Marek’s disease virus 1 (MDV-1), an oncogenic α-herpesvirus that induces T-cell lymphomas in chickens, serves as model system to study transformation by lymphotropic herpesviruses. Like the oncogenic human γ-herpesviruses Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV), MDV-1 encodes several viral microRNAs (miRNAs). One MDV-1 miRNA, miR-M4, shares the same “seed” targeting sequence with both a KSHV miRNA, miR-K11, and cellular miR-155. Importantly, miR-M4 plays a critical role in T-cell transformation by MDV-1, while miR-K11 and cellular miR-155 are thought to play key roles in B-cell transformation by KSHV and EBV, respectively. Here, we present an analysis of the mRNAs targeted by viral miRNAs expressed in the chicken T-cell line MSB1, which is naturally coinfected with MDV-1 and the related nonpathogenic virus MDV-2. Our analysis identified >1,000 endogenous mRNAs targeted by miRNAs encoded by each virus, many of which are targeted by both MDV-1 and MDV-2 miRNAs. We present a functional analysis of an MDV-1 gene, RLOF8, targeted by four MDV-1 miRNAs and a cellular gene, encoding interleukin-18 (IL-18) and targeted by both MDV-1 and MDV-2 miRNAs, and show that ectopic expression of either protein in a form resistant to miRNA inhibition results in inhibition of cell proliferation. Finally, we present a restricted list of 9 genes targeted by not only MDV-1 miR-M4 but also KSHV miR-K11 and human miR-155. Given the critical role played by miR-155 seed family members in lymphomagenesis in humans and chickens, these mRNA targets may contain genes whose inhibition plays a conserved role in herpesvirus transformation.

**IMPORANCE** Herpesviruses cause lymphomas in both humans and chickens, and in both cases, evidence indicates that virally encoded miRNAs, or virally subverted cellular miRNAs, belonging to the miR-155 seed family, play a critical role in this process. However, because each miRNA regulates numerous cellular mRNAs species, it has been difficult to elucidate which miRNA targets are important. Given the evolutionary distance between chickens and humans and the observation that miR-155 is neverthe- 

less highly conserved in both species, we reasoned that the identification of shared miR-155 targets might shed light on this pro-

cess. Here, we present an analysis of the mRNAs targeted by miRNAs encoded by the oncogenic avian herpesvirus MDV-1 in transformed chicken T cells, including a short list of mRNAs that are also targeted by miR-155 seed family members in EBV- or KSHV-transformed human B cells, and present an initial functional analysis of some of these miRNA targets.
cellular miRNAs, often encoding factors with antiviral potential, as well as in some cases regulating the expression of viral miRNAs, including miRNAs encoding factors involved in the latent-to-lytic transition of viral infections (6–9). Chickens are subject to infection by two members of the \textit{Mardivirus} genus of avian \( \alpha \)-herpesviruses, Marek’s disease virus type 1 (MDV-1) and MDV-2 (10). MDV-1 causes an economically important poultry disease (11) and induces rapid-onset T-cell lymphomas within weeks of infection of susceptible chickens (12). In contrast, the related virus MDV-2 is nonpathogenic and is widely used as a vaccine to prevent disease caused by MDV-1. MDV-1 and MDV-2 have previously been reported to encode 13 and 18 distinct premiRNAs, respectively, that give rise to numerous miRNAs and miRNA passenger strands, with some of the latter being expressed at substantial levels (13–18). However, little is known about the miRNAs targeted by these viral miRNAs, although some of the encoded antisense to viral coding sequences and therefore clearly have the potential to restrict the expression of specific viral proteins (19). One MDV-1 miRNA that has drawn significant attention is miR-M4, as it has the same seed sequence as both the cellular miRNA miR-155 (17, 19), conserved in both humans and chickens, and another viral miRNA, miR-K11, expressed by the oncogenic human \( \gamma \)-herpesvirus Kaposi’s sarcoma-associated virus (KSHV) (20, 21). Importantly, previous work has clearly demonstrated that KSHV miR-K11 and human miR-155 can indeed target very similar populations of cellular miRNAs (20, 21) and that miR-K11 can even substitute for miR-155 in promoting the normal development of B cells in vivo (22, 23).

While miR-155 is required for normal function of lymphoid cells, inappropriate expression is associated with B- and T-cell lymphoma development (24). Moreover, while for technical reasons it remains unclear whether miR-K11 promotes oncogenesis by KSHV, it is known that B-cell transformation by Epstein-Barr virus (EBV), a second oncogenic human \( \gamma \)-herpesvirus, requires the massive transcriptional upregulation of cellular miR-155 expression (25, 26), presumably to compensate for the fact that EBV, unlike KSHV, does not encode a miR-155 analog. Finally, analysis of miR-M4 function has shown that MDV-1 mutants lacking miR-M4 are severely attenuated in their ability to induce T-cell lymphomas in chickens, even though viral replication per se was largely unaffected (27). Therefore, these data indicate that transformation of B cells by human \( \gamma \)-herpesviruses, and transformation of T cells by MDV-1, requires the downregulation of one or more cellular mRNA species by a miR-155 seed family miRNA. As chicken and human miR-155 are essentially identical, we hypothesize that the same key cellular miRNAs are involved in transformation by EBV, KSHV, and MDV-1.

Previously, we sought to identify miRNA targets for KSHV miR-K11 and human miR-155 by photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) analysis of RISC binding sites in KSHV- or EBV-transformed human B-cell lines (28, 29), and similar studies have also been performed in latently KSHV- or EBV-infected B cells using the similar HITS-CLIP (high-throughput sequencing of RNA isolated by CLIP) technology (30, 31). PAR-CLIP and HITS-CLIP are techniques that allow the recovery of RISC binding sites by inducing cross-linking of miRNAs to bound RISC by UV irradiation followed by recovery, reverse transcription, and deep sequencing of these RNA binding sites (28–33). The millions of reads obtained are then bioinformatically analyzed (34), to identify RISC binding clusters on miRNAs and to assign the miRNA that has guided RISC to that cluster. Using this approach, we were previously able to identify \(~500\) mRNAs targeted by miR-155 and \(~410\) mRNAs targeted by miR-K11 (28, 29), of which at least \(\geq231\) were targeted by both miRNA species. However, this is still an unwieldy number. We therefore were interested in whether analysis of miR-M4 target sites in an MDV-1-infected chicken T-cell lymphoma would identify a smaller number of miRNAs that are targeted not only by miR-155 and miR-K11 in human cells but also by miR-M4 in chicken cells. As chicken mRNA 3′ UTRs are generally quite divergent from human mRNA 3′ UTRs, this seemed likely to be the case.

In this article, we present an analysis of the expression and miRNA targetome of MDV-1 and MDV-2 miRNAs present in the chicken T-cell lymphoma cell line MSB1 (15, 35), which is naturally coinfected by MDV-1 and MDV-2. We did not identify any novel MDV-1 or MDV-2 pre-miRNAs but did confirm the existence of the previously reported 13 MDV-1 and 18 MDV-2 premiRNAs (13–18). Interestingly, we demonstrate that three MDV-1 miRNAs and four MDV-2 miRNAs exist in two isoforms that differ by one or two nucleotides at their 5′ ends. This gives rise to two different seed sequences, and we demonstrate that each of these six viral miRNAs actually targets two overlapping but distinct sets of cellular miRNAs. Moreover, MDV-1 miR-M9-5p has the same seed sequence as the longer isoform of MDV-2, miR-M28-5p, which represents the first demonstration that MDV-1 and MDV-2 miRNAs have the potential to target some of the same sites on mRNAs. Indeed, PAR-CLIP analysis of the 3′ UTRs of cellular mRNAs bound by MDV-1 and MDV-2 miRNAs shows that these extensively overlap, albeit in most cases at distinct sites.

In terms of target identification, our data confirm that MDV-1 miR-M4, miR-M2, miR-M3, and miR-M12, located antisense to the viral RLORF8 gene, indeed restrict RLORF8 expression and demonstrate that ectopic RLORF8 expression, in a form resistant to miRNA inhibition, is toxic for MSB1 cells. We also identify the chicken interleukin-18 (IL-18) mRNA 3′ UTR as a target for two MDV-1 and five MDV-2 miRNAs and show that ectopic expression of IL-18 in a form lacking its 3′ UTR is also inhibitory for MSB1 cell growth. Finally, we identify a restricted set of nine mRNA 3′ UTRs targeted not only by miR-155 and miR-K11 in human B cells but also by miR-M4 in chicken T cells and demonstrate that these miRNAs are indeed susceptible to inhibition by both miR-155 and miR-M4. This will hopefully inform and facilitate our ongoing efforts to define the miRNA targets for miR-K11 and miR-155 that are important for lymphomagenesis in humans.

RESULTS

Analysis of the MDV-1 and MDV-2 microRNA expression profile. Conventional and deep sequencing analyses of the viral miRNAs expressed by MDV-1 and MDV-2 have previously identified 13 pre-miRNAs encoded by MDV-1 and 18 pre-miRNAs encoded by MDV-2 (13–18). While these miRNAs are derived from analogous regions of the viral genome, this work did not identify any MDV-1 and MDV-2 miRNAs with the same seed sequence and hence the potential to target a similar set of mRNA transcripts.

We performed deep sequencing of small, \(~18\)- to \(~25\)-nt-long RNAs expressed in MSB1 cells, a chicken lymphoblastoid cell line previously shown to be naturally infected by both MDV-1 and MDV-2 (14, 15). This generated 186,022,013 reads, of which 184,005,445 could be aligned to either the chicken genome or the
MDV-1 or MDV-2 genome. Of these, 161,068,074 (88%) aligned to known chicken, MDV-1, or MDV-2 pre-miRNAs, as listed in miRBase version 19. Reads aligning to known chicken miRNAs contributed 49% of the total miRNAs detected, while MDV-1 and MDV-2 contributed 42% and 9%, respectively (Fig. 1A). The most highly expressed single miRNA, chicken miR-21, contributed ~22% of all miRNAs detected, followed by MDV-1 miR-M4 (10%), MDV-1 miR-M3 (10%), chicken miR-142 (7%), and MDV-1 miR-M1 (6%) (Fig. 1B). The most highly expressed MDV-2 miRNA, miR-M29, contributed 4% of all miRNA reads.

Analysis of all small RNA reads that mapped to the MDV-1 or MDV-2 genome failed to identify any novel viral miRNAs and confirmed the expression of miRNAs and, in most cases, miRNA passenger strands derived from all of the 13 previously reported MDV-1 pre-miRNAs and 18 MDV-2 pre-miRNAs (13–18). The major isoforms of the MDV miRNAs and miRNA passenger strands derived from these 31 pre-miRNA precursors are listed in Tables 1 and 2. Given the key role played by the miRNA seed region (1), nucleotides 2 through 8, in mRNA target identification, we were not surprised to find that viral miRNA sequence variation was primarily restricted to the 3’ end, which plays a less important role in target selection. However, we did note several viral miRNAs that were processed into two different miRNA species that differed at their 5’ ends and therefore would be predicted to target overlapping but distinct mRNA populations. These included MDV-1 miR-M3-5p, where a 5’ end variant of the dominant miRNA provided 16% of all reads, as well as MDV-1 miR-M7-5p (38%), and miR-M12-3p (10%) (Table 1). In MDV-2, major 5’ variants were detected for miR-14-5p (31%), miR-M18-5p (14%), miR-M24-3p (13%), and miR-M28-5p (29%) (Table 2). To confirm that viral miRNAs are indeed expressed in two major isoforms that differ at their 5’ ends, we performed primer extension analysis for several of these viral miRNAs. These data confirmed the existence of two isoforms of MDV-1 miR-M3-5p and miR-M7-5p, as well as MDV-2 miR-M18-5p, that differ by 1 nt and two isoforms of MDV-2 miR-M28-5p that differ by 2 nt at their 5’ ends (Fig. 1C). Interestingly, the novel, extended isoform of MDV-2 miR-28-5p has the same predicted seed sequence as MDV-1 miR-M9-5p (Fig. 1D), and this therefore represents the first example of an MDV-1 and MDV-2 miRNA pair with the same seed sequence and hence, presumably, a similar mRNA target population.

The mRNA targetome of MDV microRNAs. Having identified all viral miRNA species expressed in the MSB1 lymphoblastoid cell line, we next sought to identify the cellular and viral mRNA populations targeted by these miRNAs using the previously described photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) procedure (28, 29, 32). For this purpose, MSB1 transcripts were labeled by growth of cells in 4-thiouridine, followed by cross-linking of RNAs to bound proteins by irradiation at 365 nm. After lysis, RNA fragments bound to RISC were isolated by immunoprecipitation using a pan-Ago antibody followed by RNase treatment, linker ligation, reverse transcription, PCR amplification, and deep sequencing. Analysis of the PAR-CLIP reads obtained, using the previously described PARalyzer software (34), identified 16,585 RISC-binding clusters which mapped primarily to mRNA coding regions, 3’ UTRs, and intergenic regions (Fig. 2A). (Note that the chicken transcriptome is currently not well defined, so intergenic regions are expected to be overrepresented.) A list of all RISC binding clusters identified, with their predicted miRNAs, is given in Table S1 in the supplemental material. Of these 16,585 binding
clusters, PARalyzer predicted 1,104 cellular miRNAs that were bound by one or more MDV-1 miRNAs, while 1,183 cellular miRNAs were predicted to be bound by one or more MDV-2 miRNAs. The lack of correlation between the relative abundance of the MDV-1 and MDV-2 miRNAs (Fig. 1A) and the number of miRNA binding clusters, while to some extent unexpected, has nevertheless also been observed in previous efforts to define miRNA target sites using CLIP (36). Of these viral miRNA binding clusters, PARalyzer predicted 1,104 cellular mRNAs that were bound by one or more MDV-1 miRNAs, while 1,183 cellular mRNAs were predicted to be bound by one or more MDV-2 miRNAs.

### TABLE 1 MDV-1 miRNAs expressed in MSB-1 cells

| miRNA     | Sequence                          | Length | Position               | Frequency |
|-----------|-----------------------------------|--------|------------------------|-----------|
| MDV1-M1-5p| TGGCTGTTCACGTGGCCGCCATT           | 22     | 4725-4746              | 5,220,661 | 51  |
|           | TGGCTGTTCACGTGGCCGCCATT          | 23     | 4724-4746              | 3,483,446 | 34  |
| MDV1-M1-3p| ATGCTGGCCATGAAGAGCCGA            | 21     | 4687-4707              | 38,270    | 68  |
| MDV1-M2-5p| GTGTATTCTCGCCGCTTGTGGCTTGTG     | 24     | 7365-7388              | 773,504   | 43  |
|           | GTGTATTCTCGCCGCTTGTGGCTTGTG     | 25     | 7364-7388              | 420,366   | 24  |
| MDV1-M2-3p| CGACTCCGGAGGATAGCGTT            | 21     | 7328-7348              | 285,919   | 56  |
| MDV1-M3-5p| AGGAAGGGAGAAGAACCCTCCTGC        | 24     | 7516-7538              | 8,707,716 | 54  |
|           | AGGAAGGGAGAAGAACCCTCCTGC        | 23     | 7516-7538              | 2,554,622 | 16  |
| MDV1-M3-3p| TGGGGGTCTCCCATTTTTGAGTT         | 22     | 7479-7500              | 129       | 70  |
| MDV1-M4-5p| TTAATCTGATACGACAGCTTTCACTT      | 25     | 7227-7251              | 7,837,697 | 47  |
|           | TTAATCTGATACGACAGCTTTCACTT      | 24     | 7228-7251              | 4,059,588 | 25  |
|           | TTAATCTGATACGACAGCTTTCACTT      | 23     | 7229-7251              | 2,754,899 | 17  |
| MDV1-M4-3p| AATGTTCTTGGACACAGTGC            | 21     | 7194-7214              | 42,714    | 40  |
| MDV1-M5-5p| CGATGCGACACATCATTTGACG          | 22     | 7990-8011              | 867       | 52  |
|           | CGATGCGACACATCATTTGACG          | 23     | 7950-7972              | 1,069,623 | 84  |
| MDV1-M6-5p| TGTTGGCCCTGTTGCTTGTGGCTTGTG    | 21     | 176086-176106          | 3,255,817 | 65  |
|           | TGTTGGCCCTGTTGCTTGTGGCTTGTG    | 22     | 176085-176106          | 1,045,694 | 21  |
| MDV1-M6-3p| AGATCCCCGGAATGACAGT             | 21     | 176053-176073          | 76,487    | 77  |
| MDV1-M7-5p| GTATATCTGAGGAGGATCTCTTAG        | 22     | 175913-175934          | 1,527,552 | 39  |
|           | GTATATCTGAGGAGGATCTCTTAG        | 22     | 175914-175935          | 1,490,315 | 38  |
|           | GTATATCTGAGGAGGATCTCTTAG        | 21     | 175914-175934          | 525,225   | 15  |
| MDV1-M7-3p| GAGATCTCTACGAGATTACACTG         | 22     | 175874-175895          | 26,918    | 43  |
| MDV1-M8-5p| TATGCTTCTGGTTGTTGTTGCACT        | 23     | 176205-176227          | 346,016   | 51  |
|           | TATGCTTCTGGTTGTTGTTGCACT        | 23     | 176205-176227          | 803,359   | 71  |
| MDV1-M8-3p| TGACCTTCTACGGAACATAGT           | 21     | 176164-176184          | 126,023   | 11  |
| MDV1-M9-5p| TTGCCTCTCCCGCCGGAGAGTCTC        | 23     | 8223-8245              | 564,857   | 72  |
|           | TTGCCTCTCCCGCCGGAGAGTCTC        | 22     | 8184-8205              | 13,504    | 51  |
| MDV1-M9-3p| AAACCTCCAGGAGGAGAAAG            | 22     | 175795-175817          | 11,127    | 51  |
| MDV1-M10-5p| GCTCTGATGAGGTTTTCCCCGA         | 23     | 175753-175774          | 1,290,839 | 86  |
|           | GCTCTGATGAGGTTTTCCCCGA         | 23     | 175754-175774          | 16,899    | 80  |
|           | GCTCTGATGAGGTTTTCCCCGA         | 22     | 175754-175774          | 2,499     | 12  |
| MDV1-M11-5p| TTTTCTTACGCTAGCTTTAGA         | 23     | 5544-5566              | 16,899    | 80  |
|           | TTTTCTTACGCTAGCTTTAGA         | 22     | 5545-5566              | 2,499     | 12  |
| MDV1-M11-3p| TGGTTACATGGTCAGGGATT          | 22     | 5506-5527              | 264       | 77  |
| MDV1-M12-5p| AGCCCTCCGATTAACTGTAAT          | 22     | 7706-7727              | 195       | 66  |
|           | AGCCCTCCGATTAACTGTAAT          | 23     | 7672-7694              | 5,136,011 | 73  |
| MDV1-M12-3p| TGCATAATATCAGGAGGAGCTGTG      | 23     | 7672-7694              | 736,934   | 10  |
|           | TGCATAATATCAGGAGGAGCTGTG      | 23     | 7672-7694              | 7,837,697 | 47  |
| MDV1-M2-3p| CGACTCCGGAGGATAGCGTT          | 21     | 5053-5075              | 368       | 68  |
| MDV1-M3-3p| GTGCTACGTCGAAGAGGCTAC          | 23     | 5016-5038              | 25,188    | 30  |
|           | GTGCTACGTCGAAGAGGCTAC          | 21     | 5018-5038              | 19,551    | 23  |
|           | GTGCTACGTCGAAGAGGCTAC          | 22     | 5017-5038              | 16,728    | 20  |

*a List of MDV-1 miRNAs detected by deep sequencing showing their prevalence as well as all the viral miRNA isoforms that contribute ≥10% of the total reads for that miRNA.

**Viral miRNA targets of MDV-1 and MDV-2 miRNAs.** Analysis of miRNA binding clusters on the MDV-1 genome identified five very strong binding clusters between positions ~7200 and ~7800, near the 5' end of the MDV-1 genome (Fig. 3A). These clusters essentially coincide with, but are antisense to, the highly expressed MDV-1 miRNAs miR-M2, miR-M3, miR-M4, and miR-M12, which are adjacent to each other and all located antisense to an MDV-1 mRNA that encodes the viral RLORF8 protein (Fig. 3A). Similarly, we also detected very strong binding clusters near the 5' end of the MDV-2 genome, coincident with, but antisense to, the MDV-2 miRNAs miR-M24, miR-M28, and miR-M29, which are adjacent to each other and all located antisense to an MDV-1 mRNA that encodes the viral RLORF8 protein (Fig. 3A).
are, in fact, able to repress RLORF8 expression. For this purpose, we generated an indicator construct containing the entire RLORF8 open reading frame and 445 bp of the 3′ UTR, encompassing all four predicted MDV-1 miRNA binding sites (Fig. 3A), inserted 3′ to the firefly luciferase indicator gene (fluc), as well as expression vectors encoding MDV-1 miR-M2, miR-M3, miR-M4, or miR-M12. Analogous vectors containing two perfect artificial target sites for each miRNA inserted 3′ to fluc served as positive

|TABLE 2| MDV-2 miRNAs expressed in MSB-1 cells$^a$ |
|---|---|---|---|---|
|miRNA | Sequence | Length | Position | Frequency |
| | | | Beginning | End |
|MDV2-M14-5p | GTGTGTCACGGTGCACCCTGAGA | 23 | 6911 | 6933 |
| | | | 85,064 | 31 |
|MDV2-M14-3p | TCGAAGACGCGGCCGCAAA | 21 | 6876 | 6896 |
| | | | 7,775 | 37 |
|MDV2-M15-5p | GTGGTGCTGACCCTGAGA | 22 | 6683 | 6703 |
| | | | 3,270 | 71 |
|MDV2-M15-3p | GTGGTGCTGACCCTGAGA | 22 | 6640 | 6661 |
| | | | 345,988 | 71 |
|MDV2-M16-5p | ATCCCTCCTTCCACGATGTCAG | 23 | 5547 | 5569 |
| | | | 1,323,176 | 73 |
|MDV2-M16-3p | ATCCCTCCTTCCACGATGTCAG | 22 | 5160 | 5181 |
| | | | 183,305 | 76 |
|MDV2-M17-5p | TAGGACAACGCGGCCGACAC | 22 | 5126 | 5147 |
| | | | 201,610 | 66 |
|MDV2-M17-3p | GTGGTGCTGACCCTGAGA | 23 | 5001 | 5023 |
| | | | 214,786 | 31 |
|MDV2-M18-5p | CCTCCTGACCCTGAGA | 24 | 4961 | 4981 |
| | | | 10,011 | 70 |
|MDV2-M18-3p | CATGCCCCCCTCCGAGGGTAGC | 22 | 4804 | 4825 |
| | | | 353 | 85 |
|MDV2-M19-5p | CCCCTCGGCCGATGTCAGG | 21 | 4844 | 4864 |
| | | | 1,267 | 53 |
|MDV2-M19-3p | CCCCTCGGCCGATGTCAGG | 20 | 4845 | 4864 |
| | | | 408 | 17 |
|MDV2-M20-5p | TCCTTAGCGTGCTGCTGAGA | 21 | 4694 | 4714 |
| | | | 555,354 | 73 |
|MDV2-M20-3p | TCCTTAGCGTGCTGCTGAGA | 20 | 4696 | 4715 |
| | | | 200,784 | 32 |
|MDV2-M21-5p | TCCTCCTTCGCGGGGTGCTTGA | 22 | 4559 | 4580 |
| | | | 175,317 | 74 |
|MDV2-M21-3p | GCAGCAACCGCTGCTGAGGAG | 23 | 4517 | 4539 |
| | | | 9,299 | 28 |
|MDV2-M22-5p | TCTTACACGCACGTCACTCTGGT | 23 | 4279 | 4301 |
| | | | 314,713 | 49 |
|MDV2-M22-3p | TCTTACACGCACGTCACTCTGGT | 24 | 4278 | 4301 |
| | | | 200,784 | 32 |
|MDV2-M23-5p | ATGGTCCGTGGTACGGTGTCCT | 22 | 4130 | 4151 |
| | | | 2,266 | 54 |
|MDV2-M23-3p | GTCTCCGTACACCGGACCATCG | 22 | 4094 | 4115 |
| | | | 2,975 | 48 |
|MDV2-M24-5p | TTTTTCTCTGACCCTGAGAACC | 22 | 3539 | 3561 |
| | | | 96,126 | 75 |
|MDV2-M24-3p | TTAGATCGCTCAGGAGATGAT | 22 | 3353 | 3374 |
| | | | 291 | 24 |
|MDV2-M25-5p | CTTTCGTCCGGTTCTGACG | 21 | 3121 | 3141 |
| | | | 50,748 | 67 |
|MDV2-M25-3p | CTTTCGTCCGGTTCTGACG | 22 | 3120 | 3141 |
| | | | 15,467 | 20 |
|MDV2-M27-5p | TCTTGTCAGTGCTGCCG | 21 | 2870 | 2890 |
| | | | 1,242 | 20 |
|MDV2-M27-3p | TCTTGTCAGTGCTGCCG | 22 | 2870 | 2890 |
| | | | 1,242 | 20 |
|MDV2-M28-5p | TCTCTACCTCAGGCTGCTGAC | 21 | 2578 | 2598 |
| | | | 489,862 | 36 |
|MDV2-M28-3p | TCTCTACCTCAGGCTGCTGAC | 22 | 2578 | 2598 |
| | | | 226,111 | 16 |
|MDV2-M29-5p | TGGAGGATGAGTGCTGCTGAC | 20 | 2201 | 2221 |
| | | | 182,831 | 13 |
|MDV2-M29-3p | TGGAGGATGAGTGCTGCTGAC | 21 | 2201 | 2221 |
| | | | 151,642 | 11 |
|MDV2-M30-5p | CGAGGATGAGTGCTGCTGAC | 20 | 1979 | 2000 |
| | | | 46,233 | 62 |
|MDV2-M30-3p | CGAGGATGAGTGCTGCTGAC | 21 | 1979 | 2000 |
| | | | 15,164 | 11 |
|MDV2-M32-5p | TTCTTCACGTACCTCTCTATGGC | 22 | 1650 | 1670 |
| | | | 372,131 | 58 |
|MDV2-M32-3p | TTCTTCACGTACCTCTCTATGGC | 23 | 1650 | 1670 |
| | | | 152,551 | 24 |
|MDV2-M33-5p | CTAGCAGTGGGCGAGGGTAGC | 22 | 1320 | 1340 |
| | | | 818,313 | 11 |
|MDV2-M33-3p | CTAGCAGTGGGCGAGGGTAGC | 23 | 1320 | 1340 |
| | | | 151,642 | 11 |

$^a$ As in Table 1, but showing all observed MDV-2 miRNAs and their isoforms.
controls. Finally, we also generated derivatives in which each predicted RLORF8 mRNA target site was mutated in the seed complement. As may be observed in Fig. 4A, cotransfection of each MDV-1 miRNA expression vector into 293T cells along with the positive-control indicator vectors resulted in a clear inhibition in FLuc expression, relative to a cotransfected Renilla luciferase (RLuc) internal control vector. Comparison of each wild-type or mutant vector containing the MDV-1 RLORF8 gene sequence showed significant down regulation of the fluc vectors containing the wild-type RLORF8 sequence by all four MDV-1 miRNAs. This inhibition was partially (miR-M2 and miR-M4) or totally (miR-M3 and miR-M12) rescued by mutation of each single antisense RLORF8 miRNA target site. These data are consistent with the hypothesis that MDV-1 mRNAs encoding RLORF8 are indeed targets for repression by some or all of these viral miRNAs.

We next generated derivatives of the above-described indicator construct, containing fluc linked to the RLORF8 open reading frame and 3′ UTR, that bore seed mutations in all four antisense MDV-1 RNA targets or only in the two targets present in either the RLORF8 open reading frame or the 3′ UTR and transduced these into MSB1 cells, or MDV-negative DT40 cells as a control. As shown in Fig. 4B, the indicator construct bearing mutations in all four of the hypothetical target sites for MDV miR-M2, -M3, -M4, and -M12, gave rise to significantly higher levels of FLuc expression than did the wild-type fluc vector, while the indicators containing mutations in only the target sites for miR-M2 and miR-M4 in the RLORF8 ORF or miR-M3 and miR-M12 in the RLORF8 3′ UTR gave intermediate phenotypes. These data again strongly suggest that RLORF8 mRNAs are bona fide targets for repression by MDV-1-encoded miRNAs and indicate that physiological levels of these viral miRNAs, expressed in MDV-1-infected cells, are sufficient to repress RLORF8 mRNA expression.

The finding that expression of the MDV-1 RLORF8 gene product is subject to suppression by as many as four different viral miRNAs suggests that RLORF8 has the potential to reduce MDV-1 replication and/or induce toxicity when expressed inappropriately. To test this hypothesis, we generated a Tet-inducible lentiviral expression vector encoding the RLORF8 open reading frame with both potential internal MDV-1 miRNA target sites mutated and used this to transduce MSB1 cells. After selection for antibiotic resistance, we then added doxycycline (Dox) to induce RLORF8 expression and compared the growth phenotype of MSB1 cells expressing ectopic RLORF8 with that of control cells transduced with a similar lentiviral vector expressing green fluorescent protein (GFP). As may be observed in Fig. 4C, MSB1 cells expressing ectopic RLORF8 indeed grew substantially more slowly than the control cells, consistent with the idea that inappropriate overexpression of RLORF8 can indeed exert a deleterious effect. We have not, however, attempted to determine the molecular basis for this effect, and to our knowledge, the role of RLORF8 in the MDV-1 life cycle remains undetermined.

Ectopic expression of cellular mRNAs targeted by multiple MDV microRNAs. We were intrigued by the apparent ability of ectopic RLORF8, which is targeted by four MDV-1 miRNAs in MSB1, to reduce the growth of MSB1 cells in culture and wondered if other, cellular genes that are targeted by multiple viral miRNAs might exert a similar effect. In Table S2 in the supplemental material, we list 12 cellular genes that are targeted in their 3′ UTRs by five or more MDV-1 or MDV-2 miRNAs, as determined by PAR-CLIP. Of these, two cellular genes, encoding interleukin-18 (IL-18) and CD200R1, are predicted to be targeted by up to seven MDV-1 or MDV-2 miRNAs. IL-18 is a proinflammatory cytokine that can stimulate gamma interferon (IFN-γ) production in T cells (38–40), while CD200R1 is a cell surface
clearly grew more slowly than the controls, thus suggesting that the IL-18 cells transduced with the chicken IL-18 expression vector lacking the presence of Dox. Interestingly, even in the absence of Dox, the IL-18 mRNA 3' UTR grew most rapidly, even in the presence of Dox. Cells transduced with the lentiviral vector containing the IL-18 gene linked to its 3' UTR grew at a level comparable to that of the control in the absence of Dox (Fig. 5). Therefore, these data indicate that ectopic expression of chicken IL-18 reduces the replication of MSB1 cells and that this effect is greatly ameliorated if the IL-18 mRNA 3' UTR, which is detected as a target for several MDV miRNAs by PAR-CLIP, is present in the introduced IL-18 expression vector.

Conserved mRNA targets for miR-155 seed family microRNAs. As discussed above, MDV-1 encodes a miRNA, miR-M4, that shares full seed homology not only to human and chicken miR-155 but also to the KSHV miRNA miR-K11 (Fig. 6A). Although the role of miR-K11 in transformation of B cells by KSHV remains unclear, it is known that B-cell transformation by EBV, which does not encode a miR-155 analog, is dependent on viral induction of high levels of cellular miR-155 expression (26). Finally, transformation of T cells by MDV-1 has been reported to require miR-M4 function (27). As these miR-155 seed family members all appear to play a key role in herpesviral transformation of lymphoid cells, we reasoned that we might be able to gain important insights into the underlying mechanism by identifying mRNA targets of miR-155, miR-K11, and miR-M4 that are evolutionarily conserved from chickens to humans.

A comparison of mRNA targets for miR-155, miR-K11, and miR-M4, identified by previously reported PAR-CLIP analysis of KSHV-transformed or EBV-transformed human B cells and here for MDV-1 miR-M4 in the MSB1 lymphoblastoid cell line, showed that 9 mRNA targets were detected in all three cell settings, while 4 additional mRNA targets were also conserved in both MDV-1-infected MSB1 cells and EBV-transformed lymphoblastoid cell lines (LCLs) (Fig. 6B) (see Table S3 in the supplemental material for a full listing of mRNA targets for miR-M4 in MSB1 cells). We therefore cloned the 3' UTRs of all nine mRNAs targeted by all three of these miR-155 seed family members as well as one additional miRNA, encoding JARID2, that is conserved between miR-155 in LCLs and miR-M4 in MSB1 cells but not in PELs. These 3' UTRs were inserted 3' to fluc and cotransfected into MSB1 cells along with an internal control RLuc vector. In 8 out of 10 cases, we also generated mutant derivatives of these 3' UTRs in which the predicted MDV-1 miR-M4 target or targets (JARID2 has two predicted miR-M4 targets in its 3' UTR) were mutated in their region of seed homology. As may be observed, we detected significant and selective repression of FLuc expression mediated by these 3' UTRs that was partially or entirely alleviated by mutation of the predicted miR-M4/miR-155 target sites. Therefore, although these data did not reveal a statistically significant decline in response to miR-M4 and/or miR-155 expression in all cases, they are consistent with the hypothesis that targeting of the 3' UTRs of these genes by miR-155 seed family members is conserved between humans and chickens, an evolutionary distance of ~300 million years.

**DISCUSSION**

The goal of this study was to perform an analysis of the mRNA targetomes regulated by miRNAs encoded by the oncogenic avian herpesvirus MDV-1, and its nonpathogenic relation MDV-2, and in particular to identify mRNAs targeted by MDV miR-M4, which has previously been shown to play a key role in the initiation of T-cell transformation in MDV-1 infected chickens (27). Our interest in miR-M4 was prompted by the previous observation that this inducible expression vector is “leaky” and expresses a low level of IL-18 even in the absence of added Dox. As one would predict, this inhibitory effect became even more severe in the presence of Dox (Fig. 5). Therefore, these data indicate that ectopic expression of chicken IL-18 reduces the replication of MSB1 cells and that this effect is greatly ameliorated if the IL-18 mRNA 3’ UTR, which is detected as a target for several MDV miRNAs by PAR-CLIP, is present in the introduced IL-18 expression vector.

**FIG 3** Map of RISC binding clusters that align to the MDV-1 (A) or MDV-2 (B) genome. The size of the peaks is proportional to the read depth of the clusters. A closeup of a 5' fragment of the MDV-1 and MDV-2 genome that contains the most reads is presented. Open reading frames located on the viral plus strand are marked in blue, viral miRNAs encoded on the minus strand are marked in red, and the approximate location of relevant miRNA binding clusters, identified by PAR-CLIP, is indicated in green.

glycoprotein generally expressed on myeloid cells (41) that has been proposed to play a role in the response to viral infection (42). Of these two genes, the IL-18 gene seemed most likely to exert an antiviral effect in culture, and this mRNA’s 3’ UTR was predicted to be targeted by two MDV-1 miRNAs (miR-M2-3p and miR-M9-5p) and as many as five MDV-2 miRNAs (miR-M18-5p, miR-M18-5p, miR-M26-5p, miR-M28-5p, and miR-M30-3p). We therefore generated Tet-inducible lentiviral vectors containing either the chicken IL-18 open reading frame or the IL-18 open frame linked to its cognate 3’ UTR, including all the predicted MDV miRNA target sites. These vectors, plus a control GFP-expressing lentivirus, were transduced into MSB1 cells, and puromycin-resistant transductants were selected. These were then analyzed for their growth potential in the presence and absence of Dox (Fig. 5). As may be observed, the control transduced cells grew most rapidly, even in the presence of Dox. Cells transduced with the lentiviral vector containing the IL-18 gene linked to its 3’ UTR grew at a level comparable to that of the control in the absence of Dox but showed a significant reduction in replication in the presence of Dox. Interestingly, even in the absence of Dox, cells transduced with the chicken IL-18 expression vector lacking the IL-18 3’ UTR, and hence all five MDV miRNA target sites, clearly grew more slowly than the controls, thus suggesting that
miR-M4 shows full seed homology with both the KSHV-encoded miRNA miR-K11 and with miR-155 (19–21), a cellular miRNA that is highly conserved from birds to mammals (Fig. 6A). As discussed above, it seems highly probable that KSHV miR-K11 and human miR-155 play a key role in B-cell transformation mediated by KSHV and EBV, respectively, and, given the high conservation of miR-155 across vertebrate evolution, we therefore hypothesized that the identification of mRNA targets that are targeted by not only miR-K11 and miR-155 in human B cells but also miR-M4 in chicken lymphoblastoid cells might identify a small set of mRNAs that might play a particularly important role in lymphoid transformation by these diverse herpesviruses.

As a first step in this project, we used deep sequencing to define the viral miRNAs expressed in the chicken lymphoblastoid cell line MSB1, which is naturally infected with both MDV-1 and MDV-2. Our analysis did not identify any novel viral pre-miRNAs beyond the 13 previously identified in MDV-1 and the 18 identified in MDV-2 (14–18). We did, however, demonstrate that several MDV-1 and MDV-2 miRNAs exist in two isoforms differing by 1 or 2 nt at their 5′ ends (Tables 1 and 2 and Fig. 1C). These viral miRNAs therefore exist in two isoforms with two different seed sequences that are predicted to target overlapping but distinct populations of mRNA transcripts. Our subsequent PAR-CLIP analysis of the mRNAs targeted by the MDV-1 and MDV-2 miRNA expressed in MSB1 cells indicated that this was indeed the case (Fig. 2C).

PAR-CLIP identified a list of 1,104 cellular mRNAs targeted by MDV-1 miRNAs and a list of 1,183 mRNAs targeted by MDV-2 miRNAs in MSB1, of which 419 mRNA targets were shared (Fig. 2B). This is reminiscent of our previous analysis of a human PEL cell line coinfected by KSHV and EBV, which identified an

**Figure Legend Continued**

and this is presented relative to the value for the “ALL MUTANTS” clone, which was set at 1.0. Data are averages for three independent samples with SD. (C) Induced expression of a mutated RLORF8 open reading frame lacking MDV-1 miRNA binding sites inhibits MSB1 growth. Data are averages from five independent experiments with SD.
extensive overlap between the cellular mRNA species targeted by EBV and KSHV miRNAs, even though these viral miRNAs lack any significant homology (28). This suggests that herpesviruses that target the same cell type have undergone convergent evolution leading to the expression of distinct viral miRNAs that nevertheless target overlapping sets of cellular factors via different 3’UTR target sites.

Given the very large number of mRNA targets identified by PAR-CLIP analysis of the MDV-1 and MDV-2 miRNA targetome (see Table S1 in the supplemental material), we have as yet performed only a limited validation and phenotypic analysis of these targets. One mRNA target that was validated is the MDV-1 mRNA encoding the viral RLORF8 protein, which lies antisense to no fewer than four MDV-1 miRNAs in the MDV-1 genome. As predicted by this location, we observed the most intense RISC binding clusters detected on the MDV-1 genome at the location of the RLORF8 transcript (Fig. 3A) and analysis of indicator constructs in cotransfected 293T cells (Fig. 4A) or transduced MSB1 cells (Fig. 4B) confirmed that RLORF8 is indeed targeted by all four of these MDV-1 miRNAs. Interestingly, expression of the MDV-1 RLORF8 gene in a form lacking the viral miRNA binding sites caused a substantial reduction in MSB1 cell growth (Fig. 4C), consistent with the idea that dysregulated expression of RLORF8 can exert a deleterious effect in MDV-1-infected T cells. However, the function of RLORF8 in the viral life cycle is currently unknown, and we do not yet know why overexpression inhibits the growth of MDV-infected cells. The same pattern is observed in MDV-2, where five viral miRNAs are transcribed from the DNA strand located antisense to RLORF2 (Fig. 3B). Of note, the RLORF2 and RLORF8 proteins do not share significant amino acid sequence homology, and we were also unable to detect any significant homology between these two MDV proteins and proteins encoded by other herpesvirus species. Nevertheless, it remains possible that MDV-1 RLORF8 and MDV-2 RLORF2 do have similar functions. Targeting of MDV-1 genes by MDV-1-encoded miRNAs is not restricted to miRNAs transcribed from the antisense strand, as MDV1-M7-5p was previously shown to downregulate the MDV-1 immediate early genes ICP-4 and ICP-27 (43). Interestingly, in the human α-herpesvirus HSV-1, viral miRNAs are transcribed antisense to ICP0, a viral immediate-early gene, and ICP34.5, a late gene, and downregulate the expression of these genes (8, 37). Although the physiological

FIG 6 Conserved cellular mRNA targets of miR-M4. (A) MDV1 miR-M4 seed homology with human and chicken miR-155 and KSHV miR-K11. (B) Venn diagram of overlapping targets of miR-M4, miR-155, and KSHV miR-K11. (C) Luciferase reporter assay. 3’UTRs of genes predicted to be targeted by MDV-1 miR-M4 were cloned with or without mutation of the predicted M4 target site into the 3’UTR of an fluc indicator plasmid. This plasmid, together with an RLuc internal control and the relevant miRNA expression vector, was then cotransfected into 293T cells. The FLuc-to-RLuc ratios observed for each indicator vector and miRNA were normalized to the ratio in cells that were transfected with pLCE without miRNA. The values obtained with cells expressing only FLuc without 3’UTR were set at 100%. The error bars indicate SD (n = 3).
relevance of the regulation of viral transcripts by viral miRNAs is not currently clear, it is possible that downregulation of viral early genes helps to maintain the latent phase, while the targeting of the late genes contributes to maintaining the regulated expression of immediate early, early, and late genes upon lytic infection.

Analysis of cellular mRNAs targeted by MDV-1 and/or MDV-2 miRNAs identified several that were targeted by five or more different viral miRNAs in their 3′ UTRs (see Table S2 in the supplemental material), suggestive of a possibly important role in restricting innate antiviral immunity. One factor that is expressed from an mRNA containing as many as seven viral miRNA target sites in its 3′ UTR is the chicken IL-18 gene. IL-18 is a proinflammatory cytokine that is induced upon infection by several different viruses and stimulates IFN-γ production from T cells (40). As the MSB1 cell line is of T-cell origin, we were interested in whether ectopic expression of IL-18 would indeed result in reduced cell growth. In fact, MSB1 cell growth proved to be highly sensitive to inhibition by ectopic IL-18 expression, though it remains to be determined whether this is indeed due to induction of chicken IFN-γ expression (Fig. 5). Thus, our data expose a second way in which MDV manipulates the host cell immune response, in addition to expressing a viral interleukin-8 (vIL-8) protein that is required for disease progression and tumor development (44, 45).

As noted above, we were particularly interested in identifying chicken mRNAs targeted by MDV-1 miR-M4, and PAR-CLIP indeed resulted in the identification of 73 mRNA 3′ UTR targets for miR-M4, of which 9 had previously been identified as targets for miR-155 or KSHV miR-K11 in EBV-transformed human B cells and KSHV-transformed human B cells, respectively (see Table S3 in the supplemental material). Indicator analysis of these 3′ UTRs, several of which have previously been validated as miR-K11 or miR-155 targets, gave data consistent with the hypothesis that these miRNAs are indeed targets for both miR-M4 in MSB1 and miR-155 in human B-cell lymphomas. Particularly interesting shared gene targets for miR-155/miR-M4/miR-K11 include JA,RID2, a histone methyltransferase and a known target for miR-155 in avian cells that has been shown to promote apoptosis and decrease cell survival when ectopically expressed (46), and LATS (large tumor suppressor), a protein kinase component of the Hippo pathway that inhibits YAP transcriptional activity and therefore both inhibits cell proliferation and promotes apoptosis. LATS sequence and function is evolutionarily conserved from avians to mammals and its loss is observed in many human cancers, including acute lymphoblastic leukemia (47). These activities suggest a potential selective advantage for MDV-1 in inducing downregulation of LATS. Another interesting miR-155/miR-M4/miR-K11 target, NF-κB-inducing kinase (MAP3K14/NIK), was reported to be an important regulator of the noncanonical NF-κB pathway (48). NIK is important for the development of both B cells and T cells (49-51) and may also have the potential to inhibit viral replication. It will therefore be of interest to test whether knockdown of these mRNAs using siRNAs can compensate for the previously reported loss of cell growth seen in EBV-transformed human B cells upon inhibition of miR-155 expression (26).

MATERIALS AND METHODS

Small RNA and PAR-CLIP libraries. Small RNA and PAR-CLIP libraries were constructed as previously described (32, 52). A 3-ml MSB1 cell pellet was used for PAR-CLIP, and the RISC pull-down was performed with a pan-Ago antibody (Abcam: AB 57113) that also recognizes chicken Ago proteins (data not shown). The resulting reads were pre-processed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads longer than 15 nt were aligned to the chicken (GalUs_gallus-4.0-ENSEMBLE), MDV-1 (GenBank accession no. AF243438.1) or MDV-2 (GenBank accession no. AB049735.1) genome using Bowtie (53). One mis-match was allowed in the small RNA library alignments and three in the PAR-CLIP library alignments.

For the analysis of PAR-CLIP data, we used PARalyzer version 1.1 (34) with the following parameters: a minimum cluster read depth of 15 and at least 2 T-to-C conversion locations were required. miRNA assignments to each cluster, based on the small RNA libraries, were designated according to standard nomenclature (1) with a minimal seed match of “7mer1A.” Human orthologs of chicken genes were assigned based on ENSEMBLE annotation. In order to avoid possible misannotation of the chicken genome, we included in our annotation 500 bp downstream of the predicted 3′ UTR, as detailed in Table S1 in the supplemental material. The data reported in this paper include this extension as part of the 3′ UTR. In order to generate Fig. 3, we used the Integrative Genomics Viewer (IGV) (54, 55).

Cell culture and transduction. MSB1 cells (a gift from Hsiao-Ching Liu) were grown in RPMI 1640 supplemented with penicillin, 10% fetal bovine serum (FBS), 10% tryptose phosphate broth, and 1% sodium pyruvate at 37°C. 293T cells were grown in Dulbecco’s modified Eagle medium (Sigma) supplemented with 10% FBS.

Packaging of pGIPZ or pL-CMV-GL3-derived lentiviral vectors was performed in 293T cells using pMDLgPRe, pRSV-Rev, and pVSV-G (56). pTRIPZ-derived lentiviral vectors were packaged using pCMVΔR8.75 and pMD2G. These plasmids were cotransfected into 293T cells (10-cm plate) using calcium phosphate, and virus was collected after 48 and 72 h to infect 1.5 × 106 MSB1 cells in 6-well plates. Cells were grown for 3 days before selection using puromycin.

Growth curves. Newly transduced MSB1 cells were grown in the presence of puromycin (Sigma) at a final concentration of 1 μg/ml for 14 days to select transductants. Doxycycline (Sigma) at a final concentration of 1 μg/ml was used to induce expression for 3 to 5 days before the initiation of each growth experiment, when MSB1 cells were diluted to 4 × 105 cells/ml. The cells were split into conditioned media several times during the experiment, when the cell concentration exceeded 8 × 105 cells/ml. Cells were counted using FACS Canto and FlowJo software.

Primer extension. Total RNA from 293T cells and MSB1 cells was prepared using TRizol (Invitrogen). Primer extension was performed using 8 μg of total RNA using an avian myeloblastosis virus primer extension kit (Promega), according to the manufacturer’s protocol. Reaction products were analyzed on 15% Tris-borate-EDTA-urea polyacrylamide gels.

Plasmids. DNA fragments containing the pri-miRNA stem-loops of MDV-1 miR-M2, miR-M3, miR-M4, and miR-M12, as well as up to 150 bp of viral genomic flanking sequences, were PCR amplified from total DNA purified from MSB1 cells, using primers listed in Table S4 in the supplemental material. These DNA fragments were then cloned into the previously described pLCE vector (57) using Xhol and EcoRI. For each of the four miRNAs, we cloned two perfect targets into the XhoI and KpnI sites present in the fluc-based indicator plasmid CMV-GL3 (58) by annealing two complementary primers (see Table S4). The miR-155 expression plasmid and the indicator plasmid containing perfect targets for miR-155 were described previously (20).

Chicken genes targeted by MDV1 miR-M4 and human miR-155 were cloned into the Xhol and EcoRI sites present in the fluc indicator vector pL-CMV-GL3 using primers listed in Table S4 in the supplemental material. These DNA fragments include part of the 500-bp 3′ UTR extension in relevant cases. All forward primers contained an XhoI site, while reverse primers contained an EcoRI site. Primers were chosen using the IDT PrimerQuest website. In order to create mutations in the predicted binding sites of MDV1-miR-M4, the six nucleotides that align to the miR-M4 seed region (nt 2 to 7) in the 3′ UTRs of ROP, WEE1, CSNK, and
JARID2 were replaced with an SpeI restriction site (5’ ATCATG 3’). The sequence 5’ TTAA 3’ in the 3’ UTRs of LATS and Orf103 was replaced with 5’ TTTA 3’. The MYB 3’ UTR was cloned twice, first with a 5’ primer starting at the beginning of the 3’ UTR and second with a primer that starts 40 bp downstream of the 3’ UTR start site and does not contain the predicted MDV1 miR-M4 target site.

An ~820 bp DNA fragment containing RLRORF8 and its 3’ UTR were cloned into pL-CMV-GL3 using XhoI and EcoRI. Mutations in the predicted miRNA binding sites in the coding and 3’ UTR regions of RLRORF8 were generated with primers listed in Table S4 in the supplemental material. These mutations modify part of the seed binding site and part of the region 3’ of the binding site in order to prevent the formation of a stem-loop that may be generated from an internal promoter on the minus strand. The pGIPZ vector used in this study contains GFP instead of RFP and is based on pTRIPZ (Open Biosystems), as previously described (58).

For the growth experiment whose results are shown in Fig. 4C, the RLRORF8 open reading frame, including the stop codon, was cloned into pGIPZ in place of gfp using AgeI and EcoRI. The introduced mutations in the predicted seed binding sites of MDV1 miR-M2 and miR-M4 in the RLRORF8 ORF preserved the original amino acid sequence. The chicken IL-18 open reading frame was cloned without the 3’ UTR or with the 3’ UTR, including a 500-bp extension, into pGIPZ in place of the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17:3011–3016. http://dx.doi.org/10.1101/gad.1158803.

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