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Reciprocal Inhibition between Intracellular Antiviral Signaling and the RNAi Machinery in Mammalian Cells

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SUMMARY

RNA interference (RNAi) is an established antiviral defense mechanism in plants and invertebrates. Whether RNAi serves a similar function in mammalian cells remains unresolved. We find that in some cell types, mammalian RNAi activity is reduced shortly after viral infection via poly-ADP-ribosylation of the RNA-induced silencing complex (RISC), a core component of RNAi. Well-established antiviral signaling pathways, including RIG-I/MAVS and RNaseL, contribute to inhibition of RISC. In the absence of virus infection, microRNAs repress interferon-stimulated genes (ISGs) associated with cell death and proliferation, thus maintaining homeostasis. Upon detection of intracellular pathogen-associated molecular patterns, RISC activity decreases, contributing to increased expression of ISGs. Our results suggest that, unlike in lower eukaryotes, mammalian RISC is not antiviral in some contexts, but rather RISC has been co-opted to negatively regulate toxic host antiviral effectors via microRNAs.

INTRODUCTION

RNA interference (RNAi) is a major regulator of gene expression throughout almost all eukaryotes. The small dsRNA effectors arise by Dicer processing, and one strand of the duplex becomes stably incorporated into the multiprotein RNA-induced silencing complex (RISC). The mechanism by which RISC silences a target mRNA depends on the degree of complementarity between the small RNA and its binding site. Small interfering RNAs (siRNAs) typically bind with perfect complementarity and direct RISC-mediated cleavage of the mRNA (Martinez et al., 2002). Alternatively, miRNAs (derived from endogenous transcripts) typically bind to the 3′ untranslated region (3′ UTR) of mRNAs with imperfect complementarity. This imperfect base pairing prevents RISC-mediated cleavage, instead resulting in translation inhibition and increased turnover of the transcript (Bartel, 2009).

RNAi functions directly as an antiviral defense in some eukaryotes, including nematodes, plants, and insects. In these organisms, strong genetic and biochemical evidence exists supporting the antiviral role of RNAi (Ding and Voinnet, 2007). First, mutations of the RNAi machinery render these organisms more susceptible to virus infection. Second, many viruses known to infect these cells encode inhibitors of RNAi machinery. Mutation of these inhibitors causes defects in viral replication that can be rescued in cells with reduced RNAi activity. Third, siRNAs derived from viruses are readily detectable (Hamilton and Baulcombe, 1999) and typically direct cleavage of viral transcripts (Umbach and Cullen, 2009). Thus, it is widely accepted that RNAi is an antiviral response in some eukaryotes.

However, in mammalian cells, the antiviral role of RNAi remains controversial (Cullen, 2006; de Vries and Berkhout, 2008; Jeang, 2012; Umbach and Cullen, 2009). There are reports that mammalian cells defective for RNAi components are more susceptible to viral replication. However, these data are confined to a select few viruses that seem to be coincidentally, or possibly advantageously, regulated by host miRNAs (Cullen, 2006; Lecellier et al., 2005; Otsuka et al., 2007; Umbach and Cullen, 2009). Additionally, proteins of some mammalian viruses are able to inhibit RNAi when expressed in noninfectious contexts (Bennasser et al., 2005; Haasnoot et al., 2007; Lecellier et al., 2005; Li et al., 2004). However, most of these proteins are known to bind double-stranded RNA as part of well-defined, RNAi-independent functions (e.g., virus replication, evading the IFN response [Umbach and Cullen, 2009]). Significantly, the replication defects of mammalian viruses with mutant RNA-binding proteins have not been shown to be rescued in host cells with defective RNAi. Although transfecting mammalian cells with synthetic siRNAs clearly inhibits viral replication (Gitlin et al., 2002), there is little biochemical evidence that natural siRNAs arise during infection (Lin et al., 2010; Parameswaran et al., 2010; Pfeffer et al., 2005; Umbach and Cullen, 2009). Therefore, the role of RNAi during the mammalian antiviral response remains unresolved.

In contrast to lower eukaryotes, mammals possess large-scale, protein-based innate and adaptive antiviral responses. The interferon (IFN) response is a key player in this antiviral defense. Type I IFNs are secreted proteins that, in a paracrine and autocrine fashion, trigger the expression of hundreds of antiviral genes called IFN-stimulated genes (ISGs). Many of these proteins combat virus infection by stalling the cell cycle, preventing translation, and promoting cell death (Sadler and Williams, 2008; Schoggins et al., 2011). As might be expected, such potentially cytotoxic gene products are under multiple layers of regulation. ISGs are transcriptionally regulated by transcription

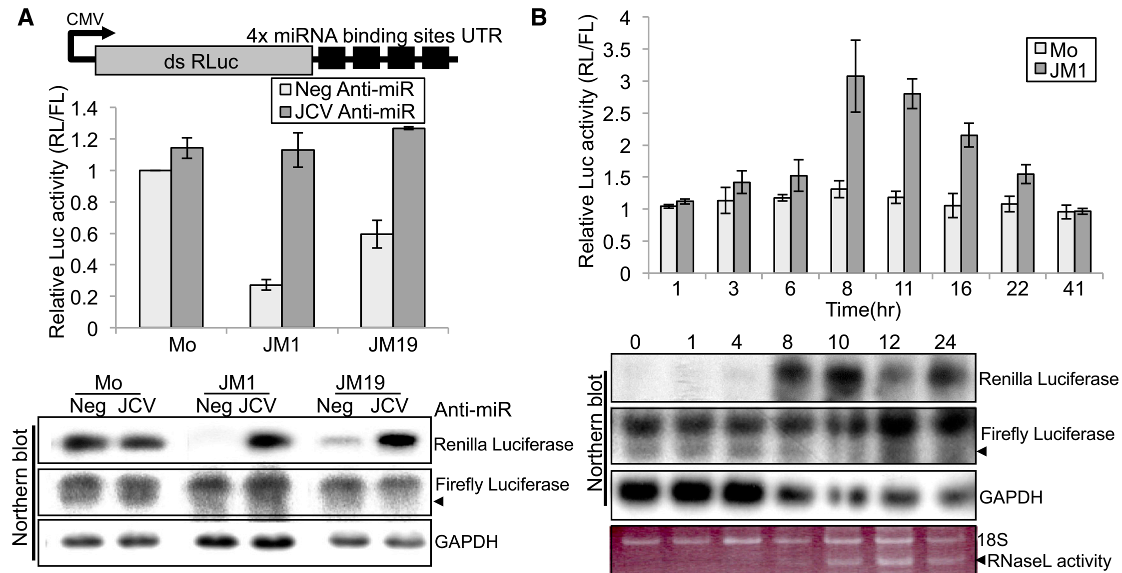

Figure 1. Triggering the Antiviral Response Inhibits RNAi

(A) Reporter cell lines effectively monitor RNAi. Schematic of *Renilla* luciferase (RLuc) reporter construct with four perfectly complementary JCV miRNA binding sites in the 3′ UTR is shown (top panel). Firefly luciferase (ffLuc) reporter lacks miRNA binding sites. JM cell lines stably express ffLuc and RLuc reporters and the JCV miRNA; Mo cell line is identical but lacks the JCV miRNA. Luciferase assays following transfection of JCV and negative control (Neg) anti-miRs confirm specific regulation of the RLuc reporter (middle). Relative luciferase activity is the ratio of luciferase activity (RLuc/ffLuc) in JCV anti-miR-transfected cells normalized to Neg anti-miR-treated cells. Northern blot analysis was performed to confirm downregulation of the RLuc reporter mRNA (bottom). GAPDH serves as a load control.

(B) Triggering the antiviral response with poly I:C inhibits RNAi. RNAi activity was measured by luciferase assay at the indicated times posttransfection with poly I:C (top). Relative luciferase activity is the ratio (RL/FL) of poly I:C-transfected cells normalized to untreated cells. Northern blot analysis confirms increase in RLuc reporter mRNA. The appearance of 18S rRNA cleavage fragment is consistent with RNaseL activity (bottom). Bar graphs depict mean; error bars represent SD. Figure 1 is related to Figure S1.

factors, particularly those responsive to JAK/STAT and NF-κB antiviral signaling pathways. Activation of some ISG products (e.g., PKR, RNaseL) requires detection of intracellular pathogen-associated molecular patterns (PAMPs) by a pattern recognition receptor (PRR). The translation of at least some ISG transcripts is regulated posttranscriptionally by miRNAs (Cloonan et al., 2008; Gregersen et al., 2010; Lee et al., 2012; Ostermann et al., 2012). Here, we test the hypothesis that antiviral signaling in mammalian cells alters the RNAi response, thereby altering expression of components of the IFN response.

RESULTS

Triggering the Antiviral Response Inhibits RNAi

An antiviral role for mammalian RNAi remains controversial. To quantitate RNAi activity when antiviral signaling pathways are activated, we developed stable HEK293 cell lines expressing luciferase-based reporters (Figure 1A). Specifically, cells were engineered to coexpress an exogenous miRNA derived from the JC virus (JCV) alongside short half-life *Renilla* luciferase (RLuc) with perfectly complementary binding sites for the JCV miRNA in its 3′ UTR. We previously demonstrated that this construct is negatively regulated by cleavage of the RLuc mRNA by RISC (McClure et al., 2011b; Seo et al., 2008). Clonal lines with varying degrees of repression were obtained, including JM1 (high) and JM19 (intermediate). Given its larger dynamic range, we utilized JM1. The parental cell line (mother, “Mo”) lacks the JCV miRNA and expresses only the reporter. We show that transfection of an anti-miR that specifically inhibits the JCV miRNA restores luciferase activity in the JM1 line to the levels of the Mo line (Figure 1A). Northern blot analysis confirms that the changes in normalized luciferase signal correspond to the intensity of the RLuc mRNA (Figure 1A, bottom panel). Thus, we have developed a quantitative assay for RNAi activity in mammalian cells.

Several PRRs recognize intracellular dsRNA, a common byproduct of viral replication, as a PAMP. We transfected JM1 cells with poly inosine:cytosine (I:C), a mimic of dsRNA. Poly I:C transfection resulted in inhibition of RNAi activity that peaked at 8 hr posttransfection and lasted the duration of the experiment (Figure 1B). This treatment had no effect on RLuc expression in the Mo cell line. Inhibition of RNAi coincides with the induction of RNaseL activity, as evidenced by cleavage of 18S rRNA (Figure 1B). In the absence of transfection, treatment of cells with poly I:C was not sufficient to alter RNAi activity (data not shown). Competitive binding of the RNAi machinery to poly I:C could possibly account for the reduced silencing activity. To rule this out, we transfected a double-stranded miRNA mimic in molar excess (relative to the amount of poly I:C) and saw no effect on silencing (Figure S1A). We observed no changes in the level of key protein effectors of RNAi (Figure S1B), and the addition of the translation elongation inhibitor cycloheximide affected the reporter (RLuc) and control (Firefly, FF) luciferase levels nearly equally (Figure S1C). Taken together, these data indicate that

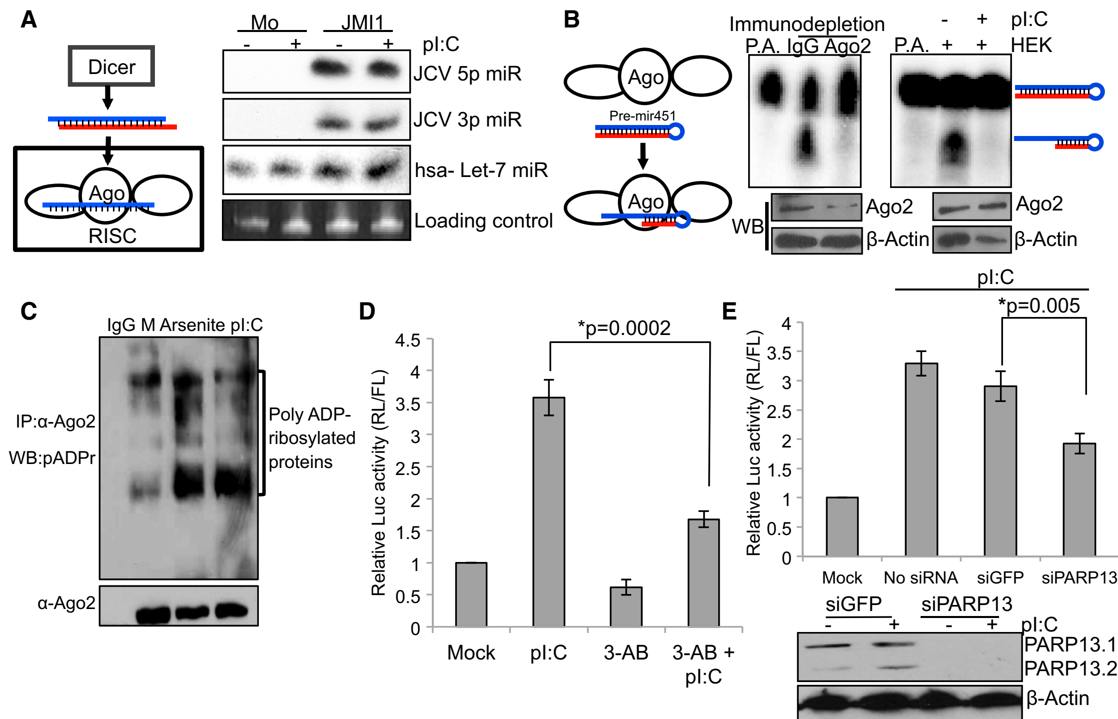

Figure 2. Activating the Antiviral Response Inhibits Argonaute-Mediated Cleavage via Poly-ADP-Ribosylation of RISC-Associated Proteins

(A) Schematic of the canonical miRNA biogenesis pathway (left panel). Northern blot analysis shows that steady-state levels of Dicer products are unaffected by poly I:C transfection (right). Ethidium bromide-stained, low-molecular-weight RNA serves as a load control.

(B) Schematic of Ago2 substrate pre-miR-451 and the derivative Ago2 cleavage product (left). Radiolabeled pre-miR-451 was incubated with HEK293 cellular extracts and resolved by denaturing PAGE. Immunodepletion of Ago2 results in decreased product. Preimmune IgG was used as a control (middle). Lysates from cells transfected with poly I:C for 8 hr show reduced RISC-mediated cleavage of pre-miR-451 (right). Probe alone (P.A., no lysate added) and mock transfected lysates were used as a control. Immunoblots confirming levels of Ago2 and β-actin are shown below.

(C) pADP-r modification of RISC-associated proteins. Lysates were prepared from cells treated with sodium arsenite (positive control), transfected with poly I:C, or mock treated (M). Ago2 was immunoprecipitated (preimmune IgG used as control, lane 1). Bound fraction was immunoblotted with pADP-r antibody. Immunoblot for Ago2 was performed as a control. pADP-r-modified proteins are indicated with a bracket.

(D) Analysis of RNAi activity in the presence of a PARP inhibitor. RNAi activity was assayed using JM1 cells transfected with poly I:C and/or treated with 20 mM 3-aminobenzamide (3-AB), a general PARP inhibitor.

(E) Role of PARP13 in poly I:C-mediated RNAi Inhibition. RNAi activity was assayed using JM1 cells following transfection of siRNAs and poly I:C. Relative luciferase activity is normalized to untreated reporter cells. siRNA knockdown of PARP13 was confirmed by immunoblotting (shown below). Bar graphs depict mean; error bars represent SD.

the inhibition of RNAi was not due to a nonspecific block of translation. Furthermore, RNAi inhibition was not unique to one specific reporter or miRNA. We observed similar effects in a different RNAi reporter cell line expressing the exogenous Merkel Cell Polyomavirus miRNA and corresponding RLuc reporter (Seo et al., 2009) (Figure S1D). Additionally, cells transiently transfected with plasmids expressing the JCV miRNA and a reporter subject to cleavage-independent repression showed decreased repression when cotransfected with poly I:C (Figure S1E). These results demonstrate that the inhibition of RNAi is not restricted to RISC-mediated mRNA cleavage. We conclude that detection of intracellular poly I:C leads to a previously uncharacterized inhibition of RNAi in mammalian cells.

Poly I:C Triggers Poly-ADP-Ribosylation and Inhibition of RISC

Transfection of poly I:C resulted in inhibition of RNAi (Figure 1B). To determine which component(s) of the RNAi machinery were inhibited, we first performed northern blot analysis on the effector RNAs. The steady-state levels of neither exogenous (JCV miRNA 5p or 3p derivatives) nor endogenous (miR-let7) miRNAs were altered (Figure 2A). As steady-state levels of Dicer products were unaffected by poly I:C, we predicted that a downstream component of RNAi is inhibited by mammalian antiviral signaling.

RISC functions downstream of Dicer. Therefore, we sought to determine whether human Argonaute2 (Ago2), a key component of RISC, is inhibited by antiviral signaling. We adapted an in vitro RISC cleavage assay from zebrafish (Cifuentes et al., 2010). Treatment of substrate RNA with cell lysate resulted in specific cleavage (Figure 2B). Immunodepletion of Ago2 confirms the specificity of our assay (Figure 2B, left panel). Lysate prepared from cells transfected with poly I:C was defective in RISC-mediated cleavage activity (Figure 2B, right panel), demonstrating that poly I:C-mediated effects inhibit RISC.

Recently, Leung and colleagues demonstrated that treatment of cells with oxidative and other stressors induced poly-ADP-ribosylation (pADP-r) of human Ago1–Ago4, relieving

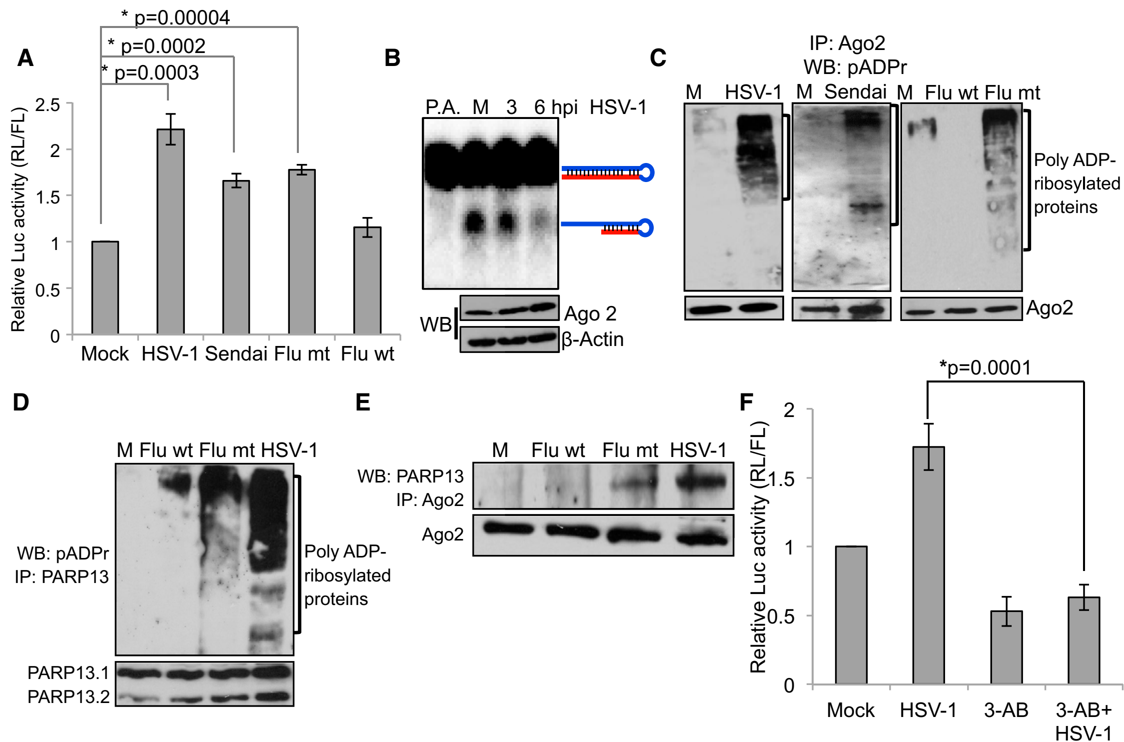

Figure 3. Viral Infection Inhibits RNAi Activity via Poly-ADP-Ribosylation of RISC-Associated Proteins

(A) RNAi activity was assayed in JM1 cells infected with HSV-1, Sendai, or WT Influenza A (Flu WT), or an NS1 mutant Influenza A (S42D) (Flu mt). Relative luciferase (RL/FL) is normalized to mock infected cells.

(B) In vitro RISC cleavage is inhibited following HSV-1 infection. Radiolabeled pre-miR-451 was incubated with cellular extracts prepared from HSV-1-infected HEK293 cells at 3 and 6 hr postinfection. Lysate from mock infected (M) or probe alone (P.A.) are shown as controls. Immunoblot confirms similar Ago2 protein levels following infection (below).

(C) pADP-r modification of RISC-associated proteins from virus-infected cells. Ago2 immunoprecipitated from cells was infected as indicated. Bound fraction was immunoblotted with pADP-r antibody. Immunoblot for Ago2 was performed as a control. pADP-r-modified proteins are indicated with a bracket.

(D) pADP-r modification of PARP13-associated proteins. HEK293 cells were infected as indicated. PARP13 was immunoprecipitated and the bound fraction was immunoblotted for pADP-r. Immunoblot for PARP13 was performed as a control.

(E) Ago2 and PARP13 interact in virus-infected cells. HEK293 cells were infected as indicated and Ago2 was immunoprecipitated. Bound fractions were immunoblotted for PARP13 (and Ago2 as a control).

(F) PARP inhibitor blocks virus-induced inhibition of RNAi. Luciferase assay was performed using JM1 cells infected with HSV-1 treated with 20 mM 3-AB. Relative luciferase activity is normalized to untreated reporter cells. Bar graphs depict mean; error bars represent SD. Figure 3 is related to Figure S2.

miRNA-mediated repression (Leung et al., 2011). Given that viral infection can trigger stress responses, we asked if the decreased RISC activity that we observe could be associated with pADP-r of Ago. We immunoprecipitated Ago2 followed by immunoblot against pADP-r-modified proteins. Consistent with Leung et al., we observed a basal amount of pADP-r-modified proteins in the Ago2 immunoprecipitate (Figure 2C, lane 2). However, upon transfection of poly I:C, a profound increase in the amount of pADP-r-modified proteins was detected in the Ago2 immunoprecipitate. The degree of poly-ADP-r observed here is on par with the reported effects of treatment with the oxidative stressor sodium arsenite (Leung et al., 2011; Figure 2C). We next determined if poly-ADP-ribose polymerase (PARP) activity was required for antiviral signaling-dependent relief of RNAi. Performing our RNAi activity assay in the presence of a chemical inhibitor of PARP (3-Aminobenzamide, 3-AB) resulted in less poly I:C-induced inhibition of RNAi (Figure 2D). Leung et al. demonstrated that the cytoplasmic PARP13 is a key component of stress-induced relief of RISC activity (Leung et al., 2011). Inhibition of RNAi activity in response to poly I:C was prevented following siRNA-mediated knockdown of PARP13 (Figure 2E). Combined, these results show that triggering the mammalian intracellular antiviral response results in pADP-r of Ago2 and/or its associated proteins and that inhibition of RISC activity is at least partly dependent on PARP13.

Infection with Viruses that Activate the Antiviral Response Inhibits RNAi

We next asked whether infection with virus inhibited mammalian RNAi. We chose two different viruses known to elicit antiviral signaling: herpes simplex virus 1 (HSV-1), a DNA virus; and Sendai virus (SenV), an RNA virus. To probe the import of the antiviral response in inactivating RNAi, we also used a mutant influenza A virus (Flu mt) that induces an increased antiviral response compared to the wild-type virus (Flu WT) (Hale et al., 2008; Hsiang et al., 2012). Infection with HSV-1, SenV, and Flu mt resulted in reduced RISC activity (Figure 3A), while infection with Flu WT only minimally relieved RISC activity. These data

demonstrate that the inhibitory effect on RISC is due to activation of the host antiviral signaling, rather than being a result of viral gene products directly blocking RISC. Infection of the Mo cell line with any of the four viruses had no effect on reporter-specific expression (data not shown). In vitro cleavage assays confirmed that infection with HSV-1 inhibits Ago2-mediated cleavage (Figure 3B). Furthermore, proteins associated with immunoprecipitated Ago2 displayed increased pADP-r in response to infection with HSV-1, SenV, or Flu mt but not with Flu WT (Figure 3C). PARP13-associated proteins are also poly-ADP-ribosylated in response to infection with Flu mt or HSV-1 (Figure 3D). Furthermore, PARP13 is observed in greater association with Ago2 upon infection (Figures 3E and S2A), and transfection of a plasmid expressing PARP13 augments the inhibitory effects of poly I:C on RNAi (Figure S2B). Our data show an increased association between PARP13 and Ago2. These findings connect PARP13 activity and association with Ago2 activity, providing evidence of a regulatory role for PARP13 in RISC function during viral infection. To determine if pADP-r is required for viral-mediated relief of RISC activity, we treated our RNAi reporter cells with the pADP-r inhibitor 3-AB during infection with HSV-1. Treatment with 3-AB suppressed the RNAi relief observed with HSV-1 infection (Figure 3F), correlating in degree with the amount of pADP-r detected on Ago2-associated proteins (Figure S2C). Therefore, we conclude that the response of mammalian cells to infection by diverse viruses involves pADP-r of PARP13- and Ago2-associated proteins, with the latter resulting in reduced RNAi activity.

Antiviral Signaling Pathways Contribute to Inhibition of RISC

Next, we investigated what components of the antiviral response are important for relief of RISC-mediated repression. Treatment with IFN was not sufficient to relieve repression by RISC, but augmented the effect of poly I:C (Figure S3A). Mitochondrial antiviral signaling adaptor (MAVS) and RNaseL are both indirectly activated by dsRNA. Using a genetic approach, we tested whether these pathways have a role in inhibition of RNAi. Mouse embryo fibroblast (MEF) cell lines devoid of MAVS or RNaseL both show reduced pADP-r of Ago2-associated proteins upon infection with HSV-1 (Figure 4). siRNA-mediated knockdown of MAVS in JM1 cells decreases poly I:C-induced inhibition of RISC activity (Figure S3B). RIG-I is a PRR that binds to dsRNA and activates MAVS. To determine if activating MAVS was sufficient to inhibit RNAi, we expressed an amino-terminal fragment of RIG-I capable of activating MAVS in the absence of dsRNA (Saito et al., 2007). This failed to inhibit RNAi; however, it substantially augmented the effects of poly I:C (Figure S3C). These data show that different PRRs can indirectly contribute to the repression of RISC during the antiviral response, but that this inhibition requires intracellular detection of a dsRNA PAMP.

Cytotoxicity-Associated ISGs Are Particularly Amenable to miRNA-Mediated Regulation, which Is Alleviated by Inhibition of RISC during the Antiviral Response

The question arises as to what benefit (if any) mammalian cells receive from inhibiting RISC during viral infection. pADP-r of RISC-associated proteins leads to inhibition of both siRNA-mediated cleavage and miRNA-mediated repression (Leung et al., 2011; Figures 2, 3, and S2). We hypothesized that relief of miRNA-mediated regulation could allow for enhanced translation of the host mRNAs that are highly regulated by miRNAs. Many ISGs are toxic to cells (Barber, 2000), and cytotoxic gene products are typically subject to multiple layers of regulation. Therefore, we predicted that, compared to all mRNAs, miRNAs and RISC are more likely to regulate the expression of ISGs. To determine the likelihood that miRNAs regulate the IFN response, we first utilized a bioinformatic approach based on literature-annotated ISGs (http://www.lerner.ccf.org/labs/williams/isgd.cgi) and the TargetScan miRNA target prediction software (Lewis et al., 2005). The 3′ UTRs of transcripts are the predominant docking sites of the miRNA-RISC complexes, and our results show that, as a whole, ISG 3′ UTRs have an approximately 2-fold higher median of predicted evolutionarily conserved miRNA binding sites, compared to all 3′ UTRs (Figure 5A). Strikingly, when only those 136 ISGs annotated as associated with cell death and proliferation (Huang et al., 2009; Table S2) were analyzed, this difference increased to 4-fold. Thus, although these analyses revealed an underrepresentation of miRNA binding sites in some (especially canonical) ISGs (data not shown), these analyses do strongly support the hypothesis that some ISGs, especially those associated with toxicity, are particularly susceptible to miRNA-mediated regulation.

Next, we experimentally identified those ISG transcripts that are most susceptible to miRNA-mediated regulation. To do this, we took advantage of a human colorectal cancer cell line (DLD-1) with an engineered hypomorphic mutant Dicer (ex5−/−) leading to reduced miRNA levels (Cummins et al., 2006). These cells, and the corresponding WT parental cells, were treated with type I IFN and subjected to microarray-based analysis of mRNA expression. A number of ISG transcripts are expressed at higher levels in the ex5−/− cells (Figures S4A, S4B, and data not shown), demonstrating that a subset of these mRNAs are regulated by RNAi machinery.

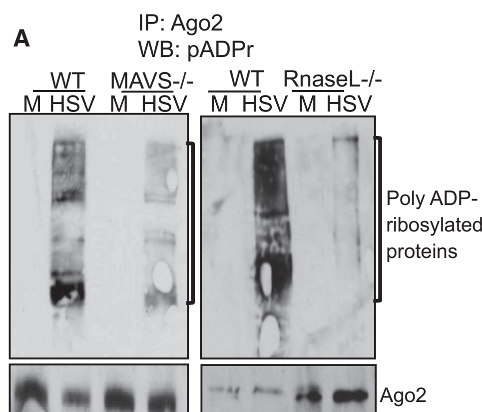

Figure 4. MAVS and RNaseL Contribute to Poly-ADP-Ribosylation of RISC-Associated Proteins

pADP-r modification of RISC-associated proteins is reduced in MAVS and RNaseL knockout MEFs. Ago2 was immunoprecipitated from MEF knockout cell lines either mock (M) infected or infected with HSV-1. Bound fractions were immunoblotted for pADP-r (and Ago2 as a control). Figure 4 is related to Figure S3.

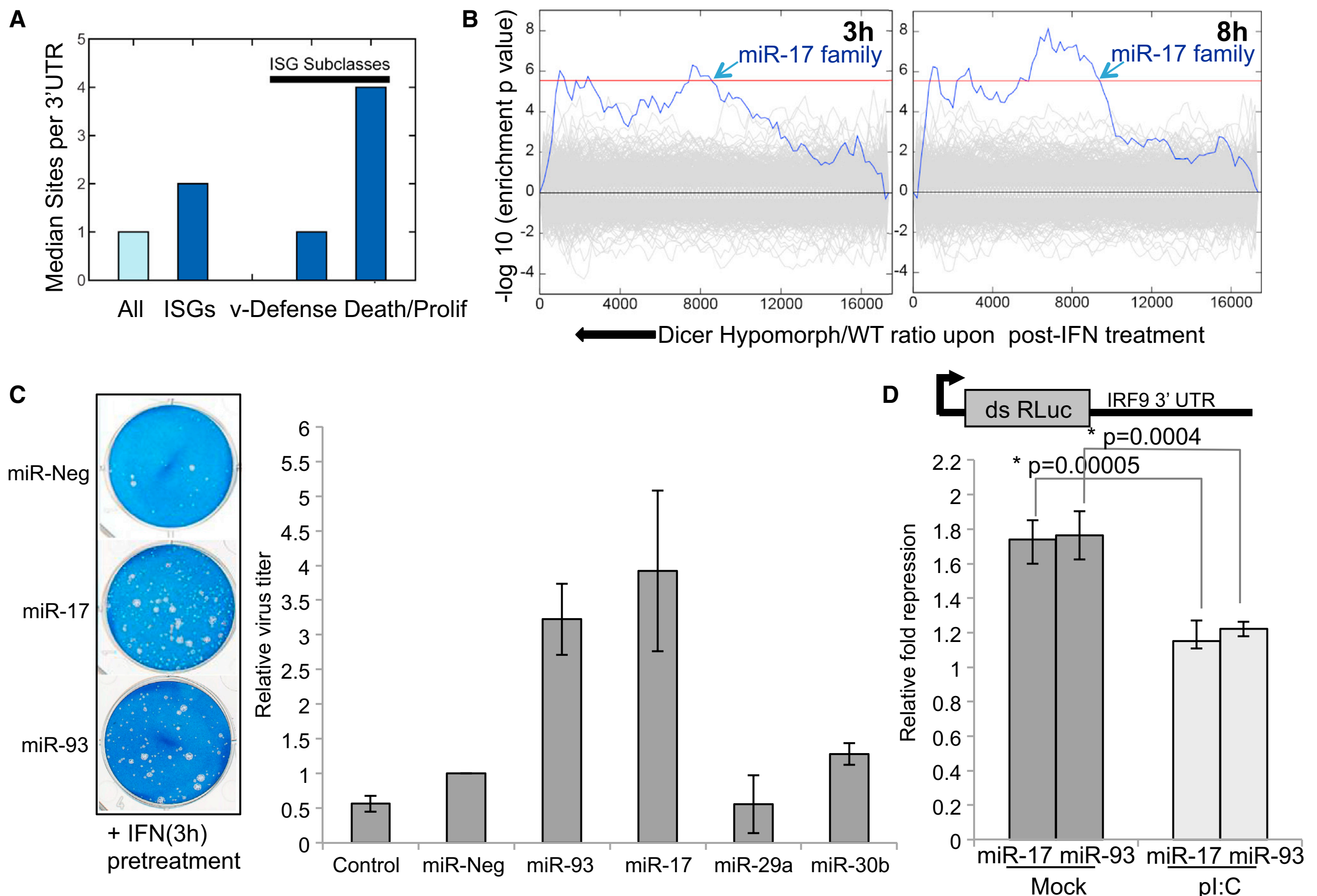

Figure 5. miR-17/93 Family Regulates IFN-Stimulated Genes and Promotes HSV-1 Infectivity

(A) Analysis of conserved miRNA targets predicted by TargetScan. The median number of predicted, conserved miRNA binding sites per 3′ UTR was calculated for all human genes (All), ISGs, the Defense Response and Response to Virus (v-Defense) subgroup, and the Regulation of Cell Death and Regulation of Cell Proliferation (Death/Prolif) subgroup.

(B) Sylamer heptamer analysis of rank-ordered microarray data from pretreatment of DLD-1 WT and Dicer ex5−/− cell lines at the indicated times. miR-17/93 family heptamer is highlighted in blue. Red line represents the threshold for a Bonferroni corrected E-value of 0.05.

(C) Analysis of miR-17 family miRNA mimics on HSV-1 infection. Dicer hypomorph cells were transfected with the indicated miRNA mimics. After 24 hr, cells were treated with 1,000 U/ml of universal type 1 IFN for 3 hr prior to infection with HSV-1. The media were collected 48 hr postinfection and virus was titered via plaque assays. A representative plaque assay is shown (left panel). Results from three independent infections were quantified (right).

(D) IRF9 3′ UTR is derepressed following poly I:C transfection. Schematic of the full-length IRF9 3′ UTR reporter construct is shown (top). HEK293T cells were cotransfected with RLuc and ffLuc reporters and miRNA mimics. Twenty-four hours later, cells were transfected with poly I:C and harvested for luciferase assays after 8 hr. Relative fold repression of luciferase activity (RL/FL) is determined by normalizing miR-17 or miR-93 mimic-transfected cells to the irrelevant miRNA mimic-transfected control cells. Bar graphs depict mean; error bars represent SD. Figure 5 is related to Figure S4.

The 5′ end of a miRNA (nucleotides 2–8), referred to as the "seed" region, plays a critical role in targeting transcripts. To determine if any of the ISG mRNAs identified above were enriched for specific miRNA seed complements, we conducted Sylamer bioinformatic analysis (van Dongen et al., 2008) on transcripts more highly expressed in the ex5−/− cells, relative to WT, following IFN treatment. Those transcripts that are more highly expressed in the ex5−/− hypomorph after IFN treatment are shown to contain significantly more binding sites for the miR-17/93 family (Figure 5B).

To determine if, as our results would predict, ISG transcripts are bona fide targets of the miR-17/93 family, we conducted a meta-analysis of published miRNA target lists identified from RISC-bound transcripts (Gottwein et al., 2011; Riley et al., 2010; Skalsky et al., 2012). Gottwein et al. and Skalsky et al. utilized PAR-CLIP, while Riley et al. utilized HITS-CLIP-based strategies to identify global miRNA target sites. Our analysis showed that many of the miR-17/93 family targets identified in these studies are annotated as ISGs (Figure S4C). Despite not being saturated, and being conducted in different lines of B cells, several ISGs were identified as targets of miR-17/93 in two or more of these studies. Next, we took an independent bioinformatic approach and interrogated the CLIP data sets to ask what miRNAs were most associated with the literature-annotated ISG transcripts (http://www.lerner.ccf.org/labs/williams/isgd.cgi). Several different miRNAs, including miR-548 and miR-142, were identified in two more of the studies (Figure S4D). These data argue that multiple different miRNAs play a role in

regulating ISGs. Strikingly, all three CLIP-based studies identified the miR-17/93 family as having the most interactions with ISG transcripts (Figure S4D). Together, our results using different cell types and completely different methodologies demonstrate that, while not the only miRNA family to target ISGs, the miR-17/93 family plays an especially important role in regulating them.

To test a functional role for the miR-17/93 family miRNAs in negatively regulating ISGs, we transfected the Dicer hypomorph DLD-1 cells with synthetic mimics of miR-17, miR-93, or any of several additional control and host miRNAs. Following 3 hr of treatment with type I IFN, sufficient time for ISG transcription, we then infected with HSV-1 or Flu mt. Production of new virus was measured 48 hr postinfection. Consistent with the notion that miR-17/93 negatively regulates components of the antiviral response, we observed a reproducible ~2- to 3-fold increase in secreted virus from cells expressing miR-17 or miR-93, but not the control miRNA mimics (Figures 5C and S4E). Similar results of slightly less magnitude were observed without IFN pretreatment (data not shown). Recent in vivo work shows that Dicer hypomorphic mice controlled viral infection better than wild-type mice at early times postinfection (Ostermann et al., 2012). This observation could be explained by the effects of miR-17/93 (or similar-functioning miRNAs) on ISG expression. Combined, these observations support an indirect role for miRNAs in promoting infection in cells undergoing an IFN response, by suppressing the translation of some ISG mRNAs.

Our data show miRNAs negatively regulate cytotoxic ISG expression. Thus, miRNAs can be considered proviral in some contexts. Consequently, we hypothesized that mammalian cells alleviate RISC to allow for increased ISG expression. We tested whether initiating the antiviral response can relieve repression of a well-established ISG transcript targeted by miR-17/93. IRF9 is a sometimes procytotoxic (Tsuno et al., 2009) transcription factor that plays a central role in the activation of promoters containing IFN-stimulated responsive elements (ISREs) (Müller et al., 1993). Thus, IRF9 is atypical, being both an ISG itself and also a transactivator of numerous other ISGs (Tsuno et al., 2009). Our analyses of both DLD-1 and B cells identified it as a target of miR-17/93 (Figures S4B and S4C and data not shown). First, we confirmed that endogenous IRF9 protein levels were higher in DLD-1 dicer hypomorph cells undergoing an IFN response (Figure S4F). Although unchanged at 4 hr post-IFN treatment (data not shown), at 6, 8, and 10 hr post-IFN treatment, IRF9 protein levels were higher in the hypomorphs (Figure S4F). Next, we cloned the entire 3′ UTR of human IRF9 behind a luciferase reporter to determine if it is regulated by either miR-17 or miR-93 in HEK293T cells. Compared to an irrelevant control miRNA, both miR-17 and miR-93 repressed luciferase activity by 1.7-fold (Figure 5D, left side). When we performed the same experiment following transfection of poly I:C, we observed reduced miRNA-mediated repression (Figure 5D, right side), as would be predicted from our above results (Figures 1B, S1D, and S1E). These data show that triggering the antiviral response can alleviate miRNA-mediated posttranscriptional regulation of an antiviral transcript. Combined, the above data demonstrate that increasing the activity of the miRNA arm of the RNAi response can be proviral in mammalian cells and, conversely, inhibiting miRNA activity can increase the protein-mediated antiviral response.

DISCUSSION

RNAi is an essential antiviral defense against viruses in plants and invertebrates. However, whether RNAi plays a similar role in mammalian cells remains debated. Here, we provide evidence that antiviral signaling attenuates RISC activity in different types of somatic mammalian cells. After viral infection, or treatment with poly I:C, RISC-associated proteins become posttranslationally modified with pADP-r. Consistent with the findings of Leung and colleagues (Leung et al., 2011), we show increased pADP-r of RISC correlates with decreased siRNA and miRNA silencing activities (Figures 2C and 3C). We demonstrate that the MAVS and RNaseL pathways are involved at least indirectly in virus-mediated inhibition of RNAi (Figures 4 and S3) and speculate that these pathways may serve to induce the levels of PARP or other factors required for ADP-ribosylation of RISC. Thus, we have identified an additional biological trigger that leads to pADP-r-mediated inhibition of RISC. pADP-r of RISC correlates with an increased association between PARP13 and the RISC complex (Figures 3E and S2A). Despite being catalytically inactive, PARP13 was shown to be an essential hub for fostering the interaction of various PARPs with RISC (Leung et al., 2011). Together, these data suggest that antiviral signaling leads to both increased PARP13 association with and pADP-r of RISC, resulting in reduced RNAi activity.

Our results are consistent with an earlier report showing that HSV-1 infection can inhibit RNAi (Wu et al., 2009). While no mechanism was provided for this observation, it was suggested that undefined, virus-encoded effectors blocked RNAi to evade its role as an antiviral defense. Previous reports have shown that infection with HSV-1 produces dsRNA (Weber et al., 2006) and elicits an antiviral signaling response (Melchjorsen et al., 2010; Rasmussen et al., 2009). Therefore, we predict that the HSV-1-mediated inhibition of RNAi is at least partly due to activation of the antiviral response (Figure 4). Consistent with this, infection with either Sendai virus, which is known to elicit an antiviral response (Seth et al., 2005), or a mutant influenza virus, known to have an enhanced antiviral response (Hale et al., 2008; Hsiang et al., 2012), results in ADP-ribosylation of RISC and inhibition of RNAi (Figure 3). Minimally, we show that infection with diverse viruses capable of triggering antiviral signaling responses results in diminished RNAi activity in mammalian cells. Combined with the difficulty of identifying siRNAs in virus-infected mammalian cells (Lin et al., 2010; Parameswaran et al., 2010; Pfeffer et al., 2005; Umbach and Cullen, 2009) and the lack of strong genetic evidence, our results argue that, at least for some cells, canonical RNAi is not an antiviral response in mammals.

What advantage is there to mammalian cells in attenuating RNAi during the antiviral response? Unlike plants and invertebrates, mammals have evolved an elaborate protein-based antiviral defense that is controlled by multiple layers of negative regulation. Once intracellular pathogens are detected, dispensing with such regulation would help ensure a robust response to infection. We demonstrate that ISGs, particularly those associated with cell proliferation and cell death, are

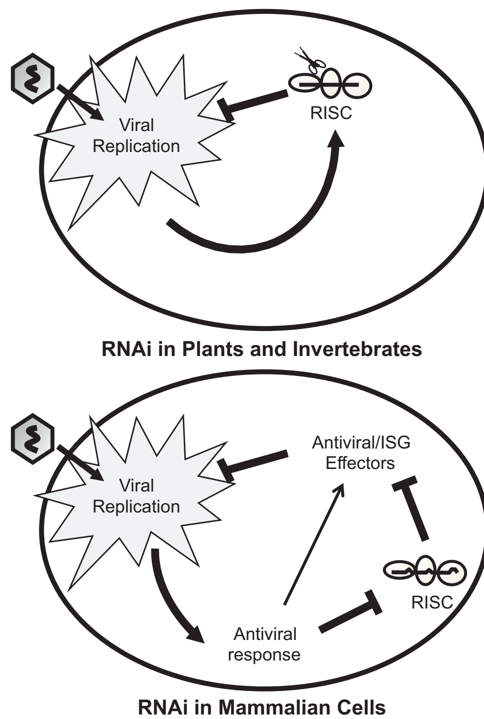

Figure 6. Model: Different Roles of RNAi Machinery in Somatic Mammalian Cells versus Plants and Invertebrates.
RNAi directly blocks viral replication in plants and invertebrate animals (top). RNAi and RISC components in somatic mammalian cells repress expression of ISG/antiviral effectors via miRNAs. RNAi is inhibited by activation of the intracellular antiviral response, allowing for optimal ISG expression, especially of cytotoxic ISGs (bottom).

significantly more likely to be regulated by miRNAs (Figure 5A). The miR-17/93 family of miRNAs can negatively regulate components of the antiviral response in diverse cell types (Figures 5B, S4C, and S4D). Consistent with this, it has previously been reported that exogenous expression of miR-93 is proviral (Santhakumar et al., 2010). Despite the clear role of miR-17/93 in regulating the antiviral response, we note that our data (Figure S4D) and others' published work (Cloonan et al., 2008; Gregersen et al., 2010; Lee et al., 2012; Ostermann et al., 2012) indicate that additional miRNAs are also involved in regulating ISGs. This point is particularly relevant to nondividing and nontransformed cells where miR-17-related miRNAs are typically not abundantly expressed.

Combined, our data support the following model (Figure 6): In many eukaryotes, RNAi serves as a primary antiviral defense by directing the RISC machinery to cleave viral RNAs, thereby hindering infection. However, the evolution of a large-scale, protein-based antiviral defense has produced an effective alternative for combating viral infection without RNAi. In somatic cells of mammals (and possibly cells of other jawed vertebrates), several different miRNAs and the RNAi machinery have been co-opted to negatively regulate expression of many of the cytotoxic effectors of this protein response, thereby allowing for responsive control of its timing, degree, and resolution. Within this model, we predict that cells receiving only transient extracellular stimulation of the IFN receptor will express ISG transcripts to prepare for a possible infection and that miRNAs will contribute to recovery from this IFN response. We note that, as miRNAs are endogenous products of the RNAi machinery, this model allows for some RNAi components to be considered proviral under certain IFN-rich conditions. If, however, the appropriate intracellular PRRs are activated, miRNA activity is reduced through posttranslational modification of RISC. This inhibition of RNAi allows enhanced expression of ISGs, resulting in increased cell death. Similar to a grenade requiring two triggers to detonate (pull the pin; release the handle), IFN signaling and inhibition of RISC combine to produce a robust, terminal IFN response. Several reports have indicated different immunostimulatory extracellular cues can induce the expression of various miRNAs (O'Connell et al., 2007; Taganov et al., 2006). We propose that these miRNAs promote an antiviral state in certain contexts, or, in other cases, serve as part of a negative feedback loop to quench the antiviral response if subsequent intracellular infection is not detected.

There are several important implications of this model. First, our work suggests that "canonical" RISC-dependent RNAi is unlikely to be an antiviral effector in cells with a strong IFN-based response, including somatic cells of mammals and other vertebrates. We note that this does not rule out a possible role for RNAi in defending against viruses in some other cell types that have a reduced IFN response (e.g., mammalian embryonic stem cells [Chen et al., 2010]).

Second, there exists an emerging model that diverse viruses utilize viral and possibly host-derived miRNAs to control the switch from latent/persistent infection to lytic/productive replication (Gunasekharan and Laimins, 2013; Kincaid and Sullivan, 2012; Murphy et al., 2008). Since it is now clear that various stressors can elicit pADP-r modification of RISC-associated proteins, our model suggests that these viruses could take advantage of changes in miRNA regulatory activity as a barometer for cellular stress. This could represent one mechanism for how these viruses promote "release" from miRNA-reinforced latency under stress conditions (e.g., a secondary infection activates the antiviral signaling pathway, inactivating RISC). Some viruses utilize miRNA activity during productive/lytic infection. Our model predicts that these viruses block antiviral signaling, thereby preventing inhibition of RISC, or that the degree of residual RISC activity is sufficient for the relevant silencing activity. We note that our model in no way precludes an antiviral role for miRNAs in certain contexts. For example, pox virus infection ablates host miRNA activity, likely to avoid direct negative regulation of its own transcripts (Backes et al., 2012). In such situations, we suggest that the benefits to the virus of removing host miRNA function outweigh the costs of any possible increased toxic ISG response.

Third, while our "grenade model" focuses on control of the IFN response, it also applies, in principle, to other stress responses reliant on expression of cytotoxic proteins. For example, severe oxidative damage could attenuate miRNA-mediated repression to increase the amount of and/or the speed with which heavily regulated cytotoxic effectors are made. Interestingly, some ISGs, including those that are cytotoxic, are also induced by other stress stimuli (Chawla-Sarkar et al., 2003). The ability of separate pathways to converge on the same cytotoxic genes both underscores the importance of careful regulation and

suggests there exists a mechanism(s) for integrating complex signals from independent signaling cascades. Consistent with this, miRNAs have been implicated in maintaining diverse cellular processes of homeostasis (Ebert and Sharp, 2012; Mendell and Olson, 2012).

In summary, we show that antiviral signaling leads to an inhibitory pADP-r modification of RISC-associated proteins. Inhibition of RISC leads to derepression of a subset of ISG transcripts. Therefore, we conclude that, in some contexts, components of the mammalian RNAi pathway do not play an antiviral role. On the contrary, miRNAs and the RNAi apparatus have been co-opted to negatively regulate toxic effectors of the protein-based IFN response.

EXPERIMENTAL PROCEDURES

Detailed methods are included in Supplemental Information.

Cell Culture and Reagents

Mo and the JM1 cell lines are HEK293 derivatives that stably express the RLuc RNAi reporter with (JM1) or without (Mo) the exogenous JC virus miRNA (McClure et al., 2011b). These and the MEF and DLD-1 cell lines and derivatives were grown under ATCC-recommended conditions. Commercial sources for chemicals and antibodies are listed in Supplemental Experimental Procedures.

Plasmid Constructs

All constructs were cloned using standard molecular biology procedures. See Supplemental Experimental Procedures for details and custom oligonucleotides used.

RNAi Activity Reporter Assay

Mo or JM1 cells were assayed with the Dual-Glo Luciferase Assay System. Poly I:C treatment: Cells were transfected with 0.5 μg poly I:C using the Lipofectamine 2000 reagent (Invitrogen) and harvested 8 hr after transfection, unless otherwise noted. Cells were infected with HSV-1 (moi 10), harvested 10 hr postinfection; influenza virus A wild-type (Flu WT) and Flu mt S42D (Hsiang et al., 2012) (moi 2), harvested 13 hr postinfection; or Sendai 50 HA/ml, harvested 6 hr postinfection. RIG-I CARD domain: The amino-terminal domain CARD domain construct (Saito et al., 2007) was provided by M. Gale (University of Washington). JM1 cells were transfected with either control pcDNA3.1 or pcDNA3.1 RIG-I CARD using Lipofectamine 2000. Forty hours posttransfection, cells were transfected with 0.5 μg poly I:C. ISG15 induction was assayed via standard semiquantitative RT-PCR with control for linearity. See Supplemental Experimental Procedures for primers and amplification conditions.

Monitoring miRNA Activity

HEK293T cells were transiently transfected with reporters and various miRNA mimics. Eighteen hours posttransfection, cells were either treated with 250 μM sodium arsenite (90 min) or transfected with poly I:C followed by luciferase assay.

Northern Blot Analysis

Total RNA was run on denaturing PAGE gels, transferred to membranes, probed with radiolabeled oligonucleotide probes, and visualized via film on Phosphorimager (McClure et al., 2011a; Seo et al., 2008).

siRNA Knockdowns

Knockdown of irrelevant controls (eGFP, GAPDH), PARP13, or MAVS was achieved through double transfection (repeat siRNA transfection after 48 hr) of 20 nM siRNAs (Table S1). Forty-eight hours after second transfection, cells were transfected with poly I:C and assayed for luciferase activity 8 hr later.

Western Blot Analysis

Proteins were extracted using cell lysis buffer consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, and the complete EDTA free protease inhibitor cocktail (Roche). Lysates were size fractionated by SDS-PAGE and immunoblotted with antibodies. See Supplemental Experimental Procedures for details of antibodies used.

In Vitro RISC Mediated Cleavage Assay

Cell extracts were exposed to a synthetic Ago2 radiolabeled substrate pre-miR-451, separated on a denaturing PAGE gel and visualized on a Phosphorimager (Cifuentes et al., 2010).

Detection of the Poly-ADP-Ribosylation of Ago2

Poly I:C-treated and infected extracts were immunoprecipitated for Ago2, and associated proteins were immunoblotted for Ago2, PARP13, or pADP-r-modified proteins (Leung et al., 2011).

Role of miR-17/93 in Virus Infection

DLD-1 Dicer hypomorph cells were transfected with miRNA mimics. Cells were treated with IFN and then infected with HSV-1 or Flu mt. Secreted infectious virus concentration was determined via plaque assay.

Microarray Analysis

Total RNA was harvested from IFN-treated DLD-1 WT and DLD-1 Dicer hypomorph cells. Illumina HumanHT-12 v4.0 microarray mRNA analysis identified transcripts higher in IFN-treated miRNA hypomorph cells. Sylamer analysis identified transcripts with 3′ UTRs significantly enriched for miRNA complementary sites (van Dongen et al., 2008).

Analysis of ISG 3′ UTRs and Predicted miRNA Target Sites

ISG names were obtained from the ISG database of Bryan Williams (http://www.lerner.ccf.org/labs/williams/isgd.cgi). Median 3′ UTR length was extracted from TargetScan 6.2 3′ UTR sequence alignment (Lewis et al., 2005). Counts of miRNA target sites were generated for each gene. Conservation of miRNA families and conservation of miRNA target sites was defined according to the TargetScan 6.2 database. G.O. analysis of the ISGs used DAVID (Huang et al., 2009). Functional annotation charts were generated using the GOTERM_BP_FAT category of terms.

Meta-Analysis of Published miRNA Target Profiles

Targets were extracted from three CLIP data sets (Gottwein et al., 2011; Riley et al., 2012; Skalsky et al., 2012), and the ISG target subset was identified bioinformatically (custom python scripts). miRNA families enriched for targeting ISGs were derived from counts of CLIP-identified targets.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.09.002.

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