repression dictated by the extent of MRE complementarity (16, 122, 296). These elegant studies began to illustrate the dynamic range of translational inhibition imposed by miRNAs, and they emphasize the cooperative ability of miRNAs to co-regulate the expression of shared targets. A similar study

suggested that miRNAs act to generate thresholds of gene expression that are specific to particular cell types and conditions (235). Given the promiscuous nature of miRNA targeting, the unique miRNA expression patterns and the dynamic range of translational regulation, it has also been proposed that miRNAs are critical to the diversification of animal species through the reciprocal evolution of MREs and mRNA UTR sequences (58, 95, 204). Taken together, these results suggest that the utility of RNAi is essential for most cellular processes and for the evolution and diversification of higher order animal species. The global and dynamic control that miRNAs exert on gene expression is illustrated by the fact that many cellular processes, biochemical pathways and diseases have been associated with characteristic miRNA expression profiles.

miRNA functions and disease associations

Since the characterization of miRNA biogenesis and targeting, miRNA-mediated regulation of gene expression has been linked to a continually expanding list of cellular pathways, processes and diseases. The first defined roles of miRNAs were related to developmental timing in *C. elegans* (232, 251, 273, 307). These studies showed that the heterochronic miRNAs lin-14 and let-7 regulated the timed expression of proteins required for proper progression through the sequential larval stages of development. Similar studies in *C. elegans* later identified the novel miRNAs *lsy-6* and *miR-273*, which were

required for asymmetric development of chemosensory neurons (55, 152). Given the relatively low abundance of these miRNAs, these findings ushered in the idea that the precise coordinated timing of miRNA expression was critical to temper the expression of their target genes. This idea was supported by similar studies in mice that identified embryonic stem cell-specific miRNAs that were thought to be involved in maintenance of pluipotency and mammalian development (135). Collectively, these works supported the idea that miRNA expression profiles were cell/tissue specific, and that their function was required to regulate many different biological processes.

The experimental validation of novel miRNA targets allowed for the refinement of miRNA targeting determinants beyond seed complementarity. These improved prediction protocols allowed for large-scale miRNA target analyses, which began to illustrate the extensive control that miRNAs potentially exerted on gene expression (92, 166, 193, 267, 315). These studies led to the discovery and characterization of many novel miRNA genes and miRNA targets. Subsequent correlative reviews revealed that miRNA-mediated regulation of gene expression was an essential cellular mechanism required for the function of many different pathways and processes including cellular differentiation, development, cell cycle, DNA damage, apoptosis and immunity (61, 136, 199, 285, 317, 337). Similarly, deregulated miRNA expression was linked to phenotypic determinants of diseases such as cancer, heart disease, metabolic disorders and other acquired or genetic diseases (94, 98, 144, 197, 208, 209,

337). The advent of microarray technology and high throughput sequencing (HTS) allowed groups to simultaneously analyze the expression profiles of miRNAs and mRNA transcripts in different tissues and cell types (11, 178, 312). These studies reinforced the idea that miRNA expression profiles were cell specific and that the kinetic nature of these profiles were critical for differentiating particular environments and cellular events. These techniques were also utilized to correlate particular diseases with unique patterns of miRNA expression (1, 21, 163, 208, 265). These expression-profiling studies illustrated that particular diseases or infections could be identified through specific miRNA expression signatures. They also showed that analyzing miRNA expression was instrumental in identifying genes and pathways that were essential for regulating the progression of particular diseases or infections.

C. Viruses and RNAi

RNAi-mediated anti-viral defense in mammals

During the initial characterization of quelling and co-suppresion in fungus and plants, it was demonstrated that actively transcribed plasmid coding sequences from transiently transduced vectors could be used as a template for mediating PTGS (65, 253). It was proposed that this could potentially be part of an antiviral response given that both viral and non-viral sequences could effectively inhibit viral replication if the complementary sequences were present in the genome of the infecting pathogen (24, 91). These theories were supported by works that illustrated both the disseminating protective capacity of PTGS and the potential for viruses to encode proteins that antagonize the accumulation of aberrant small RNAs required for mediating protective PTGS (156, 269). Collectively, this illustrated that the small RNAs produced during the antiviral RNAi response were not dependent on a particular sequence. It also suggested an evolutionary selection of this protective mechanism given the fact that viruses had evolved a defense against it.

The RNAi-mediated antiviral response is one of the main protective defense mechanisms of plants and invertebrates (reviewed in 80). Initially, it was speculated that this protective defense mechanism was universal and that it existed in many other species. In higher vertebrates, however, the evolution of

the protein-based immune system could have replaced RNAi-mediated anti-viral protection. Although mammalian host miRNA expression signatures have been correlated with attenuated replication of various viruses, the effect has not been definitively shown to be a result of the host miRNAs to directly targeting viral nucleic acid (183, 252, 255, 266, 368). These findings have contributed to the assumption that there is a very low probability that the mammalian genome codes for virus-specific miRNAs, which are intrinsic to the host anti-viral response. There is some evidence that certain viral infections in mammals elicit the production of siRNAs or miRNAs from viral RNA, but this is thought to be due to RNA secondary structure that is recognized and processed by host RNAi machinery. (10, 28, 167, 289). The capacity of these small RNAs to inhibit viral replication through targeting viral RNA has also not been substantiated, which suggests that they do not represent an RNAi-mediated anti-viral response. Vertebrates also do not retain a functional RdRP, which is required to amplify the virus-specific siRNAs generated in response to infections in invertebrates and plants. Lastly, if RNAi were a critical component of the mammalian anti-viral protective response, then one might anticipate that mammalian viruses harbor some mechanisms to counteract this host defense. A few viral proteins have been described as having RNAi suppression activity, but, these studies were often carried out in physiologically irrelevant environments and the specificity of the RNAi suppressive effects have not been defined (8, 28, 126, 151, 350, 358, 374). A KSHV gene stabilizes a viral mRNA transcript by antagonizing the

functional targeting of two host miRNAs, but this effect is not part of a general RNAi suppressive response. (154). A non-coding RNA expressed by West Nile virus (WNV) can effectively suppress RNAi in insects and mammals. However, this is not surprising given that arthropod viruses characteristically encode suppressors of RNA silencing (293).

The necessity of RNAi suppression for viral replication has not been demonstrated and the RNAi pathway itself is typically still functional during viral infections (10, 104, 167, 237, 289). Despite the fact that some viral proteins can attenuate miRNA production and the fact that small RNAs have been shown to be processed from viral nucleic acid, the collective interpretation of these studies would indicate that the mammalian the RNAi pathway does not mediate a viral-sequence-specific anti-viral protective response. This, however, should not discredit the fact that the mammalian RNAi pathway has been shown to be required for various aspects of viral replication. The contribution of the mammalian RNAi pathway to viral replication is evidenced by the expression/function of virally encoded miRNAs and by infection-dependent changes in host miRNA expression that are required for regulating various aspects of infection.

Viral miRNAs

The characterization of miRNA coding loci and requirements for biochemical processing catalyzed efforts to identify viral miRNAs. To date, a number of viruses have been shown to code for miRNAs (reviewed in 261). These are typically dsDNA viruses belonging to the *herpesviridae* family, but other viruses including adenovirus (AdV), baculovirus, human immunodeficiency virus (HIV), polyomaviruses and picornaviruses have also been shown to harbor functional miRNAs (25, 29, 33, 85, 116, 157, 231, 258, 259, 289, 305, 321). RNA Pol II mediates the transcription of viral miRNAs, but RNA Pol III-dependent transcription has also been observed in specific contexts (10, 33). In these incidences, tRNA-like non-coding RNAs are transcribed from the viral genome by RNA Pol-III and can bypass nuclear processing by the Drosha complex. Given that these viruses do not possess the factors required for processing and targeting miRNAs, they utilize host machinery to in order to direct miRNA-mediated regulation of gene expression. Viral miRNAs have been shown to regulate the expression of both host and viral genes during infection, and they contribute to various aspects of viral infection.

Work with herpes simplex virus suggested that viral miRNAs contribute to the maintenance of latency by tempering the expression of viral transactivators (130, 174, 325, 335, 336). In turn, this inhibits the reactivation of viral gene expression and prevents immunological recognition of latently infected cells.

This idea was proposed to apply to other herpesviruses as well, given miRNA complementarity to viral immediate early genes (239). Work with HCMV has shown the capacity of viral miRNAs to regulate the expression of viral genes during productive infection (117, 328). Using transient reporter assays, miR-UL112-1 was shown to regulate the expression of multiple viral genes through MREs located in 3'-UTR sequences (117). Similarly, the US7 3'-UTR harbors MREs for miR-US5-1 and miR-US5-2 with varying degrees of complementarity, and each was shown to be capable of regulating its expression (328). This is proposed to have applications relevant to both viral replication and immune evasion since these viral miRNAs control the expression of viral transactivators and immunodominant antigens. A similar mechanism was identified with simian virus 40 (SV40) wherein a viral miRNA tempers the expression of the large T antigen in order to escape immune recognition. Bioinformatics approaches suggest that HCMV miRNAs are actually required to compensate for down-regulated host miRNAs by targeting the same genes or pathways (342). Large-scale RISC-IP analysis of EBV and KSHV miRNAs suggests that they also preferentially target host genes that are involved in a variety of processes relevant to all stages of infection (83). These hypotheses are supported by the finding that both EBV and KSHV harbor human miRNA orthologues (114, 191).

Viral miRNAs have also been shown to be capable of regulating the expression of host genes during infection. Given the growing number of characterized viral miRNAs, this is speculated to be a universal trait with

commonalities in targeted transcripts and pathways (46). HCMV miRNAs have been shown to aid in immune evasion by inhibiting IL36 production and MICB expression (138, 242). Interestingly, EBV and KSHV miRNAs were also shown to repress MICB expression through similar mechanisms in order to prevent NK cell recognition (83, 243). Although most HCMV miRNAs have been shown to be non-essential for replication in culture, an MCMV knock out model suggests that the *in vivo* functionality of viral miRNAs needs to be formally addressed (78, 82). This notion is also supported by the finding that hcmv-miR-148D, a miRNA only found in clinical strains of HCMV, is also required for tempering the host immune response to infection by down-regulating levels of the chemokine RANTES (165). Similarly, an EBV miRNA has also been shown to inhibit the expression of a T-cell attracting chemokine in order to presumably escape immunological clearance (359). Expression profiling experiments using WT and miRNA-deleted viruses further suggest that the EBV miRNAs contribute to the transforming properties of the virus (71, 96). Similar studies suggest that this may be mediated by de-regulating the expression of pro-apoptotic genes and oncogenes in concert with host miRNAs (217, 277). An *in-vivo* study using a miRNA-deficient virus, however, suggests that these miRNAs play a more integral role in the establishment of acute infection rather than in oncogenesis (345). The documented functions of KSHV miRNAs have also been attributed to the establishment and maintenance of latency by regulating cell proliferation and survival (190, 191, 206).

In each case, viral miRNAs are not essential to viral replication *in-vitro*, but their necessity *in-vivo* has not been formally tested. These studies have, however, illustrated that virally encoded miRNAs are capable of regulating both host and viral gene expression. The requirement of miRNAs for viral replication is also illustrated by the fact that viral infections can impact the expression of host miRNAs that are required for efficient replication. Typically, infection-associated deregulated host miRNA expression leads to a concomitant change in target transcript translation, which is advantageous to viral replication.

Role of host miRNAs during viral infections

Host miRNAs are capable of simultaneously regulating the expression of many different genes, and their functions have been associated with essentially every cellular process and pathway studied. Given the parasitic nature of viruses, it was intuitive to predict that they were capable of manipulating the expression host miRNAs to regulate cellular processes required for efficient replication. Gene expression profiling studies defined the impact that viral infections had on host miRNA expression and subsequent studies validated host miRNA-mediated regulation of genes involved in various aspects of viral replication and pathogenesis. The roles that host miRNAs play during viral infections are still being defined, but their utility during infection is well appreciated.

Many viruses have been shown to impact the expression of host miRNAs during infection in an effort to regulate the accumulation of target proteins whose function are relevant to viral persistence. The latent membrane protein 1 (LMP1) of EBV is a viral oncogene responsible for activating cellular signaling cascades similar to the TNF-α receptor or CD40 (99). It has been shown to be capable of modulating the expression of multiple host miRNAs, and this effect contributes to immortalization and the establishment of latent infection (112, 195, 207, 365). EBV latency progresses through distinct stages marked by the transition of naive B-cells into a memory state. Unique miRNA expression profiles at the respective stages suggests that they may play a role in the establishment and maintenance of latent infection (44). Similarly, unique miRNA expression profiles in EBV-associated B-cell lymphomas suggest that deregulated miRNA expression contributes to transformation (145). Expression profiling experiments with HCMV during productive and latent infections suggest that host miRNAs play a large role in controlling the respective stages of replication (263, 316, 347). Although the mechanisms governing deregulated host miRNA expression during HCMV infection are still being deciphered, HCMV, MCMV and herpesvirus saimiri (HVS) have each been shown to post-transcriptionally downregulate the expression of host miRNAs through optimized MREs present in a viral ncRNA that act similar to "miRNA sponges" (41, 52, 186, 215). Interestingly, each virus employs this post-transcriptional mechanisms in order to downregulate a common miRNA: miR27. Other DNA viruses including herpes simplex virus, human papillomavirus and

hepatits B virus have also been shown to influence the accumulation of host proteins by deregulating host miRNA expression during infection, and these infection dependent changes in host miRNA expression are required for efficient viral replication (121, 352, 369-371).

The utility of host miRNAs in regulating viral infection is not limited to DNA viruses. Some of the pioneering work in this field was performed using hepatitis C virus (HCV) infection models. The liver-specific miR122 is capable of binding to the HCV genome, and this interaction is critical for stabilizing viral transcripts and for enhancing viral protein translation (70, 153, 300). This particular scenario is unique in the sense that direct binding of a host miRNA to viral genomic material enhances viral replication. Reconstituting miR122 expression in non-hepatic cells can even render them permissive to HCV replication, suggesting that this miRNA is a critical factor required for efficient replication (54, 108). Host miRNAs have also shown to contribute to HIV infection. Although initial studies with HIV focused on identifying host specific anti-viral miRNAs that inhibited replication by directly targeting the viral genome, it was later appreciated that HIV-1 also down-regulated the expression of the miR17/92 cluster to allow for accumulation of a viral co-factor PCAF (330). Other RNA viruses including WNV and enterovirus EV71 have also been shown to exert control over host miRNA expression and these events are required for efficient viral replication (132, 310). Although the majority of these studies focused on characterizing the involvement of host miRNAs with the replicative capacity of

human viruses, similar phenomena have also been described for viruses whose natural hosts include horses, birds and pigs (124, 318, 319).

Collectively, these works demonstrate that host mRNAs exert an important contribution to viral infections. They are essential for controlling the expression of cellular genes required for viral replication and they can potentially limit the spread of infection by controlling the expression of host immunomodulatory factors. Numerous viruses with unique tropisms, pathologies and virological characteristics have been shown to reprogram host miRNA expression, and they each employ unique strategies in order to accomplish this goal. These effects can contribute to viral replication, immune escape and pathology. This suggests that the host RNAi pathway represents an essential component of the cellular gene expression machinery that viruses have adapted to subvert in order to persist within a host.

D. Summary

HCMV is one of many viruses that have been shown to reprogram the expression of host miRNAs during infection. It utilizes the host RNAi pathway in order to implement the function of its own miRNAs and to influence the expression of genes that are involved in viral replication. To date, the mechanism by which HCMV manipulates the expression of host miRNAs is unclear, and the extent of our understanding of host miRNA-mediated regulation of gene expression during infection is incomplete. The fact that miRNAs are capable of simultaneously regulating the expression of many genes suggests that deregulated miRNA expression during HCMV infection could have dramatic effects on cellular gene expression. To this end, we sought to define the mechanisms by which HCMV reprograms host miRNA expression and to analyze the networks of genes potentially regulated by host miRNAs during infection.

Using a panel of host miRNAs whose infection-dependent change in expression was highly significant, we were able to enrich for functional transcription factor binding sites that regulate the expression of two conserved host miRNA clusters. The infection-dependent change in expression of these miRNAs was required for efficient replication, and they exhibit converse changes in expression patterns during infection. This further suggests that HCMV is manipulating the expression of host miRNAs in order to positively influence viral replication. Using the panel of infection-associated host miRNAs, we also

generated a global miRNA target network based on the enrichment of MREs using a novel target predication algorithm. This protocol illustrates the extent of regulation that host miRNAs potentially exert during HCMV infection. This protocol could easily be adapted in order to study the target networks of miRNAs in other diseases, infections or cellular processes. Given that miRNA-mediated repression of protein accumulation is relatively weak, the identification cooperative miRNA networks that co-regulate shared targets will help to accurately define the roles that miRNAs play in any scenario.

Chapter II:

Materials and Methods

Cell culture, viruses and infections:

Human embryonic lung fibroblasts (HEL, Coriell: GM01604) and the U373MG glioblastoma-derived cell lines (a gift from Eng-Shang Huang at the University of North Carolina School of Medicine) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with either ten or five percent fetal bovine serum (FBS, Invitrogen) respectively and penicillin/streptomycin antibiotics (Invitrogen). Infections were carried out by washing cell monolayers once with phosphate buffered saline (PBS, Invitrogen) and incubating the appropriate amount of human cytomegalovirus strain AD-169 (ATCC: VR-538) diluted in DMEM supplemented with two percent FBS and penicillin/streptomycin antibiotics for a 3 hour absorption period. After aspirating the infection media and washing the cell monolayers with PBS, regular growth media was added and cell pellets and supernatants were harvested as necessary for the respective experiments.

Microarray

HELs were either mock-infected or infected with AD-169 at MOI=5 for a time-course of five days. Cells were harvested at 24 hour intervals, and whole-cell RNA was purified using Trizol© (Invitrogen). 5 ug of RNA was sent to LC Sciences for microarray analysis. RNA samples were hybridized to a microarray

chip harboring probes against all known viral and cellular miRNAs based on the Sanger miRBase release version 13.0 (MRA-1041). The previous 48 hpi biological replicate array experiments were performed as described, but the microarrays harbored probes based on earlier releases of the Sanger database (miRBase release 8.2 and 9.0). Prior to examining the infection-induced changes in mean fluorescence intensity (MFI) of the individual miRNA probes, LC Sciences performed a fluorescence background subtraction through the use of internal controls and they normalized fluorescence signals based on variance between replicate probe readings. An analysis of variance (ANOVA) was performed on the corrected MFI readings to determine if a miRNA exhibited a significant mean fold-change in expression over the entire time-course of infection. A Benjamini and Hochberg post-test correction was performed to minimize the inclusion of false positive changes due to multiple comparisons. Data curated from biological replicate microarray experiments was compiled to generate a panel of miRNAs whose infection-associated change in expression was highly significant. These miRNAs each exhibited statistically significant change in expression at 48 hpi (t-test; $p<0.01$) that was reproducible among the individual experiments. They also had negligible variance in their fold-change in expression among the biological replicates (ANOVA, $p>0.05$).

Motif Enrichment

Data from triplicate independent biological replicate microarray experiments yielded both a panel of detectable miRNAs whose expression was significantly impacted by HCMV infection at 48 hpi, and a panel of miRNAs whose expression is undetectable in mock-infected and infected cells. The genomic locations of the respective miRNAs and their coding genes (if a miRNA was intragenic) were used to obtain 500bp of upstream promoter region sequence using the Ensembl BioMart (103). Enrichment of DNA motifs was performed using the multiple expectation maximization for motif elicitation suite version 4.7 (MEME) (17). The MEME algorithm was used to enrich for DNA motifs that are present only in the promoter sequences of detectable miRNAs with infection-associated changes in expression. This was done by discriminatively enriching for sequences against a background motif frequency that was generated using the promoter regions of undetectable miRNAs. The enrichment protocol outputs weighted probability motif matrices that indicate the relative frequency of each base at the respective position within the sequence. We used the motif comparison tool (TOMTOM) to align the respective motifs to known transcription factor binding site matrices found in the Transfac® database (125, 219).

qRT-PCR

Whole-cell RNA was purified from mock-infected and MOI=1 time-course infected HELs (preceded by LNA transfection where applicable) or U373MG cells using Trizol©. For miRNA expression profiling, 5 -10 ng of RNA was reverse transcribed using random hexamer primers with the Taqman© RT kit, and 2 ul of cDNA was used for qPCR analysis using the Taqman© miRNA gene expression assays (Life Technologies). For gene expression profiling, 500ng of RNA was reverse transcribed using random primers, and 2 ul of cDNA was used for qPCR analysis. Reactions were run on the StepOnePlus real-time PCR machine (Life Technologies). Fold change in gene expression was calculated relative to GAPDH using the $\Delta\Delta C_t$ method (203). Amplification threshold readings for each miRNA were first calibrated to GAPDH (ΔC_t). The corrected threshold readings for infected cells were then normalized to the corresponding values in the respective mock-infected time-points ($\Delta\Delta C_t$). For analyzing miRNA expression after LNA transfection and subsequent mock-treatment or MOI=1 infection, amplification threshold readings were calibrated first to GAPDH, and relative miRNA levels were quantitated by normalizing to the respective scramble LNA transfected time-point. Similarly, fold change in expression was calculated using the $\Delta\Delta C_t$ method. Standard curves for calculating miRNA copy number were generated using synthetic RNA oligonucleotides.

Cloning and Reporter Assays

The miR132/212 promoter region was amplified from HEL DNA via PCR using the primer set F_1508 & R_1508. The amplicon was cloned into pGL3-basic vector (Promega) by digestion with Mlu I and Bgl II and subsequent ligation with T4 DNA ligase (New England Biolabs; NEB). Promoter truncations were engineered with the same protocol using forward primer F_1508 and the respective reverse primers listed. Transcription factor binding site (TFBS) mutations were generated by PCR from T_269 template DNA using the primers listed. CRE_1/2 and SP1_1/2 double mutations required a separate set of primers due to the fact that the previous mutation overlapped the primer-binding site. Using Mlu I and Xho I digestion, the miR143/145 promoter was cloned similar to the miR132/212 promoter using the primer set F_1899 and R_1899. The subsequent truncation constructs were engineered using R_1899 and the respective forward primers listed. Mutations of the two Pax4 sites and the one AP2 site in the miR143/145 promoter were engineered similar to the miR132/212 promoter. The Pax4 binding motif consists of a paired domain (PD) and a homeo-domain (HD) (311). Mutation of each Pax4 TFBS required that we mutate each domain individually and this required the use of two separate primer pairs for each Pax4 TFBS (Pax4_1_HD & PD and Pax4_2_HD & PD). Reporter assay transfections were performed using Lipofectamine 2000 (Invitrogen). HELs were co-transfected with 250pmol of the respective truncation- or TFBS-

mutant-Firefly luciferase constructs and 250pmol of the HSV-TK-Renilla luciferase calibrator plasmid (Promega) in 24-well tissue culture plates. 24 hours post-transfection, cells were either mock-infected or infected at MOI=1 with HCMV. Cells were harvested at the respective time-points and lysed in 1x passive lysis buffer (Promega). Luciferase luminescence was activated using the Dual-Glo® luciferase assay system (Promega) and signals were read using a Synergy H4 microplate reader (Biotek). To calculate fold change in reporter activity, Firefly luciferase activity in each sample (miR-promoter) was first normalized to Renilla luciferase activity (TK-promoter). These corrected signals were then normalized to the normalized signal in un-transfected cells. Fold change in promoter activity in infected cells was calculated relative to its respective mock-infected control.

miR132/212 Truncation Primers	
Primer	**Sequence**
F_1508	GCATACGCGTAGTAAGCAGTCTAGAGCCAAG
R_1508	GCATAGATCTCACCTTCCCCAACTTCTCTG
R_988	GCATAGATCTCCGCCTCGCCACTATAAAT
R_506	GCATAGATCTAAAGTGAGGCGAAGGTGCT
R_269	GCATAGATCTAGCGGAGCTGTCCTCTCAG
R_97	GCATAGATCTGGAGGCGGAGCAGCAGAG

miR132/212 TFBS Mutant Primers	
Primer	**Sequence**
F_CRE_1	CCAGGCACGCGGCCCCTAAAATCAGAGGGCCGTGACG
R_CRE_1	CGTCACGGCCCTCTGATTTTAGGGGCCGCGTGCCTGG
F_CRE_2	CCCTGACGTCAGAGGGCCGTAAAATCAAAGATGTCCCAGAGGGG
R_CRE_2	CCCCTCTGGGACATCTTTGATTTTACGGCCCTCTGACGTCAGGG
F_SP1_1	CTTCCGGCGGGGGCGTGGGTTTTTGGCGGGACCTGGCGAGGCCC
R_SP1_1	GGGCCTCGCCAGGTCCCGCCAAAAACCCACGCCCCCGCCGGAAG
F_SP1_2	GGGGCGGGACCTGGCGAGGCCTTTTTCCCCCGGTCCTGAGAGGAC
R_SP1_2	GTCCTCTCAGGACCGGGGGAAAAAGGCCTCGCCAGGTCCCGCCCC
F_CRE_1/2	CCAGGCACGCGGCCCCTAAAATCAGAGGGCCGTAAAA
R_CRE_1/2	TTTTACGGCCCTCTGATTTTAGGGGCCGCGTGCCTGG
F_SP1_1/2	TTGGCGGGACCTGGCGAGGCCTTTTTCCCCCGGTCCTGAGAGGAC
R_SP1_1/2	GTCCTCTCAGGACCGGGGGAAAAAGGCCTCGCCAGGTCCCGCCAA

miR143/145 Truncation Primers	
Primer	**Sequence**
F_1899	GCATACGCGTCCACAGTGACCACTAAGCAATG
R_1899	GCATCTCGAGCACTTACCACTTCCAGGCTGAT
F_1497	GCATACGCGTCAGGTGAGCCATGTAGTCCA
F_999	GCATACGCGTAGCACAGGAGGAAGAGATGG
F_497	GCATACGCGTCCCAGGACTAGGGGTTGTCT
F_268	GCATACGCGTAGGCCACAGACAGGAAACAC

miR143/145 TFBS Mutant Primers	
Primer	**Sequence**
F_Pax4_1PD	GCCAGGCATGGTGGTGAGAGAGTGTAGTCCCAGCTAC
R_Pax4_1PD	GTAGCTGGGACTACACTCTCTCACCACCATGCCTGGC
F_Pax4_1HD	GTCTCTACTAAAAATACAGGGGGGAGCCAGGCATGGTGGTGAGAGAG
R_Pax4_1HD	CTCTCTCACCACCATGCCTGGCTCCCCCCTGTATTTTTAGTAGAGAC
F_Pax4_2PD	GTCTGCCCAGGACTAGAGAGAGTCTAAGGATAAGGAG
R_Pax4_2PD	CTCCTTATCCTTAGACTCTCTCGTAGTCCTGGGCAGAC
F_Pax4_2HD	GAGAGTCTAAGGATAAGGAGCTGGGGGGTTGGATGGTGAAATAACCTAAA
R_Pax4_2HD	TTTAGGTTATTTCACCATCCAACCCCCCAGCTCCTTATCCTTAGACTCTC
F_AP2	GCCAGGTTGGAGTCCCGAAAAAAACCACCAGAGCGGAGCAG
R_AP2	CTGCTCCGCTCTGGTGGTTTTTTTCGGGACTCCAACCTGGC

Reporter assays for determining the influence of MREs present in the KRas 3'-UTR were performed similar to what was described for the promoter truncation/mutations described above. Cells were co-transfected with 5 ug calibrator plasmid without the KRas 3'-UTR and with 5 ug of an experimental plasmid that harbored the KRas 3'-UTR. 24 hours after transfection, the cells were infected with HCMV AD169. Cells were harvested 48 hpi to assay for luciferase expression. The calibrator plasmid was a pGL4.10-derived vector (Promega) with the HCMV MIEP driving the expression of Firefly luciferase (pGL4.11). The HCMV MIEP was cloned by amplifying the promoter region from pGL4.75 (Promega) using the MIEP primers in the table below, and sub-cloning it into pGL4.1 using EcoRV and BglII sites. The Renilla luciferase construct containing the KRas 3'-UTR was generated by Chin *et al* 2008 and was obtained from Addgene (60). This 3'-UTR contains a single point mutation that was converted to the WT sequencing using the LCS6 primer set. The subsequent MRE mutants were engineered using the respective primer sets listed in the table below.

pGL4.11 Cloning and KRas 3'-UTR MRE Mutants	
Primer	**Sequence**
MIEP_F	GACGATATCTCAATATTGCGGATTAGCCATAT
MIEP_R	GACTCTAGAGATCTGACGGTCACTAAACTAG
LCS6_F	CTGACCTCAAGTGATTCACCCACCTTGGCCT
LCS6_R	AGGCCAAGGTGGGTGAATCACTTGAGGTCAG
m132_F	TTCCTGCTCCATGCAGACTTTTAGCTTTTACCTTAAA
m132_R	TTTAAGGTAAAAGCTAAAAGTCTGCATGGAGCAGGAA
m143.1_F	TCATGTTAAAAGAAGTCATTTCAAACTCTTAGTTTTT
m143.1_R	AAAAACTAAGAGTTTGAAATGACTTCTTTTAACATGA
m143.2_F	ACAGTTTGCACAAGTTCATTTCATTTGTATTCCATTG
m143.2_R	CAATGGAATACAAATGAAATGAACTTGTGCAAACTGT

Transfections and western blots: LNA transfections were carried out by electroporation. Cells were resuspended in Gene Pulser electroporation buffer (BioRad) and to ensure a consistent amount of nucleic acid was always transfected, the respective amounts of locked nucleic acid (LNA; Exiqon) were added for the particular conditions: [Scramble = 500pmol of scr. LNA], [miR132 = 250pmol miR132 LNA + 250pmol scr. LNA], [miR212 = 250pmol miR-212 LNA + 250pmol scr. LNA], [miR132+miR212 = 250pmol miR-132 LNA + 250pmol miR212 LNA]. Cells were electroporated in 0.1 cm cuvettes (BioRad) using a Gene Pulser II at 0.12kV, 300uF (BioRad). miRNA mimic transfections were performed using Lipofectamine 2000 (Invitrogen). Similar to the LNA transfections, equal amounts of Scramble and miRNA-specific mimics were added to ensure that a consistent amount of material was transfected in each

condition: [Scramble = 200pmol of scr. mimic], [miR143 = 100pmol miR143 mimic + 100pmol scr. mimic], [miR145 = 100pmol miR145 mimic + 100pmol scr. mimic], [miR143+miR145 = 100pmol miR143 mimic + 100pmol miR145 mimic]. Transfected cells were infected 24 hours later at an MOI=1 when applicable. Cell monolayers and supernatants were collected at 24-hour intervals for subsequent analysis by western blot and plaque assay. Whole-cell lysates were prepared by re-suspending cell pellets in radio-immuno-precipitation (RIPA) buffer (PBS, 0.1%NP-40, 1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, sodium vanadate, phenylmethylsulfonyl fluoride, and aprotinin) and incubating on ice for 1 hour with occasional vortexing. Lysates were cleared by centrifugation and 100 ug of protein were resolved by SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) and membranes were blocked in tris-buffered saline with 0.05% Tween-20 (TBST) and 0.2% milk prior to blotting for specific proteins. Immuno-blots were carried out using antibodies against IE1/IE2 (Millipore), pp65 (Virusys), gB55 (UMass Medical School, Shan Lu), actin (Sigma) and KRas (Abcam). Membranes were washed with TBST and incubated with rabbit-IgG horse raddish peroxidae (HRP) conjugated secondary antibody (GE life sciences) diluted in TBST. Blots were developed by washing membranes in TBST prior to incubation with chemilluminescent substrate (Perkin Elmer), exposing blots to imaging film (Kodak) and development. Plaque assays were performed by making triplicate serial dilutions of supernatants and subsequently infecting HEL monolayers. After a 3-hour absorption period,

infection media was removed and 20% carboxy-methyl cellulose (Sigma) prepared in growth media was added to each well. 10-14 days post infection, cell monolayers were washed and plaques were visualized with Giemsa stain (Sigma).

miRNA target enrichments: Using published microarray data that analyzed the expression of host transcripts during productive HCMV infection, we focused our target prediction analysis on genes that exhibited an MFI>200 in mock and infected cells (131). The 3'-UTR sequences for the respective genes were collected using the Ensembl BioMart (103). To identify miRNA target sites that were enriched in these sequences, we adapted a protocol that was initially utilized to calculate the likelihood of a 7mer site being present in a 3'-UTR (95). We first determined the frequency of all possible 2mer and 3mer sequences present in each 3'-UTR. This yielded a measurement of base frequency and size that would be used to calculate the probability of observing a given 6mer, 7mer or 8mer MRE. After determining the observed frequency of each MRE, we used a Markov-based analysis to calculate the probability of observing the respective sites given the size and base frequency of each 3'-UTR. We corrected those probabilities using a binomial analysis to determine the probability of observing a given miRNA target site multiple times if it was identified more than once.

Chapter III:

Human Cytomegalovirus Transcriptionally Reprograms Host Micro-RNA Expression During Productive Infection

Introduction

Gene expression profiling has allowed researchers to simultaneously evaluate the expression of essentially every human gene in various cells, tissues and conditions. These techniques have been implemented in the study of both acquired and inherited diseases, and they have contributed to the postulation that unique gene expression patterns could be used to identify particular disease states and to characterize mechanistic aspects of pathogenesis (15, 47, 208). Along these lines, unique miRNA expression profiles have been correlated with the manifestation of numerous diseases (94, 98, 144, 197, 208, 209, 337). By elucidating novel miRNA expression patterns, one can subsequently identify target transcripts whose miRNA-mediated regulation of expression is relevant to disease causality or progression (163, 178). Gene expression profiles have also proved essential in identifying co-regulated gene sets, and they ushered in new strategies to help characterize transcriptional mechanisms governing deregulated gene expression (187, 351). By combining miRNA expression profiling with global promoter sequence analyses, we sought to elucidate the complex landscape of transcriptionally mediated changes in miRNA expression that occur during HCMV infection.

HCMV has the ability to globally reprogram host gene expression in order to create an optimal environment for replication (40, 373). This infection-associated gene expression profile illustrates the result of both the cellular

response to infection and the viral manipulation of cellular gene expression in order to create a suitable environment for replication. HCMV has specifically been shown to alter the expression of host genes through the combined use of host transcription factors and viral transcriptional activator/enhancers (180, 367). Host transcription factors are also used to regulate the expression of viral genes (278, 294). Given the ability of HCMV to transcriptionally reprogram the expression of host genes that facilitate viral replication, we hypothesized that the virus similarly manipulates the expression of host miRNAs whose proper function is required for efficient viral replication.

Results

HCMV infection impacts global host miRNA expression

To determine the extent to which productive HCMV infection impacts the expression of host miRNAs, we measured the infection-associated changes in endogenous miRNA expression by utilizing a microarray platform. Human embryonic lung (HEL) fibroblasts were either mock-infected or infected with HCMV at an MOI=5, and cells were harvested every twenty-four hours for a time-course of five days. Primary fibroblasts such as HELs are the model cell type in which to study productive HCMV replication. They are fully permissive to productive infection and they are used throughout this dissertation. Purified whole-cell RNA from the individual samples was then sent to LC Sciences for microarray analysis. RNA from each sample was hybridized to independent microarrays harboring Cy5-labeled probes against all known cellular miRNAs as specified in miRBase 13 (875 mature miRNAs). Mean fluorescence intensity (MFI) readings were quantified from the individual probes in order to ascertain the fold change in miRNA expression relative to the respective mock-infected controls. Background subtraction and normalization for variance between replicate probe readings were performed prior to calculating the infection-associated fold-change in expression. An analysis of variance (ANOVA) was performed on the normalized mock-infected and infected MFI readings to

determine whether the mean fold-change in expression over the time-course of infection was statistically significant. A Benjamini and Hochberg correction was also applied to minimize the inclusion of false positive changes due to multiple comparisons.

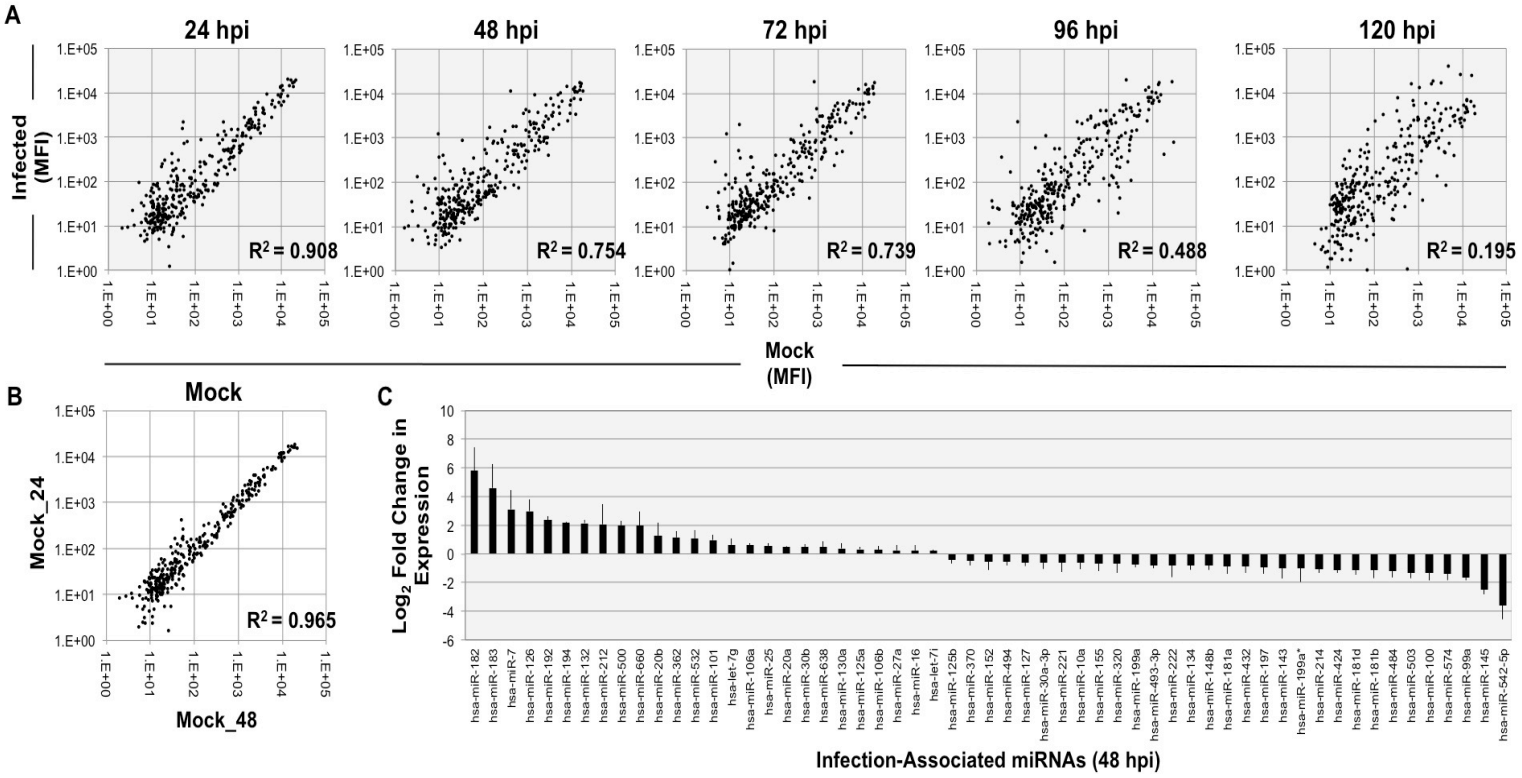


Figure 3.1: *HCMV infection reprograms the host miRNA expression profile.* (A) Each point represents a miRNA whose mean infection-associated change in expression over the time-course of infection has been deemed statistically significant (ANOVA, $p<0.01$ with a Benjamini and Hochberg correction). For each plot, the mean fluorescence intensity (MFI) of probes in mock-infected cells is plotted on the X-axis, and from the respective infected cells on the Y-axis. (B) This panel represents MFI readings from consecutive mock-infected time-points at 24 hpi (X-axis) and 48 hpi (Y-axis). The R^2 values illustrate the correlation between miRNA expression values in mock vs. infected cells. (C) A panel of statistically confident infection-associated host miRNAs at 48 hpi was complied from three biological replicate microarray experiments performed as described in (A). The miRNAs listed exhibit a statistically significant change in expression in each individual experiment (t-test, $p < 0.01$) and they display no significant variability in fold-change among the replicates (ANOVA, $p > 0.05$).

From this experiment, we observed that HCMV infection impacted the expression of over 40% of the detectable cellular miRNAs to varying degrees and with no unidirectional trend (364 miRNAs; Figure 3.1A).

As the infection time-course progressed, the impact on host miRNA expression became more readily apparent (Figure 3.1; R^2 values illustrated in the respective plots). This effect appeared to be infection-dependent as comparison of consecutive mock-infected time-points showed little change in host miRNA expression (Figure 3.1B). From three independent biological replicate microarray experiments, we assembled a cohort of host miRNAs whose infection-associated change in expression at 48 hpi was highly significant (Figure 3.1C). Each of these miRNAs exhibited a statistically significant change in expression in each individual experiment (t-test; $p<0.01$) and had negligible variance in fold change among the replicate experiments (ANOVA; $p>0.05$). This particular time-point was chosen since it embodied an ideal window in which to analyze the impact that the various aspects of viral replication could potentially have on host miRNA expression. It represents a time at which nearly all viral genes are being expressed and DNA replication is nearing peak levels. It also represents a time well after virion attachment and entry, and a time well before the effects of viral assembly and egress begin to compromise the integrity of the cell. It is important to note that this profile represents an incomplete list of host miRNAs whose expression is impacted by HCMV infection. The earliest of the replicate microarray experiments utilized probes that were based on the Sanger miRBase

release 8, and the current miRBase release 20 lists roughly triple the number of human miRNAs (2578 mature miRNAs).

These observations extend the breadth of expression profiling data from previously published work (347). We have quantified the infection-associated change in expression of many more host miRNAs over a more extensive time-course of infection and our technically optimized arrays exhibit better performance and reliability. Our findings also complement other studies through the use of a different cell line and a different strain of HCMV while still exhibiting concordance among the data (316, 347). The impact that infection had on miRNA expression levels is reminiscent of observations made with respect to changes in cellular mRNA expression during HCMV infection (373). Similar to host miRNAs, the expression of host mRNA transcripts is also extensively deregulated during infection. This suggests that the alterations in miRNA expression could either represent events influenced by the virus to create an ideal environment for replication, or the host anti-viral responses aimed at limiting viral replication. While the impact that HCMV infection has on host miRNA expression is profound, it is still unclear how infection leads to these global changes. Therefore, we sought to analyze the mechanism by which HCMV could be impacting miRNA expression.

Discriminative DNA motif enrichments identify functional transcription factor binding sites present in the promoters of infection-associated host miRNAs

HCMV specifically manipulates the expression of host transcription factors in order to regulate the expression of both host and viral genes during infection (48, 181, 294, 366, 367). These host TFBS regulate the expression of viral genes and contribute to the generation of essential host factors required for replication. Therefore, we hypothesized that the virus was also manipulating the expression of host miRNAs through the use of transcriptional elements present in their enhancer/promoter regions (hereafter referred to as promoter regions). To identify transcription factor binding sites (TFBS) that could potentially regulate miRNA expression during infection, we began by enriching for DNA motifs present in the genomic sequences upstream of our panel of infection-associated host miRNAs and their respective coding genes (Figure 3.2). Using the Ensembl BioMart, we gathered 500bp of genomic sequence upstream from the pre-miRNA hairpin loci and from their respective coding gene's transcriptional start site if the miRNA was intragenic (102). Intragenic miRNAs are generally believed to be co-transcribed with their respective coding genes. However, we did not want to ignore the fact that transcription of an intragenic miRNA could still be mediated by independent transcriptional elements present upstream of the pre-miRNA hairpin. Putative promoter region sequences were also gathered from a "background dataset" of miRNAs whose expression was undetectable in both

mock-infected and infected cells in order to establish a background frequency of non-specific DNA motifs. Promoter region enrichment analyses were performed using the MEME suite (17).

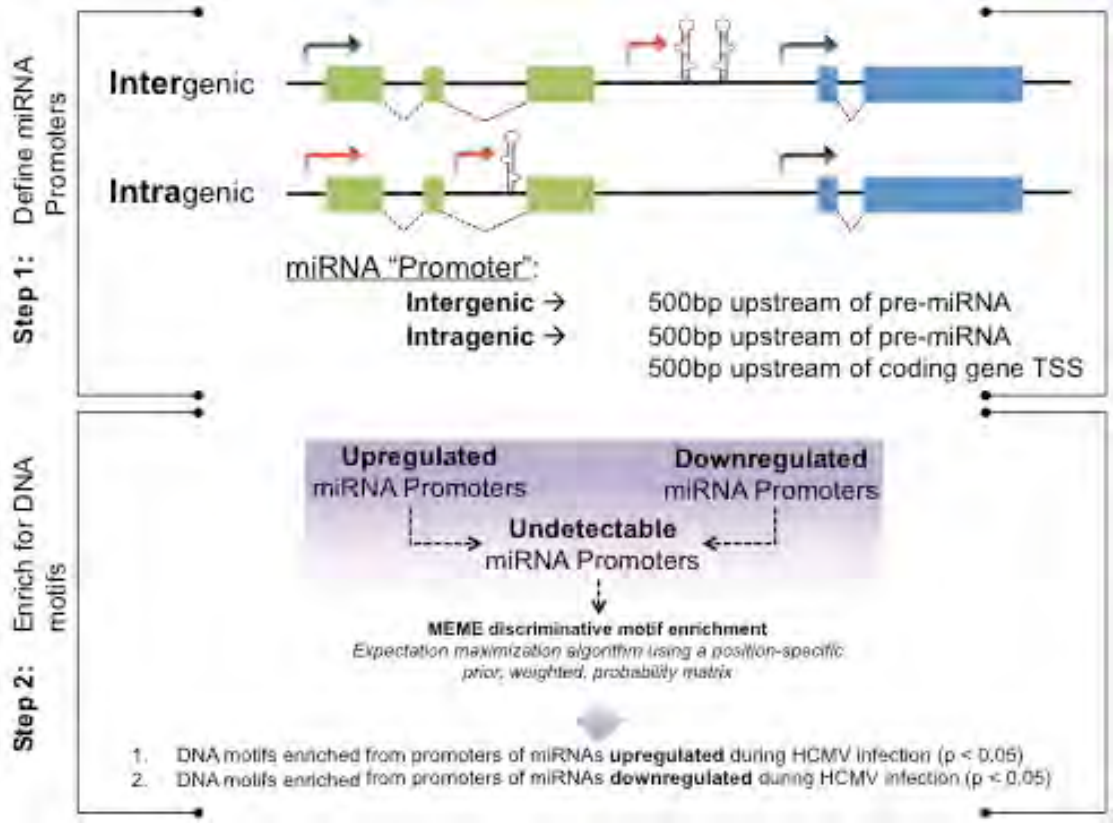


Figure 3.2: *DNA motif enrichment from infection-associated host miRNA promoter regions.* (Step 1) We began by defining potential promoter regions based on the genomic location of the respective miRNAs. If a miRNA coding locus was intergenic, we collected 500 bp of sequence upstream from the pre-miRNA hairpin. If the miRNA locus was intragenic, we collected 500 bp of sequence upstream from the pre-miRNA hairpin, and 500 bp of sequence upstream from the transcriptional start site of the respective coding gene. This generated three sequence datasets: upregulated miRNA promoters, downregulated miRNA promoters and undetectable miRNA promoters. (Step 2) We then discriminatively enriched for DNA motifs present in up- and down-regulated miRNA promoters relative to undetectable miRNA promoters. This protocol yielded a panel of position-specific, weighted probability motif-matrices. Using the TOMTOM algorithm available in the MEME suite, they were then aligned to known transcription factor binding site motif-matrices present in the Transfac® database.

The MEME algorithm uses expectation maximization to enrich for weighted motif matrices from a panel of input DNA sequences. We used our background dataset in order to discriminatively enrich for DNA motifs using position-specific prior probability matrices (18). This allowed us to identify DNA motifs that were specifically enriched in the promoter regions of infection-associated host miRNAs. DNA motifs were enriched separately from the promoter regions of miRNAs whose expression increased during infection and from miRNAs whose expression decreased during infection in order to determine whether specific DNA motifs could be associated with directional changes in miRNA expression. This protocol yielded a panel of unique DNA motif matrices that were significantly enriched in the promoters of infection-associated host miRNAs (Figure 3.3). Our analysis focused on motifs that were enriched at least 5 times from the respective datasets (Table 3.1, MEME), and were between the range of 3 - 25 bp in size. The MEME_E-value reflects the corrected probability that this same motif can be enriched from another random dataset of the same size. Given that our experimental datasets were relatively small, we felt that the enrichment E-value could be potentially misleading. To alleviate this concern, we validated the motif enrichment using the MAST module available in the MEME suite (19).

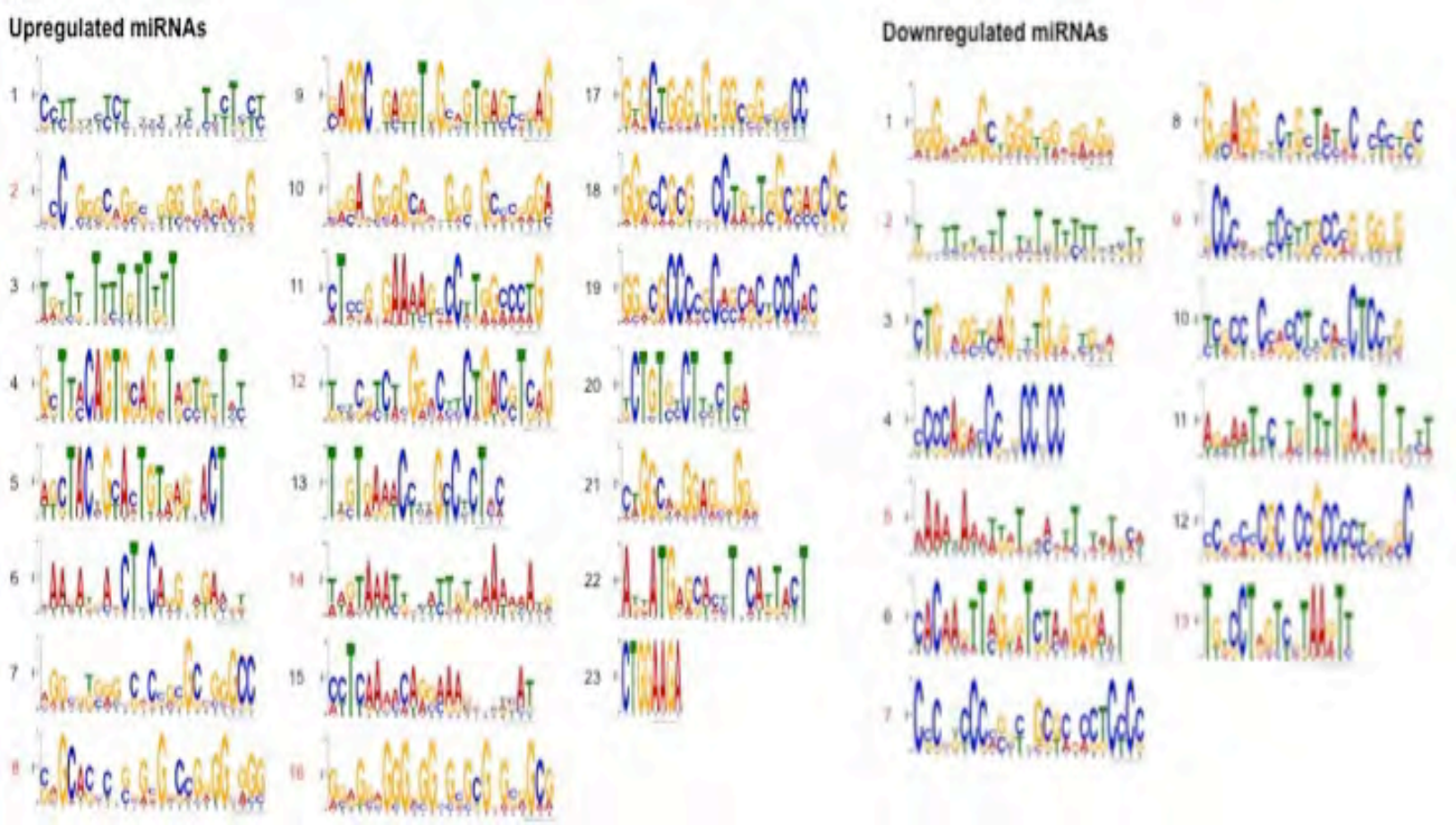


Figure 3.3: *DNA motif matrices enriched in promoter regions of infection-associated host miRNAs.* The DNA motif enrichment protocol generates position-specific, weighted probability matrices that illustrate the relative abundance of each nucleotide at the respective positions within the motif. The matrices illustrated in this figure represent the motifs that were identified at least 5 times from the respective sets of miRNA promoters. The red numbers indicate motifs that align to known TFBS sequence motifs present in the Transfac® database.

Using this algorithm, we searched for the enriched motif-matrices in our control promoter region dataset of undetectable miRNA promoter sequences ($MAST^1$) and from a dataset of eukaryotic promoter sequences ($MAST^2$). The number of promoter sequences used for the individual enrichments is listed in the respective columns. A chi-squared test was then performed using the frequency of positive motif identifications from the respective control datasets to validate that there was significant enrichment of the DNA motifs in our experimental datasets ($MAST^1\_\chi^2$ and $MAST^2\_\chi^2$). The rows highlighted in red indicate motifs that mapped to known transcription factor binding sites annotated in the Transfac® databse. The enrichment of motifs 8 and 12 in a control dataset of eukaryotic promoters ($MAST^2\_\chi^2$; upregulated table, $p>0.05$) likely reflects that these are true TFBS given that they can be found in the promoters of other random eukaryotic genes.

Upregulated miRNA Promoter Regions						
Motif	**MEME (41 sequences)**	**MEME_E-Value**	**MAST^1 (171 sequences)**	**MAST^1_ χ^2**	**MAST^2 (4806 sequences)**	**MAST^2_ χ^2**
1	39	1.90E-26	13	< 0.001	1091	< 0.001
2	23	1.00E-07	3	< 0.001	4	< 0.001
3	15	2.50E-07	5	< 0.001	0	< 0.001
4	5	1.20E-06	0	< 0.001	0	< 0.001
5	6	3.30E-05	0	< 0.001	0	< 0.001
6	15	4.70E-02	0	< 0.001	0	< 0.001
7	20	1.60E-04	19	< 0.001	1217	< 0.0012
8	13	1.60E-06	16	< 0.001	1524	N.S. > 0.05
9	6	2.20E+00	10	N.S. > 0.05	987	N.S. > 0.05
10	15	1.20E+00	2	< 0.001	3	< 0.001
11	5	5.90E+03	0	< 0.001	0	< 0.001
12	6	3.10E+05	3	< 0.019	929	N.S. > 0.05
13	7	4.30E+04	12	N.S. > 0.05	880	N.S. > 0.05
14	11	5.70E+04	0	< 0.001	1	< 0.001
15	12	3.10E+05	0	< 0.001	0	< 0.001
16	8	1.70E+06	10	< 0.013	20	< 0.001
17	9	8.80E+05	3	< 0.001	2	< 0.001
18	5	6.30E+04	0	< 0.001	0	< 0.001
19	7	7.60E+06	7	< 0.008	40	< 0.001
20	9	5.00E+06	0	< 0.001	0	< 0.001
21	17	1.20E+06	3	< 0.001	2	< 0.001
22	7	5.60E+05	0	< 0.001	0	< 0.001
23	8	4.60E+06	0	< 0.001	0	< 0.001

Downregulated miRNA Promoter Regions						
Motif	**MEME (41 sequences)**	**MEME_E-Value**	**MAST^1 (171 sequences)**	**MAST^1_ χ^2**	**MAST^2 (4806 sequences)**	**MAST^2_ χ^2**
1	31	2.20E-40	5	< 0.001	12	< 0.001
2	48	3.00E-26	10	< 0.001	604	< 0.001
3	21	2.60E-07	4	< 0.001	64	< 0.001
4	10	7.50E-07	7	< 0.0012	3	< 0.001
5	24	2.40E-06	0	< 0.001	0	< 0.001
6	7	1.00E-03	0	< 0.001	0	< 0.001
7	8	8.10E-03	6	< 0.0066	8	< 0.001
8	10	4.70E-03	0	< 0.001	2	< 0.001
9	15	2.80E-11	3	< 0.001	3	< 0.001
10	11	8.70E-05	4	< 0.001	437	< 0.0075
11	11	1.30E-04	0	< 0.001	0	< 0.001
12	8	8.80E-01	8	< 0.024	11	< 0.001
13	7	5.60E+00	0	< 0.001	0	< 0.001

Table 3.1: *Statistical enrichment of DNA motifs from the promoter regions of infection-associated host miRNAs.* These tables illustrate: the number of times each motif was enriched from the respective experimental datasets (MEME), the corrected probability that this same motif can be enriched from another random dataset of the same size (MEME_E-value), the number of times the listed motif was enriched from either our control dataset of undetectable miRNA promoters (MAST^1) or from a dataset of eukaryotic promoter sequences (MAST^2) and the significance of those respective enrichments (MAST^1_ x^2 and MAST^2_ x^2).

Using the TOMTOM algorithm available through the MEME suite, we then aligned the enriched weighted motif matrices against known transcription factor binding site (TFBS) matrices annotated in the Transfac® database (125, 219). This algorithm uses a Pearson's correlation coefficient to calculate similarities between motif matrices and calculates a probability that the queried motif aligns to any given TFBS motif. Table 3.2 illustrates the DNA motifs that aligned to annotated TFBS with high confidence.

Our alignment analyses indicated that there are both unique and shared TFBS present in the promoters of up- and down-regulated infection-associated host miRNAs. This suggests that HCMV may be utilizing combinations of transcription factors to influence the expression of host miRNAs during virus infection through conserved TFBS present in their promoter regions. It is also apparent that the majority of the enriched DNA motifs do not map to known TFBS. Given that these motifs were discriminatively enriched against background datasets of other eukaryotic promoter sequences with high confidence, it is unlikely that they represent ubiquitous transcriptional elements such as transcriptional start sites, intron acceptor/donor sites or poly-adenylation signals. It is possible, however, that they represent epigenetic DNA regulatory elements such as CpG islands. It is known that HCMV utilizes epigenetic mechanisms to influence viral gene expression during infection, so it is plausible that similar mechanisms are employed to reprogram host miRNA expression (139, 241, 272).

Upregulated miRNA Promoter Regions			
Motif	**Name**	**E – Value**	**miRNAs Harboring TFBS**
2	HIC-1	4.46E-02	**MIR101-1, MIR126, MIRLET7I, MIR25/106B, MIR7-1, MIR192/194-2, MIR183, MIR7-2, MIR132/212, MIR27A, MIR638, MIR182, MIR125A, MIR362** MIR16-1, MIR130A, MIR638, MIR16-2, MIR101-2, MIRLET7G, MIR7-1, MIR25/106b, MIR7-3
8	AP2	2.32E-02	**MIR25, MIR192/MIR194-2, MIR7-2, MIR132/212, MIR638, MIR182, MIR125A** MIR16-1, MIRLET7G, MIR7-1, MIR25/106b, MIR126, MIR7-3
12	ATF-3	1.62E-02	**MIR132/212, MIR25, MIR7-2** MIR7-3, MIRLET7G, MIR25/106b
	CREB	4.05E-02	
	ATF-1	4.77E-02	
14	Oct-1	3.40E-02	**MIR16-2, MIR101-2, MIRLET7G, MIR30B, MIR106A, MIR194-1, MIR20B, MIR532, MIR16-1, MIR362** MIR7-3
16	Zfp-219	5.32E-04	**MIRLET7I, MIR132, MIR660** MIR16-1, MIR638, MIR101-2, MIR7-1, MIR25/106b
	SP-1	3.21E-02	

Downregulated miRNA Promoter Regions			
Motif	**Name**	**E – Value**	**miRNAs Harboring TFBS**
2	Pax-4a	3.23E-02	**MIR494, MIR370, MIR424, MIR148B, MIR181D, MIR181A2, MIR127, MIR99A, MIR145, MIR197, MIR222, MIR181B2, MIR199A1, MIR181A1/B1, MIR10A, MIR542, MIR432, MIR155, MIR30A, MIR125B2, MIR221, MIR574, MIR214, MIR125B1, MIR493, MIR134, MIR100, MIR143, MIR320A, MIR320D1, MIR320B1, MIR320C2, MIR320D2, MIR320B2, MIR320C1, MIR181A2** MIR181A1/B1, MIR155, MIR125B1, MIR370, MIR152, MIR148B, MIR10A, MIR574, MIR99A, MIR503, MIR199A2, MIR484
5	Oct-1	3.42E-02	**MIR370, MIR181A2/100, MIR99A, MIR197, MIR222, MIR181A1, MIR10A, MIR542, MIR155, MIR30A, MIR214, MIR181B1, MIR134, MIR143, MIR320B1, MIR320B2, MIR320C1, MIR181A2** MIR99A,MIR181A1/B1, MIR125B1, MIR503/424, MIR199A2
7	SP-1	3.03E-03	**MIR574, MIR320A, MIR181D, MIR10A** MIR152, MIR148B, MIR574, MIR484
9	AP-2	4.30E-02	**MIR145MIR199A1, MIR574, MIR152, MIR493, MIR143, MIR320A, MIR320D2** MIR148B, MIR10A, MIR574, MIR155, MIR484, MIR503/424, MIR199A2
12	SP-1	1.74E-02	**MIR152, MIR197, MIR503, MIR320A** MIR152, MIR148B, MIR484, MIR574
	Egr-1	2.76E-02	

Table 3.2: *Transcription factor binding sites are enriched in the promoter regions of infection-associated host miRNAs.* Using the TOMTOM module of the MEME suite, position-specific, weighted, probability matrices generated from the DNA motif enrichments were aligned to known transcription factor binding site motif matrices present in the Transfac® database. The E-value represents a corrected probability that our enriched motif accurately represents the annotated TFBS motif matrix. The miRNAs harboring the indicated TFBS are listed in the far-right column (miRNAs Harboring TFBS). The names that are not bold-faced represent sites found in the promoter sequence of the cognate coding gene for the respective intragenic miRNAs.

Other DNA regulatory elements such as insulator, enhancer or repressor cassettes may also account for the DNA motifs that did not map to known TFBS. It is also possible that these matrices represent TFBS binding motifs that have yet to be characterized. All of these sequences could still potentially regulate miRNA transcription, but would not align to TFBS motifs. Given, that TFBS are enriched in the promoter sequences of our cohort of infection-associated host miRNAs, we next sought to test their functionality in regulating the expression of host miRNAs whose proper function is required for efficient replication.

HCMV reprograms the expression of two conserved host miRNA clusters whose function are required for efficient viral replication

In order to analyze the utility of the TFBS identified by our DNA motif enrichment analysis, we focused on the promoter regions of two well-conserved clusters of host miRNAs: miR132/212 and miR143/145. These two particular clusters were chosen for subsequent evaluation for a variety of reasons. Given the degree of their evolutionary conservation and their clustered genomic loci, these miRNA clusters could potentially have a larger and/or more robust cellular-targeting capacity and thus a greater potential to influence viral replication (7, 59). The miR132/212 and miR143/145 promoter regions harbor a subset of the TFBS identified from the DNA motif enrichment, and each cluster exhibits robust converse changes in expression during infection (Figure 3.4 A - F). This allowed us to investigate the influence that particular transcription factors had on changes in miRNA expression during HCMV infection. These particular miRNA clusters are also very well studied. Their validated functions are likely relevant to HCMV replication and pathogenesis, and their expression is controlled by transcriptional regulators that are required for HCMV replication (5, 168, 286, 343, 356, 360) .

To validate the infection-associated change in expression of the miR132/212 and miR143/145 clusters, we purified whole-cell RNA from mock-infected and MOI=1 infected HELs and analyzed changes in miRNA expression relative to GAPDH by Taqman qRT-PCR (Figure 3.4 E & F). In agreement with

the results of our microarray studies, miR132/212 expression increased steadily over the time-course of infection (Figure 3.4 E). The increased fold-change in miR212 expression relative to miR132 likely reflects some non-specific binding of the miR212 probe with miR132 as each mature miRNA exhibits a high degree of sequence similarity (Figure 3.4 C). Conversely, expression of the miR143/145 cluster was decreased over the time-course of infection and to a slightly greater extent than that of miR132/212 (Figure 3.4 F). We recapitulated the qRT-PCR validation for deregulated miRNA cluster expression in another permissive glial-derived cell line, U373MG (data not shown), and validated the deregulated expression of miR132 and miR145 during productive infection of HELs by northern blot (Figure 3.4 G). We also quantified the absolute copy number of each of the respective mature miRNAs in resting HELs and this revealed that miR143/145 are present at levels >2-log higher than miR132/212. (Figure 3.4 H).

These results confirm that productive HCMV infection alters the expression of two conserved host miRNA clusters. Given the extent of the global deregulation of host miRNA expression, this effect likely represents events that are associated with the host response to infection and/or the regulation of viral replication.

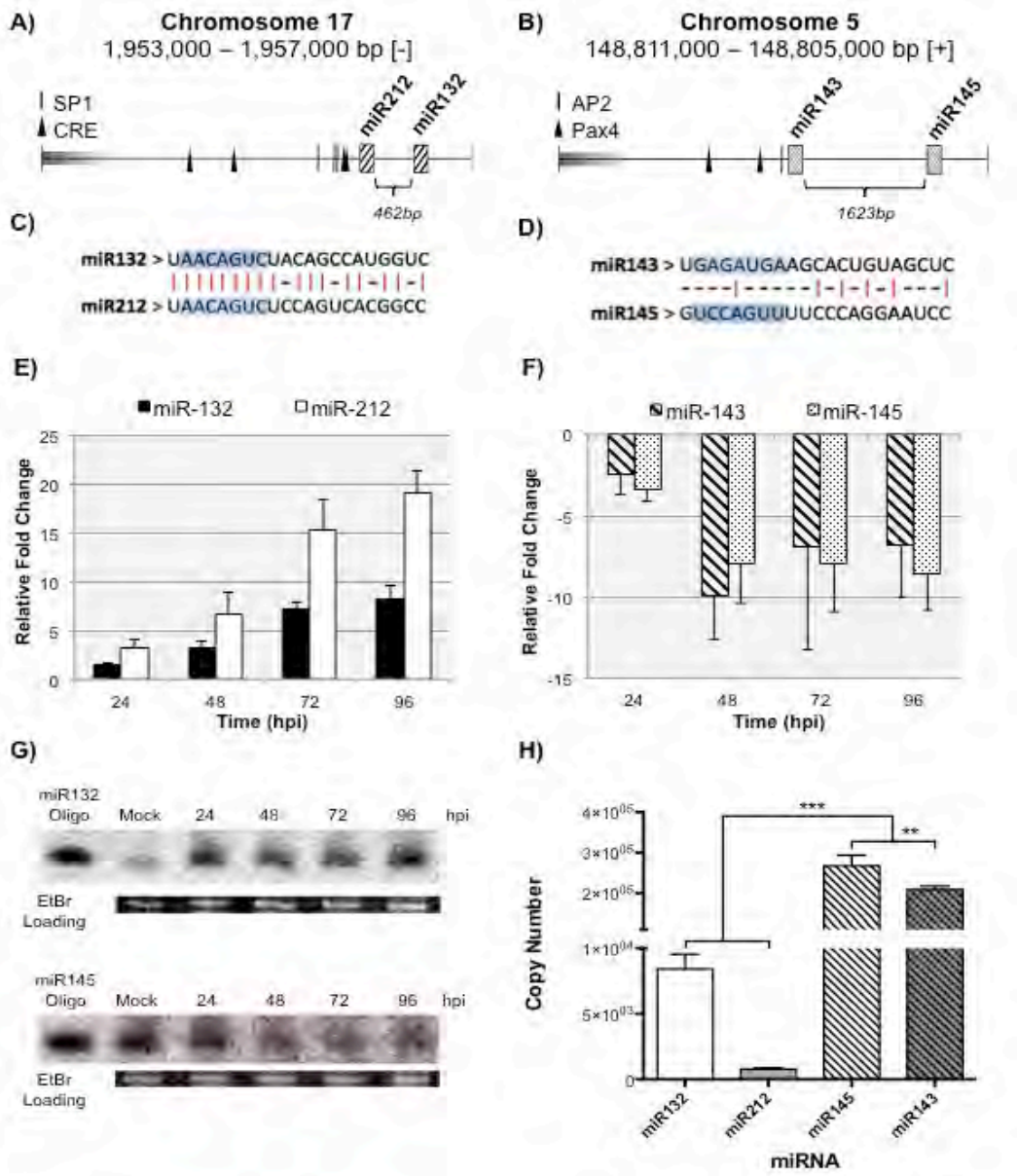


Figure 3.4: *HCMV infection impacts the expression of two conserved host miRNA clusters.* (A&B) The genomic locations of the miR132/212 (A) and the miR143/145 (B) clusters are illustrated along with pertinent TFBS identified from the DNA motif enrichments. (C&D) The sequences of the mature miRNAs from each cluster are aligned with the seed sequences highlighted in blue, and sequence homology indicated by the connecting red lines. (E&F) Expression of the miR132/212 (E) and miR143/145 (F) clusters during HCMV infection was validated by qRT-PCR in HEL fibroblasts. (G) Expression of miR132 and miR145 during HCMV infection was validated by northern blot. (H) miRNA cluster copy number was calculated in resting HELs. An ANOVA analysis and a Tukey post-test were performed to determine if the respective miRNA copy numbers were significantly different from each other (** = $p<0.01$).

In order to establish the requirement of de-regulated miRNA expression for viral replication, we counteracted the virus-induced changes in miRNA expression during infection and analyzed the impact on viral replication. To neutralize the accumulation of miR132/212, we transfected cells with miRNA-specific locked nucleic acid (LNA) prior to infection. These are chemically modified antisense oligonucleotide inhibitors that prevent miRNA function through a sequence-specific interaction with the mature miRNAs. Conversely, to counteract the infection-associated decrease in miR143/145 expression we transfected cells with synthetic miRNA mimics prior to infection. The reduction of mature miR132/212 levels after LNA transfection and the accumulation of mature miR143/145 after mimic transfection were verified pre- and post-infection by qRT-PCR (data not shown). After pre-treating HELs with either LNA or miRNA mimics, we infected with HCMV at an MOI=1 and assayed for the effects on viral replication and viral gene expression (Figure 3.5).

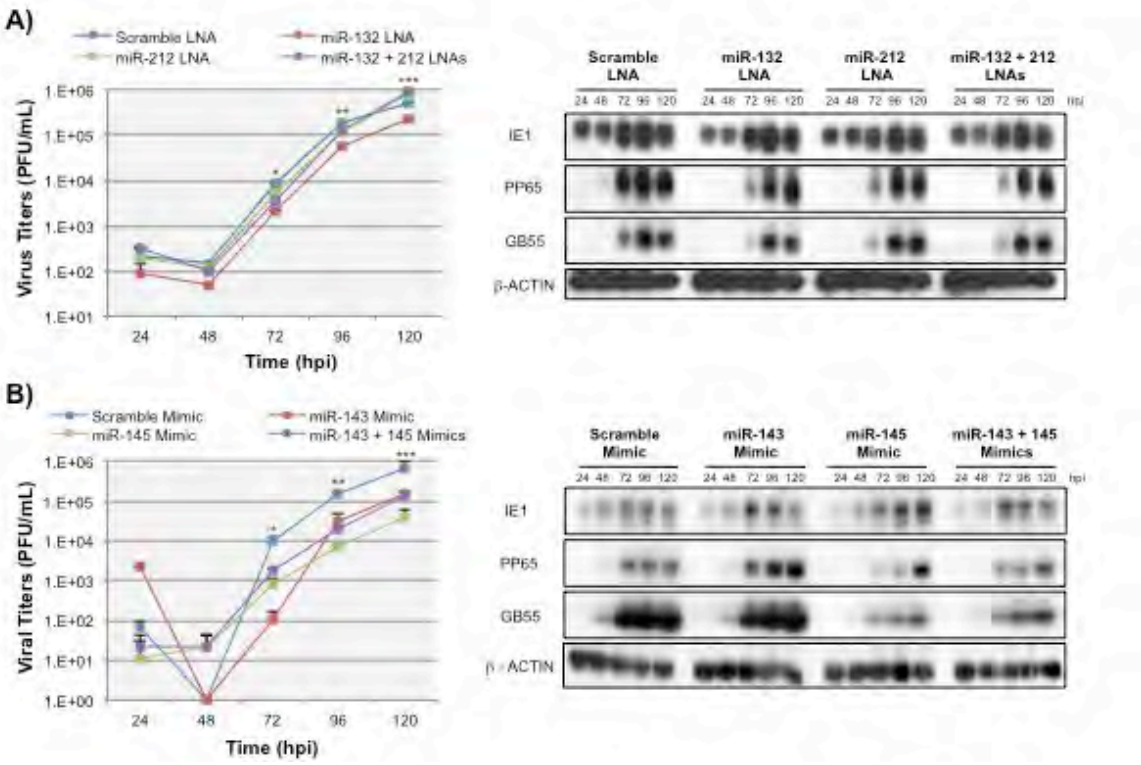


Figure 3.5: *Altered miRNA expression is required for efficient HCMV replication*. A) To inhibit accumulation of miR132/212 during HCMV infection, HEL fibroblasts were transfected with antisense LNAs and infected 24 hours later at MOI=1. Supernatants and cell monolayers were harvested at 24 h intervals for a time-course of 5 days and plaque assays and western blots were performed to assess the effects on viral replication (left panel) and markers of viral gene expression (right panel). B) To counteract the downregulation of miR143-145 expression during infection, HEL fibroblasts were transfected with miRNA mimics and infected 24 hours later at MOI=1. The same protocol was followed to assess the effects on viral replication (left panel) and viral gene expression (right panel). Mean viral titers were analyzed by ANOVA with a Tukey post-test in order to determine if the changes in titers relative to the respective scramble control were statistically significant.

A) [*, *miR132 LNA, p<0.05; miR212 LNA, p=n.s; miR132+212 LNA, p<0.05*]
[**, *miR132 LNA, p<0.001; miR212 LNA, p<0.01; miR132+212 LNA, p<0.05*]
[***, *miR132 LNA, p=n.s.; miR212 LNA p=n.s; miR132+212 LNA, p<0.05*].

B) [*, *p<0.001*]
[**, *p<0.001*]
[***, *p<0.05*].

Inhibiting the infection-associated increase in miR132/212 expression by LNA transfection resulted in a modest, but statistically significant attenuation of viral replication (Figure 3.5 A; left panel). miR132 LNA transfection repressed viral replication ~5-fold beginning at 72 hpi and continuing through 120 hpi. The reduced inhibitory effect of miR132 LNA transfection seen at 120 hpi likely reflects the turnover of the LNA given that it had been transfected 5 days earlier. This inhibitory effect of miR132 LNA transfection was ~2-fold greater than what was observed after inhibiting miR212 function. This likely reflects the fact that mature miR132 is present at levels >1-log higher than mature miR212 and that both miRNAs likely target the same network of transcripts given their conserved seed sequences (Figure 3.4 C, D & H). miR132 LNA transfection also had a greater inhibitory effect on viral replication than did miR132 + miR212 LNA transfection (Figure 3.5 A). This may represent cross-reactivity of the LNAs. Given the degree of sequence conservation between miR132 and miR212, it's possible that co-transfection of both LNAs could dilute the effects of miR132 LNA transfection alone (Figure 3.4 C). The attenuation of viral replication after LNA transfection was also reflected as a modest delay in early (pp65) and late (gb55) viral protein accumulation (Figure 3.5 A; right panel). Similar to the recovery of viral replication at later time-points, viral protein expression recovered to WT levels at the later time-points presumably as LNA was turned over. Collectively, these results support the idea that reprogramming miR132/212 expression is required for efficient replication.

Counteracting the infection-associated decrease in miR143/145 expression by miRNA mimic transfection also resulted in an attenuation of viral replication (Figure 3.5 B; left panel). miR143 mimic transfection resulted in a ~2-log decrease in viral replication at 72 hpi that progressively recovered to a ~1-log reduction at the later time-points post-infection. This effect, however, was not reflected as a defect in viral protein accumulation (Figure 3.5 B; right panel). Over-expressing miR143 during infection had little to no impact on viral protein accumulation. Since mimic transfection dramatically reduced viral replication without any noticeable impact on viral protein accumulation, miR143 may be regulating the expression of a factor(s) required for viral particle assembly or release. It is unlikely that miR143 over expression is attenuating viral DNA replication because gB55 expression has not been affected. gB is characterized as an early/late protein, and DNA replication is required in for appreciable levels of protein to accumulate. miR145 mimic transfection resulted in a ~1-log reduction in viral replication and this was also reflected by a defect in viral protein accumulation (Figure 3.5 B). Early viral protein (pp65) accumulation recovered to WT levels at 120 hpi, but early/late viral protein (gB55) accumulation was attenuated throughout the infection time-course. Inhibiting the infection-associated reduction in miR143 and miR145 had unique phenotypes with respect to viral replication and viral protein accumulation. This likely reflects the fact that each miRNA regulates a unique set of targets that contribute to infection.

These results confirm that HCMV re-programs the expression of two conserved clusters of host miRNAs during productive infection. The requirement of de-regulated miRNA expression for efficient viral replication reinforces the hypothesis that HCMV infection manipulates host miRNA expression in order to create a suitable environment for infection. The reduced inhibitory effect of miR132/212 LNA transfection relative to miR143/145 mimic transfection on viral replication may be due to the fact that miR143 and miR145 exist at much higher steady state levels in HEL fibroblasts and potentially exert a greater repressive capacity on host gene expression. Given that miR143 and miR145 mimic transfection each had unique phenotypes with respect to attenuating viral replication and viral gene expression, it is likely that each miRNA regulates a distinct network of host genes because they each harbor unique seed sequences. The mechanism by which HCMV manipulates the expression of these host miRNA clusters has not been determined. Given the utility of host transcriptional machinery in regulating viral infection, it is likely that HCMV utilizes TFBS present in the miRNA promoters to re-program their expression during infection.

TFBS identified by DNA motif enrichments contribute to deregulated host miRNA expression during HCMV infection

The genomic coding region for the miR132/212 and miR143/145 miRNA clusters are intergenic, thus leading us to presume that their expression is independently controlled by transcriptional elements that are distinct from flanking genes. In order to test the functionality of the TFBS identified by the DNA motif enrichment analysis, we designed reporter assays to analyze the activity of miRNA promoter regions during infection. We began by truncating the miR132/212 and miR143/145 promoter region in order to determine the location of the minimal essential region required for regulating miRNA expression during HCMV infection (Figure 3.6 A & 3.7 A). We then mutated the enriched TFBS contained within the minimal essential region in order to assay their functionality (Figure 3.6 B & 3.7 B). Briefly, HEL fibroblasts were co-transfected with a miRNA promoter truncation or TFBS-mutant constructs along with a calibrator reporter to control for transfection efficiency. One day post-transfection, the cells were either mock-infected or infected with HCMV at an MOI=1. Cells were then harvested at the respective time-points post-infection and promoter activity was assayed by quantifying changes in luciferase activity. Fold change in luciferase activity in all samples were first normalized to the calibrator reporter signal and this corrected signal was normalized to the same signal in un-transfected cells. The change in promoter activity during infection was then calculated relative to

the respective mock-infected control samples. After quantifying fold-change in luciferase expression, an ANOVA analysis and a Tukey post-test were performed to determine if there was a statistically significant difference in the mean fold-change in reporter activity over the time-course of infection among the respective truncated or mutated constructs.

Truncation of the miR132/212 promoter revealed that the majority of the activating potential resided in a region ~269 bp upstream of the miR212 pre-miRNA hairpin sequence (Figure 3.6 A). Each sequential truncation had little to no effect on reporter activation until the promoter was truncated to 97 bp when essentially all reporter activity was lost (Figure 3.6 A; T_97). The statistical analysis illustrated that each construct has a statistically significant difference in mean reporter activity over the time-course of infection relative to T_97. Within this promoter region were two classes of TFBS identified by our DNA motif enrichment: CREB/ATF and SP1. Mutational analysis of the respective sites revealed that the cooperative activity of both CREB and SP1 was required for maintaining promoter activity during infection (Figure 3.6 B). Individual and collective mutation of the respective CRE sites had little to no effect on promoter activation at 96 hpi (Figure 3.6 B; CRE_1, CRE_2 and CRE_1/2). However, mutation of one particular SP1 site increased luciferase expression ~3-fold, suggesting that SP1 could be exerting an inhibitory effect on promoter activity (Figure 3.6 B; SP1_2 & SP1_1/2). The collective mutation of all TFBS completely abrogated luciferase expression, further suggesting that the

cooperative effects of CREB activation and SP1 repression are required for maintaining proper miRNA expression during infection.

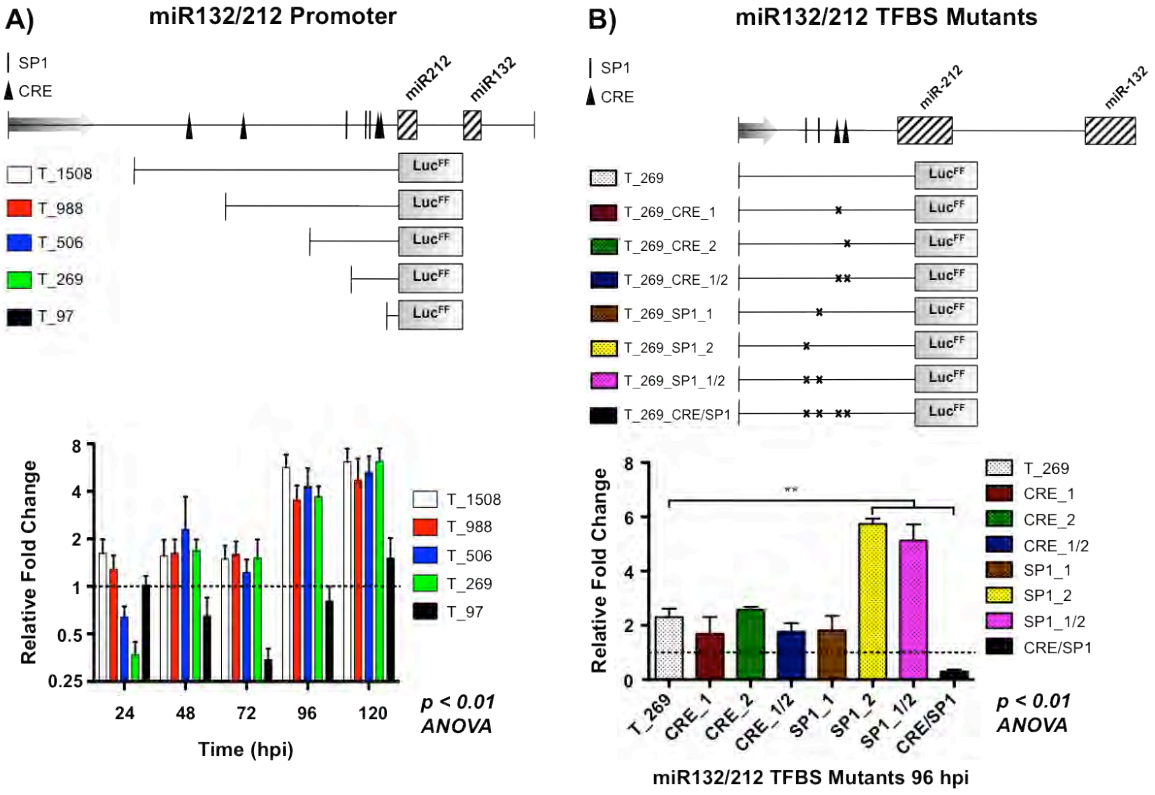


Figure 3.6: *The infection-associated change in miR132/212 expression is mediated by SP1 and CREB.* (A) Truncated miR132/212 promoters were engineered to drive the expression of luciferase in order to ascertain where the minimal essential promoter elements resided. (B) Mutations of the respective TFBS were engineered in order to test their contribution to reporter expression during infection. The dotted line on the histogram illustrates a reporter signal equal to that of the calibrator construct. Values above or below that line represent a respective increase or decrease in promoter activity during infection.

The utility of each of these particular transcription factors is supported by their established contribution to HCMV infection (180, 181, 294, 366, 367). SP1 and CREB each are capable of modulating activity of the MIEP and HCMV specifically modulates their accumulation during infection (180, 181). This effect is also required for regulating NF-κB activity during infection (366, 367). These results are further supported by the observation that CRE sites present in the miR132/212 promoter serve to activate their transcription in other contexts such as during neuronal maturation and morphogenesis (168, 343).

Truncations of the miR143/145 promoter region revealed that the repressive capacity of this promoter was progressively lost as the promoter region was shortened (Figure 3.7 A). An ANOVA and Tukey post-test analysis of the mean fold-change in promoter activity over the time-course of infection revealed that there was a significant difference among the T_1899 and T_268 truncation constructs (Figure 3.7 A). This suggested that the repressive potential of the miR143/145 promoter region was located ~1899 nucleotides upstream of the pre-miR143 hairpin sequence. Within this region were two classes of TFBS identified by our DNA motif enrichment: AP2 and Pax4a. Mutation of each individual TFBS site or of all the combined sites, however, had no effect on promoter activity (Figure 3.7 B). This, however, is not necessarily inconsistent with transcriptional repression of miR143/145 expression during infection. The initial increase in luciferase expression at 24 hpi followed by the robust repression by 48 hpi suggests that elements within the miR143/145 promoter are

capable of inhibiting promoter activity during infection (Figure 3.7 A). The fact that miR143/145 promoter activity is not efficiently repressed until 48 hpi suggests that factors required for that inhibition may not accumulate until that time post-infection. This factor could be either a host or viral gene product, but an infection-dependent event is required for eliciting the effect on miR143/145 promoter activity. It is also possible that miR143 or miR145 may regulate the host anti-viral response to infection. The initial increase in miRNA promoter activity may represent the host-response to infection, and this effect could have been attenuated at 48 hpi by the virus. The lack of functionality of the TFBS associated with down-regulated miRNAs, which were identified in our DNA motif enrichment analysis likely reflect a limitation in our TFBS enrichment protocol.

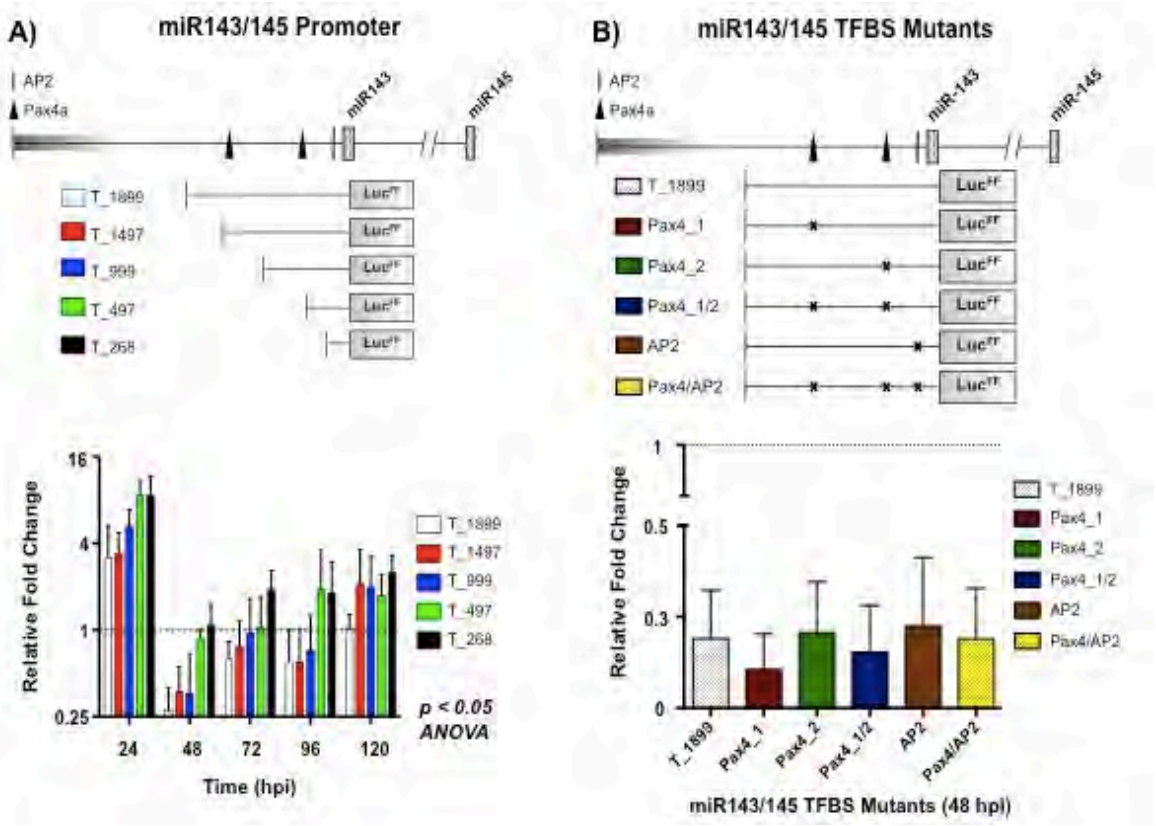


Figure 3.7: *The infection-associated change in miR143/145 expression is partially controlled at the transcriptional level.* (A) Truncations of the miR143/145 promoter were engineered to drive the expression of luciferase in order to ascertain where the minimal essential promoter elements resided. (B) Mutations of the respective TFBS were subsequently engineered in order to test their functionality during infection. The dotted line on the histogram illustrates a reporter signal equal to that of the calibrator construct. Values above or below that line represent a respective increase or decrease in promoter activity during infection.

A large portion of DNA between the miR143 and miR145 pre-miRNA hairpins was excluded from the DNA motif enrichment and the reporter construct design. Unlike miR132 and miR212, which are only separated by 472 bp, miR143 and miR145 are separated by over 1.6 kbp of genomic DNA. When we performed the DNA motif enrichments and designed the truncation constructs, we were operating under the assumption that each miRNA within the respective

clusters were co-transcribed, and we therefore limited our analysis to the sequences preceding the upstream pre-miRNA hairpin. This strategy was also used in consideration of the data size limitations imparted by the web-based DNA motif enrichment software. Even though the miR143/145 promoter region that we assayed was capable of repressing reporter activity at time-points after 24 hpi, it is possible that functional TFBS between the miRNA coding loci were excluded. p53 has also been shown to regulate miR145 expression through the use of p53 response elements present ~1500 bp upstream of miR145 (286).

p53 is relocated away from host chromatin during HCMV infection where it plays important roles in regulating viral replication (129, 234). Given the clustered miR143/145 coding loci and the proximity of p53 response elements to miR143, it is likely that either p53 or its unavailability during infection contribute to the transcriptional control of miR143 as well. It is also possible that a post-transcriptional mechanism may be influencing mature mi143/145 levels. From our microarray studies (Figure 3.1) and our qRT-PCR assays (Figure 3.4) we know that there are reduced levels of mature miR143 and miR145 at 24 hpi. Given this initial increase in promoter activation at 24 hpi it is possible that some post-transcriptional mechanism may be responsible for the decrease in mature miRNA levels. p53 has been shown to post-transcriptionally regulate the accumulation of mature miR143 and miR145 through a specific interaction with the Drosha complex (323). It is also important to mention that the DNA motif enrichment was performed using sequences ~500 bp upstream of the pre-miRNA

hairpins. The Pax4_1 site was outside of that 500 bp region, supporting the notion that it is not contributing to the deregulated expression of miR143/145 during infection. Regardless of the speculated implications, these assays show that transcriptional control of host miRNA expression is exerted by HCMV during productive infection.

Discussion

We have demonstrated that productive infection of primary fibroblasts with HCMV is sufficient to reprogram the global expression of host miRNAs (Figure 3.1). This effect was robust and reproducible, and biological replicate experiments allowed us to curate a panel of HCMV infection-associated host miRNAs. This unique expression profile was used for an informatics-based approach to understand the transcriptional mechanisms governing the change host miRNA expression during infection. The impact that HCMV infection had on host miRNA expression was extensive, and this suggested that these events represented either the host cell response to infection or the manipulation of host gene expression by the virus. The infection-associated change in expression of the miR132/212 and miR143/145 clusters was required for efficient viral replication (Figure 3.5). Using our panel of infection-associated host miRNAs, we identified DNA motifs that were specifically associated with the promoter regions of up- and downregulated miRNAs and a subset of these motifs mapped to TFBS sequences (Figure 3.3 and Table 3.2). Analysis of the miR132/212 promoter region suggested that their infection-associated change in expression was transcriptionally mediated. Mutation of enriched TFBS present in their promoter region revealed that the cooperative function of CRE and SP1 sites were required to maintain optimal promoter activity during infection. Analysis of the miR143/145 promoter region suggested that transcriptional mechanisms

were partially responsible for controlling their expression during HCMV infection. However, potential limitations in our informatics-based approach failed to identify TFBS responsible for downregulating miR143/145 during HCMV infection.

This work illustrates the extensive impact that HCMV infection has on host miRNA expression. The diverse mechanisms employed by the virus to accomplish this effect reflect the widespread control that the virus exerts host transcription in order to replicate. Little evidence exists to support the idea that host miRNAs are required for positively influencing the expression of viral genes during infection. There is also no complete curated list of HCMV transcript sequences, which makes it difficult to predict the probability of host miRNAs targeting viral genes. We therefore sought to analyze the impact that infection-associated host miRNAs exerted on global host transcript networks in order to more accurately define the relevance of de-regulated host miRNA expression during HCMV infection. Using our curated panel of infection-associated host miRNAs, we identified a vast network of host genes that harbor significantly enriched MREs. This would suggest that host miRNAs potentially exert extensive control on host gene expression during infection, and that HCMV manipulates this mechanistic control in order to positively influence infection.

Chapter IV:

Infection-Associated Host miRNAs Regulate Large Networks of Host Genes

Introduction

Gene expression profiling has helped to establish that individual cell types and tissues have unique gene expression patterns. Implicit in this theory are the inherent differences in miRNA and mRNA expression profiles, and the kinetic nature of their expression as a result of various stimuli. The distinct expression patterns of miRNAs and mRNA transcripts help sculpt a unique protein expression landscape that phenotypically distinguishes a particular environment. When determining the influence that miRNAs exert on a potential target, one must consider: 1) The relative levels of a miRNA(s) and it is cognate target mRNA; 2) The change in expression of the respective miRNAs and mRNAs; 3) The statistical confidence that the particular message harbors a functional MRE. Typically, miRNA target prediction algorithms exclude contextual information relating to relative miRNA and mRNA expression levels. Essentially, these programs analyze 3'-UTR sequences and score the predictions based on 3'-UTR architecture and MRE evolutionary conservation (73). More recently, newer models have emerged that combine sequence analysis with gene expression profiling in order to increase the likelihood of identifying functional MREs (9, 244). In the context of HCMV infection, a miRNA target prediction protocol must consider the relative expression of both miRNAs and mRNAs in uninfected and infected cells. HCMV infection characteristically impacts the expression of many cellular genes during infection in order to inhibit protective host responses and to

generate host factors required for replication (40, 131, 316, 347, 373). This creates a unique gene expression profile that is ideal for viral replication. Given the potentially extensive influence that miRNAs exert on host gene expression during HCMV infection, we thought it necessary to develop a novel protocol for identifying relevant miRNA targets.

Using a probability based MRE enrichment algorithm, we identified an extensive network of host genes whose expression is likely regulated by HCMV infection-associated host miRNAs. These mRNA targets harbor statistically significant MRE(s) identified by the enrichment of various miRNA seed types within their 3'-UTR sequences. Using the relative fold changes in miRNA and mRNA expression during infection and the statistical significance of the respective MREs, we were able to visualize the global network of miRNA target genes. The efficacy of our enrichment protocol is supported by the anecdotal identification of miRNA targets from published studies and by our experimental validation of functional miR143 MREs present in the KRas 3'-UTR. Analysis of gene ontology terms associated with the miRNA target networks revealed that host miRNAs potentially regulate many host pathways that are relevant to HCMV replication. Together, these findings suggest that host miRNAs are essential for creating a gene expression landscape that supports efficient HCMV replication.

Results

miRNA target networks can be identified using a novel probability-based MRE enrichment protocol

To begin identifying networks of host genes whose expression is regulated by infection-associated miRNAs during HCMV infection, we assembled a panel of target candidates whose expression in mock-infected and infected cells was easily detectable (Figure 4.1). Host mRNA expression data was gathered from a microarray profiling study by Hertel *et al* 2004 wherein human foreskin fibroblasts were infected with HCMV AD169 at an MOI=10 and fold changes in gene expression were observed at 50 hpi (131). Focusing our MRE predictions on genes with appreciable expression in primary fibroblasts served to increase the likelihood of identifying functional MREs. Since miRNAs can only target genes whose transcripts are coincidentally present in the same place at the same time, we can eliminate non-functional predictions and experimental noise by removing genes whose likelihood of encountering a miRNA is physiologically improbable. After assembling a cohort of target candidates, we gathered their respective 3'-UTR sequences using the Ensembl BioMart to use as the template for identifying MREs (102).

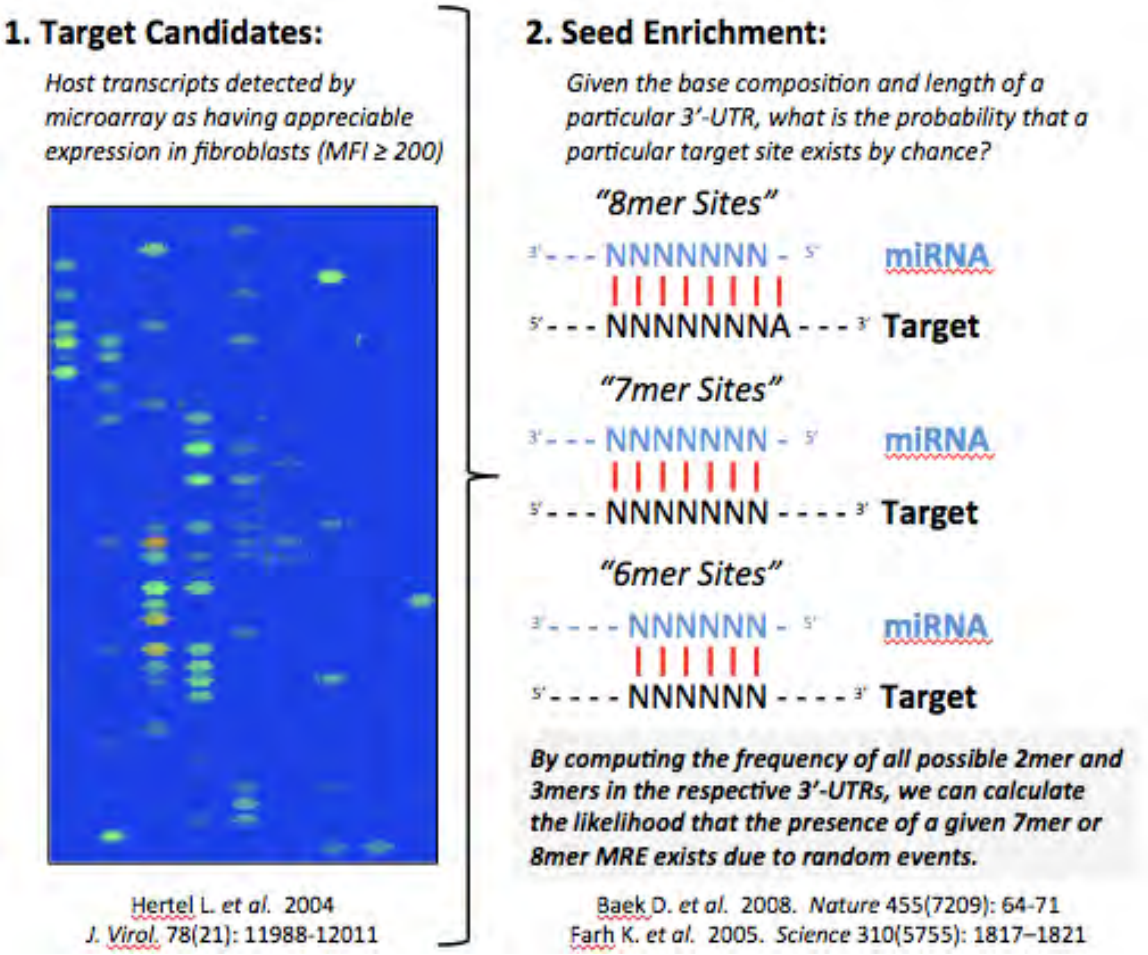


Figure 4.1: *MRE prediction protocol.* 1) In order to predict targets of HCMV infection-associated host miRNAs, we focused our enrichment analyses on candidate genes that exhibited appreciable expression in primary fibroblasts. These transcripts exhibited an MFI ≥ 200 in both mock-infected and infected cells as detected by microarray analysis. The picture illustrates a representative image generated from a microarray. 2) After gathering 3'-UTR sequences of the respective target candidates, we predicted the likelihood that the presence of an MRE represented a statistical enrichment. Our analysis focused on identifying only 6mer, 7mer and 8mer MREs and their respective orientations are illustrated.

In order to identify functional MREs, we developed a probability-based algorithm that uses 3'-UTR sequences as a template for the statistical enrichment of various MREs (6mer, 7mer and 8mer; Figure 4.1). This protocol is based on an algorithm developed by Farh *et al* 2005, where the probability of any

given 7mer being enriched in panel of 3'-UTR sequences is calculated (95). By computing the size of the 3'-UTR and the frequency of all possible 2mer and 3mers, we can use a Markov based probability calculation to determine the likelihood that the presence of a particular MRE is not due to chance. This probability can then be corrected using a binomial calculation in order to correct the significance of enrichment if multiple MREs are present in a single 3'-UTR. A significantly enriched MRE(s) would suggest that the sequence has been retained in that 3'-UTR and therefore implies some functionality. Multiple MRE types have been described, and their functional efficacy has been tested *in vitro* (23, 38). This work has led to the postulation that more extensive complementarity between nucleotides 2-8 in the miRNA seed dictates more robust regulation of mRNA translation. Although the efficacy of each MRE is not totally equivalent, miRNA-mediated repression of mRNA translation has been documented with each respective seed type. We therefore considered each MRE type when predicting targets of HCMV infection-associated host miRNAs.

Our MRE enrichment protocol is unique when considered against the available web-based prediction algorithms. It allows for batched query of contextually filtered target candidates using solely a determinant of target site retention (27). It could easily be applied to any dataset in order to identify functional miRNA targets. Once we curated our panel of potential miRNA targets based on gene expression profiling, we then gathered the seed sequences to be queried from our infection-associated host miRNAs (6mer, 7mer and 8mers) and

used batch searches in order to query all relevant miRNAs and mRNAs simultaneously. Our protocol operates under a simple assumption: if a miRNA and it is cognate target are each expressed at appreciable levels and if the mRNA 3'-UTR harbors a statistically enriched MRE(s), then the likelihood of a functional miRNA:mRNA interaction occurring is statistically probable.

The available online miRNA prediction programs typically do not allow batch searches (30, 107, 111, 118, 160, 228). Contextually filtering potential targets by using gene expression data is also not an option that is typically offered in the available web-based prediction programs. Given the fact the miRNA and mRNA expression profiles are unique to each cell type and tissue, it is essential to be able to focus MRE predictions on the appropriate candidate genes. There are, however, limitations to our approach. The stoichiometric relationship between miRNA and mRNA copy number is essential when analyzing MRE efficacy. Given that our experiments utilized baseline detection and fold-change in MFI data from microarray experiments, we were not able to weight the enriched MREs based on the relative copy number of the miRNA and their cognate targets. We were also not able to score predictions based on parameters such as MRE conservation or validated functional sites from the literature. These are all prediction parameters that could subsequently be added to the current algorithm in order to increase the likelihood of predicting functional MREs.

We initially sought to validate the efficacy of our MRE prediction algorithm by enriching for the presence of previously validated MREs from the literature. Wang *et al* 2005, showed that HCMV infection impacted the expression of host miRNA in primary fibroblasts using a similar strain of HCMV (347). The effect that infection had on host miRNA expression was comparable to what we observed although the breadth and depth of our dataset was much more extensive (Chapter 3, Figure 3.1). Using reporter assays, they showed that miR100 was capable of regulating the expression of both mTOR and Raptor, while miR101 was only capable of mediating mTOR expression (albeit much less efficiently than miR100) (347). Using our MRE enrichment algorithm, we were able to positively identify miR100 sites in both mTOR and Raptor. The miR101 site in mTOR, however, was not identified. Its lack of MRE enrichment may reflect its relatively inefficient ability to reduce mTOR reporter expression. Pickering *et al* 2009 from our own group determined that miR17 and miR20a sites present in the E2F1 3'-UTR were required for regulating a G1 cell cycle checkpoint (260). Our MRE enrichment algorithm also positively identified both the miR17-5p and the miR20a target sites. Lastly, Zheng *et al* 2011 showed that miR101 expression was reduced during HSV-1 infection and that this effect was required to increase the levels of the pro-viral mitochondrial ATP synthase subunit beta (ATPB5) (370). The MRE that was responsible for inhibiting ATPB5 protein accumulation during HSV-1 infection was also positively identified by our enrichment algorithm (370). The ability of our enrichment protocol to replicate

the observations of other groups supports the efficacy of our MRE prediction algorithm. We applied this protocol to our panel of miRNA target candidates to identify the target networks of HCMV infection-associated host miRNAs. This will allow us to begin to analyze the relevance of de-regulated host miRNA expression during HCMV infection.

Our panel of target candidates was comprised of 6723 unique genes, which exhibited appreciable expression (MFI>200) in primary fibroblast before and after infection with HCMV at an MOI=10 (131). From these genes we gathered 28,675 associated 3'-UTR sequences, which were annotated in the Ensembl BioMart. Each annotated transcript has multiple associated 3'-UTR sequences. Though many transcripts encode multiple isoforms with potentially different 3'-UTR sequences, this magnitude is also partially a product of the Ensembl automatic gene annotation system (72). In any case, duplicate enrichments are removed later in the process. The curated 3'-UTR sequences were used as the template for our MRE enrichment protocol. By assigning multiple significance cutoffs ($p<0.05$ and $p<0.01$), we were able to visualize the number of unique genes with statistically enriched MREs and the respective number of total enrichment predictions (Table 4.1). Our analysis indicates that ~40% – 80% of detectable genes expressed in primary fibroblasts are potentially regulated by HCMV infection-associated host miRNAs. In order to begin assessing the functional relevance of this extensive miRNA mediated regulation of gene expression during HCMV infection, we focused further analyses on

genes harboring 7mer and 8mer MREs with p<0.01 (hereafter referred to as the "infection-associated host miRNA target network"). The literature has shown that each MRE type has the capacity to be functional, but given the size of the dataset we wanted to focus subsequent analyses on highly confident MREs that exhibit a more robust regulatory capacity.

Significance	MRE Type	Target Predictions	Target Genes	Total Unique Genes	miRNAs with Enriched MREs	Total miRNAs
p<0.05	6mer	19,674	4,560	5,595	56	56
	7mer	16,837	4,070		51	
	8mer	12,435	3,436		56	
p<0.01	6mer	3,052	1,763	2,652	56	56
	7mer	2,915	1,823		51	
	8mer	1,812	1,812		56	

Table 4.1: *HCMV infection-associated host miRNAs potentially target vast arrays of host genes during infection.* This table summarizes the results from our MRE enrichment analysis. Illustrated are the two significance cutoffs used for identifying statistically enriched MREs (Significance), the MRE types that were being queried for the respective enrichment (MRE type), the total number of significant sites identified for that particular MRE type (Target Predictions), the number of unique genes predicted to harbor the respective MREs (Target Genes) the cumulative number of genes harboring enriched MREs (Total Unique Genes), the number of infection-associated miRNAs corresponding to the respective enriched MREs (miRNAs with Enriched MREs) and the total number of miRNAs queried for the enrichment (Total miRNAs).

The infection-associated host miRNA target network (7mer & 8mer; $p<0.01$) contains 2013 unique genes harboring 5953 enriched MREs. Roughly 37% of the predicted 7mer targets are also 8mer targets and this is reflected by the increase in probablistic confidence of those respective 7mer sites. Given that an 8mer is classified by having an adenosine at position 1 in the 5' end of the 7mer MRE, a random model would suggest that ~25% of all 7mers are also 8mers. The fact that almost 40% of our 7mer MREs are also 8mers supports the robustness of our enrichment protocol. In order to visualize the infection-associated host miRNA target network, we have plotted the miRNAs and their respective targets using Cytoscape (Figure 4.2) (64). We have illustrated the relative fold-change in gene expression using a blue-to-red color schematic that indicates downregulated to upregulated gene expression during infection. One can immediately notice that the majority of cellular transcripts exhibit downregulated expression during infection while the host miRNAs equally exhibit both increased and decreased expression. Our initial filtering of potential target candidates and the particular time point that we are analyzing may have contributed to this trend, but these observations are in agreement with other gene expression profiling experiments performed in HCMV-infected fibroblasts (40). By visualizing the probabilistic confidence of MRE enrichment using edge thickness and color, we can see that the significance of MRE enrichment is increased when multiple miRNAs targeted the same transcripts. This highlights the cooperative nature of miRNA-mediated regulation of gene expression.

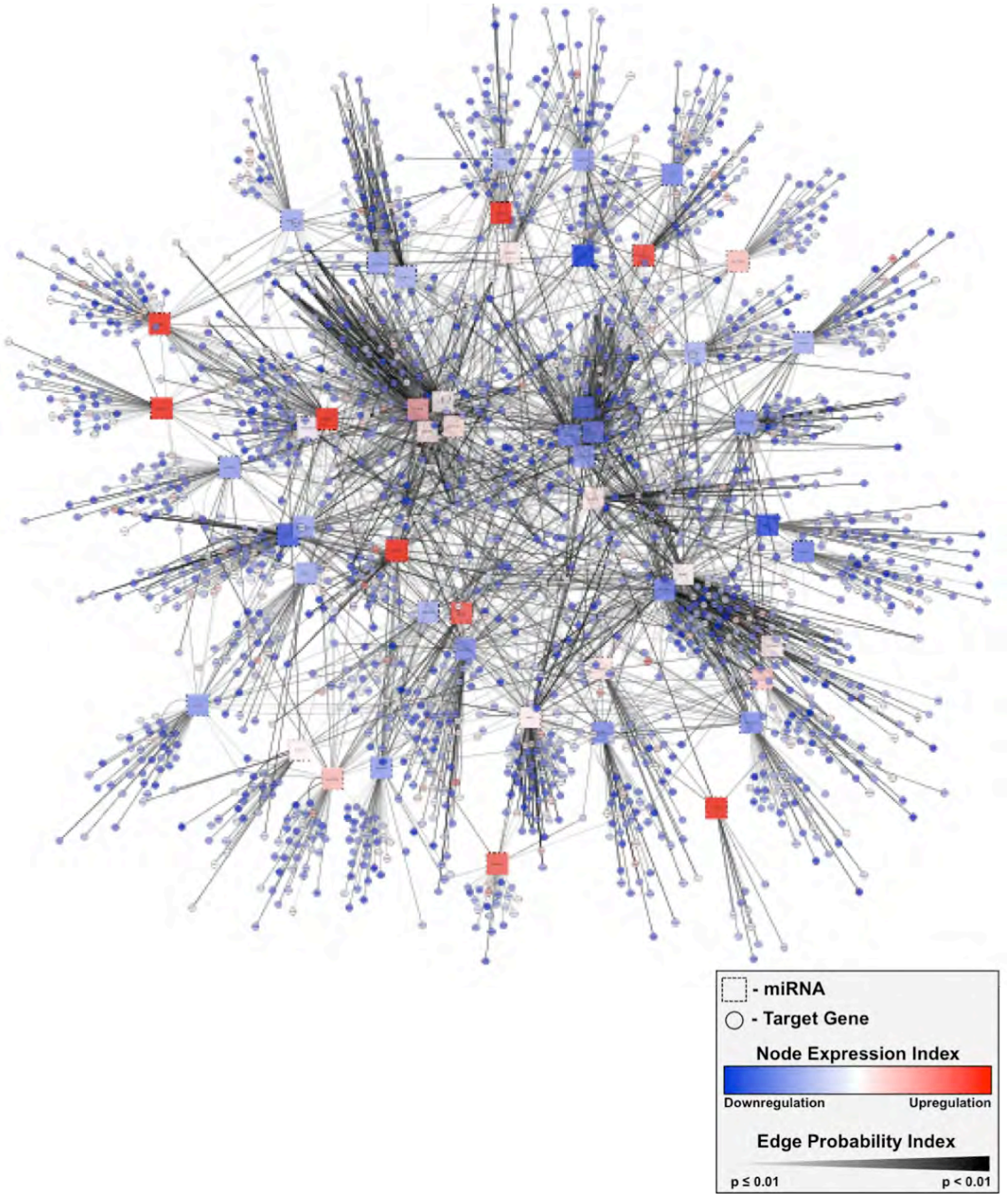


Figure 4.2: *The infection-associated miRNA target network.* Illustrated in this plot are genes harboring enriched 7mer and 8mer MREs with a p<0.01 (circular nodes) and the respective miRNAs corresponding to those MREs (square nodes). We have removed 7mer MREs that are also 8mers, and we have removed duplicate 3'-UTRs since the same MREs are typically enriched with similar confidence. Fold-change in gene expression is illustrated with the color scheming shown in the legend (red = upregulation and blue = downregulation), and MRE enrichment probabilities are reflected by the edge thickness and color (dark/thick = p<0.01, light/thin = p.≤0.01). The edges are also springe embedded so that an MRE with a better enrichment probability is propelled further from the targeting miRNA in 3D.

Each infection-associated miRNA harbors a unique set of predicted target transcripts as well. We also notice that there is not always an inverse correlation between fold-change in miRNA expression and target transcript accumulation. This finding, however, should not discredit the fact that there may be a correlative change in the accumulation of the corresponding proteins. miRNA-mediated regulation of mRNA translation does not always result in a correlative increase in mRNA degradation. Therefore one will not consistently observe an inverse correlation between fold-change in miRNA and mRNA accumulation. One must also consider the influence that transcriptional elements play in regulating the expression of the predicted target genes. It is likely that a combination of transcriptional regulation and miRNA-mediated control of transcript translation cooperate to optimize gene expression levels. These considerations are criterion that can be added to weight probabilities generated by the MRE enrichment algorithm.

The stoichiometric relationship between miRNA and mRNA copy number is a more critical determinant for predicting the probability of miRNA-mediated regulation of gene expression. For example, if the relative fold-change in expression of a particular miRNA decreases during infection, the absolute number of accumulated miRNA molecules may still be sufficient to inhibit the translation of a cognate target transcript. One must also consider the cooperative action of miRNAs in regulating the translation of target transcripts. The field of miRNA biology has been largely focused on validating the effect that

a single miRNA has on the regulation of a single transcript. This work was essential to establish the biochemical mechanisms of miRNA targeting, but it is likely that miRNAs cooperatively target large networks of genes in order to regulate gene expression. The fact that each transcript potentially harbors up to hundreds of MREs and therefore could potentially be simultaneously regulated by hundreds of miRNAs is an aspect of miRNA biology that should be considered further. In order to illustrate the cooperative capacity of deregulated miRNAs during HCMV infection, we sought to analyze the regulatory capacity of miR132/212 and miR143 on a shared target: *KRas*. These miRNAs exhibit converse changes in expression during infection, yet KRas harbors statistically enriched MREs for each respective miRNA suggesting that they are both capable of regulating KRas mRNA translation during HCMV infection.

Functional MREs are enriched in the KRas 3'-UTR

Using our MRE enrichment protocol, we observed that the miR132/212 and miR143/145 clusters each harbor a unique set of predicted targets (Figure 4.3 A). There are, however, a limited number of shared targets and among them is KRas. KRas mRNA expression increases ~2 – 4 fold over the course of productive HCMV infection, but protein accumulation is reduced at each respective time-point (Figure 4.3 B & C). This would indicate that a post-transcriptional mechanism is responsible for downregulating KRas protein accumulation during infection. The KRas 3'-UTR harbors multiple MREs corresponding to 7 different miRNAs, each of which exhibit unique changes in expression during infection (Figure 4.3 D). Given that it also contains statistically confident 8mer MREs corresponding to miR132/212 and miR143, and that miR143/145 are capable of regulating KRas expression in other contexts, we hypothesized that these miRNAs were contributing to the repression of KRas mRNA translation observed during infection (159). We sought to assay the ability of each miRNA to regulate KRas expression during HCMV infection using a reporter assay (Figure 4.4).

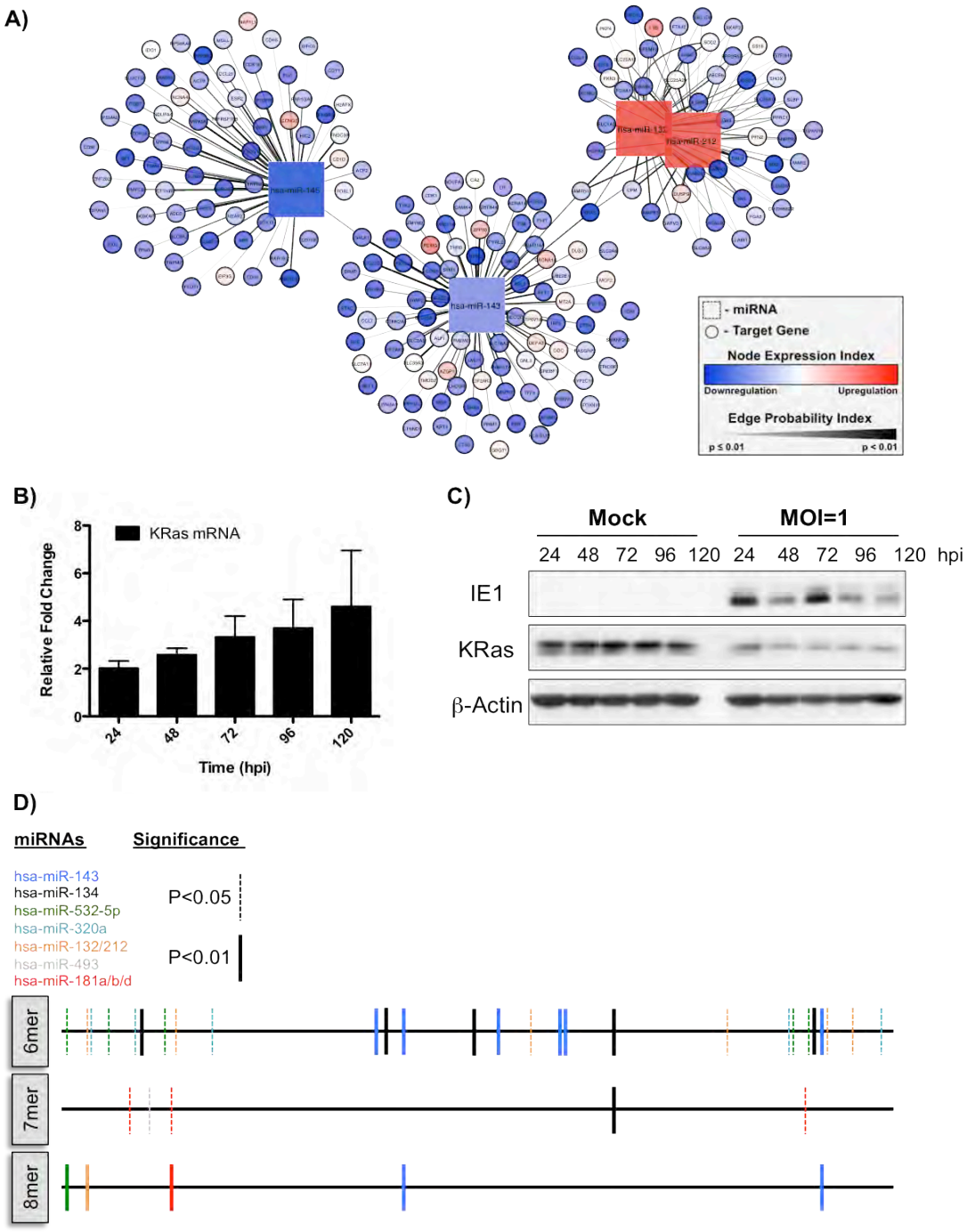


Figure 4.3: *KRas mRNA is a target of multiple infection-associated host miRNAs.* (A) The target networks of miR132/212 and miR143/145 (7mer & 8mer MREs, p<0.01). KRas is highlighted in green. (B) The increase in KRas mRNA expression during HCMV infection was quantified relative to GAPDH by qRT-PCR. (C) The decrease in KRas protein accumulation during HCMV infection was determined by western blot. IE1 detection is used as a positive control for infection. (D) All of the enriched MREs present in the KRas 3'-UTR are illustrated relative to the 3910 bp amplicon used in the subsequent reporter assays (Figure 4.4). The corresponding miRNAs are color-coded and the significance of their enrichment is represented by line thickness (Figure legend).

Briefly, HELs were co-transfected with a Renilla luciferase construct containing the KRas 3'-UTR and with a calibrator Firefly luciferase construct lacking the UTR. 24 hours after transfection, the cells were infected with HCMV and luciferase activity was assayed 48 hpi. This allowed us to quantify the repressive capacity that the KRas 3'-UTR exerted on reporter activity by calculating the fold change in luciferase expression relative to the calibrator construct. By engineering reporter constructs harboring the KRas 3'-UTR with mutant MREs, we tested the function of the respective sites by quantifying the attenuation of reporter repression. Cloning the KRas 3'-UTR downstream of luciferase was sufficient to reduce reporter expression ~10-fold during HCMV infection (Fiigure 4.4 B; WT). This was a positive indication that elements present in the KRas 3'-UTR were sufficient to inhibit reporter expression. As each respective MRE was mutated, the repressive capacity of the KRas 3'-UTR was progressively lost. Mutation of both miR143 MREs reduced reporter repression ~4-fold (Figure 4.4; m143.1/2). In agreement with the greater repression exerted by miR143 than miR132/212, a higher probability of enrichment was predicted for miR143 suggesting that the informatics approach is predictive of empirical results. Mutating each MRE, however, was not sufficient to completely alleviate repression of luciferase expression. It is likely that other miRNAs are exerting a repressive effect on reporter expression given that we had a statistical enrichment of many other MREs in the KRas 3'-UTR (Figre 4.3 D). This would suggest that the combined action of each MRE contained in the

3’-UTR is required for downregulating KRas protein levels during HCMV infection (Figure 4.3 C). These results further support the efficacy of our MRE enrichments. They also reflect the fact that the fold change in miRNA expression is not necessarily reflective of the ability of a miRNA to target a transcript.

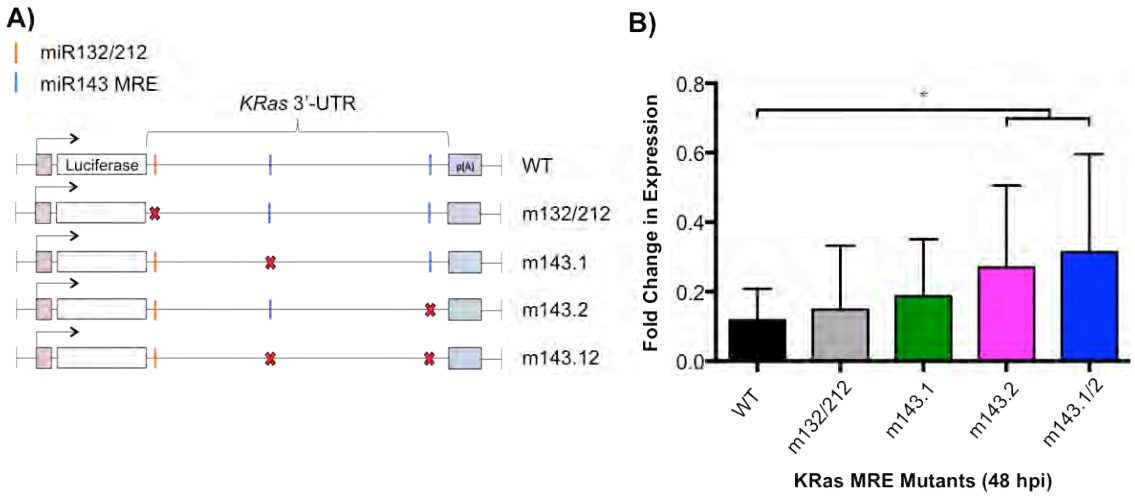


Figure 4.4: *MREs present in the KRas 3’-UTR are capable of repressing reporter expression.* (A) Reporter construct design. The KRas 3’-UTR was cloned downstream of luciferase in order to test its ability to inhibit luciferase expression. MRE mutants were engineered in order to test their contribution to reporter repression. (B) By quantifying the fold change in expression of the “KRas-UTR-containing reporter construct” relative to a calibrator reporter construct with no 3’-UTR, we calculated the relative repressive capacity of the KRas 3’-UTR and the respective MRE mutants. A t-test was performed in order to determine whether the alleviation of reporter repression was statistically significant relative to the WT construct (*=$p<0.05$).

Gene ontology term enrichments can be used to elucidate the functional relevance of de-regulated host miRNA expression during HCMV infection

To further understand the relevance of global deregulation of host miRNA expression during HCMV infection, we sought to analyze the functions of the networks of predicted miRNA targets. By enriching for ontology terms associated with each gene, we can begin to discern specific cellular mechanisms and processes that may be influenced by miRNA-mediated regulation during HCMV infection.

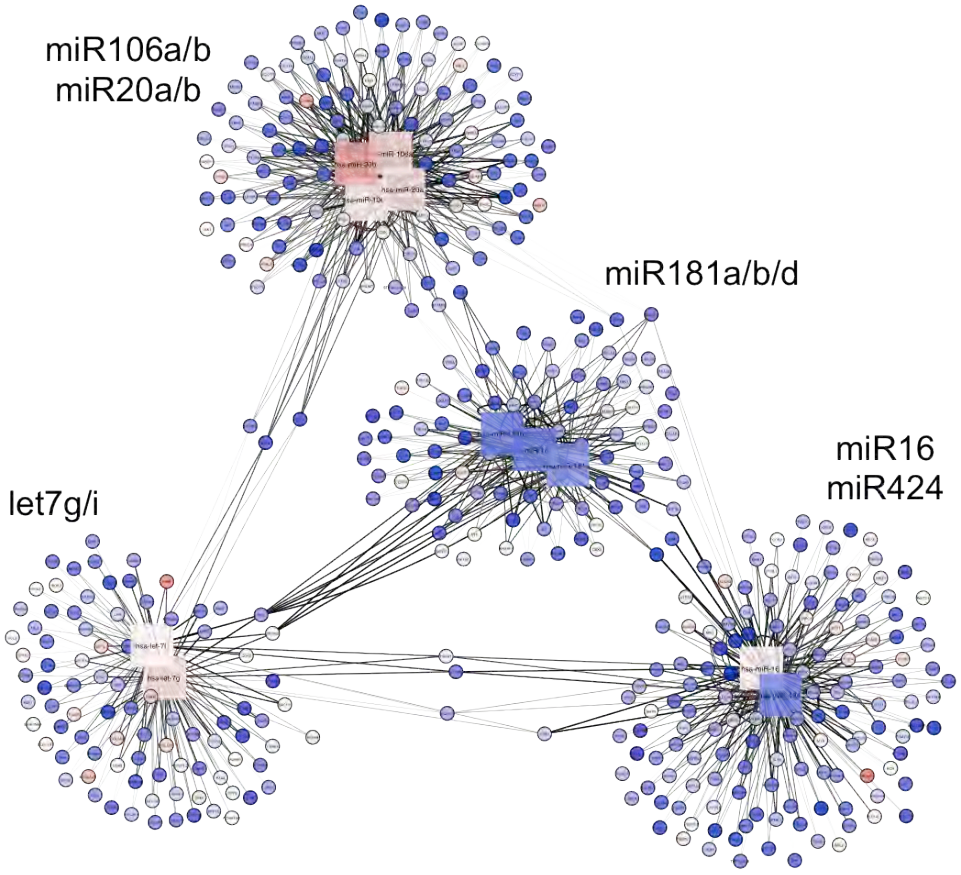
In order to analyze the potential functional associations of the predicted miRNA target genes, we analyzed associated gene ontology (GO) terms using the DAVID bioinformatics resources 6.7 (137). Using our panel of host genes harboring statistically enriched MREs, we performed a modified Fisher's exact test to determine whether particular GO terms are enriched in that that data set. This is done by observing the number of term associations in our list and comparing it against the expected GO term frequency relative to the human genome. This then allows us to compute a corrected probability to determine if there is significant term enrichment in our dataset. GO terms can specifically group genes based on aspects of their biological functions: pathway associations, protein domains, protein:protein interactions, tissue expression. They can also describe more general terms regarding the validated functions or cellular associations of a gene: metabolic process, cytosolic fraction, nucleotide binding.

GO terms enriched from our panel of infection-associated host miRNA targets revealed a number of pathways and cellular process that are known to be required for efficient HCMV replication (Table 4.2).

GO Category	Term	Count	%	P-Value	Benjamini
Pathways	Pathways in cancer	103	5.3	5.30E-10	1.00E-07
	EGF signaling pathway	32	1.6	4.00E-05	8.40E-04
	Insulin signaling pathway	43	2.2	7.80E-05	1.50E-03
	Glioma	25	1.3	8.80E-05	1.50E-03
	Cell cycle	35	1.8	4.70E-03	4.40E-02
Protein Domains	Protein kinase, core	103	5.3	1.10E-10	1.40E-07
	Serine/threonine protein kinase	58	3	5.70E-07	2.90E-04
	Tyrosine protein kinase	27	1.4	3.90E-06	4.10E-03
	EGF-like region	56	2.9	1.10E-04	2.80E-02
Tissue Expression	Epithelium	382	19.5	1.70E-13	3.40E-11
	Brain	966	49.4	2.30E-12	2.30E-10
	Placenta	454	23.2	3.40E-11	2.70E-09
	Leukocyte	35	1.8	4.20E-06	1.70E-04
	Kidney	203	10.4	2.00E-05	7.40E-04
	Bone marrow	106	5.4	3.30E-04	6.90E-03
	Fibroblast	26	1.3	1.60E-03	2.40E-02

Table 4.2: *GO terms enriched from the infection-associated host miRNA target network.* Using the DAVID bioinformatics resources, we calculated GO term enrichments using a modified Fisher's exact test. This table illustrates the number of genes (Count) associated with the particular GO term (Term), the percentage of that count relative to the entire list of miRNA target genes (%) and a p-value for that term enrichment (P-Value), which is corrected using a Benjamini and Hochberg post-test (Benjamini). Each GO term is also classified based on more broad GO categories (GO category).

The enrichment of terms associated with “pathways in cancer” and “cell-cycle” likely reflect the fact that HCMV exerts control over the host cell cycle and DNA damage response during productive infection (48, 49, 87, 129). The “EGF signaling pathway” is activated upon virus binding and the EGFR is one of the known cellular receptors of HCMV (40, 349). “Glioma” and the “pathways in cancer” also reflect the associations that have been made by correlating HCMV infection with the development of glioblastoma multiforme and the proposed transforming potential of the virus (31, 86, 314). The contribution of these pathways to viral replication is further reflected by the enrichment of GO terms associated with intercellular signaling pathway mediators like protein kinases. HCMV infection increases MAPK activity to mobilize factors such as NF-κB, ERK, ATF and CREB, each of which are each required for regulating the expression of host and viral genes during infection (48, 172, 278, 294, 366). There are also significant associations with many tissues that are relevant to HCMV pathology and disease. HCMV infection typically starts at the epithelium and congenital infections traverse the placenta to ultimately cause damage to the fetal brain or nervous system. Virus is shed in the urine and source of this virus is thought to originate in the kidney. Latent HCMV infection has been observed in subsets of CD34+ bone marrow-derived hematopoietic stem cells. The enrichment of GO terms that are associated with many essential aspects of HCMV replication supports the efficacy of our MRE enrichment protocol.



miRNA	Term	Count	%	P-Value	Benjamini
mir106a/b & mir20a/b	Phosphoprotein	63	54.8	2.20E-04	4.80E-02
let7g/i	Phosphoprotein	54	59.3	3.70E-05	7.90E-03
	Cell cycle	7	7.7	1.50E-04	1.10E-02
	Ser/Thr kinase	9	9.9	4.10E-04	4.30E-02
miR16 & miR424	Plasma membrane	36	26.3	3.00E-05	7.10E-03
	Phosphoprotein	75	54.7	5.60E-05	1.40E-02
	ATP-binding	23	16.8	1.50E-04	2.00E-02
	Ser/Thr kinase	11	8	3.80E-04	2.50E-02
	Disease mutation	24	17.5	7.60E-04	3.90E-02
	Alternative splicing	72	52.6	1.10E-03	4.70E-02
miR181a/b/d	Nucleus	36	40.4	1.60E-04	1.10E-02
	Transcription	23	25.8	1.20E-04	1.20E-02
	Phosphoprotein	50	56.2	4.70E-04	1.90E-02
	Dna-binding	20	22.5	6.70E-04	2.30E-02
	Transcription repressor activity	9	10.1	4.60E-04	4.30E-02

Figure 4.5: *GO term enrichments reveal nodes of target genes associated with cellular processes relevant to HCMV replication.* By performing GO term enrichments on the genes targeted by infection-associated host miRNAs with >100 targets, we can classify the respective miRNAs based on target gene ontology. These associations can be useful in identifying functional targets for subsequent validation and to elucidate key pathways that may be regulated by the respective miRNAs.

This analysis has helped reveal the extensive nature of miRNA-mediated regulation of host gene expression during HCMV infection, and it can also be applied on a smaller scale in order to classify specific nodes of miRNAs or genes based on their associated functions.

By enriching for GO terms associated with target genes of miRNAs with >100 predicted targets, we can identify cellular processes and pathways that may be regulated by the respective miRNAs during HCMV infection (Figure 4.5). The fact that certain MREs are enriched in a greater number of host genes suggests that the respective miRNAs may exert a greater regulatory capacity during infection. This is supported by the observation that the genes of each node are shared targets of multiple miRNAs and are therefore capable of being cooperatively regulated those miRNAs (Figure 4.5). This possibility, however, would need to be correlated with miRNA copy number in order to determine the likelihood in which the miRNAs could potentially target the respective genes. This analysis revealed that genes targeted by let7g/i, miR106a/b, miR20a/b, miR16 and miR424 are associated with cell cycle, intracellular signaling cascades and post-translational phosphorylation. Genes targeted by miR181a/b/d, however, seem to be associated with transcriptional processes and DNA binding. Given the association of miR16 and miR424 with the plasma membrane and with the propagation of intracellular signaling cascades, it is possible that proliferative signals are propagated to the targets of miR181a/b/d in order to regulate the transcription of genes required for viral replication.

The let7 family was originally identified as having roles attributed to developmental timing in C. *elegans*, and it is speculated that these functions are conserved in higher order eukaryotes given the evolutionary conservation of both miRNA and MRE sequences (283). Studies in human fibroblasts indicated that the let7 family is involved in regulating the proliferative response to growth stimuli that activate PI(3)K signaling (120). The node of genes targeted by miR106a/b and miR20a/b could also potentially contribute to the proliferative response or the cell cycle (Figure 4.5). This particular set of genes is enriched with phosphotransferases and proteins modified by phosphorylation. CDKN1A (p21) is one such gene whose expression is regulated by each of these miRNAs (148). This protein regulates cell proliferation and cell-cycle progression by inhibiting cyclin-dependent kinase activity through phosphorylation. Similar terms are enriched in the targets of miR16 and miR424, and many of the predicted targets have also been substantiated by Selbach *et al* 2008 (296). As mentioned previously, the precise control of cell-cycle gene expression is a critical aspect of HCMV replication. Upon infection, IE72 inactivates p53 and subsequently induces a p21-dependent cell cycle arrest (48, 50). Given that each of these genes are predicted targets of infection-associated host miRNAs, it is likely that HCMV utilizes host miRNAs to control cell cycle progression in an effort to generate host factors required for viral replication. Similarly, E2F1 is a transcription factor required for transactivating the expression of S-phase genes to allow for cell cycle progression. HCMV induces an E2F1-mediated pro-viral

DNA damage response during infection and precise control of E2F1 protein accumulation is critical for proper progression through S-phase (87, 260). This further supports the theory that infection-associated host miRNAs are integral to controlling host processes required for HCMV replication.

Discussion

We have developed a novel MRE enrichment protocol that identifies miRNA target sites using a probabilistic determinant of sequence retention. This algorithm differs from the available web-based protocols and it allows the user to perform large-scale batch enrichments on contextually filtered transcripts in order to maximize prediction efficacy. Using this algorithm, we have identified previously validated miRNA targets from the literature and we have shown that miR143 MREs present in the KRas 3'-UTR are capable of significantly repressing reporter expression during HCMV infection. By enriching for GO terms associated with miRNA target genes, we showed that the predicted miRNA targets are associated with many host pathways that are relevant to HCMV replication. Given the association of KRas with many of the enriched GO terms and its mechanistic regulation by miR143 during infection (and likely other miRNAs), precise miRNA-mediated control of KRas protein accumulation is likely required for supporting productive infection in primary fibroblasts. KRas is a member of the RAS family of small GTPases. It is one of the most commonly mutated genes in various cancers and it has validated functions that are related to propagating growth stimuli in order to influence cell-cycle, cell growth, motility and proliferation (280). KRas also propagates proliferative signals through cellular signaling cascades that are relevant to HCMV infection such as MAPK and PI(3)K (280). KRas function has also been linked to HCMV entry, and it

presumably propagates signaling cascades initiated by particle binding (346). KRas is also involved in regulating cell shape, motility and cytoskeletal arrangement. Given the fact that deregulated miR143 expression during HCMV infection may be relevant to viral particle assemble or release (Figure 3.5), it is likely that this may be partially regulated through it is ability to control KRas expression.

The promiscuous nature of miRNA targeting makes it difficult to identify functional MREs. The global analysis of miRNA target networks is essential when analyzing the relevance of changes in miRNA expression in any environment. We propose that analyzing the cooperative action of statistically enriched MREs, and the relative levels of miRNA and mRNA target candidates, will allow researchers to discern the complex nature of miRNA-mediated regulation of gene expression. We have used productive HCMV infection as the model in which to study this phenomenon, but the mechanistic aspects that we have described are likely ubiquitous.

Chapter 5:

Discussion

Summary

Human cytomegalovirus is an obligate intracellular pathogen. Its parasitic nature requires that it subvert host responses aimed at limiting infection and that it assume control of essential host pathways that are required for viral replication. To this end, HCMV has evolved elaborate strategies that allow it to escape immune surveillance and to reprogram host cell gene expression in order to create a suitable environment for infection. The mammalian RNAi pathway is one such mechanism that is commandeered by HCMV. The requirement of the host RNAi pathway for infection is evidenced by the necessity of host machinery for the function of viral miRNAs and by the ability of host miRNAs to influence viral replication. I propose that host miRNAs contribute to various aspects of HCMV infection, and that the virus assumes control over the host RNAi pathway in order to efficiently replicate. The data presented in this dissertation indicate that HCMV reprograms host miRNA expression during productive infection, and that this infection-dependent reprogramming is required for efficient viral replication. This effect is partially mediated through the utility of host transcription factors such as SP1 and CREB, each of which is each also required for regulating viral gene expression during infection. By identifying global infection-associated miRNA target networks, I have determined that miRNA-mediated regulation of host gene expression during infection is extensive. Nodes of predicted miRNA target genes have statistically enriched associations with

cellular functions and pathways that are relevant to HCMV replication and persistence. I also propose that correlating fold change in miRNA:mRNA expression during infection is not an effective means to identifying functional miRNA targets. This is evidenced by the functionality of miR143 MREs enriched in the KRas 3'-UTR, and the results have led me to speculate that analyzing the cooperative action of miRNAs to co-regulate shared targets is essential in determining the functional relevance of host miRNAs in regulating productive HCMV infection. The experimental work-flow utilized in this body of work could easily be applied to other investigational settings in order to examine the relevance of deregulated miRNA expression in any context.

HCMV-specific miRNA expression profiles could help elucidate specific mechanisms governing various aspects of viral infection

Our panel of statistically confident, infection-associated host miRNAs represents a novel, HMCV-specific host miRNA expression profile (Figure 3.1 C). The comparative analysis of miRNA expression profiles alone harbors great potential when characterizing host contributions to cellular tropism, disease and the various stages of infection.

The utility of host miRNAs in regulating the characteristic events of HCMV infection could be ascertained using classical determinants of viral gene expression kinetics. Alterations in host miRNA expression elicited by particle

binding could be identified by binding virus to cells at 4°C and subsequently performing gene expression profiling analyses. Studies have shown that cellular gene expression is impacted as early as 1 hpi suggesting that particle binding is sufficient to impact host gene expression (373). However, this effect was observed during productive infection and the involvement of host miRNAs was not investigated. Inhibiting cellular protein translation using cyclohexamide or inhibiting DNA replication using foscarnet could potentially characterize host miRNAs as having functions relevant to IE/E or E/DE viral replication events. The relevance of miRNAs to L events of viral replication could be inferred through the use of mutant virus strains, which are defective in capsid loading or virion egress (3, 297). These studies could also give indication as to which viral proteins are contributing to the deregulated miRNA expression at the relevant times post-infection.

Analyzing HCMV-specific miRNA expression profiles from infections in fully permissive cells against infections in cells that do not support a complete productive replication cycle could prove useful in identifying miRNA-mediated determinants of cell tropism. It is known that clinical strains of HCMV harbor ancillary genes that contribute to *in-vivo* tropism patterns (42, 53, 149). The mechanistic function of those genes is still being defined, but the potential role that host miRNAs play in this process has not been investigated. Endothelial cell tropism is dictated by events that occur after virion fusion and entry, which suggests the necessity of a specific nuclear transport mechanism (306, 308).

The contribution of specific host factors in regulating these processes has not been formally discredited, and it is therefore possible that miRNAs contribute to HCMV tropism given their mechanistic control of gene expression. An exemplary model to support this hypothesis is illustrated by miR122, a liver-specific miRNA that enhances HCV replication (70, 153). Reconstituting miR122 expression in non-permissive cell lines is sufficient to support efficient HCV replication (54, 108).

Comparison of HCMV-specific miRNA expression profiles to other virus-specific miRNA expression profiles could be used to identify miRNAs whose deregulated expression is either common to viral infections or are specific to particular classes of viruses. miRNA expression profiling of HPV(+) cervical cancer tissues and cell lines revealed that many of the deregulated miRNAs exhibited similar expression dynamics as our HCMV infection-associated host miRNAs (371). Furthermore HPV E6 protein was proposed to be responsible for upregulating miR16 expression during infection. The miR16 family regulates the cell-cycle and cell growth, and their increased expression is thought to contribute to blocking cell-cycle progression (200). Given functional similarities in HPV E6 and HCMV IE proteins, it is possible that increasing miR16 expression may serve to modify the proliferative capacity of the infected cell in order to create an environment that is suitable for replication. GO terms enriched from the predicted targets of miR16 further suggest that the control over cell cycle may be

exerted through miRNA-mediated control of signaling cascade intermediates (Figure 4.5).

Comparing the miRNA expression profiles from the different stages of HCMV infection in the relevant cell types could also help elucidate the contribution of host miRNAs in the establishment and maintenance of the respective stages: productive, persistent and latent. Studies have shown that the different stages of EBV latency can be characterized by unique miRNA expression patterns, and similar studies with HCMV have also begun to analyze the influence of host miRNAs in the establishment and maintenance of latency (44, 263). Evidence in the literature suggests that miR106a/b and miR20a/b may have roles that are relevant to maintaining latent HCMV. The miR17/92 family and its homologues are comprised of miR17, miR19a/b, miR20a/b, miR25, miR106a/b and miR93. This particular family of miRNAs is involved oncogenesis given their roles in controlling cell proliferation, differentiation and cell survival (250). Decreased miR106a/b and miR20a/b have been shown to regulate monocyte differentiation by allowing for accumulation of AML1 (RUNX1) (105). HCMV is latently maintained in CD34+ bone marrow progenitor cells, which are monocytic precursor cells. Given that increased miR106a/b and miR20a/b expression is sufficient to prevent monocyte differentiation, this may represent a mechanism by which HCMV could maintain a latent infection.

miR132/212 and miR143/145 are potentially key players in HCMV biology

We observed that productive HCMV infection results in a robust alteration of host miRNA expression (Figure 3.1 A & C). The lack of a correlation between miRNA expression in mock and infected cells indicated that this effect was infection dependent (Figure 3.1 A; R^2 values). This was also illustrated by the static nature of miRNA expression in consecutive mock-infected time-points (Figure 3.1 B). The global change in host miRNA expression during infection was extensive and robust, suggesting that their expression is specifically de-regulated during infection (Figure 3.1). We validated the infection-associated change in expression of two conserved clusters of host miRNAs (miR132/212 and miR143/145) and determined that their altered expression contributed to efficient viral replication (Figure 3.4 & 3.5). This suggested that HCMV specifically reprogrammed the expression of these host miRNAs, and it indicates that their functionality contributes to infection. Each of these miRNA clusters has validated functions that are relevant to different aspects of HCMV infection and pathogenesis.

The best characterized functions of miR132/212 are related to neuronal morphology and maturation. miR132 regulates the expression of methyl-CpG-binding protein 2 (MeCP2) and this effect impacts dendritic spine density and neuronal maturation (168, 343). This effect is interesting given the phenotypic similarities with neurological disorders associated with congenital HCMV infection.

HCMV infection is also capable of inhibiting neuronal precursor cell differentiation, and this is thought to contribute to HCMV-associated neurological diseases (248, 249). It is possible that deregulated miR132/212 expression during HCMV infection of neurons or neuronal precursor cells could contribute to these effects. The relevance of MeCP2 to HCMV replication is also intriguing given the fact that the HCMV genome is methylated and enriched in GC dinucleotides (Figure 1.1). MeCP2 can bind to a single methylated CpG residue and has functions relevant to transcriptional repression and chromatin modification (194). We determined that MeCP2 expression is not influenced by miR132/212 during productive HCMV infection in HEL fibroblasts (data not shown). We also observed a dramatic cellular re-localization of MeCP2 protein from nuclear foci to perinuclear aggregates during HCMV infection. This suggested that other mechanisms might exist to limit MeCP2 nuclear function during productive infection. However, it is still possible that miR132/212-mediated regulation of MeCP2 expression could contribute to neurological pathology that is characteristic to congenital HCMV infection. The ability of miR132/212 to influence MeCP2 expression during HCMV infection should be examined in relevant neuronal or neuronal precursor cell types or in an animal model of congenital infection (292). miR132 expression is also upregulated during KSHV, HSV-1 and HCMV infection in monocytes (177). This effect is thought to be required for regulating p300 expression in order to subvert the host interferon response. miR132 has also been shown to regulate the host inflammatory response by regulating the

expression of acetylcholinesterase (298). Taken together, these results suggest that deregulated miR132/212 expression during HCMV infection could potentially serve to mediate various aspects of viral replication, immunity and disease.

The miR143/145 cluster also has validated functions relevant to HCMV replication. Decreased expression of miR143 and miR145 is associated with the development of numerous types of cancers (5). These associations are reminiscent of the dynamic gene expression patterns, metabolic changes and intracellular signaling signatures during HCMV infection, which have garnered it the title of an “onco-modulatory” virus (22, 63, 236). miR145 expression is attenuated during HPV infection, and this is speculated to inhibit both the antiviral effect of miR145 targeting HPV genes and the ability of miR145 to regulate the cellular transcription factor KLF4 (121). Although our MRE enrichment protocol did not identify KLF4 as a predicted target of miR145, other downregulated host miRNAs including miR25 and miR7 were predicted to target KLF4. This potentially highlights the utility of KLF4 in regulating HCMV replication. miR143/145 also regulate cytoskeletal dynamics in smooth muscle cells (SMC) (360). This is interesting given that the defect in HCMV replication imposed by miR143 over-expression may be involve deregulated events involving the cytoskeleton required for virion assembly or egress (Figure 3.5). miR143/145 also affect KRas signaling by regulating the accumulation of Ras response element binding protein 1 (RRBE1) (159). Over-expression of either miRNA leads to attenuated MAPK and PI(3)K signaling, and this is contrary to what is

observed during HCMV infection. It's also possible that miR143 and/or miR145 represent a part of the host anti-viral response to infection. As mentioned in chapter 3, miR143/145 promoter activity is strongly activated at early times post-infection (Figure 3.7). This initial activation could represent the host response to infection, and the later attenuation could represent the virus combatting that response. Preliminary enrichment analyses indicated that the HCMV Merlin genome harbors statistically enriched MREs corresponding to miR143, supporting the idea that miR143 may be an anti-viral host miRNA (data not shown). This analysis, however, used the HCMV Merlin genomic sequence as a template for the enrichment of host MREs (AY446894.2). Therefore, it is still unclear whether the corresponding viral transcripts also harbor functional host MREs. Similar to the validated miR132/212 functions, deregulated miR143/145 expression during HCMV infection could potentially serve to mediate various aspects of viral replication and disease. These findings highlight the necessity of analyzing the global miRNA target networks when identifying the functional relevance of deregulated miRNA expression.

Identifying mechanisms governing deregulated host miRNA expression during HCMV infection

Identifying the regulatory mechanisms that govern miRNA expression during HCMV infection is essential to understanding their relevance to viral replication. By enriching for DNA motifs associated with infection-associated host miRNA promoters, we were able to assemble a cohort of host TFBS whose functions were likely relevant to controlling host miRNA expression during infection. We determined that deregulated miR132/212 expression during HCMV infection was transcriptionally mediated by the cooperative action CREB and SP1. The utility of these particular transcription factors is supported by their documented contribution to HCMV replication. Although we determined that transcriptional mechanisms contributed to deregulated miR143/145 expression during infection, our protocol failed to identify functional TFBS that contributed to this effect. This may reflect limitations in our experimental design.

As mentioned in Chapter 3, it is possible that the large amount of genomic sequence between the miR143 and miR145 coding loci may contain transcriptional elements that contribute to regulating miR143/145 expression during infection. Future DNA motif enrichments should not operate under the assumption that clustered miRNAs are co-transcribed in the same transcriptional unit, and thus should include genomic sequences preceding each clustered miRNA. Even if a particular set of clustered miRNA are co-expresed in the same

transcriptional unit, this optimized protocol will allow us to analyze the contribution of TFBS between the clustered miRNA in regulating their expression during HCMV infection. It is also likely that post-transcriptional mechanisms may be influencing the accumulation of miR143/145 expression during infection. This concern could be addressed by quantifying pri-miR143/145 levels during infection by qRT-PCR, but future experiments should quantify the absolute copy numbers of pri-miRNAs, mature miRNAs and mRNAs through the use of high throughput sequencing or microarray. This will effectively allow us to investigate the influence of post-transcriptional mechanisms in regulating global miRNA expression. This analysis could also strengthen the DNA motif enrichment analysis by allowing us to concentrate on miRNAs whose expression is more likely controlled at the transcriptional level.

Clustering miRNAs and host genes on the basis of infection-associated fold change in expression could also help to more accurately elucidate transcriptional mechanisms governing changes in gene expression during infection. The use of a spline-based multivariate regression method has proved useful in identifying sets of co-regulated genes (268, 320). Clustering the miRNAs on the basis of expression kinetics will allow us to discriminatively curate statistically robust groups of co-regulated genes in order perform DNA motif enrichments. This analysis can also be applied to host mRNAs to identify genes whose expression is transcriptionally modulated during infection. This criterion could subsequently be incorporated into the MRE enrichment protocol in order to

decipher the impact of miRNA-mediated vs. transcriptionally-mediated mechanisms regulating gene expression during infection. Transcription factor expression data can also be incorporated to weigh the likelihood that a particular transcription factor is functional based on its absolute expression levels and it is relative change in expression during infection. The promoter analyses should also incorporate epigenetic signatures such as CpG islands. HCMV gene expression can be influenced by epigenetic mechanisms and it is possible that similar mechanisms control host gene expression during infection (202).

Identifying infection-associated host miRNAs networks

In order to identify targets of HCMV infection-associated host miRNAs, we developed a probability-based algorithm that uses 3'-UTR sequences as a template for the statistical enrichment of various MREs. This novel protocol allows the end user to perform large-scale batch probabilistic enrichments on contextually filtered gene sets. The efficacy of this protocol was evidenced by the positive identification of validated miRNA targets from our own lab and from other published studies, and by the functionality of MREs identified in the KRas 3'-UTR. Analysis of the infection-associated host miRNA target network revealed that miRNA-mediated control of host gene expression during HCMV infection is extensive. We have also determined that correlating fold change in miRNA:mRNA expression during infection is not an effective means to identifying

functional miRNA targets. This is evidenced by the functionality of miR143 MREs enriched in the KRas 3'-UTR. miR143 expression is robustly decreased during HCMV infection (Figure 3.4). This effect, however, does not alleviate the ability of this miRNA to regulate KRas expression during infection (Figure 4.4). These results have led me to speculate that analyzing the cooperative action of miRNAs to co-regulate shared targets is essential in determining the functional relevance of host miRNAs in regulating productive HCMV infection.

Our algorithm calculates the probability that the presence of an MRE(s) in a 3'-UTR sequence is due to random events. This probability can be corrected in future software updates in order to account for the presence of other miRNA MREs and the absolute levels of the miRNA and mRNA in question. This will allow the end user to more accurately identify functional miRNA targets. The absolute copy numbers of miRNAs and their cognate target mRNAs can be used to weight the MRE enrichments based on the likelihood of a miRNA:mRNA interaction occurring. The thresholds for binning miRNAs and mRNAs based on relative copy number would have to be determined empirically, but the protocol would operate with the intention of positively weighting the enrichment probabilities of genes with desirable relative copy numbers. The enrichment probabilities can also be weighted using the number of ancillary MREs present in the given 3'-UTR. Currently, the MRE enrichment algorithm only calculates the probability of a single miRNA's MRE being present. This can be performed for the different MRE types (6mer, 7mer and 8mer) but it does not consider the

contribution of other miRNAs whose MREs are also enriched in that same 3'-UTR. The last addition to this protocol should include enrichment of other miRNA seed types described by Bartel *et al* 2009 (23). We are still operating under the same assumption: if a miRNA and it is cognate target are each expressed at appreciable levels and if the mRNA 3'-UTR harbors a statistically enriched MRE(s), then the likelihood of a functional miRNA:mRNA interaction occurring is statistically probable. However, we can now correct the enrichment probabilities using the relative copy numbers of miRNAs:mRNA and the cumulative summation of additional sites within that same 3'-UTR. This will allow the end user to more accurately predict the presence functional MREs.

In order to analyze the global implications that deregulated host miRNA expression had on HCMV replication, we enriched for GO terms associated with the predicted miRNA target networks. This analysis identified host pathways and processes that are known to contribute to various aspects of HCMV infection and it implicated host miRNAs in the regulation of HCMV replication and pathology (Table 4.2). These findings support the efficacy of our MRE enrichment protocol and they suggest that the phenotypic function of miRNAs can be inferred through GO analysis of their respective targets (Figure 4.5). Cell types and tissues are distinguished by their unique gene expression profiles. Implicit in this fact is the constant dynamic between miRNA and mRNA expression levels. The result of which, is a unique miRNA:mRNA repertoire for the respective tissues and cell types. Therefore, miRNAs could potentially have a completely unique mRNA

target network in each environment, and a completely unique phenotypic function. I propose that applying GO term enrichments to the predicted miRNA target network will allow the user to infer the phenotypic function of a miRNA (or a family/cluster of miRNAs). This information can also aid in the identification of functional miRNA target genes.

Workflow for analyzing the effects of dynamic miRNA expression

The work presented in this thesis have led us to propose that analyzing the cooperative ability of miRNAs to co-regulate shared targets is essential in determining the functional relevance of host miRNAs in regulating productive HCMV infection. To address this concern we employed a novel MRE enrichment protocol and we have validated the efficacy of this algorithm using both experimental and anecdotal measures. Future updates of this software should consider the relative copy number of miRNA:mRNA and the presence of ancillary MREs in a given 3'-UTR when correcting the enrichment probabilities. This will allow the end user to more accurately predict the presence of functional MREs. By combining this miRNA target prediction algorithm with GO term enrichments, one will also be able to infer a phenotypic relevance of the miRNA target networks and thus can more accurately identify relevant miRNA-regulated genes. I present here, a work-flow designed for studying the relevance of changes in miRNA expression in any context or environment (Figure 5.1).

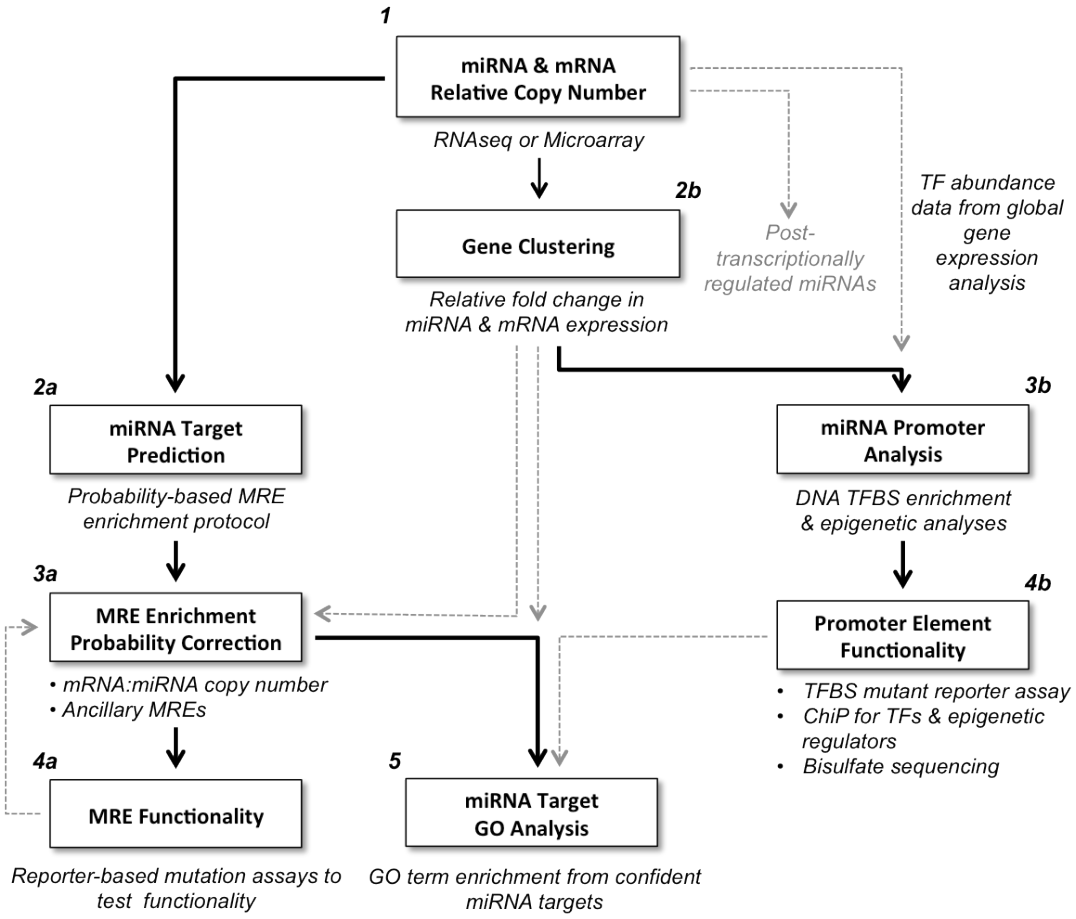


Figure 5.1: *Work-flow for investigating the relevance of degregulated miRNA expression.* The experimental protocols illustrated in this figure can be applied in any context to study the functional relevance of deregulated miRNA expression. It will address the mechanisms controlling miRNA expression (2b-4b & 5) and their phenotypic implications by analyzing miRNA target networks (2a-4a & 5).

This work-flow could be applied in order to identify functional targets of both host and viral miRNAs. Accordingly, it can also be used to identify functional MREs present in either host or viral transcripts. Given the observation the miRNAs are capable of regulating the expression of viral genes through MREs present anywhere in viral transcripts, identifying functional MREs in viral genes should not solely focus on 3'-UTR sequences (198).

Identifying functional miRNA target networks should start by quantifying absolute copy numbers and calculating relative fold change in expression of miRNAs and mRNA transcripts in infected cells (Figure 5.1; 1). After contextually filtering potential mRNA targets based on empirically determined detection limits, 3'-UTR sequences to the respective genes can be gathered and the MRE enrichments can be performed (Figure 5.1; 2a). MRE enrichment probabilities will be weighted using relative miRNA:mRNA copy numbers and by incorporating the influence of ancillary MREs present in the same 3'-UTR (Figure 5.1; 3a). Subsequent MRE validation can also be used as a means to weigh MRE enrichment probabilities using experimentally validated data (Figure 5.1; 4a). Predicted target networks can then be used as the template for GO term enrichments (Figure 5.1; 5). This will help decipher the phenotypic relevance of individual miRNAs or clusters/families of miRNAs based on the GO term associations of their respective target network genes.

Fold change in expression data can be used to cluster genes based on similar expression kinetics during infection, and upstream genomic sequence from these clusters can be used as the template for performing DNA motif enrichment analyses (Figure 5.1; 2b). Prior to this, however, pri-miRNA accumulation can be examined in order to remove the promoter sequences of miRNAs whose expression is regulated post-transcriptionally. This will allow the user to focus on genes that have a higher likelihood of being transcriptionally regulated during infection. Also, transcription factor abundance and fold-change

in expression can be utilized to assign significance to the respective TFBS being investigated. DNA motif enrichment will be used in order to identify functional TFBS that potentially influence gene expression during infection and GC islands can also be identified to test the contribution of methylation to the deregulation of miRNA and mRNA expression during HCMV infection (Figure 5.1; 3b). Functionality of the identified promoter elements can subsequently be validated and the utility of particular TFs can be incorporated into the GO analysis.

The promiscuous nature of miRNA-mediated regulation of gene expression makes it difficult to characterize the relevance of deregulated miRNA expression to any process or pathway. I propose that the combined use of bioinformatics and experimental molecular biology will help elucidate the complex network of influences that miRNAs exert of host gene expression.

Appendix I

HCMV miRNAs Expressed During Productive Infection Display Sequence Variability

Introduction

After the characterization of miRNA coding determinants and biochemical processing, there was a significant effort to determine whether viral genomes also harbored miRNA coding loci. This was typically done by identifying potential miRNA hairpin structures *in silico* using RNA folding prediction algorithms, and subsequently cloning small RNA fractions from infected cells in order to validate the mature miRNA sequences. Many of the initial studies focused on large dsDNA viruses such as the herpesviruses, but various groups have shown that many other viral species also express mature miRNAs during infection (261).

One of the first viruses shown to express mature miRNAs during infection was HCMV (258). These miRNAs were identified during the productive infection of multiple relevant cell types with both lab-adapted strains and clinical isolates of virus (85, 116, 225, 258). Small RNA fractions were cloned from infected cells and the predicted mature miRNAs were identified through sequencing or northern blot. A subset of these miRNAs were also classified as having IE, E/DE or L expression kinetics based on the requirement of protein synthesis or DNA replication for their expression (116). The recent utilization of high throughput RNA sequencing revealed that both the “guide” and “passenger” strands can accumulate to appreciable levels during HCMV infection (225, 316). Currently, the Sanger miRBase lists 15 viral miRNA coding loci present in the HCMV genome and 26 detectable mature miRNAs processed from the respective

hairpins (173). Only a fraction of these miRNAs have been functionally validated, but they have been shown to be capable of regulating the expression of both host and viral genes during infection (332).

There are still some unanswered concerns regarding the presence or absence of a number of miRNA coding loci predicted by Grey *et. al.* 2005, and the presence of alternatively processed miRNAs known as “isomiRs”. Therefore, we sought to validate the expression and mature sequence of the predicted HCMV miRNAs using a microarray “probe walking” experiment that detected the accumulation of consecutive 22 nt sequences along the predicted miRNA hairpin during productive infection. This work was done in concert with a former graduate student of the Kowalik Lab, Dr. Bradford Stadler, and with the help of a former post-doctoral fellow Herve Seitz.

Results

The 15 HCMV miRNAs listed in the Sanger database were identified in 4 landmark studies (Table A1) (85, 116, 225, 258). Cumulatively, these works predicted the presence of over 20 viral miRNAs. Hairpin structures predicted by Grey *et al* 2005 corresponding to regions in UL31, UL53, UL54, UL102 and UL111 were never further investigated beyond their predication. The detection of miR-UL70 was also speculated to have been an artifact given that it was detected in mock-infected cells and because it is detection was not consistently replicated by other groups. The advent of high throughput sequencing ushered in the idea that there is some degree of variability in the mature miRNA sequences (184, 355, 372). The differential processing of pre-miRNAs has been observed and this can lead to sequence and length variability at both the 3' and 5' ends of the miRNA (109, 184, 355). The mechanistic aspects of this variability are beginning to be discerned, but this phenomenon has important implications in miRNA functions since the production of these isomiRs could potentially result in the regulation of novel targets through miRNA seed variability. To this end, we designed an experimental protocol that employed microarray "probe walking" in order to analyze the accumulation of sequences along each of the predicted miRNA hairpins during productive infection. This experimental design would also allow us to investigate the potential for variability in these same sequences through differential miRNA processing.

Accession[1]	ID[2]	Mature ID[3]	Publication[4]
MI0001680	hcmv-mir-UL112	hcmv-miR-UL112-3p	Pfeffer 2005 (258)
		hcmv-miR-UL112-5p	
MI0001681	hcmv-mir-UL148D	hcmv-miR-UL148D	Pfeffer 2005 (258)
MI0001678	hcmv-mir-UL22A	hcmv-miR-UL22A-3p	Pfeffer 2005 (258)
		hcmv-miR-UL22A-5p	Dunn 2005 (85)
MI0001679	hcmv-mir-UL36	hcmv-miR-UL36-3p	Pfeffer 2005 (258)
		hcmv-miR-UL36-5p	
MI0024164	hcmv-mir-UL59	hcmv-miR-UL59	Stark 2012 (316)
MI0024165	hcmv-mir-UL69	hcmv-miR-UL69	Stark 2012 (316)
MI0003688	hcmv-mir-UL70	hcmv-miR-UL70-3p	Grey 2005 (116)
		hcmv-miR-UL70-5p	
MI0023578	hcmv-mir-US22	hcmv-miR-US22-3p	Stark 2012 (316)
		hcmv-miR-US22-5p	
MI0001684	hcmv-mir-US25-1	hcmv-miR-US25-1-3p	Pfeffer 2005 (258)
		hcmv-miR-US25-1-5p	Dunn 2005 (85)
MI0001685	hcmv-mir-US25-2	hcmv-miR-US25-2-3p	Pfeffer 2005 (258)
		hcmv-miR-US25-2-5p	
MI0023579	hcmv-mir-US29	hcmv-miR-US29-3p	Grey 2005 (116)
		hcmv-miR-US29-5p	
MI0001686	hcmv-mir-US33	hcmv-miR-US33-3p	Pfeffer 2005 (258)
		hcmv-miR-US33-5p	
MI0003687	hcmv-mir-US4	hcmv-miR-US4-3p	Grey 2005 (116)
		hcmv-miR-US4-5p	
MI0001682	hcmv-mir-US5-1	hcmv-miR-US5-1	Pfeffer 2005 (258)
MI0001683	hcmv-mir-US5-2	hcmv-miR-US5-2-3p	Pfeffer 2005 (258)
		hcmv-miR-US5-2-5p	
Predicted	*hcmv-mir-UL31*	-	Grey 2005 (116)
Predicted	*hcmv-mir-UL53*	-	Grey 2005 (116)
Predicted	*hcmv-mir-UL54*	-	Grey 2005 (116)
Predicted	*hcmv-mir-UL102-1*	-	Grey 2005 (116)
Predicted	*hcmv-mir-UL102-2*	-	Grey 2005 (116)
Predicted	*hcmv-mir-UL111A*	-	Grey 2005 (116)

Table A.1: *The HCMV genome harbors novel viral miRNAs.* Numerous groups have predicted the presence of miRNAs in the HCMV genome using *in-silico* prediction algorithms to identify miRNA hairpin structures. The expression and function of a subset of those miRNAs have been validated during infection, but the expression of a number of predicted miRNAs from Grey *et al* 2005 have not yet been substantiated. Included in this table are all of the validated viral miRNAs listed in the Sanger miRBase release 20 (Accession[1]) and the unsubstantiated miRNAs predicted by Grey *et al* 2005. Also illustrated are the miRNA identifiers (ID[2]), the mature miRNAs processed from the respective hairpins (Mature ID[3]) and the relevant publications that identified the respective viral miRNAs. The rows highlighted in green indicate the miRNAs included in our analysis.

In order to analyze the expression and sequences of the predicted viral miRNAs during productive infection, we designed 22 nt custom microarray probes staggered by 1 base that spanned the entire pre-miRNA hairpin (Figure A.1). It is of note to mention that miR-UL59, miR-UL69 and miR-US22 were discovered after the design of this experiment and were therefore excluded from our analysis. Similar to the microarray studies in Chapter 3, whole-cell RNA was purified from mock-infected and MOI = 5 infected HEL fibroblasts at 48 hpi. Small RNAs were fractionated from the whole-cell RNA and they were subsequently hybridized to our custom microarray. This allowed us to analyze the accumulation of sequences along the entire pre-miRNA hairpin by quantifying the fluorescence generated from the binding of the consecutive array probes (Figure A1).

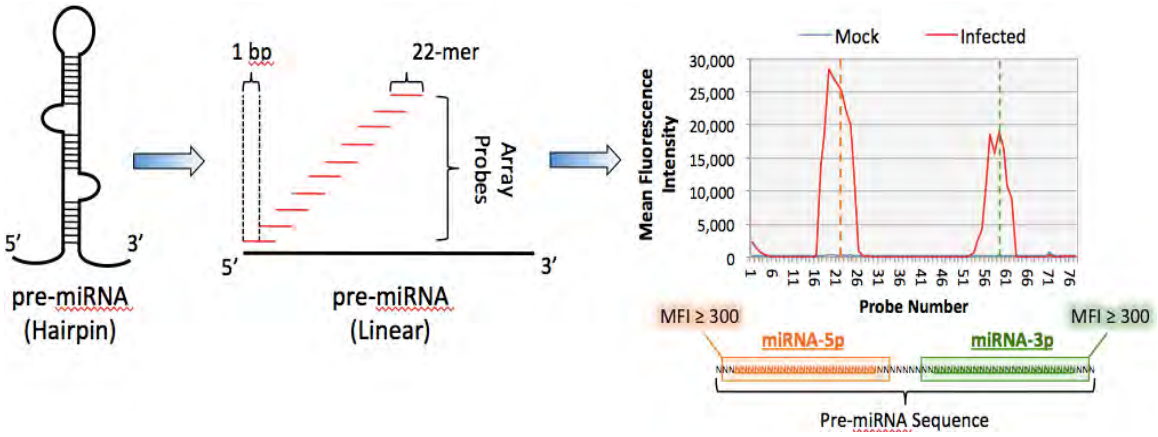


Figure A.1: *Viral miRNA "probe walking" design.* The viral pre-miRNA sequences were used as the template to design 22 nt probes, staggered by 1 base, which covered the entire pre-miRNA sequence. After hybridizing RNA to our custom array, we calculated the relative accumulation of small RNAs with complementary sequences by quantifying the mean fluorescence intensity generated by the binding of the respective array probes. Below each plot is the pre-miRNA sequence annotated in the Sanger miRBase 20. The bold-faced/underlined sequence represents the annotated mature miRNAs, and the boxed areas represent sequences corresponding to probes that generated an MFI>300. The dotted lines on the MFI plots illustrate the location of a probe corresponding to the mature miRNA sequences listed in miRBase 20. Each plot illustrates MFI signal generated by the same probe-sets in mock (blue) and infected (red) cells.

Upon investigating the accumulation transcripts corresponding to the unsubstantiated miRNAs predicted by Grey *et al* 2005 (miR-UL31, miR-UL53, miR-UL54, miR-UL102-1, miR-UL102-2 and miR-UL111A), we determined that these predicted hairpins did not represent true virally encoded miRNAs (Figure A.2 A). The majority of the array probes corresponding to the predicted pre-miRNA hairpins generated little to no detectable fluorescence, and those that did fluoresce had equivalent signals in both the mock-infected and infected samples. This would indicate that no complementary small viral RNA sequences exist, and that the array probes are detecting cellular artifacts. Given that the RNA hybridized to the arrays was size-selected to enrich for small RNAs, it is unlikely

that these probes are detecting cellular transcripts. Some of the probes do harbor limited complementarity to cellular miRNAs, but it is difficult to determine whether they are binding to miRNAs with limited complementarity or to some other small RNAs that could have arisen from RNA degradation. A similar pattern was also observed for HCMV-miR-UL70 (Figure A.2 B). Although miR-UL70 is listed as a viral miRNA in the Sanger miRBase 20, its detection during infection was not consistently replicated. More recent works that utilized deep sequencing to analyze the HCMV miRNA transcriptome were also unable to detect miRNAs corresponding to miR-UL70 (225, 316). These findings support the notion that miR-UL70 is not an HCMV-encoded miRNA.

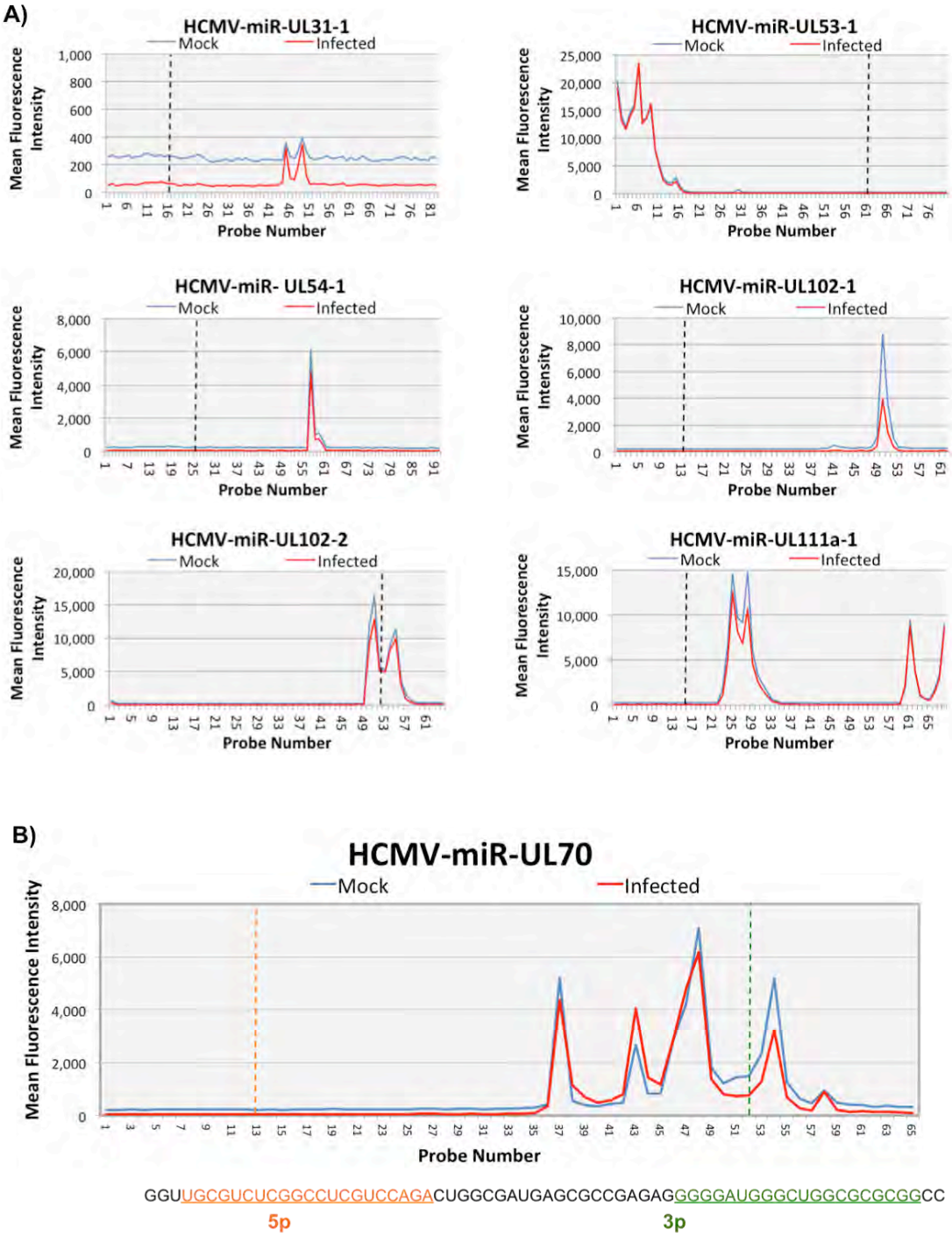
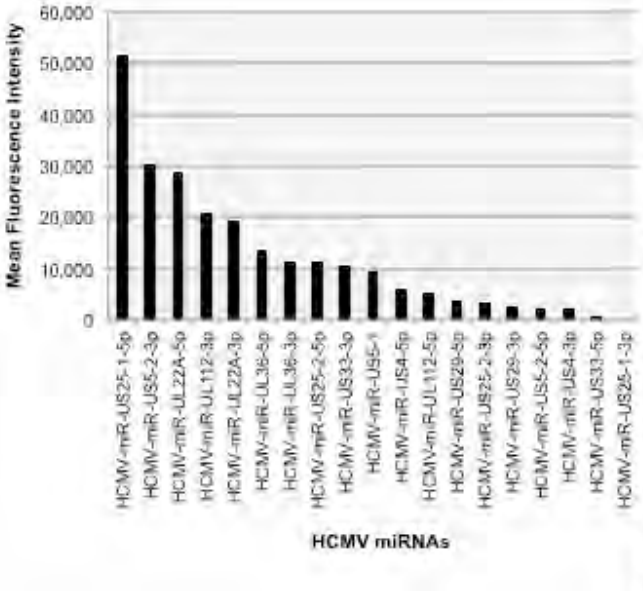


Figure A.2: *Predicted HCMV pre-miRNAs and miR-UL70 are not virally encoded miRNAs.* (A) Using the custom microarray "probe walking" protocol, we detected the accumulation of transcripts corresponding to the predicted pre-miRNAs from Grey *et al* 2005: miR-UL31-1, miR-UL53-1, miR-UL54-1, miR-UL102-1, miR-UL102-2 and miR-UL111A. (B) The same protocol was used to detect mature miRNAs processed from the miR-UL70 pre-miRNA as well. Based on the limited detection and equivalent signals in mock-infected and infected cells, these results suggest that none of these transcripts represent true viral miRNAs.

Using the miRNA probes that generated the highest MFI readings, we compared the relative accumulation of each mature viral miRNA with respect to what was observed by other groups (Figure A.3). Generally, these trends were in concordance with observations made by Stark *et al* 2012 and Meshesha *et al* 2012, but there were differences in the ranking of each mature viral miRNA among the respective groups (225, 316). The lack of concordance in the ranked accumulation of the respective mature miRNAs in each study suggests the potential for variability in either regulation of miRNA expression or processing in the respective experimental conditions.

A)



B)

Rank by Abundance	Stark *et al* 2012	Meshesha *et al* 2012
US25-1-5p	US5-2-3p	UL36-5p
US5-2-3p	UL36-5p	UL22A-3p
UL22A-5p	UL22A-3p	US25-1-5p
UL112-3p	US25-1-5p	US25-2-5p
UL22A-3p	UL22A-5p	US25-2-3p
UL36-5p	US25-2-3p	UL22A-5p
UL36-3p	US25-2-5p	US5-2-3p
US25-2-5p	US29-5p	UL112-3p
US33-3p	US33-5p	US29-5p
US5-1	UL112-3p	US33-5p
US4-5p	*US22-5p*	US5-1-3p
UL112-5p	UL36-3p	UL36-3p
US29-5p	US4-5p	US25-1-3p
US25-2-3p	US29-3p	US33-3p
US29-3p	*US22-3p*	UL112-5p
US5-2-5p	*UL148D*	US4-5p
US4-3p	US5-1	US4-3p
US33-5p	*US25-1-3p*	US29-3p
US25-1-3p	US33-3p	US5-2-5p
	US5-2-5p	*US5-1-5p*
	UL112-5p	
	US4-3p	

Figure A.3: *Relative accumulation of HCMV miRNAs.* Using the microarray probes that generated the highest MFI readings as a determinant of the most abundant viral miRNA, we compared the relative accumulation of each viral miRNA to other published studies. Generally, the rankings were similar among the individual groups. However, differences in the accumulation of the respective mature miRNAs indicate to potential variability in miRNA expression or processing in the respective experimental conditions. miRNAs highlighted in green were not included in our study.

Using the “probe-walking” protocol, we also mapped the mature sequences of the remaining HCMV miRNAs listed in Table A1 (Table A2). A general trend that emerged when comparing probes that generated the highest MFI readings to the annotated mature miRNA sequences listed in the Sanger miRBase 20 was that these probes typically corresponded to sequences that were offset from the miRBase annotations by 1-3 bases at the 5’ end of the mature miRNA (Table A2). Assuming that there is an average GC content among the template RNA being hybridized to the array, probes with 100% complementarity to the target template will exhibit the highest fluorescence readings. As the probes walk along the pre-miRNA sequence and complementarity is lost, the fluorescence signal will begin to decrease. Although this is not necessarily a linear loss of signal, we can assume that peak fluorescence signals will give an accurate representation of target accumulation. A representative set of MFI plots in Figure A.4 illustrates the broad and/or staggered peak readings, which indicate the potential variability in mature miRNA sequence and/or length. Given that each respective set of observations were made using unique experimental conditions, it is tempting to speculate that productive HCMV can potentially generate unique sets of mature viral miRNAs depending on the virus strain and cell type being infected. However, given the concordance among Stark *et al* 2012 and Meshesha *et al* 2012 and the increased sensitivity of their deep sequencing protocols, further sequence-based experimentation would be required to substantiate this theory.

miRNA	Sequence	Source
HCMV-miR-UL22A-5p	UAACUAGCCUUCCCGUGAGA UAACUAGCCUUCCCGUGAGA UAACUAGCCUUCCCGUGAGA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GUCUAACUAGCCUUCCCGUGAG	
HCMV-miR-UL22A-3p	UCACCAGAAUGCUAGUUUGUAG UCACCAGAAUGCUAGUUUGUAG UCACCAGAAUGCUAGUUUGUAG	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	UCACCAGAAUGCUAGUUUGUAG	
HCMV-miR-UL36-5p	UCGUUGAAGACACCUGGAAAGA UCGUUGAAGACACCUGGAAAGA UCGUUGAAGACACCUGGAAAGA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GUCGUUGAAGACACCUGGAAAG	
HCMV-miR-UL36-3p	UUUCCAGGUGUUUUCAACGUGC UUUCCAGGUGUUUUCAACGUG UUUCCAGGUGUUUUCAACGUG	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CUUUCCAGGUGUUUUCAACGUG	
HCMV-miR-UL112-5p	CCUCCGGAUCACAUGGUUACUCA CCUCCGGAUCACAUGGUUACUCA CCUCCGGAUCACAUGGUUACUCA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CCUCCGGAUCACAUGGUUACUC	
HCMV-miR-UL112-3p	AAGUGACGGUGAGAUCCAGGCU AAGUGACGGUGAGAUCCAGGCU AAGUGACGGUGAGAUCCAGGC	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	UAAGUGACGGUGAGAUCCAGGC	
HCMV-miR-US33-5p	GAUUGUGCCCGGACCGUGGGCG GAUUGUGCCCGGACCGUGGGCG GAUUGUGCCCGGACCGUGGGCG	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GAUUGUGCCCGGACCGUGGGCG	
HCMV-miR-US33-3p	UCACGGUCCGAGCACAUCCAA UCACGGUCCGAGCACAUCCAA UCACGGUCCGAGCACAUCCA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CCGUCACGGUCCGAGCACAUCC	
HCMV-miR-US5-1	UGACAAGCCUGACGAGAGCGU UGACAAGCCUGACGAGAGCGU UGACAAGCCUGACGAGAGCGU	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CCAUGACAAGCCUGACGAGAGC	
HCMV-miR-US25-1-5p	AACCGCUCAGUGGCUCGGACC AACCGCUCAGUGGCUCGGACC AACCGCUCAGUGGCUCGGACC	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GAACCGCUCAGUGGCUCGGACC	
HCMV-miR-US25-1-3p	UCCGAACGCUAGGUCGGUUCU GUCCGAACGCUAGGUCGGUUCU UCCGAACGCUAGGUCGGUUCU	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
HCMV-miR-US25-2-5p	AGCGGUCUGUUCAGGUGGAUGA AGCGGUCUGUUCAGGUGGAUGA AGCGGUCUGUUCAGGUGGAUGA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	UUAGCGGUCUGUUCAGGUGGAU	
HCMV-miR-US25-2-3p	AUCCACUUGGAGAGCUCCCGCGGU AUCCACUUGGAGAGCUCCCGCGGU AUCCACUUGGAGAGCUCCCGCGGU	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CCACUUGGAGAGCUCCCGCGGU	
HCMV-miR-US4-5p	UGGACGUGCAGGGGGAUGUCUG UGGACGUGCAGGGGGAUGUCUG UGGACGUGCAGGGGGAUGUC	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CAUGGACGUGCAGGGGGAUGUC	
HCMV-miR-US4-3p	UGACAGCCCGCUACACCUCU UGACAGCCCGCUACACCUCUCU UGACAGCCCGCUACACCUCU	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GUGACAGCCCGCUACACCUCUC	

miRNA	Sequence	Source
HCMV-miR-US5-2-5p	CUUUCGCCACACCUAUCCUGAAAG CUUUCGCCACACCUAUCCUGAAAG CUUUCGCCACACCUAUCCUGAAAG	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CUUUCGCCACACCUAUCCUGAA	
HCMV-miR-US5-2-3p	UAUGAUAGGUGUGACGAUGUCU UAUGAUAGGUGUGACGAUGUCU UUAUGAUAGGUGUGACGAUGUC	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	UUUAUGAUAGGUGUGACGAUGU	
HCMV-miR-US29-5p	UGGAUGUGCUCGGACCGUGACG UGGAUGUGCUCGGACCGUGACG UGGAUGUGCUCGGACCGUGACG	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	GGAUGUGCUCGGACCGUGACGG	
HCMV-miR-US29-3p	CCCACGGUCCGGGCACAAUCA CCCACGGUCCGGGCACAAUCA CCCACGGUCCGGGCACAAUCA	miRBase 20 Stark *et al* 2012 Meshesha *et al* 2012
	CGCCCACGGUCCGGGCACAAUC	

Table A.2: *HCMV miRNAs exhibit sequence variability.* This table illustrates the mature sequences of the HCMV miRNAs as designated by the respective publications, miRBase 20 and from our microarray experiment. The sequences from Stark *et al* 2012 and Meshesha *et al* 2012 were determined by deep sequencing small RNAs from HCMV infected cells, and our sequence was determined by the probe generating the highest MFI reading.

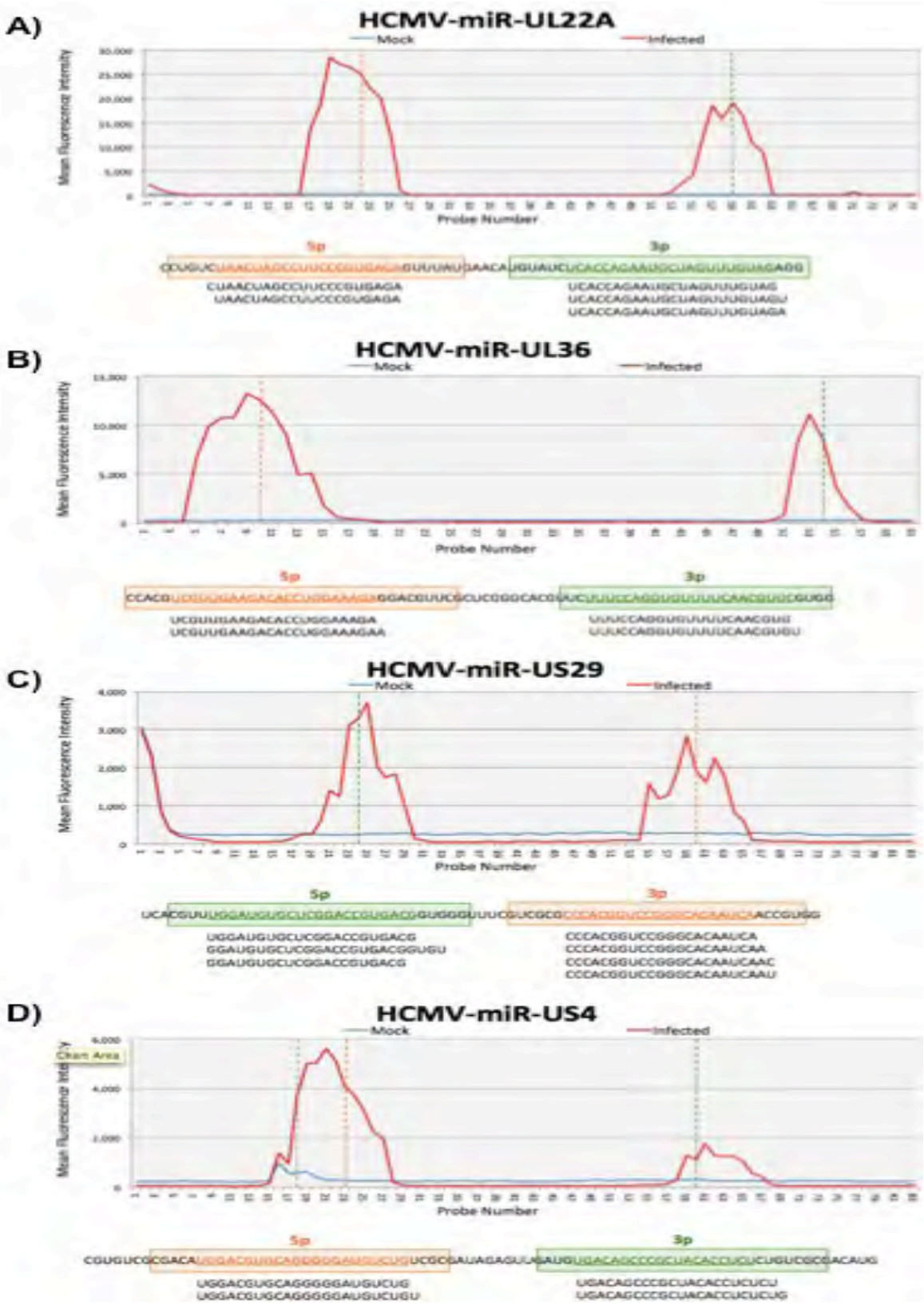

A)
HCMV-miR-UL22A
Mock
Infected
Mean Fluorescence Intensity
Probe Number
5p
3p
CUAACUAGCCUUCCCGUGAGA
UAACUAGCCUUCCCGUGAGA
UCACCAGAAUGCUAGUUUGUAG
UCACCAGAAUGCUAGUUUGUAGU
UCACCAGAAUGCUAGUUUGUAGA
B)
HCMV-miR-UL36
Mock
Infected
Mean Fluorescence Intensity
Probe Number
5p
3p
UCGUUGAAGACACCUGGAAAGA
UCGUUGAAGACACCUGGAAAGAA
UUUCCAGGUGUUUUCAACGUG
UUUCCAGGUGUUUUCAACGUGU
C)
HCMV-miR-US29
Mock
Infected
Mean Fluorescence Intensity
Probe Number
5p
3p
UGGAUGUGCUCGGACCGUGACG
GGAUGUGCUCGGACCGUGACGGUGU
GGAUGUGCUCGGACCGUGACG
CCCACGGUCCGGGCACAAUCA
CCCACGGUCCGGGCACAAUCAA
CCCACGGUCCGGGCACAAUCAAC
CCCACGGUCCGGGCACAAUCAAU
D)
HCMV-miR-US4
Mock
Infected
Clean Area
Probe Number
5p
3p
UGGACGUGCAGGGGGAUGUCUG
UGGACGUGCAGGGGGAUGUCUGU
UGACAGCCCGCUACACCUCUCU
UGACAGCCCGCUACACCUCUCUG

Figure A.4: *Mature HCMV miRNAs exhibit variability in sequence and length.* This figure illustrates a representative panel of HCMV miRNAs that potentially exhibit variability in length due to alternative pre-miRNA processing. The MFI profiles are illustrated as in Figure A1, but listed below each pre-miRNA sequence are the alternative mature miRNAs that were detected by Stark *et al* 2012. The grey dotted line shown in the MFI profile of miR-US4-5p represents the original mature sequence of miR-US4 that was recently corrected by miRBase 20.

Discussion

Using our "probe-walking" microarray platform, we were able to monitor the accumulation of sequences along each of the HCMV pre-miRNA hairpins (Figure A.1). This allowed us to establish that the predicted miRNAs published by Grey *et al* 2005 (miR-UL31-1, miR-UL53-1, miR-UL54-1, miR-UL102-1, miR-UL102-2, miR-UL111a-1 and miRUL70) did not represent true virally encoded miRNAs (Figure A.2). Probes corresponding to the respective pre-miRNA hairpins either generated little to no fluorescence or had equivalent signal in both mock and infected cells. This indicated that the probe-sets were detecting mRNA artifacts that did not correspond to mature viral miRNAs. Using the miRNA probes corresponding to the highest MFI readings, we were also able to rank the relative accumulation of each mature viral miRNA (Figure A.3). These rankings were generally conserved among published findings from Stark *et al* 2012 and Meshesha *et al* 2012, but the lack of direct concordance in each study suggests the potential for variability in either the regulation of miRNA expression or miRNA processing. Given that each assay was performed using unique experimental conditions, it is possible the different experimental environments allowed for

variability in the accumulation of mature miRNAs through either transcriptional or post-transcriptional mechanisms. The potential for variability in miRNA processing is supported by our findings in Table A2. The sequences corresponding to probes generating the highest MFI readings consistently reflected variability in the 5' end of the mature miRNAs when compared to the mature miRNA sequences annotated in miRBase 20. Stark *et al* 2012 observed that mature HCMV miRNAs accumulating at 24 and 72 hpi exhibited variability in length at both the 5' and 3' ends (316). MFI profiles from a representative panel of HCMV miRNAs support the idea that alternatively processed miRNAs exist in HCMV infected cells (Figure A.4). Given the consistency in the detection of mature HCMV miRNAs with sequences offset at the 5' ends, it is tempting to speculate that productive infection of HEL fibroblasts with AD169 generates a unique set of isomiRs relative to that seen during infections of human foreskin fibroblasts (HFF) with HCMV Towne (Stark *et al* 2012) or AD169 (Meshesha *et al* 2012). However, given the sensitivity of deep sequencing it is difficult to substantiate this idea without sequencing the mature viral miRNAs that accumulate during productive infection of HELs with AD169. Although, we cannot definitively postulate that the mature miRNAs detected by our microarray "probe walking" experiment represent a completely unique set of mature HCMV miRNAs, our findings support the hypothesis that productive HCMV infection yields mature miRNAs with variable length and sequence.

Chapter VI:

References

1. **Abu-Elneel, K., T. Liu, F. S. Gazzaniga, Y. Nishimura, D. P. Wall, D. H. Geschwind, K. Lao, and K. S. Kosik.** 2008. Heterogeneous dysregulation of microRNAs across the autism spectrum. Neurogenetics **9:**153-61.
2. **Acheson, N. H.** 2011. Fundamentals of molecular virology, 2nd ed. John Wiley & Sons, Hoboken, NJ.
3. **Adamo, J. E., J. Schroer, and T. Shenk.** 2004. Human cytomegalovirus TRS1 protein is required for efficient assembly of DNA-containing capsids. J Virol **78:**10221-9.
4. **Ahlfors, K., S. A. Ivarsson, and S. Harris.** 1999. Report on a long-term study of maternal and congenital cytomegalovirus infection in Sweden. Review of prospective studies available in the literature. Scand J Infect Dis **31:**443-57.
5. **Akao, Y., Y. Nakagawa, and T. Naoe.** 2006. MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. Oncol Rep **16:**845-50.
6. **Alba, M. M., R. Das, C. A. Orengo, and P. Kellam.** 2001. Genomewide function conservation and phylogeny in the Herpesviridae. Genome Res **11:**43-54.
7. **Altuvia, Y., P. Landgraf, G. Lithwick, N. Elefant, S. Pfeffer, A. Aravin, M. J. Brownstein, T. Tuschl, and H. Margalit.** 2005. Clustering and conservation patterns of human microRNAs. Nucleic Acids Res **33:**2697-706.
8. **Andersson, M. G., P. C. Haasnoot, N. Xu, S. Berenjian, B. Berkhout, and G. Akusjarvi.** 2005. Suppression of RNA interference by adenovirus virus-associated RNA. J Virol **79:**9556-65.
9. **Antonov, A. V., S. Dietmann, P. Wong, D. Lutter, and H. W. Mewes.** 2009. GeneSet2miRNA: finding the signature of cooperative miRNA activities in the gene lists. Nucleic Acids Res **37:**W323-8.
10. **Aparicio, O., N. Razquin, M. Zaratiegui, I. Narvaiza, and P. Fortes.** 2006. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. J Virol **80:**1376-84.
11. **Arora, A., and D. A. Simpson.** 2008. Individual mRNA expression profiles reveal the effects of specific microRNAs. Genome Biol **9:**R82.
12. **Asher, Y., M. Heller, and Y. Becker.** 1969. Incorporation of lipids into herpes simplex virus particles. J Gen Virol **4:**65-76.
13. **AuCoin, D. P., G. B. Smith, C. D. Meiering, and E. S. Mocarski.** 2006. Betaherpesvirus-conserved cytomegalovirus tegument protein ppUL32 (pp150) controls cytoplasmic events during virion maturation. J Virol **80:**8199-210.
14. **Aujeszky, A.** 1902. Über eine neue infektionskrankheit bei haustieren. Zbl Bakt 1 Orig. **32:**353-357.

15. **Baechler, E. C., F. M. Batliwalla, A. M. Reed, E. J. Peterson, P. M. Gaffney, K. L. Moser, P. K. Gregersen, and T. W. Behrens.** 2006. Gene expression profiling in human autoimmunity. Immunol Rev **210:**120-37.
16. **Baek, D., J. Villen, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel.** 2008. The impact of microRNAs on protein output. Nature **455:**64-71.
17. **Bailey, T. L., M. Boden, F. A. Buske, M. Frith, C. E. Grant, L. Clementi, J. Ren, W. W. Li, and W. S. Noble.** 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res **37:**W202-8.
18. **Bailey, T. L., M. Boden, T. Whitington, and P. Machanick.** 2010. The value of position-specific priors in motif discovery using MEME. BMC Bioinformatics **11:**179.
19. **Bailey, T. L., and M. Gribskov.** 1998. Combining evidence using p-values: application to sequence homology searches. Bioinformatics **14:**48-54.
20. **Baldwin, K. J., and K. L. Roos.** 2011. Neuroinfectious diseases in pregnancy. Semin Neurol **31:**404-12.
21. **Bar, M., S. K. Wyman, B. R. Fritz, J. Qi, K. S. Garg, R. K. Parkin, E. M. Kroh, A. Bendoraite, P. S. Mitchell, A. M. Nelson, W. L. Ruzzo, C. Ware, J. P. Radich, R. Gentleman, H. Ruohola-Baker, and M. Tewari.** 2008. MicroRNA discovery and profiling in human embryonic stem cells by deep sequencing of small RNA libraries. Stem Cells **26:**2496-505.
22. **Barami, K.** 2010. Oncomodulatory mechanisms of human cytomegalovirus in gliomas. J Clin Neurosci **17:**819-23.
23. **Bartel, D. P.** 2009. MicroRNAs: target recognition and regulatory functions. Cell **136:**215-33.
24. **Baulcombe, D. C.** 1996. Mechanisms of Pathogen-Derived Resistance to Viruses in Transgenic Plants. Plant Cell **8:**1833-1844.
25. **Bauman, Y., D. Nachmani, A. Vitenshtein, P. Tsukerman, N. Drayman, N. Stern-Ginossar, D. Lankry, R. Gruda, and O. Mandelboim.** 2011. An identical miRNA of the human JC and BK polyoma viruses targets the stress-induced ligand ULBP3 to escape immune elimination. Cell Host Microbe **9:**93-102.
26. **Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork, and E. Izaurralde.** 2006. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev **20:**1885-98.
27. **Bell, G. W., and F. Lewitter.** 2009. Resources for small regulatory RNAs. Curr Protoc Mol Biol **Chapter 19:**Unit19 8.
28. **Bennasser, Y., S. Y. Le, M. Benkirane, and K. T. Jeang.** 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity **22:**607-19.
29. **Besecker, M. I., M. E. Harden, G. Li, X. J. Wang, and A. Griffiths.** 2009. Discovery of herpes B virus-encoded microRNAs. J Virol **83:**3413-6.

30. **Betel, D., M. Wilson, A. Gabow, D. S. Marks, and C. Sander.** 2008. The microRNA.org resource: targets and expression. Nucleic Acids Res **36:**D149-53.
31. **Bhattacharjee, B., N. Renzette, and T. F. Kowalik.** 2012. Genetic analysis of cytomegalovirus in malignant gliomas. J Virol **86:**6815-24.
32. **Bissinger, A. L., C. Sinzger, E. Kaiserling, and G. Jahn.** 2002. Human cytomegalovirus as a direct pathogen: correlation of multiorgan involvement and cell distribution with clinical and pathological findings in a case of congenital inclusion disease. J Med Virol **67:**200-6.
33. **Bogerd, H. P., H. W. Karnowski, X. Cai, J. Shin, M. Pohlers, and B. R. Cullen.** 2010. A mammalian herpesvirus uses noncanonical expression and processing mechanisms to generate viral MicroRNAs. Mol Cell **37:**135-42.
34. **Bohnsack, M. T., K. Czaplinski, and D. Gorlich.** 2004. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA **10:**185-91.
35. **Boppana, S. B., R. F. Pass, W. J. Britt, S. Stagno, and C. A. Alford.** 1992. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. Pediatr Infect Dis J **11:**93-9.
36. **Borchert, G. M., W. Lanier, and B. L. Davidson.** 2006. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol **13:**1097-101.
37. **Boyle, K. A., R. L. Pietropaolo, and T. Compton.** 1999. Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. Mol Cell Biol **19:**3607-13.
38. **Brennecke, J., A. Stark, R. B. Russell, and S. M. Cohen.** 2005. Principles of microRNA-target recognition. PLoS Biol **3:**e85.
39. **Britt, W. J., and S. Boppana.** 2004. Human cytomegalovirus virion proteins. Hum Immunol **65:**395-402.
40. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J Virol **75:**12319-30.
41. **Buck, A. H., J. Perot, M. A. Chisholm, D. S. Kumar, L. Tuddenham, V. Cognat, L. Marcinowski, L. Dolken, and S. Pfeffer.** 2010. Post-transcriptional regulation of miR-27 in murine cytomegalovirus infection. RNA **16:**307-15.
42. **Bughio, F., D. A. Elliott, and F. Goodrum.** 2013. An endothelial cell-specific requirement for the UL133-UL138 locus of human cytomegalovirus for efficient virus maturation. J Virol **87:**3062-75.
43. **Cai, X., C. H. Hagedorn, and B. R. Cullen.** 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA **10:**1957-66.
44. **Cameron, J. E., C. Fewell, Q. Yin, J. McBride, X. Wang, Z. Lin, and E. K. Flemington.** 2008. Epstein-Barr virus growth/latency III program alters cellular microRNA expression. Virology **382:**257-66.

45. **Cannon, M. J., D. S. Schmid, and T. B. Hyde.** 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol **20:**202-13.
46. **Carl, J. W., Jr., J. Trgovcich, and S. Hannenhalli.** 2013. Widespread evidence of viral miRNAs targeting host pathways. BMC Bioinformatics **14 Suppl 2:**S3.
47. **Carr, K. M., M. Bittner, and J. M. Trent.** 2003. Gene-expression profiling in human cutaneous melanoma. Oncogene **22:**3076-80.
48. **Castillo, J. P., F. M. Frame, H. A. Rogoff, M. T. Pickering, A. D. Yurochko, and T. F. Kowalik.** 2005. Human cytomegalovirus IE1-72 activates ataxia telangiectasia mutated kinase and a p53/p21-mediated growth arrest response. J Virol **79:**11467-75.
49. **Castillo, J. P., and T. F. Kowalik.** 2004. HCMV infection: modulating the cell cycle and cell death. Int Rev Immunol **23:**113-39.
50. **Castillo, J. P., and T. F. Kowalik.** 2002. Human cytomegalovirus immediate early proteins and cell growth control. Gene **290:**19-34.
51. **Castillo, J. P., A. D. Yurochko, and T. F. Kowalik.** 2000. Role of human cytomegalovirus immediate-early proteins in cell growth control. J Virol **74:**8028-37.
52. **Cazalla, D., and J. A. Steitz.** 2010. Down-regulation of a host microRNA by a viral noncoding RNA. Cold Spring Harb Symp Quant Biol **75:**321-4.
53. **Cha, T. A., E. Tom, G. W. Kemble, G. M. Duke, E. S. Mocarski, and R. R. Spaete.** 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol **70:**78-83.
54. **Chang, J., J. T. Guo, D. Jiang, H. Guo, J. M. Taylor, and T. M. Block.** 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. J Virol **82:**8215-23.
55. **Chang, S., R. J. Johnston, Jr., C. Frokjaer-Jensen, S. Lockery, and O. Hobert.** 2004. MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. Nature **430:**785-9.
56. **Chen, J., and M. F. Stinski.** 2002. Role of regulatory elements and the MAPK/ERK or p38 MAPK pathways for activation of human cytomegalovirus gene expression. J Virol **76:**4873-85.
57. **Chen, Y., Y. Tang, C. Zhang, R. Lin, T. Liu, and S. Shang.** 2010. Severe primary cytomegalovirus pneumonia in a 5-year-old immunocompetent child. Indian J Pediatr **77:**708.
58. **Cheng, C., N. Bhardwaj, and M. Gerstein.** 2009. The relationship between the evolution of microRNA targets and the length of their UTRs. BMC Genomics **10:**431.
59. **Chhabra, R., R. Dubey, and N. Saini.** Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. Mol Cancer **9:**232.
60. **Chin, L. J., E. Ratner, S. Leng, R. Zhai, S. Nallur, I. Babar, R. U. Muller, E. Straka, L. Su, E. A. Burki, R. E. Crowell, R. Patel, T. Kulkarni, R.**

Homer, D. Zelterman, K. K. Kidd, Y. Zhu, D. C. Christiani, S. A. Belinsky, F. J. Slack, and J. B. Weidhaas. 2008. A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. Cancer Res **68:**8535-40.

61. **Chivukula, R. R., and J. T. Mendell.** 2008. Circular reasoning: microRNAs and cell-cycle control. Trends Biochem Sci **33:**474-81.
62. **Chou, S. W.** 1986. Acquisition of donor strains of cytomegalovirus by renal-transplant recipients. N Engl J Med **314:**1418-23.
63. **Cinatl, J., Jr., J. U. Vogel, R. Kotchetkov, and H. Wilhelm Doerr.** 2004. Oncomodulatory signals by regulatory proteins encoded by human cytomegalovirus: a novel role for viral infection in tumor progression. FEMS Microbiol Rev **28:**59-77.
64. **Cline, M. S., M. Smoot, E. Cerami, A. Kuchinsky, N. Landys, C. Workman, R. Christmas, I. Avila-Campilo, M. Creech, B. Gross, K. Hanspers, R. Isserlin, R. Kelley, S. Killcoyne, S. Lotia, S. Maere, J. Morris, K. Ono, V. Pavlovic, A. R. Pico, A. Vailaya, P. L. Wang, A. Adler, B. R. Conklin, L. Hood, M. Kuiper, C. Sander, I. Schmulevich, B. Schwikowski, G. J. Warner, T. Ideker, and G. D. Bader.** 2007. Integration of biological networks and gene expression data using Cytoscape. Nat Protoc **2:**2366-82.
65. **Cogoni, C., J. T. Irelan, M. Schumacher, T. J. Schmidhauser, E. U. Selker, and G. Macino.** 1996. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J **15:**3153-63.
66. **Cogoni, C., and G. Macino.** 1999. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature **399:**166-9.
67. **Cogoni, C., and G. Macino.** 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. Proc Natl Acad Sci U S A **94:**10233-8.
68. **Cogoni, C., and G. Macino.** 1999. Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science **286:**2342-4.
69. **Colberg-Poley, A. M.** 1996. Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36-38, UL115-119, TRS1/IRS1 and US3 loci. Intervirology **39:**350-60.
70. **Conrad, K. D., F. Giering, C. Erfurth, A. Neumann, C. Fehr, G. Meister, and M. Niepmann.** 2013. microRNA-122 Dependent Binding of Ago2 Protein to Hepatitis C Virus RNA Is Associated with Enhanced RNA Stability and Translation Stimulation. PLoS One **8:**e56272.
71. **Cosmopoulos, K., M. Pegtel, J. Hawkins, H. Moffett, C. Novina, J. Middeldorp, and D. A. Thorley-Lawson.** 2009. Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma. J Virol **83:**2357-67.

72. **Curwen, V., E. Eyras, T. D. Andrews, L. Clarke, E. Mongin, S. M. Searle, and M. Clamp.** 2004. The Ensembl automatic gene annotation system. Genome Res **14:**942-50.
73. **Dai, Y., and X. Zhou.** 2010. Computational methods for the identification of microRNA targets. Open Access Bioinformatics **2:**29-39.
74. **Dalmay, T., A. Hamilton, S. Rudd, S. Angell, and D. C. Baulcombe.** 2000. An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101:**543-53.
75. **Davison, A. J., R. Eberle, B. Ehlers, G. S. Hayward, D. J. McGeoch, A. C. Minson, P. E. Pellett, B. Roizman, M. J. Studdert, and E. Thiry.** 2009. The order Herpesvirales. Arch Virol **154:**171-7.
76. **de Carvalho, F., G. Gheysen, S. Kushnir, M. Van Montagu, D. Inze, and C. Castresana.** 1992. Suppression of beta-1,3-glucanase transgene expression in homozygous plants. EMBO J **11:**2595-602.
77. **Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting, and G. J. Hannon.** 2004. Processing of primary microRNAs by the Microprocessor complex. Nature **432:**231-5.
78. **Dhuruvasan, K., G. Sivasubramanian, and P. E. Pellett.** 2011. Roles of host and viral microRNAs in human cytomegalovirus biology. Virus Res **157:**180-92.
79. **Dimitropoulou, P., R. Caswell, B. P. McSharry, R. F. Greaves, D. A. Spandidos, G. W. Wilkinson, and G. Sourvinos.** 2010. Differential relocation and stability of PML-body components during productive human cytomegalovirus infection: detailed characterization by live-cell imaging. Eur J Cell Biol **89:**757-68.
80. **Ding, S. W.** 2010. RNA-based antiviral immunity. Nat Rev Immunol **10:**632-44.
81. **Doench, J. G., and P. A. Sharp.** 2004. Specificity of microRNA target selection in translational repression. Genes Dev **18:**504-11.
82. **Dolken, L., A. Krmpotic, S. Kothe, L. Tuddenham, M. Tanguy, L. Marcinowski, Z. Ruzsics, N. Elefant, Y. Altuvia, H. Margalit, U. H. Koszinowski, S. Jonjic, and S. Pfeffer.** 2010. Cytomegalovirus microRNAs facilitate persistent virus infection in salivary glands. PLoS Pathog **6:**e1001150.
83. **Dolken, L., G. Malterer, F. Erhard, S. Kothe, C. C. Friedel, G. Suffert, L. Marcinowski, N. Motsch, S. Barth, M. Beitzinger, D. Lieber, S. M. Bailer, R. Hoffmann, Z. Ruzsics, E. Kremmer, S. Pfeffer, R. Zimmer, U. H. Koszinowski, F. Grasser, G. Meister, and J. Haas.** 2010. Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay. Cell Host Microbe **7:**324-34.
84. **Drew, W. L., E. S. Sweet, R. C. Miner, and E. S. Mocarski.** 1984. Multiple infections by cytomegalovirus in patients with acquired

immunodeficiency syndrome: documentation by Southern blot hybridization. J Infect Dis **150:**952-3.

85. **Dunn, W., P. Trang, Q. Zhong, E. Yang, C. van Belle, and F. Liu.** 2005. Human cytomegalovirus expresses novel microRNAs during productive viral infection. Cell Microbiol **7:**1684-95.
86. **Dziurzynski, K., S. M. Chang, A. B. Heimberger, R. F. Kalejta, S. R. McGregor Dallas, M. Smit, L. Soroceanu, and C. S. Cobbs.** Consensus on the role of human cytomegalovirus in glioblastoma. Neuro Oncol **14:**246-55.
87. **E, X., M. T. Pickering, M. Debatis, J. Castillo, A. Lagadinos, S. Wang, S. Lu, and T. F. Kowalik.** 2011. An E2F1-mediated DNA damage response contributes to the replication of human cytomegalovirus. PLoS Pathog **7:**e1001342.
88. **Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411:**494-8.
89. **Elbashir, S. M., W. Lendeckel, and T. Tuschl.** 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev **15:**188-200.
90. **Elmayan, T., S. Balzergue, F. Beon, V. Bourdon, J. Daubremet, Y. Guenet, P. Mourrain, J. C. Palauqui, S. Vernhettes, T. Vialle, K. Wostrikoff, and H. Vaucheret.** 1998. Arabidopsis mutants impaired in cosuppression. Plant Cell **10:**1747-58.
91. **English, J. J., E. Mueller, and D. C. Baulcombe.** 1996. Suppression of Virus Accumulation in Transgenic Plants Exhibiting Silencing of Nuclear Genes. Plant Cell **8:**179-188.
92. **Enright, A. J., B. John, U. Gaul, T. Tuschl, C. Sander, and D. S. Marks.** 2003. MicroRNA targets in Drosophila. Genome Biol **5:**R1.
93. **Fagard, M., S. Boutet, J. B. Morel, C. Bellini, and H. Vaucheret.** 2000. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc Natl Acad Sci U S A **97:**11650-4.
94. **Farazi, T. A., J. I. Hoell, P. Morozov, and T. Tuschl.** 2013. MicroRNAs in human cancer. Adv Exp Med Biol **774:**1-20.
95. **Farh, K. K., A. Grimson, C. Jan, B. P. Lewis, W. K. Johnston, L. P. Lim, C. B. Burge, and D. P. Bartel.** 2005. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. Science **310:**1817-21.
96. **Feederle, R., S. D. Linnstaedt, H. Bannert, H. Lips, M. Bencun, B. R. Cullen, and H. J. Delecluse.** 2011. A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus. PLoS Pathog **7:**e1001294.
97. **Feire, A. L., H. Koss, and T. Compton.** 2004. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc Natl Acad Sci U S A **101:**15470-5.

98. **Fernandez-Hernando, C., C. M. Ramirez, L. Goedeke, and Y. Suarez.** 2013. MicroRNAs in metabolic disease. Arterioscler Thromb Vasc Biol **33:**178-85.
99. **Fields, B. N., D. M. Knipe, and P. M. Howley.** 2007. Fields' virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
100. **Fire, A., D. Albertson, S. W. Harrison, and D. G. Moerman.** 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle. Development **113:**503-14.
101. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.** 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391:**806-11.
102. **Flicek, P., I. Ahmed, M. R. Amode, D. Barrell, K. Beal, S. Brent, D. Carvalho-Silva, P. Clapham, G. Coates, S. Fairley, S. Fitzgerald, L. Gil, C. Garcia-Giron, L. Gordon, T. Hourlier, S. Hunt, T. Juettemann, A. K. Kahari, S. Keenan, M. Komorowska, E. Kulesha, I. Longden, T. Maurel, W. M. McLaren, M. Muffato, R. Nag, B. Overduin, M. Pignatelli, B. Pritchard, E. Pritchard, H. S. Riat, G. R. Ritchie, M. Ruffier, M. Schuster, D. Sheppard, D. Sobral, K. Taylor, A. Thormann, S. Trevanion, S. White, S. P. Wilder, B. L. Aken, E. Birney, F. Cunningham, I. Dunham, J. Harrow, J. Herrero, T. J. Hubbard, N. Johnson, R. Kinsella, A. Parker, G. Spudich, A. Yates, A. Zadissa, and S. M. Searle.** 2013. Ensembl 2013. Nucleic Acids Res **41:**D48-55.
103. **Flicek, P., M. R. Amode, D. Barrell, K. Beal, S. Brent, Y. Chen, P. Clapham, G. Coates, S. Fairley, S. Fitzgerald, L. Gordon, M. Hendrix, T. Hourlier, N. Johnson, A. Kahari, D. Keefe, S. Keenan, R. Kinsella, F. Kokocinski, E. Kulesha, P. Larsson, I. Longden, W. McLaren, B. Overduin, B. Pritchard, H. S. Riat, D. Rios, G. R. Ritchie, M. Ruffier, M. Schuster, D. Sobral, G. Spudich, Y. A. Tang, S. Trevanion, J. Vandrovcova, A. J. Vilella, S. White, S. P. Wilder, A. Zadissa, J. Zamora, B. L. Aken, E. Birney, F. Cunningham, I. Dunham, R. Durbin, X. M. Fernandez-Suarez, J. Herrero, T. J. Hubbard, A. Parker, G. Proctor, J. Vogel, and S. M. Searle.** Ensembl 2011. Nucleic Acids Res **39:**D800-6.
104. **Flores, O., S. Nakayama, A. W. Whisnant, H. Javanbakht, B. R. Cullen, and D. C. Bloom.** 2013. Mutational Inactivation of Herpes Simplex Virus 1 MicroRNAs Identifies Viral mRNA Targets and Reveals Phenotypic Effects in Culture. J Virol **87:**6589-603.
105. **Fontana, L., E. Pelosi, P. Greco, S. Racanicchi, U. Testa, F. Liuzzi, C. M. Croce, E. Brunetti, F. Grignani, and C. Peschle.** 2007. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol **9:**775-87.
106. **Fowler, K. B., S. Stagno, R. F. Pass, W. J. Britt, T. J. Boll, and C. A. Alford.** 1992. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. N Engl J Med **326:**663-7.

107. **Friedman, R. C., K. K. Farh, C. B. Burge, and D. P. Bartel.** 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res **19:**92-105.
108. **Fukuhara, T., H. Kambara, M. Shiokawa, C. Ono, H. Katoh, E. Morita, D. Okuzaki, Y. Maehara, K. Koike, and Y. Matsuura.** 2012. Expression of microRNA miR-122 facilitates an efficient replication in nonhepatic cells upon infection with hepatitis C virus. J Virol **86:**7918-33.
109. **Fukunaga, R., B. W. Han, J. H. Hung, J. Xu, Z. Weng, and P. D. Zamore.** 2012. Dicer partner proteins tune the length of mature miRNAs in flies and mammals. Cell **151:**533-46.
110. **Furlong, D., H. Swift, and B. Roizman.** 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J Virol **10:**1071-4.
111. **Gaidatzis, D., E. van Nimwegen, J. Hausser, and M. Zavolan.** 2007. Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinformatics **8:**69.
112. **Gatto, G., A. Rossi, D. Rossi, S. Kroening, S. Bonatti, and M. Mallardo.** 2008. Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. Nucleic Acids Res **36:**6608-19.
113. **Ghildiyal, M., and P. D. Zamore.** 2009. Small silencing RNAs: an expanding universe. Nat Rev Genet **10:**94-108.
114. **Gottwein, E., N. Mukherjee, C. Sachse, C. Frenzel, W. H. Majoros, J. T. Chi, R. Braich, M. Manoharan, J. Soutschek, U. Ohler, and B. R. Cullen.** 2007. A viral microRNA functions as an orthologue of cellular miR-155. Nature **450:**1096-9.
115. **Gregory, R. I., K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, and R. Shiekhattar.** 2004. The Microprocessor complex mediates the genesis of microRNAs. Nature **432:**235-40.
116. **Grey, F., A. Antoniewicz, E. Allen, J. Saugstad, A. McShea, J. C. Carrington, and J. Nelson.** 2005. Identification and characterization of human cytomegalovirus-encoded microRNAs. J Virol **79:**12095-9.
117. **Grey, F., H. Meyers, E. A. White, D. H. Spector, and J. Nelson.** 2007. A human cytomegalovirus-encoded microRNA regulates expression of multiple viral genes involved in replication. PLoS Pathog **3:**e163.
118. **Griffiths-Jones, S.** 2004. The microRNA Registry. Nucleic Acids Res **32:**D109-11.
119. **Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim, and D. P. Bartel.** 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell **27:**91-105.
120. **Gu, J., and V. R. Iyer.** 2006. PI3K signaling and miRNA expression during the response of quiescent human fibroblasts to distinct proliferative stimuli. Genome Biol **7:**R42.

121. **Gunasekharan, V., and L. A. Laimins.** 2013. Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol **87:**6037-43.
122. **Guo, H., N. T. Ingolia, J. S. Weissman, and D. P. Bartel.** 2010. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature **466:**835-40.
123. **Guo, S., and K. J. Kemphues.** 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:**611-20.
124. **Guo, X. K., Q. Zhang, L. Gao, N. Li, X. X. Chen, and W. H. Feng.** 2013. Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol **87:**1159-71.
125. **Gupta, S., J. A. Stamatoyannopoulos, T. L. Bailey, and W. S. Noble.** 2007. Quantifying similarity between motifs. Genome Biol **8:**R24.
126. **Haasnoot, J., W. de Vries, E. J. Geutjes, M. Prins, P. de Haan, and B. Berkhout.** 2007. The Ebola virus VP35 protein is a suppressor of RNA silencing. PLoS Pathog **3:**e86.
127. **Hagemeier, C., S. M. Walker, P. J. Sissons, and J. H. Sinclair.** 1992. The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently trans-activate the c-fos, c-myc and hsp70 promoters via basal promoter elements. J Gen Virol **73 (Pt 9):**2385-93.
128. **Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin, and V. N. Kim.** 2004. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev **18:**3016-27.
129. **Hannemann, H., K. Rosenke, J. M. O'Dowd, and E. A. Fortunato.** 2009. The presence of p53 influences the expression of multiple human cytomegalovirus genes at early times postinfection. J Virol **83:**4316-25.
130. **Held, K., A. Junker, K. Dornmair, E. Meinl, I. Sinicina, T. Brandt, D. Theil, and T. Derfuss.** 2011. Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates. J Virol **85:**9680-5.
131. **Hertel, L., and E. S. Mocarski.** 2004. Global analysis of host cell gene expression late during cytomegalovirus infection reveals extensive dysregulation of cell cycle gene expression and induction of Pseudomitosis independent of US28 function. J Virol **78:**11988-2011.
132. **Ho, B. C., S. L. Yu, J. J. Chen, S. Y. Chang, B. S. Yan, Q. S. Hong, S. Singh, C. L. Kao, H. Y. Chen, K. Y. Su, K. C. Li, C. L. Cheng, H. W. Cheng, J. Y. Lee, C. N. Lee, and P. C. Yang.** 2011. Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. Cell Host Microbe **9:**58-69.
133. **Hobom, U., W. Brune, M. Messerle, G. Hahn, and U. H. Koszinowski.** 2000. Fast screening procedures for random transposon libraries of

cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J Virol **74:**7720-9.
134. **Hon, L. S., and Z. Zhang.** 2007. The roles of binding site arrangement and combinatorial targeting in microRNA repression of gene expression. Genome Biol **8:**R166.
135. **Houbaviy, H. B., M. F. Murray, and P. A. Sharp.** 2003. Embryonic stem cell-specific MicroRNAs. Dev Cell **5:**351-8.
136. **Hu, H., and R. A. Gatti.** 2011. MicroRNAs: new players in the DNA damage response. J Mol Cell Biol **3:**151-8.
137. **Huang da, W., B. T. Sherman, and R. A. Lempicki.** 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc **4:**44-57.
138. **Huang, Y., Y. Qi, Y. Ma, R. He, Y. Ji, Z. Sun, and Q. Ruan.** 2013. The expression of interleukin-32 is activated by human cytomegalovirus infection and down regulated by hcmv-miR-UL112-1. Virol J **10:**51.
139. **Hummel, M., S. Yan, Z. Li, T. K. Varghese, and M. Abecassis.** 2007. Transcriptional reactivation of murine cytomegalovirus ie gene expression by 5-aza-2'-deoxycytidine and trichostatin A in latently infected cells despite lack of methylation of the major immediate-early promoter. J Gen Virol **88:**1097-102.
140. **Humphreys, D. T., B. J. Westman, D. I. Martin, and T. Preiss.** 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A **102:**16961-6.
141. **Hutto, C., E. A. Little, R. Ricks, J. D. Lee, and R. F. Pass.** 1986. Isolation of cytomegalovirus from toys and hands in a day care center. J Infect Dis **154:**527-30.
142. **Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl, and P. D. Zamore.** 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science **293:**834-8.
143. **Hutvagner, G., and P. D. Zamore.** 2002. A microRNA in a multiple-turnover RNAi enzyme complex. Science **297:**2056-60.
144. **Ikeda, S., S. W. Kong, J. Lu, E. Bisping, H. Zhang, P. D. Allen, T. R. Golub, B. Pieske, and W. T. Pu.** 2007. Altered microRNA expression in human heart disease. Physiol Genomics **31:**367-73.
145. **Imig, J., N. Motsch, J. Y. Zhu, S. Barth, M. Okoniewski, T. Reineke, M. Tinguely, A. Faggioni, P. Trivedi, G. Meister, C. Renner, and F. A. Grasser.** 2011. microRNA profiling in Epstein-Barr virus-associated B-cell lymphoma. Nucleic Acids Res **39:**1880-93.
146. **Isomura, H., M. F. Stinski, A. Kudoh, T. Daikoku, N. Shirata, and T. Tsurumi.** 2005. Two Sp1/Sp3 binding sites in the major immediate-early proximal enhancer of human cytomegalovirus have a significant role in viral replication. J Virol **79:**9597-607.

147. **Istas, A. S., G. J. Demmler, J. G. Dobbins, and J. A. Stewart.** 1995. Surveillance for congenital cytomegalovirus disease: a report from the National Congenital Cytomegalovirus Disease Registry. Clin Infect Dis **20:**665-70.
148. **Ivanovska, I., A. S. Ball, R. L. Diaz, J. F. Magnus, M. Kibukawa, J. M. Schelter, S. V. Kobayashi, L. Lim, J. Burchard, A. L. Jackson, P. S. Linsley, and M. A. Cleary.** 2008. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol **28:**2167-74.
149. **Jarvis, M. A., and J. A. Nelson.** 2002. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. Curr Opin Microbiol **5:**403-7.
150. **Jim, W. T., C. H. Shu, N. C. Chiu, H. A. Kao, H. Y. Hung, J. H. Chang, C. C. Peng, W. S. Hsieh, K. C. Liu, and F. Y. Huang.** 2004. Transmission of cytomegalovirus from mothers to preterm infants by breast milk. Pediatr Infect Dis J **23:**848-51.
151. **Jing, X. L., M. N. Fan, G. Jia, L. W. Liu, L. Ma, C. C. Zheng, X. P. Zhu, H. M. Liu, and X. Y. Wang.** 2011. A multifunctional protein encoded by turkey herpesvirus suppresses RNA silencing in Nicotiana benthamiana. J Virol **85:**12792-803.
152. **Johnston, R. J., and O. Hobert.** 2003. A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature **426:**845-9.
153. **Jopling, C. L., M. Yi, A. M. Lancaster, S. M. Lemon, and P. Sarnow.** 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science **309:**1577-81.
154. **Kang, J. G., N. Pripuzova, V. Majerciak, M. Kruhlak, S. Y. Le, and Z. M. Zheng.** 2011. Kaposi's sarcoma-associated herpesvirus ORF57 promotes escape of viral and human interleukin-6 from microRNA-mediated suppression. J Virol **85:**2620-30.
155. **Karlin, S., E. S. Mocarski, and G. A. Schachtel.** 1994. Molecular evolution of herpesviruses: genomic and protein sequence comparisons. J Virol **68:**1886-902.
156. **Kasschau, K. D., and J. C. Carrington.** 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell **95:**461-70.
157. **Kaul, D., A. Ahlawat, and S. D. Gupta.** 2009. HIV-1 genome-encoded hiv1-mir-H1 impairs cellular responses to infection. Mol Cell Biochem **323:**143-8.
158. **Kennerdell, J. R., and R. W. Carthew.** 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell **95:**1017-26.
159. **Kent, O. A., K. Fox-Talbot, and M. K. Halushka.** 2013. RREB1 repressed miR-143/145 modulates KRAS signaling through downregulation of multiple targets. Oncogene **32:**2576-85.

160. **Kertesz, M., N. Iovino, U. Unnerstall, U. Gaul, and E. Segal.** 2007. The role of site accessibility in microRNA target recognition. Nat Genet **39:**1278-84.
161. **Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, and R. H. Plasterk.** 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev **15:**2654-9.
162. **Ketting, R. F., T. H. Haverkamp, H. G. van Luenen, and R. H. Plasterk.** 1999. Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell **99:**133-41.
163. **Kim, A. H., M. Reimers, B. Maher, V. Williamson, O. McMichael, J. L. McClay, E. J. van den Oord, B. P. Riley, K. S. Kendler, and V. I. Vladimirov.** 2010. MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. Schizophr Res **124:**183-91.
164. **Kim, V. N., J. Han, and M. C. Siomi.** 2009. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol **10:**126-39.
165. **Kim, Y., S. Lee, S. Kim, D. Kim, J. H. Ahn, and K. Ahn.** 2012. Human cytomegalovirus clinical strain-specific microRNA miR-UL148D targets the human chemokine RANTES during infection. PLoS Pathog **8:**e1002577.
166. **Kiriakidou, M., P. T. Nelson, A. Kouranov, P. Fitziev, C. Bouyioukos, Z. Mourelatos, and A. Hatzigeorgiou.** 2004. A combined computational-experimental approach predicts human microRNA targets. Genes Dev **18:**1165-78.
167. **Klase, Z., P. Kale, R. Winograd, M. V. Gupta, M. Heydarian, R. Berro, T. McCaffrey, and F. Kashanchi.** 2007. HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. BMC Mol Biol **8:**63.
168. **Klein, M. E., D. T. Lioy, L. Ma, S. Impey, G. Mandel, and R. H. Goodman.** 2007. Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci **10:**1513-4.
169. **Klemola, E., R. Von Essen, G. Henle, and W. Henle.** 1970. Infectious-mononucleosis-like disease with negative heterophil agglutination test. Clinical features in relation to Epstein-Barr virus and cytomegalovirus antibodies. J Infect Dis **121:**608-14.
170. **Knight, S. W., and B. L. Bass.** 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science **293:**2269-71.
171. **Kopp, M., H. Granzow, W. Fuchs, B. Klupp, and T. C. Mettenleiter.** 2004. Simultaneous deletion of pseudorabies virus tegument protein UL11 and glycoprotein M severely impairs secondary envelopment. J Virol **78:**3024-34.

172. **Kowalik, T. F., B. Wing, J. S. Haskill, J. C. Azizkhan, A. S. Baldwin, Jr., and E. S. Huang.** 1993. Multiple mechanisms are implicated in the regulation of NF-kappa B activity during human cytomegalovirus infection. Proc Natl Acad Sci U S A **90:**1107-11.
173. **Kozomara, A., and S. Griffiths-Jones.** 2011. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res **39:**D152-7.
174. **Kramer, M. F., I. Jurak, J. M. Pesola, S. Boissel, D. M. Knipe, and D. M. Coen.** 2011. Herpes simplex virus 1 microRNAs expressed abundantly during latent infection are not essential for latency in mouse trigeminal ganglia. Virology **417:**239-47.
175. **Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel, and N. Rajewsky.** 2005. Combinatorial microRNA target predictions. Nat Genet **37:**495-500.
176. **Krosky, P. M., M. C. Baek, and D. M. Coen.** 2003. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. J Virol **77:**905-14.
177. **Lagos, D., G. Pollara, S. Henderson, F. Gratrix, M. Fabani, R. S. Milne, F. Gotch, and C. Boshoff.** miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat Cell Biol **12:**513-9.
178. **Landgraf, P., M. Rusu, R. Sheridan, A. Sewer, N. Iovino, A. Aravin, S. Pfeffer, A. Rice, A. O. Kamphorst, M. Landthaler, C. Lin, N. D. Socci, L. Hermida, V. Fulci, S. Chiaretti, R. Foa, J. Schliwka, U. Fuchs, A. Novosel, R. U. Muller, B. Schermer, U. Bissels, J. Inman, Q. Phan, M. Chien, D. B. Weir, R. Choksi, G. De Vita, D. Frezzetti, H. I. Trompeter, V. Hornung, G. Teng, G. Hartmann, M. Palkovits, R. Di Lauro, P. Wernet, G. Macino, C. E. Rogler, J. W. Nagle, J. Ju, F. N. Papavasiliou, T. Benzing, P. Lichter, W. Tam, M. J. Brownstein, A. Bosio, A. Borkhardt, J. J. Russo, C. Sander, M. Zavolan, and T. Tuschl.** 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell **129:**1401-14.
179. **Landthaler, M., A. Yalcin, and T. Tuschl.** 2004. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol **14:**2162-7.
180. **Lang, D., S. Gebert, H. Arlt, and T. Stamminger.** 1995. Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. J Virol **69:**6030-7.
181. **Lashmit, P., S. Wang, H. Li, H. Isomura, and M. F. Stinski.** 2009. The CREB site in the proximal enhancer is critical for cooperative interaction with the other transcription factor binding sites to enhance transcription of the major intermediate-early genes in human cytomegalovirus-infected cells. J Virol **83:**8893-904.

182. **Lazzarotto, T., and M. Lanari.** Why is cytomegalovirus the most frequent cause of congenital infection? Expert Rev Anti Infect Ther **9:**841-3.
183. **Lecellier, C. H., P. Dunoyer, K. Arar, J. Lehmann-Che, S. Eyquem, C. Himber, A. Saib, and O. Voinnet.** 2005. A cellular microRNA mediates antiviral defense in human cells. Science **308:**557-60.
184. **Lee, H. Y., and J. A. Doudna.** 2012. TRBP alters human precursor microRNA processing in vitro. RNA **18:**2012-9.
185. **Lee, R. C., R. L. Feinbaum, and V. Ambros.** 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75:**843-54.
186. **Lee, S., J. Song, S. Kim, J. Kim, Y. Hong, Y. Kim, D. Kim, D. Baek, and K. Ahn.** 2013. Selective Degradation of Host MicroRNAs by an Intergenic HCMV Noncoding RNA Accelerates Virus Production. Cell Host Microbe **13:**678-90.
187. **Lee, T. I., and R. A. Young.** 2013. Transcriptional regulation and its misregulation in disease. Cell **152:**1237-51.
188. **Lee, Y., K. Jeon, J. T. Lee, S. Kim, and V. N. Kim.** 2002. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J **21:**4663-70.
189. **Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek, and V. N. Kim.** 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J **23:**4051-60.
190. **Lei, X., Z. Bai, F. Ye, J. Xie, C. G. Kim, Y. Huang, and S. J. Gao.** 2010. Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. Nat Cell Biol **12:**193-9.
191. **Lei, X., Y. Zhu, T. Jones, Z. Bai, Y. Huang, and S. J. Gao.** 2012. A Kaposi's sarcoma-associated herpesvirus microRNA and its variants target the transforming growth factor beta pathway to promote cell survival. J Virol **86:**11698-711.
192. **Lewis, B. P., C. B. Burge, and D. P. Bartel.** 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell **120:**15-20.
193. **Lewis, B. P., I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge.** 2003. Prediction of mammalian microRNA targets. Cell **115:**787-98.
194. **Lewis, J. D., R. R. Meehan, W. J. Henzel, I. Maurer-Fogy, P. Jeppesen, F. Klein, and A. Bird.** 1992. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell **69:**905-14.
195. **Li, G., Z. Wu, Y. Peng, X. Liu, J. Lu, L. Wang, Q. Pan, M. L. He, and X. P. Li.** 2010. MicroRNA-10b induced by Epstein-Barr virus-encoded latent membrane protein-1 promotes the metastasis of human nasopharyngeal carcinoma cells. Cancer Lett **299:**29-36.

196. **Li, W. X., and S. W. Ding.** 2001. Viral suppressors of RNA silencing. Curr Opin Biotechnol **12:**150-4.
197. **Li, Y., and K. V. Kowdley.** 2012. MicroRNAs in common human diseases. Genomics Proteomics Bioinformatics **10:**246-53.
198. **Lin, H. R., and D. Ganem.** 2011. Viral microRNA target allows insight into the role of translation in governing microRNA target accessibility. Proc Natl Acad Sci U S A **108:**5148-53.
199. **Lindsay, M. A.** 2008. microRNAs and the immune response. Trends Immunol **29:**343-51.
200. **Linsley, P. S., J. Schelter, J. Burchard, M. Kibukawa, M. M. Martin, S. R. Bartz, J. M. Johnson, J. M. Cummins, C. K. Raymond, H. Dai, N. Chau, M. Cleary, A. L. Jackson, M. Carleton, and L. Lim.** 2007. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol **27:**2240-52.
201. **Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, and G. J. Hannon.** 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science **305:**1437-41.
202. **Liu, X. F., X. Wang, S. Yan, Z. Zhang, M. Abecassis, and M. Hummel.** 2013. Epigenetic control of cytomegalovirus latency and reactivation. Viruses **5:**1325-45.
203. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-8.
204. **Loh, Y. H., S. V. Yi, and J. T. Streelman.** 2011. Evolution of microRNAs and the diversification of species. Genome Biol Evol **3:**55-65.
205. **Long, D., R. Lee, P. Williams, C. Y. Chan, V. Ambros, and Y. Ding.** 2007. Potent effect of target structure on microRNA function. Nat Struct Mol Biol **14:**287-94.
206. **Lu, F., W. Stedman, M. Yousef, R. Renne, and P. M. Lieberman.** 2010. Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. J Virol **84:**2697-706.
207. **Lu, F., A. Weidmer, C. G. Liu, S. Volinia, C. M. Croce, and P. M. Lieberman.** 2008. Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. J Virol **82:**10436-43.
208. **Lu, J., G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz, and T. R. Golub.** 2005. MicroRNA expression profiles classify human cancers. Nature **435:**834-8.
209. **Lu, M., Q. Zhang, M. Deng, J. Miao, Y. Guo, W. Gao, and Q. Cui.** 2008. An analysis of human microRNA and disease associations. PLoS One **3:**e3420.

210. **Lurain, N. S., B. A. Hanson, J. Martinson, S. E. Leurgans, A. L. Landay, D. A. Bennett, and J. A. Schneider.** 2013. Virological and Immunological Characteristics of Human Cytomegalovirus Infection Associated With Alzheimer Disease. J Infect Dis.
211. **Lytle, J. R., T. A. Yario, and J. A. Steitz.** 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A **104:**9667-72.
212. **Maidji, E., S. Tugizov, T. Jones, Z. Zheng, and L. Pereira.** 1996. Accessory human cytomegalovirus glycoprotein US9 in the unique short component of the viral genome promotes cell-to-cell transmission of virus in polarized epithelial cells. J Virol **70:**8402-10.
213. **Malm, G., and M. L. Engman.** 2007. Congenital cytomegalovirus infections. Semin Fetal Neonatal Med **12:**154-9.
214. **Manicklal, S., V. C. Emery, T. Lazzarotto, S. B. Boppana, and R. K. Gupta.** 2013. The "silent" global burden of congenital cytomegalovirus. Clin Microbiol Rev **26:**86-102.
215. **Marcinowski, L., M. Tanguy, A. Krmpotic, B. Radle, V. J. Lisnic, L. Tuddenham, B. Chane-Woon-Ming, Z. Ruzsics, F. Erhard, C. Benkartek, M. Babic, R. Zimmer, J. Trgovcich, U. H. Koszinowski, S. Jonjic, S. Pfeffer, and L. Dolken.** 2012. Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication in vivo. PLoS Pathog **8:**e1002510.
216. **Maroney, P. A., Y. Yu, J. Fisher, and T. W. Nilsen.** 2006. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol **13:**1102-7.
217. **Marquitz, A. R., A. Mathur, C. S. Nam, and N. Raab-Traub.** 2011. The Epstein-Barr Virus BART microRNAs target the pro-apoptotic protein Bim. Virology **412:**392-400.
218. **Martinez, J., A. Patkaniowska, H. Urlaub, R. Luhrmann, and T. Tuschl.** 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563-74.
219. **Matys, V., O. V. Kel-Margoulis, E. Fricke, I. Liebich, S. Land, A. Barre-Dirrie, I. Reuter, D. Chekmenev, M. Krull, K. Hornischer, N. Voss, P. Stegmaier, B. Lewicki-Potapov, H. Saxel, A. E. Kel, and E. Wingender.** 2006. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res **34:**D108-10.
220. **McGeoch, D. J., S. Cook, A. Dolan, F. E. Jamieson, and E. A. Telford.** 1995. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J Mol Biol **247:**443-58.
221. **McGeoch, D. J., F. J. Rixon, and A. J. Davison.** 2006. Topics in herpesvirus genomics and evolution. Virus Res **117:**90-104.
222. **McVoy, M. A., and S. P. Adler.** 1994. Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. J Virol **68:**1040-51.

223. **Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl.** 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell **15:**185-97.
224. **Mendelson, M., S. Monard, P. Sissons, and J. Sinclair.** 1996. Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. J Gen Virol **77 (Pt 12):**3099-102.
225. **Meshesha, M. K., I. Veksler-Lublinsky, O. Isakov, I. Reichenstein, N. Shomron, K. Kedem, M. Ziv-Ukelson, Z. Bentwich, and Y. S. Avni.** 2012. The microRNA Transcriptome of Human Cytomegalovirus (HCMV). Open Virol J **6:**38-48.
226. **Mettenleiter, T. C.** 2004. Budding events in herpesvirus morphogenesis. Virus Res **106:**167-80.
227. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2006. Herpesvirus assembly: a tale of two membranes. Curr Opin Microbiol **9:**423-9.
228. **Miranda, K. C., T. Huynh, Y. Tay, Y. S. Ang, W. L. Tam, A. M. Thomson, B. Lim, and I. Rigoutsos.** 2006. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell **126:**1203-17.
229. **Montague, M. G., and C. A. Hutchison, 3rd.** 2000. Gene content phylogeny of herpesviruses. Proc Natl Acad Sci U S A **97:**5334-9.
230. **Morgan, C., H. M. Rose, M. Holden, and E. P. Jones.** 1959. Electron microscopic observations on the development of herpes simplex virus. J Exp Med **110:**643-56.
231. **Morgan, R., A. Anderson, E. Bernberg, S. Kamboj, E. Huang, G. Lagasse, G. Isaacs, M. Parcells, B. C. Meyers, P. J. Green, and J. Burnside.** 2008. Sequence conservation and differential expression of Marek's disease virus microRNAs. J Virol **82:**12213-20.
232. **Moss, E. G., R. C. Lee, and V. Ambros.** 1997. The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell **88:**637-46.
233. **Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo, and H. Vaucheret.** 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:**533-42.
234. **Muganda, P., O. Mendoza, J. Hernandez, and Q. Qian.** 1994. Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts. J Virol **68:**8028-34.
235. **Mukherji, S., M. S. Ebert, G. X. Zheng, J. S. Tsang, P. A. Sharp, and A. van Oudenaarden.** 2011. MicroRNAs can generate thresholds in target gene expression. Nat Genet **43:**854-9.
236. **Munger, J., S. U. Bajad, H. A. Coller, T. Shenk, and J. D. Rabinowitz.** 2006. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog **2:**e132.

237. **Murakami, Y., H. H. Aly, A. Tajima, I. Inoue, and K. Shimotohno.** 2009. Regulation of the hepatitis C virus genome replication by miR-199a. J Hepatol **50:**453-60.
238. **Muranyi, W., J. Haas, M. Wagner, G. Krohne, and U. H. Koszinowski.** 2002. Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina. Science **297:**854-7.
239. **Murphy, E., J. Vanicek, H. Robins, T. Shenk, and A. J. Levine.** 2008. Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. Proc Natl Acad Sci U S A **105:**5453-8.
240. **Murphy, F. A.** 2012. The Foundations of Virology: Discoverers and Discoveries, Inventors and Inventions, Developers and Technologies. Infinity Publishing, 1094 New Dehaven Street, Suite 100.
241. **Murphy, J. C., W. Fischle, E. Verdin, and J. H. Sinclair.** 2002. Control of cytomegalovirus lytic gene expression by histone acetylation. EMBO J **21:**1112-20.
242. **Nachmani, D., D. Lankry, D. G. Wolf, and O. Mandelboim.** 2010. The human cytomegalovirus microRNA miR-UL112 acts synergistically with a cellular microRNA to escape immune elimination. Nat Immunol **11:**806-13.
243. **Nachmani, D., N. Stern-Ginossar, R. Sarid, and O. Mandelboim.** 2009. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe **5:**376-85.
244. **Nam, S., M. Li, K. Choi, C. Balch, S. Kim, and K. P. Nephew.** 2009. MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression. Nucleic Acids Res **37:**W356-62.
245. **Napoli, C., C. Lemieux, and R. Jorgensen.** 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell **2:**279-289.
246. **Nielsen, C. B., N. Shomron, R. Sandberg, E. Hornstein, J. Kitzman, and C. B. Burge.** 2007. Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. RNA **13:**1894-910.
247. **Nykanen, A., B. Haley, and P. D. Zamore.** 2001. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107:**309-21.
248. **Odeberg, J., N. Wolmer, S. Falci, M. Westgren, A. Seiger, and C. Soderberg-Naucler.** 2006. Human cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells. J Virol **80:**8929-39.
249. **Odeberg, J., N. Wolmer, S. Falci, M. Westgren, E. Sundtrom, A. Seiger, and C. Soderberg-Naucler.** 2007. Late human cytomegalovirus (HCMV) proteins inhibit differentiation of human neural precursor cells into astrocytes. J Neurosci Res **85:**583-93.

250. **Olive, V., I. Jiang, and L. He.** 2010. mir-17-92, a cluster of miRNAs in the midst of the cancer network. Int J Biochem Cell Biol **42:**1348-54.
251. **Olsen, P. H., and V. Ambros.** 1999. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol **216:**671-80.
252. **Otsuka, M., Q. Jing, P. Georgel, L. New, J. Chen, J. Mols, Y. J. Kang, Z. Jiang, X. Du, R. Cook, S. C. Das, A. K. Pattnaik, B. Beutler, and J. Han.** 2007. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity **27:**123-34.
253. **Palauqui, J. C., T. Elmayan, J. M. Pollien, and H. Vaucheret.** 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J **16:**4738-45.
254. **Pasquinelli, A. E.** 2012. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet **13:**271-82.
255. **Pedersen, I. M., G. Cheng, S. Wieland, S. Volinia, C. M. Croce, F. V. Chisari, and M. David.** 2007. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature **449:**919-22.
256. **Penfold, M. E., and E. S. Mocarski.** 1997. Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. Virology **239:**46-61.
257. **Petersen, C. P., M. E. Bordeleau, J. Pelletier, and P. A. Sharp.** 2006. Short RNAs repress translation after initiation in mammalian cells. Mol Cell **21:**533-42.
258. **Pfeffer, S., A. Sewer, M. Lagos-Quintana, R. Sheridan, C. Sander, F. A. Grasser, L. F. van Dyk, C. K. Ho, S. Shuman, M. Chien, J. J. Russo, J. Ju, G. Randall, B. D. Lindenbach, C. M. Rice, V. Simon, D. D. Ho, M. Zavolan, and T. Tuschl.** 2005. Identification of microRNAs of the herpesvirus family. Nat Methods **2:**269-76.
259. **Pfeffer, S., M. Zavolan, F. A. Grasser, M. Chien, J. J. Russo, J. Ju, B. John, A. J. Enright, D. Marks, C. Sander, and T. Tuschl.** 2004. Identification of virus-encoded microRNAs. Science **304:**734-6.
260. **Pickering, M. T., B. M. Stadler, and T. F. Kowalik.** 2009. miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. Oncogene **28:**140-5.
261. **Plaisance-Bonstaff, K., and R. Renne.** 2011. Viral miRNAs. Methods Mol Biol **721:**43-66.
262. **Poma, E. E., T. F. Kowalik, L. Zhu, J. H. Sinclair, and E. S. Huang.** 1996. The human cytomegalovirus IE1-72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. J Virol **70:**7867-77.

263. **Poole, E., S. R. McGregor Dallas, J. Colston, R. S. Joseph, and J. Sinclair.** 2011. Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34(+) progenitors. J Gen Virol **92:**1539-49.
264. **Popovic, M., K. Smiljanic, B. Dobutovic, T. Syrovets, T. Simmet, and E. R. Isenovic.** Human cytomegalovirus infection and atherothrombosis. J Thromb Thrombolysis **33:**160-72.
265. **Porkka, K. P., M. J. Pfeiffer, K. K. Waltering, R. L. Vessella, T. L. Tammela, and T. Visakorpi.** 2007. MicroRNA expression profiling in prostate cancer. Cancer Res **67:**6130-5.
266. **Potenza, N., U. Papa, N. Mosca, F. Zerbini, V. Nobile, and A. Russo.** 2011. Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. Nucleic Acids Res **39:**5157-63.
267. **Rajewsky, N., and N. D. Socci.** 2004. Computational identification of microRNA targets. Dev Biol **267:**529-35.
268. **Ramsey, S. A., S. L. Klemm, D. E. Zak, K. A. Kennedy, V. Thorsson, B. Li, M. Gilchrist, E. S. Gold, C. D. Johnson, V. Litvak, G. Navarro, J. C. Roach, C. M. Rosenberger, A. G. Rust, N. Yudkovsky, A. Aderem, and I. Shmulevich.** 2008. Uncovering a macrophage transcriptional program by integrating evidence from motif scanning and expression dynamics. PLoS Comput Biol **4:**e1000021.
269. **Ratcliff, F., B. D. Harrison, and D. C. Baulcombe.** 1997. A similarity between viral defense and gene silencing in plants. Science **276:**1558-60.
270. **Raup, D. M.** 1986. Biological extinction in earth history. Science **231:**1528-33.
271. **Raup, D. M., and J. J. Sepkoski, Jr.** 1982. Mass extinctions in the marine fossil record. Science **215:**1501-3.
272. **Reeves, M. B.** 2011. Chromatin-mediated regulation of cytomegalovirus gene expression. Virus Res **157:**134-43.
273. **Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun.** 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature **403:**901-6.
274. **Revello, M. G., and G. Gerna.** 2004. Pathogenesis and prenatal diagnosis of human cytomegalovirus infection. J Clin Virol **29:**71-83.
275. **Reynolds, D. W., S. Stagno, T. S. Hosty, M. Tiller, and C. A. Alford, Jr.** 1973. Maternal cytomegalovirus excretion and perinatal infection. N Engl J Med **289:**1-5.
276. **Rigoutsos, I.** 2009. New tricks for animal microRNAS: targeting of amino acid coding regions at conserved and nonconserved sites. Cancer Res **69:**3245-8.
277. **Riley, K. J., G. S. Rabinowitz, T. A. Yario, J. M. Luna, R. B. Darnell, and J. A. Steitz.** 2012. EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J **31:**2207-21.

278. **Rodems, S. M., C. L. Clark, and D. H. Spector.** 1998. Separate DNA elements containing ATF/CREB and IE86 binding sites differentially regulate the human cytomegalovirus UL112-113 promoter at early and late times in the infection. J Virol **72:**2697-707.
279. **Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst, and A. Bradley.** 2004. Identification of mammalian microRNA host genes and transcription units. Genome Res **14:**1902-10.
280. **Rodriguez-Viciana, P., C. Sabatier, and F. McCormick.** 2004. Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol **24:**4943-54.
281. **Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology **16:**201-17.
282. **Romano, N., and G. Macino.** 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol Microbiol **6:**3343-53.
283. **Rougvie, A. E.** 2001. Control of developmental timing in animals. Nat Rev Genet **2:**690-701.
284. **Ryckman, B. J., M. A. Jarvis, D. D. Drummond, J. A. Nelson, and D. C. Johnson.** 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. J Virol **80:**710-22.
285. **Saba, R., and G. M. Schratt.** 2010. MicroRNAs in neuronal development, function and dysfunction. Brain Res **1338:**3-13.
286. **Sachdeva, M., S. Zhu, F. Wu, H. Wu, V. Walia, S. Kumar, R. Elble, K. Watabe, and Y. Y. Mo.** 2009. p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc Natl Acad Sci U S A **106:**3207-12.
287. **Saetrom, P., B. S. Heale, O. Snove, Jr., L. Aagaard, J. Alluin, and J. J. Rossi.** 2007. Distance constraints between microRNA target sites dictate efficacy and cooperativity. Nucleic Acids Res **35:**2333-42.
288. **Sambucetti, L. C., J. M. Cherrington, G. W. Wilkinson, and E. S. Mocarski.** 1989. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J **8:**4251-8.
289. **Sano, M., Y. Kato, and K. Taira.** 2006. Sequence-specific interference by small RNAs derived from adenovirus VAI RNA. FEBS Lett **580:**1553-64.
290. **Savva, G., A. Pachnio, B. Kaul, K. Morgan, F. Huppert, C. Brayne, and P. Moss.** 2013. Cytomegalovirus infection is associated with increased mortality in the older population. Aging Cell.
291. **Scheffczik, H., C. G. Savva, A. Holzenburg, L. Kolesnikova, and E. Bogner.** 2002. The terminase subunits pUL56 and pUL89 of human

cytomegalovirus are DNA-metabolizing proteins with toroidal structure. Nucleic Acids Res **30:**1695-703.
292. **Schleiss, M. R.** 2006. Nonprimate models of congenital cytomegalovirus (CMV) infection: gaining insight into pathogenesis and prevention of disease in newborns. ILAR J **47:**65-72.
293. **Schnettler, E., M. G. Sterken, J. Y. Leung, S. W. Metz, C. Geertsema, R. W. Goldbach, J. M. Vlak, A. Kohl, A. A. Khromykh, and G. P. Pijlman.** 2012. Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and Mammalian cells. J Virol **86:**13486-500.
294. **Schwartz, R., B. Helmich, and D. H. Spector.** 1996. CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. J Virol **70:**6955-66.
295. **Schwarz, D. S., G. Hutvagner, T. Du, Z. Xu, N. Aronin, and P. D. Zamore.** 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell **115:**199-208.
296. **Selbach, M., B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky.** 2008. Widespread changes in protein synthesis induced by microRNAs. Nature **455:**58-63.
297. **Seo, J. Y., and W. J. Britt.** 2007. Cytoplasmic envelopment of human cytomegalovirus requires the postlocalization function of tegument protein pp28 within the assembly compartment. J Virol **81:**6536-47.
298. **Shaked, I., A. Meerson, Y. Wolf, R. Avni, D. Greenberg, A. Gilboa-Geffen, and H. Soreq.** 2009. MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity **31:**965-73.
299. **Shenk, T., and M. Stinski.** 2008. Human cytomegalovirus. Springer, Berlin.
300. **Shimakami, T., D. Yamane, C. Welsch, L. Hensley, R. K. Jangra, and S. M. Lemon.** 2012. Base pairing between hepatitis C virus RNA and microRNA 122 3' of its seed sequence is essential for genome stabilization and production of infectious virus. J Virol **86:**7372-83.
301. **Siegert, R., and D. Falke.** 1966. [Electron microscopic studies on the development of Herpesvirus hominis in tissue culture cells]. Arch Gesamte Virusforsch **19:**230-49.
302. **Sijen, T., J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plasterk, and A. Fire.** 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:**465-76.
303. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. J Virol **77:**10594-605.
304. **Sinclair, J., and P. Sissons.** 2006. Latency and reactivation of human cytomegalovirus. J Gen Virol **87:**1763-79.

305. **Singh, C. P., J. Singh, and J. Nagaraju.** 2012. A baculovirus-encoded MicroRNA (miRNA) suppresses its host miRNA biogenesis by regulating the exportin-5 cofactor Ran. J Virol **86:**7867-79.
306. **Sinzger, C., M. Kahl, K. Laib, K. Klingel, P. Rieger, B. Plachter, and G. Jahn.** 2000. Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus. J Gen Virol **81:**3021-35.
307. **Slack, F. J., M. Basson, Z. Liu, V. Ambros, H. R. Horvitz, and G. Ruvkun.** 2000. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell **5:**659-69.
308. **Slobbe-van Drunen, M. E., A. T. Hendrickx, R. C. Vossen, E. J. Speel, M. C. van Dam-Mieras, and C. A. Bruggeman.** 1998. Nuclear import as a barrier to infection of human umbilical vein endothelial cells by human cytomegalovirus strain AD169. Virus Res **56:**149-56.
309. **Smardon, A., J. M. Spoerke, S. C. Stacey, M. E. Klein, N. Mackin, and E. M. Maine.** 2000. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. Curr Biol **10:**169-78.
310. **Smith, J. L., F. E. Grey, J. L. Uhrlaub, J. Nikolich-Zugich, and A. J. Hirsch.** 2012. Induction of the cellular microRNA, Hs_154, by West Nile virus contributes to virus-mediated apoptosis through repression of antiapoptotic factors. J Virol **86:**5278-87.
311. **Smith, S. B., H. C. Ee, J. R. Conners, and M. S. German.** 1999. Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol Cell Biol **19:**8272-80.
312. **Sood, P., A. Krek, M. Zavolan, G. Macino, and N. Rajewsky.** 2006. Cell-type-specific signatures of microRNAs on target mRNA expression. Proc Natl Acad Sci U S A **103:**2746-51.
313. **Soroceanu, L., A. Akhavan, and C. S. Cobbs.** 2008. Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. Nature **455:**391-5.
314. **Soroceanu, L., and C. S. Cobbs.** Is HCMV a tumor promoter? Virus Res **157:**193-203.
315. **Stark, A., J. Brennecke, R. B. Russell, and S. M. Cohen.** 2003. Identification of Drosophila MicroRNA targets. PLoS Biol **1:**E60.
316. **Stark, T. J., J. D. Arnold, D. H. Spector, and G. W. Yeo.** 2012. High-resolution profiling and analysis of viral and host small RNAs during human cytomegalovirus infection. J Virol **86:**226-35.
317. **Stefani, G., and F. J. Slack.** 2008. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol **9:**219-30.
318. **Stewart, C. R., G. A. Marsh, K. A. Jenkins, M. P. Gantier, M. L. Tizard, D. Middleton, J. W. Lowenthal, J. Haining, L. Izzard, T. J. Gough, C. Deffrasnes, J. Stambas, R. Robinson, H. G. Heine, J. A. Pallister, A. J.**

Foord, A. G. Bean, and L. F. Wang. 2013. Promotion of Hendra virus replication by microRNA 146a. J Virol **87:**3782-91.
319. **Stik, G., G. Dambrine, S. Pfeffer, and D. Rasschaert.** 2013. The oncogenic microRNA OncomiR-21 overexpressed during Marek's disease lymphomagenesis is transactivated by the viral oncoprotein Meq. J Virol **87:**80-93.
320. **Storey, J. D., W. Xiao, J. T. Leek, R. G. Tompkins, and R. W. Davis.** 2005. Significance analysis of time course microarray experiments. Proc Natl Acad Sci U S A **102:**12837-42.
321. **Sullivan, C. S., A. T. Grundhoff, S. Tevethia, J. M. Pipas, and D. Ganem.** 2005. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. Nature **435:**682-6.
322. **Sundaram, G. M., J. E. Common, F. E. Gopal, S. Srikanta, K. Lakshman, D. P. Lunny, T. C. Lim, V. Tanavde, E. B. Lane, and P. Sampath.** 2013. 'See-saw' expression of microRNA-198 and FSTL1 from a single transcript in wound healing. Nature **495:**103-6.
323. **Suzuki, H. I., K. Yamagata, K. Sugimoto, T. Iwamoto, S. Kato, and K. Miyazono.** 2009. Modulation of microRNA processing by p53. Nature **460:**529-33.
324. **Syggelou, A., N. Iacovidou, S. Kloudas, Z. Christoni, and V. Papaevangelou.** 2010. Congenital cytomegalovirus infection. Ann N Y Acad Sci **1205:**144-7.
325. **Tang, S., A. S. Bertke, A. Patel, K. Wang, J. I. Cohen, and P. R. Krause.** 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. Proc Natl Acad Sci U S A **105:**10931-6.
326. **Taylor-Wiedeman, J., J. G. Sissons, L. K. Borysiewicz, and J. H. Sinclair.** 1991. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. J Gen Virol **72 (Pt 9):**2059-64.
327. **Tejedor Cerdena, M. A., A. Velasco Guardado, A. Fernandez Prodomingo, M. C. Concepcion Pinero Perez, R. Calderon, A. B. Prieto Bermejo, A. Sanchez Garrido, J. Martinez Moreno, F. Geijo Martinez, O. J. Blanco Munez, and A. Rodriguez Perez.** 2011. Cytomegalovirus ileitis in an immunocompetent patient. Rev Esp Enferm Dig **103:**154-6.
328. **Tirabassi, R., L. Hook, I. Landais, F. Grey, H. Meyers, H. Hewitt, and J. Nelson.** 2011. Human cytomegalovirus US7 is regulated synergistically by two virally encoded microRNAs and by two distinct mechanisms. J Virol **85:**11938-44.
329. **Tomtishen, J. P., 3rd.** 2012. Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28). Virol J **9:**22.
330. **Triboulet, R., B. Mari, Y. L. Lin, C. Chable-Bessia, Y. Bennasser, K. Lebrigand, B. Cardinaud, T. Maurin, P. Barbry, V. Baillat, J. Reynes, P.**

Corbeau, K. T. Jeang, and M. Benkirane. 2007. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. Science **315:**1579-82.

331. **Tsai, W. P., M. H. Chen, M. H. Lee, K. H. Yu, M. W. Wu, and L. B. Liou.** 2012. Cytomegalovirus infection causes morbidity and mortality in patients with autoimmune diseases, particularly systemic lupus: in a Chinese population in Taiwan. Rheumatol Int **32:**2901-8.
332. **Tuddenham, L., and S. Pfeffer.** 2011. Roles and regulation of microRNAs in cytomegalovirus infection. Biochim Biophys Acta **1809:**613-22.
333. **Tugizov, S., E. Maidji, and L. Pereira.** 1996. Role of apical and basolateral membranes in replication of human cytomegalovirus in polarized retinal pigment epithelial cells. J Gen Virol **77 (Pt 1):**61-74.
334. **Tumilowicz, J. J., M. E. Gawlik, B. B. Powell, and J. J. Trentin.** 1985. Replication of cytomegalovirus in human arterial smooth muscle cells. J Virol **56:**839-45.
335. **Umbach, J. L., M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature **454:**780-3.
336. **Umbach, J. L., M. A. Nagel, R. J. Cohrs, D. H. Gilden, and B. R. Cullen.** 2009. Analysis of human alphaherpesvirus microRNA expression in latently infected human trigeminal ganglia. J Virol **83:**10677-83.
337. **Urbich, C., A. Kuehbacher, and S. Dimmeler.** 2008. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res **79:**581-8.
338. **van der Krol, A. R., L. A. Mur, M. Beld, J. N. Mol, and A. R. Stuitje.** 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:**291-9.
339. **Varmuza, S. L., and J. R. Smiley.** 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell **41:**793-802.
340. **Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp, 2nd, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson.** 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. J Virol **78:**10960-6.
341. **Vasudevan, S., Y. Tong, and J. A. Steitz.** 2007. Switching from repression to activation: microRNAs can up-regulate translation. Science **318:**1931-4.
342. **Veksler-Lublinsky, I., Y. Shemer-Avni, E. Meiri, Z. Bentwich, K. Kedem, and M. Ziv-Ukelson.** 2012. Finding quasi-modules of human and viral miRNAs: a case study of human cytomegalovirus (HCMV). BMC Bioinformatics **13:**322.

343. **Vo, N., M. E. Klein, O. Varlamova, D. M. Keller, T. Yamamoto, R. H. Goodman, and S. Impey.** 2005. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proc Natl Acad Sci U S A **102:**16426-31.
344. **Voinnet, O.** 2009. Origin, biogenesis, and activity of plant microRNAs. Cell **136:**669-87.
345. **Wahl, A., S. D. Linnstaedt, C. Esoda, J. F. Krisko, F. Martinez-Torres, H. J. Delecluse, B. R. Cullen, and J. V. Garcia.** 2013. A cluster of virus-encoded microRNAs accelerates acute systemic Epstein-Barr virus infection but does not significantly enhance virus-induced oncogenesis in vivo. J Virol **87:**5437-46.
346. **Wang, A., L. Ren, and H. Li.** 2011. A systemic network triggered by human cytomegalovirus entry. Adv Virol **2011:**262080.
347. **Wang, F. Z., F. Weber, C. Croce, C. G. Liu, X. Liao, and P. E. Pellett.** 2008. Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. J Virol **82:**9065-74.
348. **Wang, X., D. Y. Huang, S. M. Huong, and E. S. Huang.** 2005. Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. Nat Med **11:**515-21.
349. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature **424:**456-61.
350. **Wang, Y., N. Kato, A. Jazag, N. Dharel, M. Otsuka, H. Taniguchi, T. Kawabe, and M. Omata.** 2006. Hepatitis C virus core protein is a potent inhibitor of RNA silencing-based antiviral response. Gastroenterology **130:**883-92.
351. **Wang, Z., H. Yao, S. Lin, X. Zhu, Z. Shen, G. Lu, W. S. Poon, D. Xie, M. C. Lin, and H. F. Kung.** 2013. Transcriptional and epigenetic regulation of human microRNAs. Cancer Lett **331:**1-10.
352. **Wei, X., C. Tan, C. Tang, G. Ren, T. Xiang, Z. Qiu, R. Liu, and Z. Wu.** 2013. Epigenetic repression of miR-132 expression by the hepatitis B virus x protein in hepatitis B virus-related hepatocellular carcinoma. Cell Signal **25:**1037-43.
353. **Wolf, D. G., C. T. Courcelle, M. N. Prichard, and E. S. Mocarski.** 2001. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc Natl Acad Sci U S A **98:**1895-900.
354. **Wolfstein, A., C. H. Nagel, K. Radtke, K. Dohner, V. J. Allan, and B. Sodeik.** 2006. The inner tegument promotes herpes simplex virus capsid motility along microtubules in vitro. Traffic **7:**227-37.
355. **Wu, H., C. Ye, D. Ramirez, and N. Manjunath.** 2009. Alternative processing of primary microRNA transcripts by Drosha generates 5' end variation of mature microRNA. PLoS One **4:**e7566.

356. **Wu, J., and X. Xie.** 2006. Comparative sequence analysis reveals an intricate network among REST, CREB and miRNA in mediating neuronal gene expression. Genome Biol **7:**R85.
357. **Wu, L., J. Fan, and J. G. Belasco.** 2006. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A **103:**4034-9.
358. **Wu, Z., Y. Zhu, D. M. Bisaro, and D. S. Parris.** 2009. Herpes simplex virus type 1 suppresses RNA-induced gene silencing in mammalian cells. J Virol **83:**6652-63.
359. **Xia, T., A. O'Hara, I. Araujo, J. Barreto, E. Carvalho, J. B. Sapucaia, J. C. Ramos, E. Luz, C. Pedroso, M. Manrique, N. L. Toomey, C. Brites, D. P. Dittmer, and W. J. Harrington, Jr.** 2008. EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res **68:**1436-42.
360. **Xin, M., E. M. Small, L. B. Sutherland, X. Qi, J. McAnally, C. F. Plato, J. A. Richardson, R. Bassel-Duby, and E. N. Olson.** 2009. MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev **23:**2166-78.
361. **Yamamoto, A. Y., R. A. Castellucci, D. C. Aragon, and M. M. Mussi-Pinhata.** 2012. Early high CMV seroprevalence in pregnant women from a population with a high rate of congenital infection. Epidemiol Infect**:**1-5.
362. **Yekta, S., I. H. Shih, and D. P. Bartel.** 2004. MicroRNA-directed cleavage of HOXB8 mRNA. Science **304:**594-6.
363. **Yi, R., Y. Qin, I. G. Macara, and B. R. Cullen.** 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev **17:**3011-6.
364. **Yu, B., and H. Wang.** 2010. Translational inhibition by microRNAs in plants. Prog Mol Subcell Biol **50:**41-57.
365. **Yu, H., J. Lu, L. Zuo, Q. Yan, Z. Yu, X. Li, J. Huang, L. Zhao, H. Tang, Z. Luo, Q. Liao, Z. Zeng, J. Zhang, and G. Li.** 2012. Epstein-Barr virus downregulates microRNA 203 through the oncoprotein latent membrane protein 1: a contribution to increased tumor incidence in epithelial cells. J Virol **86:**3088-99.
366. **Yurochko, A. D., T. F. Kowalik, S. M. Huong, and E. S. Huang.** 1995. Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. J Virol **69:**5391-400.
367. **Yurochko, A. D., M. W. Mayo, E. E. Poma, A. S. Baldwin, Jr., and E. S. Huang.** 1997. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-kappaB promoters. J Virol **71:**4638-48.
368. **Zhang, G. L., Y. X. Li, S. Q. Zheng, M. Liu, X. Li, and H. Tang.** 2010. Suppression of hepatitis B virus replication by microRNA-199a-3p and microRNA-210. Antiviral Res **88:**169-75.

369. **Zhang, X., E. Zhang, Z. Ma, R. Pei, M. Jiang, J. F. Schlaak, M. Roggendorf, and M. Lu.** 2011. Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. Hepatology **53:**1476-85.
370. **Zheng, S. Q., Y. X. Li, Y. Zhang, X. Li, and H. Tang.** 2011. MiR-101 regulates HSV-1 replication by targeting ATP5B. Antiviral Res **89:**219-26.
371. **Zheng, Z. M., and X. Wang.** 2011. Regulation of cellular miRNA expression by human papillomaviruses. Biochim Biophys Acta **1809:**668-77.
372. **Zhou, H., M. L. Arcila, Z. Li, E. J. Lee, C. Henzler, J. Liu, T. M. Rana, and K. S. Kosik.** 2012. Deep annotation of mouse iso-miR and iso-moR variation. Nucleic Acids Res **40:**5864-75.
373. **Zhu, H., J. P. Cong, G. Mamtora, T. Gingeras, and T. Shenk.** 1998. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. Proc Natl Acad Sci U S A **95:**14470-5.
374. **Zhu, Y., N. C. Cherukuri, J. N. Jackel, Z. Wu, M. Crary, K. J. Buckley, D. M. Bisaro, and D. S. Parris.** 2012. Characterization of the RNA silencing suppression activity of the Ebola virus VP35 protein in plants and mammalian cells. J Virol **86:**3038-49.