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# Degradation of Host MicroRNAs by Poxvirus Poly(A) Polymerase Reveals Terminal RNA Methylation as a Protective Antiviral Mechanism

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## SUMMARY

The life cycle of several viruses involves host or virally encoded small noncoding RNAs, which play important roles in posttranscriptional regulation. Small noncoding RNAs include microRNAs (miRNAs), which modulate the transcriptome, and small interfering RNAs (siRNAs), which are involved in pathogen defense in plants, worms, and insects. We show that insect and mammalian poxviruses induce the degradation of host miRNAs. The virally encoded poly(A) polymerase, which polyadenylates viral transcripts, also mediates 3′ polyadenylation of host miRNAs, resulting in their degradation by the host machinery. In contrast, siRNAs, which are protected by 2′O-methylation (2′OMe), were not targeted by poxviruses. These findings suggest that poxviruses may degrade host miRNAs to promote replication and that virus-mediated small RNA degradation likely contributed to 2′OMe evolution.

## INTRODUCTION

Nucleic acid recognition is at the heart of self/non-self-discrimination. Organisms from bacteria to plants and humans sense foreign invaders such as viruses using a variety of nucleic acid sensors. These sensors distinguish particular nucleic acid structures, which either leads to the direct degradation of the molecule or the induction of signaling cascades that induce antipathogen effectors. One such pathway used by plants, worms, and insects is mediated by the small interfering RNAs (siRNAs) derived from exogenous sources such as the invading pathogen (viral siRNAs [vsiRNAs]), or from endogenous double-stranded RNA derived from convergently transcribed mRNAs (endogenous siRNAs [siRNAs]) (Carthew and Sontheimer, 2009). These small RNAs are subsequently used to induce RNA interference (RNAi) to target and degrade viral RNAs or complementary genome-encoded transcripts (Ding, 2010). A related process, active in the germline of insects and mammals, is the Piwi-interacting RNA (piRNA) pathway; piRNAs are a distinct class of small noncoding RNAs implicated in silencing transposable elements in order to protect the organism from another example of self-replicating nucleic acids (Siomi et al., 2011).



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Interestingly, while chordates have maintained piRNA-mediated defenses, the somatic response to virus infection is thought to be independent of small RNA biogenesis. In place of RNAi, chordates respond to virus infection through the recognition of viral nucleic acids to induce type I interferons (IFN-I), cytokines responsible for the upregulation of >250 genes, which confer an antiviral state on the cell and its neighbors (Kawai and Akira, 2006). While the underlying reason as to why chordates seemingly replaced RNAi with IFN-I remains unknown, it is clear that small RNAs, such as microRNAs (miRNAs), have the potential to restrict virus infection (Lecellier et al., 2005). Perhaps this is best illustrated by the fact that viruses can be engineered to be targeted by miRNAs (Barnes et al., 2008; Edge et al., 2008; Kelly et al., 2008; Perez et al., 2009; Langlois et al., 2012b; Pham et al., 2012).

miRNAs are genome-encoded small RNA species generally derived from RNA Polymerase II transcripts and are modulated at the levels of transcription and processing (Hagan et al., 2009; Han et al., 2011; Heo et al., 2009; Johnson et al., 2003; Liu et al., 2011). In brief, the primary transcript (pri-miRNA) is recognized by a nuclear Microprocessor complex, which includes the RNase III enzyme, Drosha, and its binding partner DGCR8 in mammals and pasha in insects (Bartel, 2004). The nuclear Microprocessor cleaves the pri-miRNA into a precursor miRNA (pre-miRNA) hairpin structure with a 2 nucleotide (nt) 3′ overhang (Siomi and Siomi, 2010). The pre-miRNA is exported from the nucleus and is subsequently cleaved by a cytoplasmic RNase III enzyme, Dicer, to generate a duplex RNA of ~22 nt, flanked with 2 nt 3′ overhangs (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001). Duplex RNA is then loaded into an RNA-induced silencing complex (RISC), a multisubunit structure that includes argonaute proteins (Czech and Hannon, 2011). RISC performs strand selection of the RNA duplex, based at least in part on the thermodynamic stability of the respective 5′ ends, and uses the mature miRNA as a guide to induce posttranscriptional silencing (PTS) on target mRNAs (Bartel, 2004). In addition to the canonical biogenesis pathway, select miRNAs can be generated in the absence of the Microprocessor or Dicer (Yang and Lai, 2011).

In general, miRNAs modulate transcript levels by less than 2-fold (Baek et al., 2008; Selbach et al., 2008) and have little impact on the acute cellular response to virus infection (Cullen, 2011). In contrast, there is significant interplay between miRNAs and chronic viral infections. For example, hepatitis C virus exploits tissue-specific miR-122 to enhance replication (Jopling et al., 2006), while a number of nuclear DNA viruses and retroviruses have usurped this pathway by expressing virus-encoded miRNAs to manipulate the host transcriptome (Gottwein et al., 2011; Kincaid et al., 2012; Pfeffer et al., 2004). Taken together, these observations suggest that viral utilization of miRNAs is reserved for microbes with sustained replication cycles and that virus-encoded miRNAs are restricted to pathogens with large DNA genomes (Cullen, 2011). One interesting exception to this generalization is the family of poxviruses, a unique group of DNA viruses replicating exclusively in the cytoplasm.

We asked why poxviruses, such as vaccinia virus (VACV), have not been reported to exploit the cellular miRNA pathway. These large, double-stranded DNA viruses transcribe genes with long 3′ UTRs (Yang et al., 2010, 2011) and could therefore benefit from the posttranscriptional regulation provided by miRNAs. Since cytoplasmic-mediated synthesis of miRNAs has now been widely demonstrated (Langlois et al., 2012a; Rouha et al., 2010; Shapiro et al., 2010, 2012) this does not explain the apparent lack of encoded miRNAs. Thus, we investigated the interplay between small RNAs and poxviruses. To this end, we analyzed small RNAs from poxvirus-treated mammalian and insect cells and discovered that infection induced tailing and subsequent degradation of host-derived miRNAs mediated by the virally encoded poly(A) polymerase. Interestingly, this activity was not observed on endogenous siRNAs (esiRNAs) that contain a terminal 2′O-methyl group. Indeed, miRNAs

with this modification were also protected. Given that 2′O-methylation (2′OMe) of host mRNAs and tRNAs has been implicated in discerning between host and pathogen RNA (Daffis et al., 2010), we propose that the evolution of this RNA modification has evolved more broadly as a tool to combat infection.

## RESULTS

### Poxvirus-Mediated Degradation of miRNAs in Insects

Poxviruses are an expansive class of pathogens that infect diverse animals and encode many host evasion factors (McFadden, 2005; Moss, 2007). Little is known about insect-borne poxviruses and whether they target antiviral defenses in their insect hosts. One major antiviral defense strategy in insects is RNA silencing, which uses small RNAs to target viral replication (Ding, 2010). Furthermore, since insect cells utilize both miRNAs and siRNAs, we used this model system to begin investigating the interplay between poxviruses and these diverse small RNAs. To determine whether poxviruses interfere with RNA silencing, we sequenced the small RNA population of *Drosophila melanogaster* cells in response to VACV infection and compared this to infection by Sindbis virus (SINV), a virus that does not impact small RNA silencing (Myles et al., 2008). Interestingly, we observed an addition of nontemplated adenosines at the 3′ end of miRNAs such as miR-34 (Figures 1A and 1B). In contrast, esiRNAs were unaffected by VACV infection (Figure 1B). In VACV-infected cells, we observed that all miRNAs (bantam, miR-7, and miR-34 are shown) had the addition of nontemplated adenosines at the 3′ end (Figures 1A, 1B, S1A, and S1B and Table S1). Northern blot analysis revealed that miRNAs from VACV-infected cells migrated more slowly than in uninfected cells, and the reduced mobility of these miRNAs was coupled with a decrease in miRNA levels, suggesting that the modified miRNAs were degraded (Figure 1C). This activity is reminiscent of tailing and trimming of small RNAs in *Drosophila*, which has been described previously (Ameres et al., 2010).

We next set out to determine whether miRNA polyadenylation was dependent upon either of the two argonautes present in these cells, Ago1 and Ago2, which mediate miRNA and siRNA silencing, respectively (Czech et al., 2009; Kim et al., 2009; Okamura et al., 2004). While the loss of Ago2 did not impact the polyadenylation status of miRNAs in VACV-infected cells, depletion of Ago1 led to a decrease in nontemplated polyA tailing (Figure 1B and Table S1). Interestingly, extensive tailing was not observed on miRNA star strands or endogenous siRNA (esiRNA) esi1.1 and esi2.1 (Figures 1B and S1C and Tables S1A and S1B), although the basal levels of tailing likely reflect a small degree of cross-loading of each of these small RNAs into Ago1 versus Ago2 (Czech et al., 2009; Förstemann et al., 2007; Ghildiyal et al., 2010; Okamura et al., 2009; Tomari et al., 2007). Further, upon loss of Ago2 we observed an increase in the tailing of esiRNAs, again reflecting the cross-loading of these small RNAs into an Ago1-containing complex in the absence of Ago2 (Figure 1B). Together, these data suggest that Ago1-loaded small RNAs are tailed and degraded during poxvirus infection.

Given the artificial nature of mammalian poxvirus infection of insect cells, we tested a bona fide insect- poxvirus pair. We challenged the tiger moth *Amsacta moorei* cells with *Amsacta moorei* entomopoxvirus (AMEV) and found that infection also resulted in the specific loss of all mature miRNAs tested, including miR-11, miR-34, and miR-184 (Figure 1D). Other small RNAs such as U6 were unaffected by AMEV infection (Figure 1D).

### VACV-Mediated Degradation of miRNAs in Mammalian Cells

We next set out to determine whether VACV, which normally infects mammals, also targets mammalian miRNAs for degradation. As previous studies have established that cytoplasmic

RNA viruses can be engineered to encode and generate functional miRNAs (Langlois et al., 2012a; Rouha et al., 2010; Shapiro et al., 2010, 2012), we engineered VACV to synthesize the neuronal-specific miR-124 (VV124) as a tool to study virus-mediated tailing. To obtain expression throughout the viral replication cycle, we placed the miRNA under transcriptional control of a VACV synthetic early-late promoter that is transcribed by the virus-encoded DNA-dependent RNA polymerase (Moss, 2007). This allowed us to directly compare plasmid-derived miR-124 (p124) transfections and cells infected with either SINV or Vesicular Stomatitis Virus (VSV) expressing miR-124 (SV124 and VSV124, respectively) (Figure 2A). Interestingly, while SV124, VSV124, and p124 demonstrated robust miRNA accumulation, VV124 infections resulted in low levels of miR-124 synthesis and a nearly complete loss of endogenous miR-93, while U6 RNA levels were unaffected. Furthermore, we observed that while the pri- and pre-miR-124 derived from VV124 ran as a single species, mature miR-124 products ranged from ~22–35 nt, suggesting improper processing or a posttranscriptional modification similar to our observations in insect cells.

To explore this phenomenon further, VV124 infection kinetics were performed (Figure 2B). These data demonstrated production of a single miR-124 species of ~22 nt as early as 3 hr postinfection (hpi). Levels of miR-124 increased at 6 hpi, and we also observed the generation of the slower migrating products ranging from ~20–35 nt, reminiscent of the miRNA tailing that results following perfect mRNA binding (Ameres et al., 2010). Beyond 6 hpi, total levels of the mature miR-124 stabilized, but the majority of VV124-derived small RNAs were greater than 22 nt, with a dominant species migrating at ~30 nt in length. The appearance of the 30 nt product correlated with the loss of endogenous miR-93, where we also observed a slower migrating product. This activity was not cell type- or species-specific, as it was equally apparent in other cell types (Figures 2A, 2B, S2A, and S2B). Furthermore, degradation of endogenous miR-93 was not specific to VV124 infection, as wild-type VACV, lacking the miR-124 locus (VVctrl), also resulted in loss of this endogenous miRNA (Figures 2B and S2).

Next, we determined the mechanism by which VACV-mediated miR-124 synthesis occurred. Processing could be mediated in a canonical manner, as observed for engineered influenza A virus expressing miR-124 (IAV124), although influenza replicates in the nucleus and VACV replicates exclusively in the cytoplasm. Alternatively, noncanonical processing was observed for cytoplasmic RNA viruses such as SV124 and VSV124 (Langlois et al., 2012a; Rouha et al., 2010; Shapiro et al., 2010, 2012). VV124 infection of wild-type murine fibroblasts resulted in the production of miR-124 species ranging from ~22 nt to greater than 30 nt in length with a corresponding decrease in endogenous miR-93 expression (Figure S2B). DGCR8, which is essential for canonical processing of pri- to pre-miRNA (Wang et al., 2007), was dispensable. Interestingly, despite normal pre-miR-124 levels, VACV-derived mature miR-124 was completely absent in these conditions, suggesting that the lack of endogenous miRNAs may accelerate miR-124 degradation. This was supported by kinetic experiments in cells treated with Cre to disrupt DGCR8 expression, in which low levels of VACV-mediated miR-124 is detectable, even in the absence of endogenous miR-93 (Figure S2C). Furthermore, as expected, no VACV-mediated miRNA production was evident in the absence of Dicer, which is required both for canonical and noncanonical processing (Figure S2A). Taken together, these data demonstrate that VV124-mediated miRNA generation requires a noncanonical processing pathway similar to that described for other cytoplasmic viruses (Langlois et al., 2012a; Rouha et al., 2010; Shapiro et al., 2010,2012).

## An Early Viral Factor Mediates VACV-Induced miRNA Modifications

Expression of the ~200 VACV-encoded proteins occurs sequentially through the regulation by stage-specific promoters and transcription factors; immediately after infection, early

genes are expressed, followed by viral genome replication and subsequently intermediate followed by late gene expression (Moss, 2007). VACV infection of *Drosophila* cells results in early gene expression, but arrests prior to intermediate gene expression (Bengali et al., 2011; Moser et al., 2010), suggesting a requirement for an early gene product. To verify this hypothesis, we used cytosine arabinoside (AraC) and cycloheximide (CHX) to block late and early-late protein synthesis, respectively. Treatment of VV124-infected cells with CHX allowed for robust expression of the miRNA and blocked laddering of miR-124 and the degradation of endogenous miR-93 (Figure 3). In contrast, loss of endogenous miR-93 was observed upon treatment with AraC, which only allows for early gene expression. Western blot analysis of lysates obtained from these samples validated the block of early/late (E3/A27) and late (A27) protein synthesis caused by CHX and AraC, respectively (Figure S3). Taken together, these data suggest that the posttranscriptional modification or improper processing of host miRNAs is dependent on an early viral gene product or an encapsidated virion protein released upon uncoating in both insect and mammalian cells (Joklik and Becker, 1964).

## VACV Infection Induces miRNA-Specific Polyadenylation

In an effort to determine the nature of the slower migrating VACV-derived miR-124 products, we deep sequenced the 20–35 nt RNAs from VV124-infected cells and analyzed the small RNA populations by deep sequencing. This analysis demonstrated that greater than 50% of VACV-derived miR-124 contained 7–9 adenosines on the 3′ end (Figure 4A and Table S2). Interestingly, this same level of polyadenylation was not observed on miR-124 star. As this pattern of polyadenylation could, in part, be explained by tailing of the pre-miRNAs prior to Dicer cleavage, we subsequently analyzed miRNAs produced from the 5′ end of the hairpin, such as miR-31. Analysis of small RNAs ranging from 20 to 35 nt revealed that, similar to miR-124, miR-31, as well as all other host miRNAs, were polyadenylated with greater than 50% of the captured reads containing 7–9 adenosines (Figure 4B and Table S2). Furthermore, we found little evidence for the polyadenylation of miRNA star strands, nor non-miRNAs captured in this RNA fraction (Table S2). The polyadenylation of miR-124 and miR-31 was also evident by northern blot, demonstrating tailing in the presence of VV124 infection (Figure 4C), whereas pre-miR-124, miR-124*, tRNA, and U6 all appeared unmodified (Figure 4D).

## VACV-Induced miRNA Polyadenylation Results in Degradation

To determine whether the degradation of endogenous miRNAs was biased in any way, we performed a second round of small RNA deep sequencing on the 19–22 nt fraction of VV124-infected murine embryonic fibroblasts (Tables S3A and S3B). This analysis demonstrated that infection led to a 30-fold reduction of all endogenous miRNAs, with the exception of VACV-produced miR-124, while still maintaining the same overall small RNA profile, similar to our results in *Drosophila* cells (Figure 5A and Table S1A). Again, we did not observe any modification of non-miRNAs in this fraction (Table S3B).

Nontemplated tailing is emerging as a general mechanism used to degrade miRNAs (Ameres et al., 2010; Kim et al., 2009; Li et al., 2005; Marcinowski et al., 2012; Shen and Goodman, 2004; van Wolfswinkel et al., 2009). In order to investigate whether the polyadenylation of miRNAs was directly responsible for degradation and to discern whether a cellular- or a virus-encoded nuclease was involved, we designed miR-124 mimetics containing no (124), one (124 + 1A), or seven (124 + 7A) nontemplated adenosines at the 3′ end (Figure S4) and transfected these into mammalian cells. Small RNA northern blot analyses of these miR-124 mimetics demonstrated that the degradation of tailed miRNAs was directly related to polyadenylation and was not the result of a virus-encoded nuclease, as the synthetic tailed miR-124 containing seven adenosines was degraded in the absence of

infection (Figure 5B). Altogether, these data suggest that poxvirus-induced polyadenylation is specific for miRNAs, does not display a sequence-specific bias, and results in degradation by the cell.

## VACV VP55 Is Both Necessary and Sufficient for miRNA Polyadenylation

VACV polyadenylates its own transcripts to generate mature viral mRNAs, and the virus-encoded heterodimeric poly(A) polymerase is responsible for this activity. The larger subunit, VP55, initiates polyadenylation and possesses all of the catalytic functions, whereas the processivity factor, VP39, extends the poly(A) tail (Gershon et al., 1991; Moss et al., 1975). As the modification of miRNAs was limited to the addition of less than ten adenosines, we speculated that VP55, which is also an early VACV gene product and is encapsulated in the virion (Assarsson et al., 2008; Chung et al., 2006; Moss et al., 1975), was the probable virus component responsible. To test this, we transfected either control (scrambled [scbl]) or VP55-specific siRNAs and infected with VV124. Control siRNAs had no effect on the polyadenylation of VACV-derived miR-124 or the degradation of endogenous miR-93 (Figure 6A). In contrast, cells treated with siRNAs against VP55 produced VACV-derived miR-124 with no evidence of modification and did not lead to the degradation of endogenous miR-93 (Figure 6A). While these data suggest that VP55 is necessary for the polyadenylation and degradation of miRNAs in VACV-infected cells, loss of VP55 also resulted in an expected block in VACV replication (compare early protein expression E3 to the late A27 virus product) (Figure S5A).

Thus, we next determined whether VP55 was sufficient to confer this activity. Flag-tagged VP39 and/or VP55 were transfected into fibroblasts in the presence or absence of plasmid-derived miR-124 (Figure S5B). At 36 hr posttransfection (hpt), northern blot analysis revealed that expression of VP55 alone was sufficient to induce specific tailing of miR-124 (Figure 6B). Furthermore, endogenous miR-93 was lost upon expression of VP55 (Figure 6C). In contrast, VP39 had no impact on either miRNA (Figures 6B and 6C). Again, VP55 expression did not impact miR-124*, tRNA, or U6, demonstrating a similar specificity profile as observed during VACV infection. Taken together, these results demonstrate that VACV VP55 is both necessary and sufficient for the specific polyadenylation of miRNAs.

To determine whether polyadenylation had an impact on miRNA-mediated silencing, we cotransfected fibroblasts with epitope-tagged VP55 together with a miR-124-expressing plasmid (p124) and a miR-124-targeted GFP (GFP-124T) reporter construct containing four perfect miR-124 target sites (Figure 6D). As expected, plasmid-derived miR-124 expression was robust and was extensively modified in the presence of VP55. Moreover, expression of miR-124 resulted in a complete loss of GFP, whereas coexpression of VP55 rescued GFP expression, suggesting that miRNA tailing rendered miR-124 nonfunctional (Figure 6D).

As VP55 represents a critical component of the poxviral replication machinery, determining the physiological requirement for this protein in the context of miRNA degradation is difficult, as these activities cannot be separated. As such, in an effort to determine whether host miRNAs interfere with poxvirus replication, we reconstituted miRNAs in VACV-infected cells with miRNA duplex mimetics or control mimetics and determined the impact on virus replication. To this end, we transfected a pool of the let-7 family, miR-21, miR-22, and miR-93. We chose this group because they represent greater than 50% of total miRNAs found in fibroblasts (Table S3B). While VACV infection reduced endogenous miRNAs to undetectable levels, the transfected mimetics were not degraded, even at 60 hpi (Figures S5C). In comparing virus replication in miRNA-reconstituted and control cells, we monitored the two distinct infectious forms of VACV: the more abundant intracellular mature virus (IMV) that remains in the infected cell until lysis, and an extracellular enveloped virus (EEV) that is propelled along actin tails to infect more distant cells or is

released (Moss, 2007). At 60 hpi, total virus levels (IMV and EEV) were significantly reduced ($p = 0.0038$), demonstrating approximately half the presence of total virus in cells reconstituted with miRNA mimetics (Figure S5D). Importantly, we observed the same reduction in EEV titer in the supernatants of miRNA mimetic-transfected cells compared to control transfections (Figure S5E). Given that EEVs play a fundamental role in virus dissemination, a 50% loss of EEVs would likely result in a significant in vivo attenuation of the virus (Payne, 1980; Payne and Kristensson, 1985). We conclude from these data that host miRNAs, including the let-7 family, miR-21, miR-22, and miR-93, reduce the production of infectious virions, either directly or indirectly, and that VACV counteracts this activity.

### 3′-Terminal Nucleotide Methylation Protects Small RNAs from VACV-Mediated Ago-Dependent Polyadenylation

With no evidence of miRNA star modifications, we hypothesized that polyadenylation occurred after strand selection in RISC. To determine whether VACV-modified miRNAs were loaded into RISC, we immunoprecipitated Ago2-associated RNA fractions from VV124-infected cells (Figure 7A). Input RNA from epitope-tagged Ago2- or GFP-expressing cells demonstrated the characteristic polyadenylation of VACV-derived miR-124 and loss of endogenous miR-93. Furthermore, RNA from corresponding immunoprecipitations demonstrated that, while no miRNAs associated with GFP, nonadenylated miR-124*, miR-93, and all species of miR-124 were associated with Ago2. These data implicate that miRNA tailing occurs sometime after strand selection by VP55 interacting with Ago or another component of mature RISC.

To further explore the mechanism underlying the specificity of VP55-mediated miRNA polyadenylation, we examined the differences between miRNAs and other cellular small RNAs. Our deep-sequencing results from *Drosophila* cell infections demonstrated that, while miRNAs were degraded, esiRNAs were spared (Figures 1B and S1C). *Drosophila* siRNAs are selectively 2′O-methylated (2′OMe) on the 3′-terminal nucleotide (Horwich et al., 2007; Kawamura et al., 2008; Saito et al., 2007), and studies have shown that 2′OMe protects small RNAs from degradative pathways including 3′-end uridylation-dependent decay and 3′-to-5′ exonuclease-mediated degradation (Ameres et al., 2010; Li et al., 2005; Yu et al., 2005). We therefore reasoned that the VACV-mediated polyadenylation-dependent decay pathway may also be blocked by this modification. Indeed, VACV infection of cells transfected with miR-124/miR-124* duplexes with 2′OMe modifications on miR-124 were completely protected from VACV-mediated polyadenylation, while the unmethylated form was targeted (Figures 7B and 7C). miR-124*, despite the lack of a 3′ 2′OMe conjugation, also showed no evidence of tailing, suggesting that protection of the passenger strand may be due to the fact that polyadenylation occurs after strand selection, in agreement with results observed from de novo infections where star strands are not modified (Figures 1B, 4D, and 7A). These findings suggest that poxviruses inactivate Ago-loaded regulatory small RNAs unless they are protected by terminal 2′OMe. VP55-mediated destruction of miRNAs offers an explanation why poxviruses, with their large DNA genomes, seemingly do not encode and utilize miRNAs in similar ways as many herpesviruses do (Cullen, 2011).

## DISCUSSION

Following the discovery that cytoplasmic viruses were capable of generating functional miRNAs, we set out to explore the interplay between poxviruses and miRNAs. This led to the discovery that poxviruses tailed miRNAs and induced their degradation. As miRNA tailing was due to 3′ polyadenylation, this implicated the catalytic subunit of the viral poly(A) polymerase, VP55. Importantly, VP55 is both an early gene product and is

encapsulated in the virion and is both necessary and sufficient for miRNA tailing. VP55 induced the specific polymerization of 2–7 adenosines onto miRNAs after strand selection, as neither the star strand nor comparably sized cellular RNAs were tailed. Furthermore, we found that endogenous siRNAs were protected from polyadenylation. In contrast to miRNAs, siRNAs are known to be methylated, which led to the discovery that 2′OMe protects these small antiviral siRNAs of the cell.

VP55-mediated destruction of miRNAs could have arisen for two distinct reasons. First, it may have evolved to evade miRNA-mediated restriction either as a result of its long 3′ UTRs or more indirectly through the control of endogenous cellular factors. Consistent with this is our observation that restoring miRNA function during VACV infection reduces viral replication (Figures S5D and S5E). Alternatively, it may have evolved to evade more generalized antiviral small RNA restriction present in lower organisms such as insects, as observed for AMEV infection of *Amsacta moorei* cells (Figure 1D). This evolutionary maintenance would then suggest that 2′OMe evolved as a cellular response to protect antipathogen small RNAs during poxvirus infection. Certainly the fact that poxviruses, as a family, can infect both invertebrate and vertebrate hosts suggests that a common ancestor may have evolved this antagonistic activity as a means of innate immune evasion. In either case, the activity of VP55 would have provided the virus an environment that lacked a large pool of non-2′OMe small noncoding RNAs, which may have resulted in a dependency on this activity, regardless of its original necessity.

Interestingly, the utilization of a small RNA methyltransferase appears relatively recently in our evolutionary history. The relationships between different eukaryotic lineages of HEN1, the methyl transferase of siRNAs and piRNAs in *Drosophila*, suggest that it originates in a common ancestor of Eukaryota, before the divergence of the Plantae and Metazoa/Fungi (Tkaczuk et al., 2006). This is supported by the fact that HEN1 orthologs have not be detected in Archaea or in primitive Eukaryota and is in stark contrast to the Ago protein family, which is more deeply conserved (Murphy et al., 2008). Equally interesting is the fact that HEN1 appears to have been horizontally transferred to bacteria (Tkaczuk et al., 2006), suggesting a common evolutionary pressure resulted in convergent utilization of 2′OMe for defense. Indeed, recent studies have suggested that *Escherichia coli* utilize 2′OMe to mask their tRNA from being recognized as foreign by TLR-7 (Gehrig et al. 2012; Jöckel et al., 2012). Furthermore, plants also use 2′OMe as a means of microbial protection. Loss of HEN1 renders *Arabidopsis* hypersusceptible to Cucumber Mosaic Virus (Boutet et al., 2003). In addition, Carnation Italian ringspot virus (CIRV), Tobacco etch virus (TEV), and Zucchini yellow mosaic virus (ZYMV) have all been found to inhibit HEN1 activity (Jamous et al., 2011; Lózsa et al., 2008).

Thus, 2′OMe may be a prevalent mechanism used to protect small RNAs and allow them to target pathogens for defense. Indeed, the interplay between 2′OMe and the antiviral response is not limited to small RNA-mediated silencing. Recently, the 5′ cap of mRNA was also demonstrated to contain a 2′OMe modification as a means of distinguishing cellular from viral RNA (Daffis et al., 2010; Züst et al., 2011). In turn, viruses have evolved mechanisms to 2′O-methylate their own messages. Indeed, VP39, the other component of the viral poly(A) polymerase, also has methyltransferase activity and modifies VACV mRNAs to escape detection of this cap-sensing pathway (Lockless et al., 1998). Thus the complex VP55/VP39 provides both functions required for replication (capping and polyadenylation) as well as evasion tactics (degradation of small RNAs and methylation of mRNAs). Given the interplay between microbes and 2′OMe modification, it is tempting to speculate that RNA methylation emerged in a response to evolutionary pressure, as part of the timeless struggle between host and pathogen. Clearly, identification of these interactions and the scope of 2′OMe utilization in the antiviral response warrant further investigation.

# EXPERIMENTAL PROCEDURES

## Virus Design and Infections

Recombinant VACV expressing miR-124 (VV124) was generated as described previously (Blasco and Moss, 1995). A mutant non-plaque-forming VACV (strain Western Reserve) deficient of the F13L gene (vRB12), and the VACV transfer plasmid pRB21 containing a functional F13L gene, a VACV synthetic early/late promoter, and flanking sequences of VACV DNA for homologous recombination have been described previously (Blasco and Moss, 1995). F13L encodes a component of the viral outer envelope required for efficient viral spread. The miR-124-2 murine locus (chr3:17,695,454–17,696,037) was cloned into pRB21 and transfected into vRB12-infected cells. Recombinant viruses were identified and isolated by their ability to form plaques. VVctrl contains the miR-124-2 locus in inverse orientation under control of the synthetic early-late promoter. Viruses were propagated, purified, and titrated using standard methodology.

## Cells, Infections, and Transfections

BSC-1, BHK21, and murine fibroblasts were grown at 37°C in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). *Drosophila* DL1 cells were grown and maintained at 25°C in complete *Drosophila* media (Schneider's *Drosophila* medium [GIBCO]) containing 10% FBS, GlutaMAX (GIBCO), and 1% P/S (GIBCO). *Amsacta moorei* Ld652 cells were obtained from Rollie Clem (Kansas State University, Manhattan, KS) and grown and maintained at 27°C in Grace's insect media supplemented with 10% FBS and 1% P/S (GIBCO). VACV infections of mammalian and *Drosophila* cells were performed at an moi of 10 at 37°C and at an moi of 300 at 25°C, respectively (Bengali et al., 2011; Moser et al., 2010). Cytosine β-D-arabinofuranoside (AraC; Sigma-Aldrich) and cycloheximide (CHX; Sigma-Aldrich) were used (40 μg/ml and 100 μg/ml, respectively). Infections with Sindbis virus expressing miR-124 (SV124) (Shapiro et al., 2010) and vesicular stomatitis virus expressing miR-124 (VSV124) (Langlois et al., 2012a) were performed at an moi of 1. AMEV was obtained from S.A. Perera (Great Lakes Forestry Centre, Ontario, Canada). AMEV infections of Ld652 were performed at an moi of 10 at 27°C for 48 hr (Becker et al., 2004).

## Transfections and RNA Interference

Plasmids were transfected into cells in suspension using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Unmodified, 3′-2′O-methylated, or polyadenylated miR-124 and miR-124* (Integrated DNA Technologies) or siRNAs (Thermo Scientific) were transfected into BHK cells in suspension using RNAiMAX (Invitrogen). Cells were infected with VACV 6 hr posttransfection and harvested 16 hr posttransfection. dsRNA for RNAi in *Drosophila* were designed using the SnapDragon design tool (http://www.flyrnai.org/), and PCR amplification was performed using *Drosophila* genomic DNA as a template. PCR products were then used as templates for in vitro transcription using the MEGAScript T7 kit (Ambion) as per manufacturer's instructions. dsRNA was purified using an RNeasy Kit (QIAGEN) following the manufacturer's protocol. For knockdown experiments, cells were passaged into serum-free media and plated into wells containing dsRNA at 4 μg/2.5 × $10^6$ cells in six-well plates. One hour later, complete media was added. Three days later, the cells were infected with VACV at the indicated moi in 2% FBS.

## Small RNA Northern Blot

Northern blots were reproduced a minimum of three independent times. Depicted blots reflect a representation of triplicate results. Small RNA northern blots were performed as

described previously (Pall and Hamilton, 2008). Probes used can be found in Supplemental Experimental Procedures.

### Western Blot

Western blots were generated from total protein separated on a 15% SDS-PAGE gel. Resolved protein was transferred to nitrocellulose (Bio-Rad), blocked for 1 hr with 5% skim milk at 25°C, and then incubated with the indicated antibody overnight at 4°C. The VACV E3L (BEI Resources, NR-4547) and A27L antibodies (BEI Resources, NR-567) were used at a concentration of 1 μg/ml. VACV E1L antibody (kind gift from R. Condit, University of Florida, Gainesville) was used (1:5,000). Anti-Flag (Sigma), anti-GFP (Santa Cruz Biotechnology), and anti-pan-actin (NeoMarkers) antibodies were used (0.04 μg/ml). Secondary mouse and rabbit antibodies (GE Healthcare) were used at a 1:5,000 dilution for 1 hr at 25°C. All antibodies were diluted in 5% skim milk. Immobilon Western Chemoluminescent HRP substrate (Millipore) was used as directed.

### Immunoprecipitations

BHK cells were transfected with either Flag-tagged Ago or Flag-tagged GFP (Addgene Cat Numbers 19888 and 22612, respectively) and, where indicated, cotransfected with a plasmid expressing pri-miR-124. Twenty-four hours posttransfection, cells were infected with VV124 (moi of 10), and protein extracts were harvested at the indicated time points. Immunoprecipitations were performed overnight at 4°C using 10 μg anti-Flag antibody (Sigma) and protein G PLUS Agarose (Santa Cruz Biotechnology). Beads were washed and RNA was extracted with TRIzol (Ambion) and analyzed by small RNA northern blot.

### Deep Sequencing

Deep sequencing in mammalian cells was performed on BHK21 (20–35 nt fraction) and on wild-type murine fibroblasts (19–22 nt fraction) infected with VV124 at an moi of 10 for 24 hr. Isolation, purification, and amplification of small RNA species were performed as previously described (Langlois et al., 2012a). Small RNA libraries were generated as previously described (Pfeffer et al., 2004). Briefly, total RNA from indicated samples was isolated using TRIzol (Invitrogen) and spiked with radiolabeled size markers prior to size fractionation on a 12% denaturing Tris-urea gel (SequaGel, National Diagnostics). Deep sequencing of *Drosophila* cells was performed with 40 μg total RNA from virus-infected DL1 cells. RNA was separated by electrophoresis on a 15% TBE-urea gel, and RNA molecules ~15–25 nt were excised and eluted from the gel fragments. Following ethanol precipitation, smRNA-seq libraries were produced using the Small RNA Sample Prep v1.5 kit (Illumina, San Diego, CA) as per manufacturer's instructions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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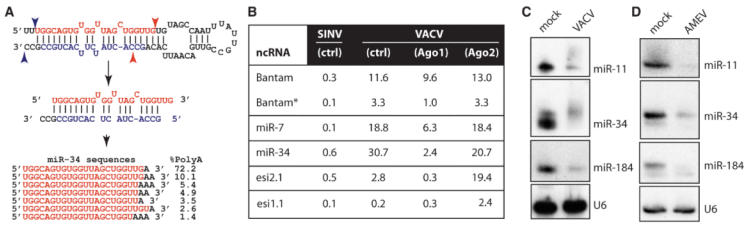


| ncRNA | SINV (ctrl) | VACV (ctrl) | VACV (Ago1) | VACV (Ago2) |
|---|---|---|---|---|
| Bantam | 0.3 | 11.6 | 9.6 | 13.0 |
| Bantam* | 0.1 | 3.3 | 1.0 | 3.3 |
| miR-7 | 0.1 | 18.8 | 6.3 | 18.4 |
| miR-34 | 0.6 | 30.7 | 2.4 | 20.7 |
| esi2.1 | 0.5 | 2.8 | 0.3 | 19.4 |
| esi1.1 | 0.1 | 0.2 | 0.3 | 2.4 |

**Figure 1. Poxvirus Infection Results in Loss of Host miRNAs in Insects**

(A) Schematic of miR-34 processing and corresponding miRNA duplex (middle) and mature miRNAs (bottom). The mature miRNA sequence-specific reads were determined by deep sequencing of the 15–25 nt fraction of VACV-infected cells. Adenosines (A) in black depict nontemplated bases and percent representation reflects the portion of the corresponding sequence in the total miRNA-specific tailed fraction.

(B) Deep-sequencing analysis of small RNA fractions of SINV-infected control (LacZ knockdown) and VACV-infected Ago1, Ago2, and control (GFP) knockdown *Drosophila* cells 72 hpi (moi of 300). Numbers reflect the percentage of polyadenylated species in relation to the unmodified mature species.

(C) Northern blot of mock or VACV-infected *Drosophila* cells analyzed for miR-11, miR-34, miR-184, and U6 (72 hpi).

(D) Northern blot of mock or AMEV-infected *Amsacta moorei* cells (moi of 10) analyzed for miR-11, miR-34, miR-184, and U6 (48 hpi). See also Figure S1 and Table S1.

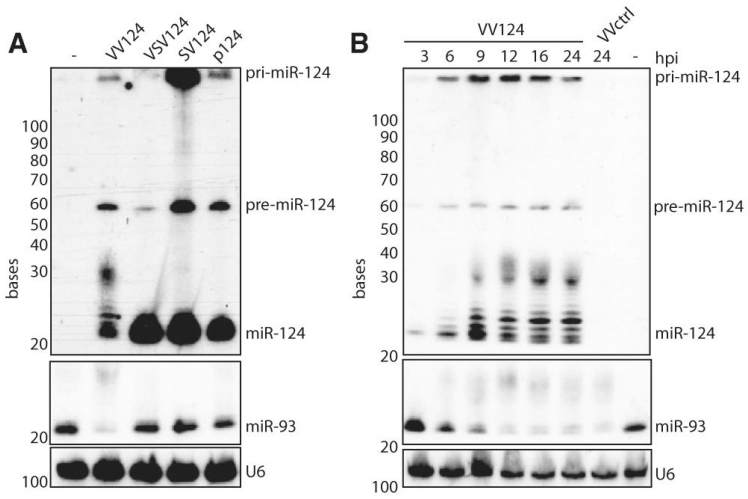


**Figure 2. Vaccinia Virus-Mediated Degradation of miRNAs**

(A) Northern blot of RNA derived from BHK cells transfected with miR-124 expressing plasmid (p124) or infected with VACV, VSV, and SV expressing miR-124 (VV124, VSV124, and SV124, respectively) for 18 hr. Northern blots were probed for miR-124, miR-93, and U6.

(B) Northern blot of RNA derived from BSC-1 cells infected with VV124 or VVctrl and analyzed for miR-124, miR-93, and U6 at the indicated time points. See also Figure S2.

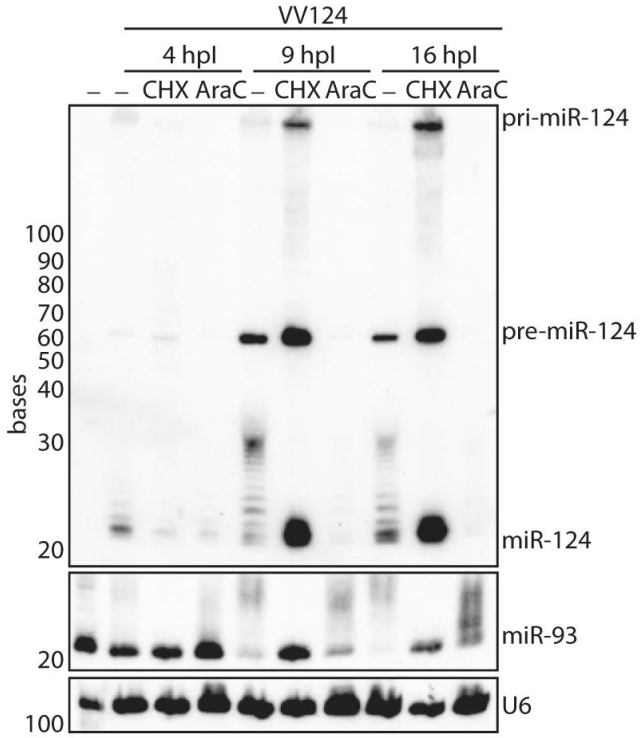


**Figure 3. An Early Viral Factor Mediates VACV-Induced miRNA Modification**

Northern blot of RNA derived from BHK cells mock treated or infected with VV124 at an moi of 10 in presence or absence of cytosine arabinoside (AraC) or cyclohexamide (CHX) and harvested at the indicated hours postinfection (hpi). Northern blots were probed for miR-124, miR-93, and U6. See also Figure S3.

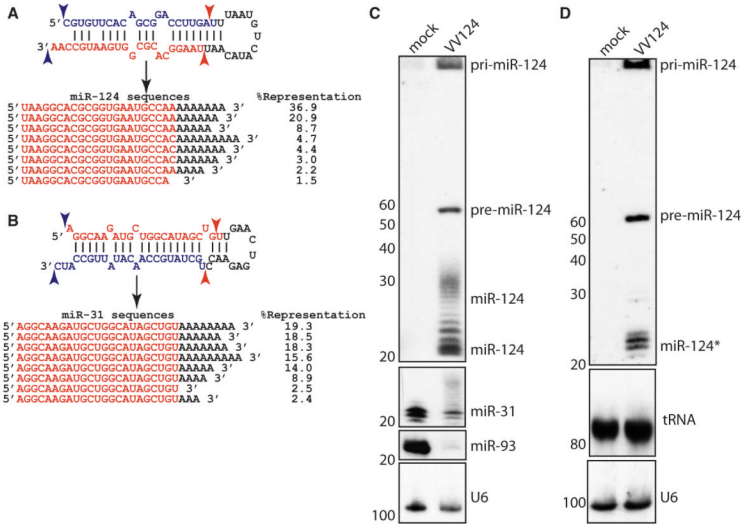


**Figure 4. VACV Infection Induces miRNA-Specific Polyadenylation**

(A and B) Schematic of pre-miR-124 (A) and pre-miR-31 (B) processing into miRNA duplex (middle) and mature miRNA (bottom). miRNA sequence-specific reads were determined by deep sequencing of the 20–35 nt fraction of VV124-infected BHK cells. Adenosines (A) in black depict nontemplated bases, and percent representation reflects the portion of the corresponding sequence represented in the total miRNA-specific tailed fraction.

(C) Northern blot analysis of RNA derived from BHK cells mock treated or infected with VV124 for 18 hr (moi of 10). Small northern blots were probed for miR-124, miR-31, and U6.

(D) Same as in (C) but probed for miR-124*, tRNA, and U6. See also Table S2.

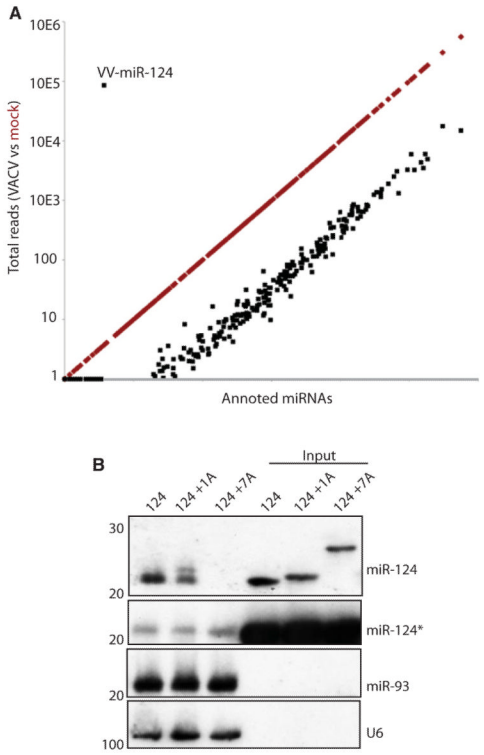


**Figure 5. VACV-Mediated miRNA Degradation Is Unbiased**

(A) Deep-sequencing analysis (19–22 nt fraction) of mock and VV124-infected murine embryonic fibroblasts, 24 hpi (moi of 10). VACV-derived miR-124 (VV-miR-124) is depicted.

(B) Northern blot of mammalian cells transfected with synthetic wild-type (miR-124) or tailed (miR-124(+1A)/miR-124(+7A)) miRNAs for 16 hr and probed for miR-124, miR-124*, miR-93, and U6. See also Figure S4 and Table S3.

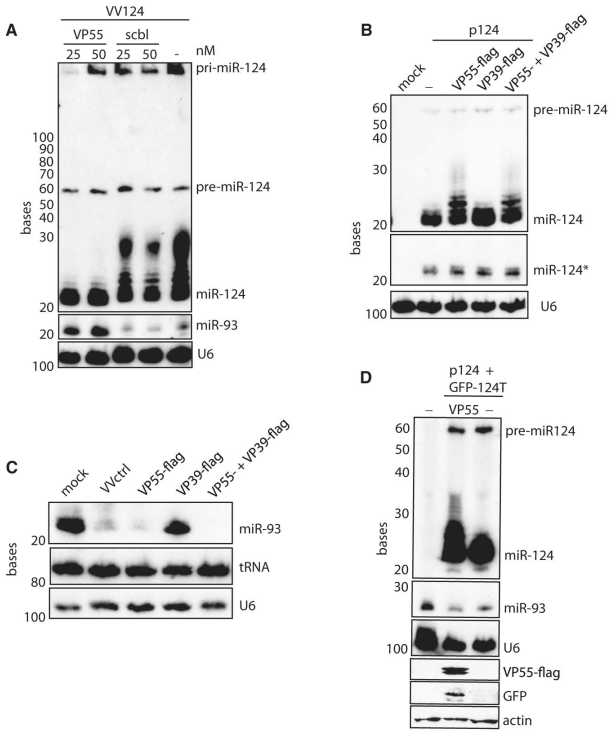


**Figure 6. VACV VP55-Mediated Tailing Inhibits miRNA Function**

(A) Analysis of BHK cells transfected with scrambled (scbl) or VP55-specific siRNAs. Six hours posttransfection, cells were mock treated or infected with VV124 for 16 hr (moi of 10). Small northern blots were probed for miR-124, miR-93, and U6.

(B) Analysis of BHK cells mock treated or transfected with plasmids expressing miR-124 (p124) and Flag-tagged VP55 and/or VP39 for 36 hr. Northern blots were probed for miR-124, miR-124*, and U6.

(C) Analysis of BHK cells mock treated, infected with VVctrl, or transfected with plasmids expressing Flag-tagged VP55 and/or VP39 for 36 hr. Northern blots were probed for miR-93, tRNA, and U6.

(D) BHK cells transfected with plasmids expressing miR-124 (p124), GFP-containing four tandem miR-124 target sites in the 3′ UTR (GFP-124T), and control or Flag-tagged VP55 for 36 hr. Top three panels: northern blot probed for miR-124, miR-93, and U6. Bottom three panels: immunoblots probed for Flag, GFP, and actin. See also Figure S5.

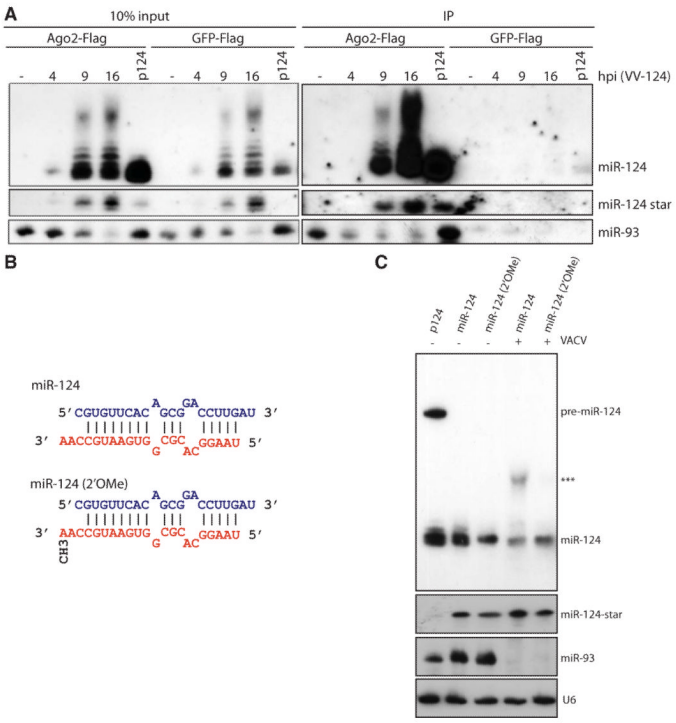


**Figure 7. 3′-Terminal Nucleotide Methylation Protects Small RNAs from VACV-Mediated Ago-Dependent Polyadenylation**

(A) Northern blot analysis of RNA derived from BHK cells transfected with plasmids expressing miR-124 (p124) and Flag-tagged Ago2 or GFP. Twenty-four hours posttransfection, cells were mock infected or infected with VV124 (moi of 10). Cell lysates were subjected to coimmunoprecipitations at the indicated hours postinfection (hpi) using anti-Flag antibody. Blots were probed for miR-124, miR-124*, and miR-93.

(B) Schematic of synthetic miR-124 duplexed miRNAs including unmethylated passenger strand (top) and 2′OMe miRNA-124 guide strand (bottom).

(C) Northern blot of RNA derived from BHK cells transfected with the miRNAs depicted in (B). Transfected cells were mock treated or VVctrl infected for 16 hr. Northern blot were probed for miR-124, miR-124*, miR-93, and U6.