A conserved miRNA-183 cluster regulates the innate antiviral response

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Upon immune recognition of viruses, the mammalian innate immune response activates a complex signal transduction network to combat infection. This activation requires phosphorylation of key transcription factors regulating IFN production and signaling, including IFN regulatory factor 3 (IRF3) and STAT1. The mechanisms regulating these STAT1 and IRF3 phosphorylation events remain unclear. Here, using human and mouse cell lines along with gene microarrays, quantitative RTPCR, viral infection and plaque assays, and reporter gene assays, we demonstrate that a microRNA cluster conserved among bilaterian animals, encoding miR-96, miR-182, and miR-183, regulates IFN signaling. In particular, we observed that the miR-183 cluster promotes IFN production and signaling, mediated by enhancing IRF3 and STAT1 phosphorylation. We also found that the miR-183 cluster activates the IFN pathway and inhibits vesicular stomatitis virus infection by directly targeting several negative regulators of IRF3 and STAT1 activities, including protein phosphatase 2A (PPP2CA) and tripartite motif–containing 27 (TRIM27). Overall, our work reveals an important role of the evolutionarily conserved miR-183 cluster in the regulation of mammalian innate immunity.

The innate immune system represents the immediate and nonspecific response to pathogens in almost all multicellular organisms (1). This first line of defense coordinates protection against a variety of both single-stranded and double-stranded DNA and RNA viruses as well as intracellular bacteria and parasites via the action of a class of cytokines called IFNs. Interferons induce the expression of numerous antiviral effector proteins, and IFN production relies on multiple phosphorylation events, including C-terminal phosphorylation of IFN regulatory factor 3 (IRF3) (2, 3), a transcription factor that regulates the expression of both type I and III IFNs (4). An integrated gene network of both positive and negative regulators is crucial for proper dynamic activation and resolution of IFN signaling.

Recent work has highlighted a group of small RNAs, called microRNAs (miRNAs) as key regulatory layer in cell intrinsic immunity and the inflammatory response (5). Generally, miRNAs posttranscriptionally regulate gene expression relevant to diverse cellular processes by base-pairing with the 3′ UTR of mRNAs and mediating mRNA destabilization and translational repression (6). MicroRNA expression can be modulated during viral infection or the innate immune response (5, 7) and can serve to either potentiate or dampen the immune response (8, 9, 10). The functional relevance of miRNAs in shaping the innate antiviral response is not completely understood.

When examining miRNA signatures associated with viral infection, we identified that the highly conserved miR-183 cluster (miR-96, miR-182, and miR-183; Fig. 1A) is dysregulated in the context of different viral infections, including both DNA and RNA viruses (11–15). Furthermore, genetic variation in this miRNA cluster is associated with increased susceptibility to inflammatory and autoimmune disease (16). Despite these connections, the role of the miR-183 cluster in innate immunity is unclear.

Here, we examined the role of the miR-183 cluster in the innate immune response. We found that all three miRNAs encoded in the cluster promoted IFN signaling and production through repression of key negative regulators of IRF3 and STAT1 signaling. Our work demonstrates a novel role of this miRNA cluster in cooperative regulation of the IFN pathway.

Results

The miR-183 cluster suppresses viral replication

To characterize the miR-183 cluster’s role in the innate antiviral response, we first examined the influence of the cluster on virus production. As previous work has shown dysregulation of

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6 The abbreviations used are: miRNA, microRNA; VSV, vesicular stomatitis virus; ISG, IFN-stimulated gene; qPCR, quantitative RT-PCR; TLR, Toll-like receptor; LMW, low-molecular-weight; NEAA, nonessential amino acid; Con-miR, control microRNA.
the cluster’s expression in the context of viral infection of the liver (13, 14, 17), we performed our initial studies in IFN signaling–competent HepG2 hepatoma cells (18). We transfected HepG2 cells with mimics of each miRNA in the cluster and subsequently infected the cells with GFP-encoding vesicular stomatitis virus (VSV-GFP). miR-183 mimics transfection elicited the most potent antiviral effect, inducing a more than 2-log drop in virus production (Fig. 1B). Fluorescence microscopy confirmed decreased GFP production in miR-183–transfected cells infected with the VSV reporter virus (Fig. 1C). We also observed an analogous antiviral effect in MCF7 breast cancer cells (Fig. 1D) and immortalized mouse embryonic fibroblasts (Fig. S1), suggesting that the cluster’s antiviral effects are conserved across mice and humans. A comparison of the cluster’s inhibitory effects against VSV in the three tested models revealed that miR-183 was consistently the most potent antiviral miRNA. To further confirm the miR-183 cluster’s antiviral effects, we examined VSV production in miRNA inhibitor–treated MCF7 breast cancer cells 72 h post-transfection with miRNA mimics or inhibitors and 24 h after infection. In B and E, error bars represent mean ± S.E. from three independent experiments. In D, data represent the means ± S.E. of two biological replicates.

Figure 1. An immune-regulated miRNA cluster inhibits viral infection. A, schematic depicting the immunoregulatory role of the miR-183 cluster. Conservation of miR-96, miR-182, and miR-183 is shown below. B and C, HepG2 cells were transfected with mimics of miR-96, miR-182, or miR-183, along with a negative control mimic at a final concentration of 50 nM. 48 h post-transfection, cells were infected with VSV-GFP (multiplicity of infection = 0.01) for 24 h. The effects of the miRNAs on virus production were assessed by plaque assays for VSV-GFP (B) and VSV-GFP–infected cells (C) and visualized 72 h post-transfection and 24 h post-infection using fluorescence microscopy; representative images are shown for Con-miR– and miR-183–transfected cells. Scale bar = 100 μm. D and E, plaque assay results showing the influence of miR-183 cluster mimics (D) and inhibitors (E) on VSV-GFP production in MCF7 breast cancer cells 72 h post-transfection with miRNA mimics or inhibitors and 24 h after infection. In B and E, error bars represent mean ± S.E. from three independent experiments. In D, data represent the means ± S.E. of two biological replicates.
profiling on HepG2 cells treated with miR-183 cluster mimics and subsequently immunostimulated with poly(I:C)/LyoVec. This poly(I:C)/LyoVec complex acts as an agonist of the cytoplasmic sensor of viral RNA, retinoic acid-inducible gene I (RIG-I). Among the genes activated by more than 8-fold by each miR-96/182/183 mimics independently were several well-established interferon-stimulated genes (ISGs), including OAS2, RSAD2, DDX60, and IFITM1 (Fig. 2A).

Gene ontology enrichment analysis of genes up-regulated more than 1.5-fold in expression revealed that biological processes related to type I IFN signaling were activated by each miRNA of the cluster (Fig. 2B and Tables S1–S3). Data from qPCR experiments of type I and III IFN (IFNB1 and IFNL1) as well as ISG (STAT1, DDX58, and RSAD2) expression levels in poly(I:C)-stimulated HepG2 cells confirmed enhanced IFN signaling and production in miR-183 cluster–treated cells (Fig. 2, C and D). Additionally, a similar activation of IFN and ISG expression was observed in VSV-infected MCF7 breast cancer cells treated with the miR-183 mimics (Fig. S2, A and B). Conversely, inhibition of the miR-183 cluster produced an opposite effect, decreasing IFNB1 and RSAD2 expression (Fig. S3). Analogous levels of ISG expression were observed in HepG2 cells following VSV infection.
These data strongly validate the regulatory role of the miR-183 cluster in IFN signaling.

miR-183 promotes Jak/STAT signaling

Type I and III IFN stimulation activates the JAK/STAT pathway (20), which yields phosphorylation-dependent activation of STAT1, a transcription factor required for ISG induction. Interestingly, we observed increased STAT1 mRNA expression in miR-183–treated cells, suggesting that miR-183 activates constitutive Jak/STAT signaling in the absence of immune stimulation (Fig. S5). Activation of STAT1 depends on phosphorylation at a C-terminal tyrosine residue (Tyr-701), whereas an additional phosphorylation at Ser-727 is thought to be required for maximal activation of the transcription factor (21). We examined the miR-183 cluster’s effect on these two STAT1 phosphorylation sites. As miR-183 exhibited the most potent antiviral effect (Fig. 1, B–D, and Fig. S1), we focused our efforts on characterizing this miRNA’s effects on Jak/STAT signaling. miR-183–transfected HepG2 cells showed increased STAT1 phosphorylation at Tyr-701 and Ser-727 during poly(I:C)/LyoVec and IFN-α immunostimulation at various time points (Fig. 3, A−C), consistent with enhanced Jak/STAT signaling.

We performed short pulses of IFN-α to distinguish changes in phosphorylation status from overall increases in STAT1 expression levels. The results showed that the increase in phosphorylation by miR-183 occurred prior to any change in expression of STAT1 (Fig. 3B). Consistent with this, miR-183 was shown to be able to enhance IFN-stimulated response element (ISRE)-driven reporter activity during TBK1 and IKKε overexpression in HEK293T cells (Fig. S6). Measurements by qPCR also confirmed that the miRNA cluster activated ISG expression during IFN-α stimulation, with DDX58, RSAD2, and STAT1 expression increasing over 4-fold in cells transfected with each mimic independently (Fig. 3D).

Increased STAT1 Tyr-701 phosphorylation was also observed in the context of VSV-GFP infection; however, no significant difference in phosphorylation at the Ser-727 site was seen (Fig. 3E). The use of an attenuated VSV strain, defective in its capacity to evade IFN signaling because of a deletion in the matrix (M) protein (22), rescued the miR-183–mediated effect.
on Ser-727 phosphorylation. This suggests that WT VSV M protein partially impairs miR-183’s effect on STAT1; however, because it has been shown that it is possible to achieve STAT1 activation independent of the phosphorylation at Ser-727 (21), there is only partial impairment. Maximal activation of STAT1 is achieved when phosphorylation at Ser-727 is also maximized (21). Furthermore, similar results were observed in poly(I:C)/LyoVec-treated A549 lung cancer cells (Fig. S7). Collectively, our observations revealed that miR-183 enhances Jak/STAT signaling under both basal and virus-stimulated conditions.

Viral RNA can also be sensed by Toll-like receptors (TLRs) 3, 7, and 8 in the endosomal compartment, and ligand engagement of these TLRs results in downstream IFN production and signaling. We examined whether the cluster could influence TLR3 signaling in MCF7 cells by treating miR-183 cluster mimic–transfected cells with naked poly(I:C) and probing STAT1 phosphorylation levels. Our immunoblot analyses revealed that miR-96 and miR-183 promoted TLR3 agonist–induced STAT1 phosphorylation (Fig. S8). Taken together, these results demonstrate that the miR-183 cluster activates both TLR and RIG-I–induced Jak/STAT signaling.

**miR-183 represses negative regulators of IFN production**

To characterize the direct targets of miR-183 contributing to its stimulatory effects on IFN signaling/production and antiviral effects, we utilized the gene expression profiling data to identify potential miR-183 targets. To do this, we examined the overlap between miR-183 predicted targets from TargetScan (23) and genes whose expression was down-regulated by miR-183 mimic transfection in poly(I:C)/LyoVec–stimulated HepG2 cells (>1.5-fold) (Fig. 4A). This produced a list of 413 genes, and we examined this list for genes described previously...
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Figure 5. A schematic of the proposed model of the mechanism by which miR-183 regulates the innate antiviral immune response. The miR-183 cluster positively regulates type I interferon and ISG production by repressing negative regulators of the antiviral response. Specifically, miR-183 targets PPP2CA, which is known to negatively regulate IRF3 and STAT1 phosphorylation (24, 26), and miR-183 also targets TRIM27 and, as a result, impairs the degradation of TBK1 and enhances IRF3 signaling in the initiation of type I IFN production (25). This establishes a model whereby miR-183’s repression of PPP2CA and TRIM27 enhances the activation of IFN and ISG production.

To be negative regulators of IFN production and signaling, miR-183 repressed the expression of serine/threonine protein phosphatase 2A catalytic subunit α isoform (PPP2CA) (24) and tripartite motif-containing 27 (TRIM27) (25), genes shown previously to inhibit IFN production with putative miR-183 binding sites in their 3’ UTRs (Fig. 4B).

We then validated repression of miR-183 regulated genes in poly(I:C)/LyoVec-immunostimulated HepG2 cells transfected with mimics of the miR-183 cluster. qPCR confirmed that miR-183 inhibited mRNA levels of these predicted targets by over 50% in poly(I:C)/LyoVec-treated HepG2 cells (Fig. 4C). In parallel, decreased protein phosphatase 2A (PP2A) catalytic subunit α protein abundance was confirmed by Western blot analysis (Fig. 4D) and PPP2CA was validated as a direct target of miR-183 (Fig. S9). These results suggest that the miR-183 cluster represses the expression of negative regulators of IRF3 phosphorylation to mediate their inhibitory effects on IFN production.

PP2A and TRIM27 inhibit interferon production by decreasing IRF3 phosphorylation (24, 25). Phosphorylation of IRF3 at the C terminus is required for the transcription factor to homo/heterodimerize and translocate to the nucleus to mediate transcriptional activation of IFN genes and ISGs. Therefore, we hypothesized that miR-183 activates IFN production and signaling through increased IRF3 activation and examined the phosphorylation status of IRF3 in miR-183–transfected cells. Western blot analyses confirmed increased IRF3 phosphorylation and down-regulated expression of PPP2CA in miR-183–transfected HepG2 cells during poly(I:C) treatment or VSV infection (Fig. 4, D and E). This miR-183–enhanced IRF3 phosphorylation was also observed in RIG-I agonist–treated A549 cells (Fig. S6) and TLR3 agonist–treated MCF7 cells (Fig. S7).

PP2A has also been proposed previously to impair IFN-induced STAT1 phosphorylation (26); miR-183 targeting of the phosphatase would contribute to its effects on Jak/STAT signaling (Fig. 3). Furthermore, knockdown of PPP2CA in HepG2 cells resulted in the dampening of the miR-183-mediated effect on STAT1 phosphorylation following poly(I:C) stimulation (Fig. S10). As predicted, both miR-183 and specific siRNA targeting PPP2CA led to lower levels of protein expression and a commensurate increase in phosphorylation of STAT1.

Treatment with PPP2CA siRNA dampens the effects of miR-183 by lowering the mRNA levels of the key miRNA target (Fig. S10). Additionally, it is evident that, with siRNA knockdown of PPP2CA, the magnitude of difference in STAT1 phosphorylation (Tyr-701 and Ser-727) between Con-miR– and miR-183–transfected HepG2 cells is not as significant relative to what is observed in naïve Con-siRNA–transfected HepG2 cells (Fig. S10, lanes 1 and 2 versus lanes 3 and 4). This further validates that miR-183’s effect on Jak–STAT signaling depends on PPP2CA repression. Overall, this confirms that miR-183–driven enhancement of IFN signaling and the accompanying antiviral response are mediated through PPP2CA targeting. In addition, specific induction of the miR-183 cluster by the CRISPR activation system (27) in HepG2 cells produced consistent increases in IRF3 and STAT1 phosphorylation and ISG expression as well as concomitant down-regulation of PPP2CA expression during poly(I:C)/LyoVec stimulation (Fig. S11). Therefore, our data demonstrate that the miR-183 cluster mediates its antiviral effects through repression of negative regulators of IRF3 and STAT1 phosphorylation (Fig. 5).

Discussion

Overall, our work elucidates a novel role for the miR-183 cluster in regulation of the innate antiviral response through activation of IFN production and signaling. Although previous studies have highlighted functional roles of miRNAs encoded in the miR-183 cluster in helper T lymphocyte clonal expansion (28) and natural killer cell function (29), our work highlights a novel function of this highly conserved miRNA cluster in the cell-intrinsic defense against pathogens. Furthermore, miR-183’s regulation of tonic IFN signaling could also play an important role in immune homeostasis, as increased constitutive IFN signaling has been linked to autoimmunity (30). This provides a
potential explanation for the previously reported association between increased miR-183 cluster expression and lupus disease in mice (31). The identification of the miR-183 family as a primary regulator of IFN signaling opens novel avenues for immunomodulatory strategies to treat interferonopathies and other immune disorders. This is, to the best of our knowledge, the only known example of a miRNA cluster shaping the innate immune response through cooperative regulation of the IFN pathway.

**Experimental procedures**

**Reagents**

The HepG2 hepatoma cells (HB-8065), MCF7 breast cancer cells (HTB-22), A549 lung epithelial cancer cells (CCL-185), and HEK293 human embryonic kidney cells (CRL-1573) were procured from the ATCC. Mouse embryonic fibroblast cells (32) were kindly provided by Dr. Marc Servant (Université de Montréal, Montreal, QC, Canada). VSV-GFP WT and mutant viruses were kindly provided by Dr. John C. Bell (Ottawa Hospital Research Institute, Ottawa, ON, Canada) (22). All miRNA mimics and inhibitors, along with controls, were purchased from Ambion. PPP2CA-targeting siRNA (4390824) and the negative control (4390843, Silencer Select) were purchased from Ambion. All mirVana miRNA mimics and inhibitors, along with controls, were purchased from Ambion. The PPP2CA 3’ UTR luciferase construct was purchased from Genecopoeia. Low-molecular-weight (LMW) poly(I:C) complexed with LyoVec and LMW poly(I:C) alone were purchased from Invivogen. IFN-α poly(I:C) complexed with LyoVec and LMW poly(I:C) alone were purchased from Invivogen. IFN-α was procured from PBL Assay Science. The pSRE-Luc plasmid was a kind gift from Dr. Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA). pcDNA3.1-TBK1-FLAG, pcDNA3.1-FLAG-IKBKE, and empty pcDNA3.1 were a kind gift from Dr. Daniel Lamarre (Centre hospitalier de l’Université de Montréal, Montreal, QC, Canada) (33). The lentiviral vectors encoding MS2-p65-HSF1 (61426) and dCas9-VP64 (61425) fusion proteins and the lenti sgRNA(MS2)_puro-optimized backbone (73797) were gifts from Feng Zhang (Broad Institute, Cambridge, MA), obtained from Addgene (27).

**Cell culture and transfection**

Adherent HepG2 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (PAA Laboratories), 1× nonessential amino acids (NEAAs; Gibco), 50 units/ml penicillin, and 50 mg/ml streptomycin. Adherent HEK293 cells were cultured in minimum essential medium (Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco), 1× NEAAs, 50 units/ml penicillin, and 50 mg/ml streptomycin. Adherent mouse embryonic fibroblasts, A549 cells, and Vero cells were cultured in DMEM supplemented with 10% FBS. Adherent MCF7 cells were cultured in minimum essential medium containing 10% FBS, 1% NEAAs, 1% sodium pyruvate, and 0.01 mg/ml recombinant insulin (Sigma-Aldrich). All cell culture maintenance and experiments were performed at 37 °C and 5% CO2. IFN-α was used at a working concentration of 1000 units/ml. LMW poly(I:C) complexed with LyoVec was used at 500 ng/ml, whereas LMW poly(I:C) for TLR3 agonism was used at 50 μg/ml. Transfections for miRNA mimics and inhibitors were performed using Lipofectamine RNAiMax (Life Technologies). Transfections were performed according to the manufacturer’s protocol, using 2.5 μl of RNAiMax/1 μl of 100 μM of miRNA mimic. Transfection of plasmid DNA was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.

**mRNA microarray analysis**

RNA isolation from HepG2 cells was performed with the RNeasy kit (Qiagen). Gene expression profiling was performed using Affymetrix Human Gene ST.2.0 arrays. Data were normalized and analyzed using the Affymetrix Expression Console and Transcriptome Analysis Console (v3.0) according to the manufacturer’s protocols. Gene ontology analysis was performed using the ToppGene suite (34). For ToppGene analysis, p values were adjusted with Bonferroni correction. Heatmap visualization of expression data was done on datasets filtered for genes activated over 8-fold under all conditions (HepG2 cells transfected with mimics of miR-96, miR-182, or miR-183 individually at 50 nM). Because multiple transcript cluster IDs correspond to TRANK1, we show the -fold change corresponding to ID 17123262, the probe showing the highest magnitude of -fold change in expression.

**Quantitative RT-PCR**

RNA isolation from hepatocytes was performed using TRIzol (Life Technologies), RNeasy kits (Qiagen), or NucleoSpin miRNA (Macherey-Nagel) according to the manufacturer’s protocol. RNA integrity was confirmed by 0.8% agarose gel electrophoresis in 1× Tris borate-EDTA (Ambion). For mRNA profiling, 250 ng of total RNA was reverse-transcribed using the Superscript II RT Kit (Life Technologies), following the manufacturer’s instructions. qPCR was subsequently performed on an iCycler (Bio-Rad), using iQ SYBR Green SSO Advanced Supermix (Bio-Rad) according to the manufacturer’s protocol. Primer sequences are listed in Table S4. Relative miRNA levels were quantified using the TaqMan miRNA assay (Applied Biosystems), with 10 ng of total RNA used for reverse transcription using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For both mRNA and miRNA quantification, the 2−ΔΔCt method was used to calculate -fold changes in expression relative to mock- or control-treated samples (35), with 18S rRNA or RNU6B levels being used for normalization.

**Viral infection**

HepG2 cells and MCF7 cells were seeded at 5 × 10⁵ cells/well in 6-well plates and simultaneously reverse-transfected with 50 nM control; miR-96, miR-182, or miR-183 mimics; or inhibitors. Forty-eight hours post-transfection, the medium was removed, and cells were incubated at 37 °C in serum-free DMEM containing VSV-GFP particles at the appropriate multiplicity of infection. One hour post-infection, cells were washed with 1× PBS, and the medium was replaced with DMEM supplemented with 10% FBS. Twenty-four hours post-infection (VSV), cell supernatants (for plaque assays) were collected and clarified via centrifugation at 1700 × g for 10 min to remove cells and cellular debris. Adherent cells were washed once with PBS and then lysed for subsequent cellular RNA and protein analyses.
HepG2 cells were prepared in DMEM. The medium was nucleotide of the miR-183 seed sequence (forward, 5'-GGA-primer designed to introduce a point mutation in the first (Stratagene) according to the manufacturer's protocol, with a 19792

Viral plaque assays

Vero cells were seeded at 7.5 × 10^5 cells/well in 6-well plates. Twenty-four hours after seeding, 10-fold serial dilutions (1:10 to 1:10^6) of the supernatants collected from VSV-infected HepG2 cells were prepared in DMEM. The medium was removed from Vero cells and replaced with 100 μl of virus-containing supernatant (one dilution per well). Cells were incubated in virus-containing supernatant at 37 °C for 1 h. One hour post-infection, 2 ml of warm (37 °C to 40 °C) agarose medium (1 × DMEM, 10% FBS, 1% (w/v) agarose) was added to each well and allowed to solidify. After 24 h (VSV), plaques were counted by visual examination. Viral titers in supernatants were back-calculated based on serial dilutions and expressed as plaque-forming units per milliliters of supernatant.

VSV-GFP fluorescence imaging

HepG2 cells were seeded at 2 × 10^5 cells/well in 12-well plates and simultaneously reverse-transfected with Con-miR, miR-96, miR-182, or miR-183 mimics as described previously. Forty-eight hours post-transfection, cells were infected with VSV-GFP. 24 h post-infection, cells were washed twice with PBS and fixed with a solution of 4% (v/v) formaldehyde and 4% (w/v) sucrose in water for 15 min at room temperature. Fixed cells were washed twice with PBS for 3 min and stored in PBS at 4 °C until imaging. Fixed cells were mounted with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Imaging was performed using an Axiophot fluorescence microscope (Zeiss) attached to a DP-70 color charged coupled device (CCD) camera (Olympus) with a 50-watt mercury fluorescence excitation light source using either a blue or green filter cube for DAPI or GFP, respectively. Images were acquired using the ImagePro 6 software suite (MediaCybernetics) and analyzed using ImageJ (National Institutes of Health).

3' UTR luciferase reporter assay

The miR-183 binding site in a Dual-Luciferase reporter containing the 3' UTR of the protein phosphatase 2A catalytic subunit α was mutated using the QuikChange Lightning Kit (Stratagene) according to the manufacturer’s protocol, with a primer designed to introduce a point mutation in the first nucleotide of the miR-183 seed sequence (forward, 5’-GGA-CCAAAGATGTGCCATAAAATAACAAAGCC-3’; reverse, 5’-GGCTTGTATTTTATAGGCAATCTTTT-GGCC-3’). HEK293 cells were seeded at 4 × 104 cells/well in 24-well plates and transfected 24 h later with 500 ng of either the WT or mutant 3’ UTR construct as described previously. Twenty-four hours post-transfection, cells were transfected with Con-miR, miR-96, miR-182, or miR-183 mimics as described previously. Forty-eight hours post-mimic transfection, cells were lysed in 1× passive lysis buffer (Promega), and the Dual-Luciferase assay was carried out as described previously (36), using a SpectraMax L luminometer (Molecular Devices).

PPP2CA knockdown

HepG2 cells were reverse-transfected in a 10-cm dish with 50 nM control or miR-183 mimics. 24 h post-transfection, cells were reseeded in a 12-well plate and then reverse-transfected with 50 nM siRNA targeting PPP2CA or control siRNA. 24 h after siRNA transfection, cells were stimulated with poly(I:C)/LyoVec at 500 ng/ml final concentration. 24 h post-stimulation, cells were lysed in 1× SDS lysis buffer and prepared for immunoblotting.

Immunoblotting

After transfections, cells were washed twice with PBS and lysed with SDS lysis buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. A protease inhibitor mixture (Roche Diagnostics) was added to each extract. The protein concentration of each sample was quantified using the DC Protein Assay (Bio-Rad) according to the manufacturer’s protocol. Prior to loading, 10% (v/v) of DTT and bromphenol blue (1:1) was added to each sample, and 40–60 μg/well was loaded onto an SDS-PAGE gel (10% resolving, 4% stacking gel). The resolved proteins were transferred to a Hybond-P PVDF membrane (Amersham Biosciences). The membrane was probed using the following primary antibodies and corresponding volume dilutions (in 3% (w/v) BSA): rabbit anti-STAT1 (1:1000; Cell Signaling Technology, Beverly, MA; 9172), rabbit anti-phospho-STAT1 (Tyr-701, 1:1000, Cell Signaling Technology, 7649), rabbit anti-phospho-STAT1 (Ser-727, 1:1000, Cell Signaling Technology, 9177), rabbit anti-IRF3 (1:1000; Santa Cruz Biotechnology, Dallas, TX; sc-9082), anti-IRF3 (1:1000, Cell Signaling Technology, 10949S), rabbit anti-phospho-IRF3 (Ser-386, 1:1000; EMD Millipore, Etobicoke, ON, Canada; EBE501), rabbit anti-phospho-IRF3 (Ser-386, 1:1000, Abcam, ab76493), rabbit anti-PP2A (α/β catalytic subunit, 1:5000, Abcam, ab32141), rabbit anti-β-tubulin (1:1000, Santa Cruz Biotechnology, sc-904), and anti-β-tubulin (1:5000, Abcam, ab6046). β-Tubulin expression levels were used as loading controls. Membranes were then incubated with HRP-conjugated goat anti-mouse or donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Westgrove, PA). Protein bands were visualized using either Amersham Biosciences ECL Prime Western Blotting Detection Reagent (GE Healthcare) or Clarity ECL Western Blotting Substrate (Bio-Rad) according to the respective manufacturer’s protocols, depending on antibody strength and background signal.

ISRE-luciferase assay

HEK293T cells were reverse-transfected with 50 nM siRNA targeting PPP2CA or control siRNA. 24 h after siRNA transfection, cells were stimulated with poly(I:C)/LyoVec at 500 ng/ml final concentration. 24 h post-stimulation, cells were lysed with 1× passive lysis buffer (Promega), and Dual-Luciferase reporter assays were performed as described previously (36).

CRISPR activation and lentivirus generation

HEK293T cells in 10-cm dishes were transfected with the packaging vectors pMD2.G (2 μg) and psPAX2 (4 μg) and 6 μg

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Viral plaque assays

Vero cells were seeded at 7.5 × 10^5 cells/well in 6-well plates. Twenty-four hours after seeding, 10-fold serial dilutions (1:10 to 1:10^6) of the supernatants collected from VSV-infected HepG2 cells were prepared in DMEM. The medium was removed from Vero cells and replaced with 100 μl of virus-containing supernatant (one dilution per well). Cells were incubated in virus-containing supernatant at 37 °C for 1 h. One hour post-infection, 2 ml of warm (37 °C to 40 °C) agarose medium (1 × DMEM, 10% FBS, 1% (w/v) agarose) was added to each well and allowed to solidify. After 24 h (VSV), plaques were counted by visual examination. Viral titers in supernatants were back-calculated based on serial dilutions and expressed as plaque-forming units per milliliters of supernatant.
of transfer vector. Viral supernatant was collected 72 h post-transfection, filtered (0.45 μm), and used for transduction of HepG2 cells. HepG2 cells were lentivirally infected with dCas9-VP64 and MS2-p65-HSF1 and selected using blasticidin and hygromycin for more than 2 weeks; subsequently, cells were lentivirally infected with a nontargeting control (NTC) or guides targeting the promoter of miR-183. Guides were annealed and cloned into the lentigal sgRNA(M52)_puro-optimized backbone via the BsmBI sites. Five guides were tested for the capacity to mediate CRISPR activation–induced miR-183 cluster expression, and the top tested guide was identified using TaqMan miRNA qPCR assays (cloned with oligos CACCGAGGGCCCTCCGTCCAGCCGC (forward) and AAACCGGC-GCTGGACGAGGCCCCCTC (reverse)) and used for functional analysis of miR-183 cluster GOF. Nontargeting guide sequences (forward, CACCGGCGAGGTATTCGGTCCCGG; reverse, AAACCGGCAGCCGAATACCTCGGC) were used as a control.

**Statistical analysis**

Data are presented as the mean of replicates, with error bars representing the standard error of the mean. Unless otherwise stated, statistical significance was evaluated using two-tailed Student’s t test, and p values of less than 0.05 were deemed significant.

**Author contributions**

R. S., N. A., and J. P. P. conceptualization; R. S., C. Q., and P. S. data curation; R. S., N. A., C. Q., P. S., and J. P. P. formal analysis; R. S. and J. P. P. supervision; R. S. and J. P. P. funding acquisition; R. S., N. A., C. Q., P. S., C. J. A., D. G. R., and J. P. P. investigation; R. S., N. A., and J. P. P. methodology; R. S., N. A., C. Q., and J. P. P. writing–original draft; R. S. and J. P. P. project administration; N. A., C. Q., and P. S. validation; N. A., C. Q., and P. S. visualization; J. P. P. resources.

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**References**

1. Kimbrell, D. A., and Beutler, B. (2001) The evolution and genetics of innate immunity. *Nat. Rev. Genet.* 2, 256–267 CrossRef Medline
2. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) IKK and TBK1 are essential components of the IRF3 signalling pathway. *Nat. Immunol.* 4, 491–496 CrossRef Medline
3. Sharma, S., ten Oever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscock, J. (2003) Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300, 1148–1151 CrossRef Medline
4. Onoguchi, K., Yoneyama, M., Takemura, A., Taniguchi, T., Namiki, H., and Fujita, T. (2007) Viral infections activate type I and III interferon genes through a common mechanism. *J. Biol. Chem.* 282, 7576–7581 CrossRef Medline
5. Forster, S. C., Tate, M. D., and Hertzog, P. J. (2015) MicroRNA as type I interferon-regulated transcripts and modulators of the innate immune response. *Front. Immunol.* 6, 334 CrossRef Medline
6. Jonas, S., and Izaurralde, E. (2015) Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* 16, 421–433 CrossRef Medline
7. Powdrill, M. H., Desrochers, G. F., Singaravelu, R., and Pezacki, J. P. (2016) The role of microRNAs in metabolic interactions between viruses and their hosts. *Curr. Opin. Virol.* 19, 71–76 CrossRef Medline
8. O’Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., and Baltimore, D. (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1604–1609 CrossRef Medline
9. Sheedy, F. J., Palsson-McDermott, E., Hennessey, E. J., Martin, C., O’Leary, J. J., Ruan, Q., Johnson, D. S., Chen, Y., and O’Neill, L. A. (2010) Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat. Immunol.* 11, 141–147 CrossRef Medline
10. Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006) NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12481–12486 CrossRef Medline
11. Ouassaief, L., Fendri, A., Chane-Woon-Ming, B., Poiré, R., Delecouse, H. J., Joab, I., and Pfeffer, S. (2015) Modulation of microRNA cluster miR-183-96-182 expression by Epstein–Barr virus latent membrane protein 1. *J. Virol.* 89, 12178–12188 CrossRef Medline
12. Singaravelu, R., O’Hara, S., Jones, D. M., Chen, R., Taylor, N. G., Srinivasan, P., Quan, C., Roy, D. G., Steenbergen, R. H., Kumar, A., Lyn, R. K., Ozcelik, D., Rouleau, Y., Nguyen, M. A., Rayner, J. K., et al. (2015) MicroRNAs regulate the immunometabolic response to viral infection in the liver. *Nat. Chem. Biol.* 11, 988–993 CrossRef Medline
13. El Sobky, S. A., El-Ekabi, N. M., Mekky, R. Y., Elemam, N. M., Mohey Eldin, M. A., El-Sayed, M., Esmat, G., and Abdelaziz, A. I. (2016) Contradicting roles of miR-182 in both NK cells and their target hepatocytes in HCV. *Immunol. Lett.* 169, 52–60 CrossRef Medline
14. Chen, Y., Dong, X., Yu, D., and Wang, X. (2015) Serum miR-96 is a promising biomarker for hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *Int. J. Clin. Exp. Med.* 8, 18462–18468 Medline
15. Stark, T. J., Arnold, J. D., Spector, D. H., and Yeo, G. W. (2012) High-resolution profiling and analysis of viral and host small RNAs during human cytomegalovirus infection. *J. Virol.* 86, 226–235 CrossRef Medline
16. Yu, H., Liu, Y., Bai, L., Kijlstra, A., and Yang, P. (2014) Predisposition to Behçet’s disease and VKH syndrome by genetic variants of miR-182. *J. Mol. Med.* 92, 961–967 CrossRef Medline
17. Singaravelu, R., Quan, C., Powdrill, M. H., Shaw, T. A., Srinivasan, P., Lyn, R. K., Alonzi, R. C., Jones, D. M., Filip, R., Russell, R. S., and Pezacki, J. P. (2018) MicroRNA-7 mediates cross-talk between metabolic signaling pathways in the liver. *Sci. Rep.* 8, 361 CrossRef Medline
18. Mitchell, A. M., Stone, A. E., Cheng, L., Ballinger, K., Edwards, M. G., Stoddard, M., Li, H., Golden-Mason, L., Shaw, G. M., Khetani, S., and Rosen, H. R. (2015) Transmitted/founder hepatitis C viruses induce cell-type- and genotype-specific differences in innate signaling within the liver. *MBio.* 6, e02510 CrossRef Medline
19. Li, P., Sheng, C., Huang, L., Zhang, H., Huang, L., Cheng, Z., and Zhu, Q. (2014) miR-183/96-182 cluster is up-regulated in most breast cancers and increases cell proliferation and migration. *Breast Cancer Res.* 16, 473 CrossRef Medline
20. Au-Yeung, N., Mandhana, R., and Horvath, C. M. (2013) Transcriptional regulation by STAT1 and STAT2 in the interferon JAK-STAT pathway. *J. Immunol.* 190, 12481–12486 CrossRef Medline
21. Wen, Z., Zhong, Z., and Darnell, J. E. (1995) Maximal activation of transcription by STAT1 and STAT3 requires both tyrosine and serine phosphorylation. *Cell* 82, 961–967 CrossRef Medline
22. Stojdl, D. F., Lichty, B. D., ten Oever, B. R., Paterson, J. M., Power, A. T., Stoddard, M., Li, H., Golden-Mason, L., Shaw, G. M., Khetani, S., and Rosen, H. R. (2015) Transmitted/founder hepatitis C viruses induce cell-type- and genotype-specific differences in innate signaling within the liver. *MBio.* 6, e02510 CrossRef Medline
and limits type I interferon signaling. *Immunity* 40, 515–529 CrossRef Medline

25. Zheng, Q., Hou, J., Zhou, Y., Yang, Y., Xie, B., and Cao, X. (2015) Siglec1 suppresses antiviral innate immune response by inducing TBK1 degradation via the ubiquitin ligase TRIM27. *Cell Res.* 25, 1121–1136 CrossRef Medline

26. Shanker, V., Trincucci, G., Heim, H. M., and Duong, H. T. (2013) Protein phosphatase 2A impairs IFNα-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation. *J. Viral Hepat.* 20, 612–621 CrossRef Medline

27. Joung, J., Konermann, S., Gootenberg, J. S., Abudayyeh, O. O., Platt, R. J., Brigham, M. D., Sanjana, N. E., and Zhang, F. (2017) Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. *Nat. Protoc.* 12, 828–863 CrossRef Medline

28. Stittrich, A. B., Haftmann, C., Sgouroudis, E., Kühl, A. A., Hegazy, A. N., Panse, I., Riedel, R., Flossdorf, M., Dong, J., Fuhrmann, F., Heinz, G. A., Fang, Z., Li, N., Bissels, U., Hatam, F., *et al.* (2010) The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat. Immunol.* 11, 1057–1062 CrossRef Medline

29. Donatelli, S. S., Zhou, J. M., Gilvary, D. L., Eksioglu, E. A., Chen, X., Cress, W. D., Haura, E. B., Schabath, M. B., Coppola, D., Wei, S., and Djeu, J. Y. (2014) TGF-β-inducible microRNA-183 silences tumor-associated natural killer cells. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4203–4208 CrossRef Medline

30. Gough, D. J., Messina, N. L., Clarke, C. J., Johnstone, R. W., and Levy, D. E. (2012) Constitutive type I interferon modulates homeostatic balance through tonic signaling. *Immunity* 36, 166–174 CrossRef Medline

31. Dai, R., Zhang, Y., Khan, D., Heid, B., Caudell, D., Crasta, O., and Ahmed, S. A. (2010) Identification of a common lupus disease-associated microRNA expression pattern in three different murine models of lupus. *PLoS ONE* 5, e14302 CrossRef Medline

32. Clément, J.-F., Bibeau-Poirier, A., Gravel, S.-P., Grandvaux, N., Bonneil, E., Thibault, P., Meloche, S., and Servant, M. J. (2008) Phosphorylation of IRF-3 on Ser 339 Generates a Hyperactive Form of IRF-3 through Regulation of Dimerization and CBP Association. *J. Virol.* 82, 3984–3996 CrossRef Medline

33. Tremblay, N., Baril, M., Chatel-Chaix, L., Es-Saad, S., Park, A. Y., Koenekoop, R. K., and Lamarre, D. (2016) Spliceosome SNRNP200 promotes viral RNA sensing and IRF3 activation of antiviral response. *PLoS Pathog.* 12, e1005772 CrossRef Medline

34. Chen, J., Bardes, E. E., Aronow, B. J., and Jegga, A. G. (2009) ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* 37, W305–W311 CrossRef Medline

35. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408 CrossRef Medline

36. Dyer, B. W., Ferrer, F. A., Klinedinst, D. K., and Rodriguez, R. (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal. Biochem.* 282, 158–161 CrossRef Medline