Published online 3 September 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 19* ***6587–6599***
doi:10.1093/nar/gkp714

Short-hairpin RNAs delivered by lentiviral vector transduction trigger RIG-I-mediated IFN activation

Rachael Kenworthy[1], Diana Lambert[1], Feng Yang[1], Nan Wang[2], Zihong Chen[3], Haizhen Zhu[4], Fanxiu Zhu[1], Chen Liu[4], Kui Li[2] and Hengli Tang[1,*]

[1]Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, [2]Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, TN 38163, [3]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555 and [4]Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL 32611, USA

Received May 15, 2009; Revised August 13, 2009; Accepted August 14, 2009

ABSTRACT

Activation of the type I interferon (IFN) pathway by small interfering RNA (siRNA) is a major contributor to the off-target effects of RNA interference in mammalian cells. While IFN induction complicates gene function studies, immunostimulation by siRNAs may be beneficial in certain therapeutic settings. Various forms of siRNA, meeting different compositional and structural requirements, have been reported to trigger IFN activation. The consensus is that intracellularly expressed short-hairpin RNAs (shRNAs) are less prone to IFN activation because they are not detected by the cell-surface receptors. In particular, lentiviral vector-mediated transduction of shRNAs has been reported to avoid IFN response. Here we identify a shRNA that potently activates the IFN pathway in human cells in a sequence- and 5′-triphosphate-dependent manner. In addition to suppressing its intended mRNA target, expression of the shRNA results in dimerization of interferon regulatory factor-3, activation of IFN promoters and secretion of biologically active IFNs into the extracellular medium. Delivery by lentiviral vector transduction did not avoid IFN activation by this and another, unrelated shRNA. We also demonstrated that retinoic-acid-inducible gene I, and not melanoma differentiation associated gene 5 or toll-like receptor 3, is the cytoplasmic sensor for intracellularly expressed shRNAs that trigger IFN activation.

INTRODUCTION

A specific double-stranded RNA (dsRNA) structure, ~21–22 bp dsRNA with 3′ overhangs, plays a critical role in initiating both microRNA (miRNA)- and small interfering RNA (siRNA)-mediated gene silencing, as it is the structure recognized by the RNA interference (RNAi) machinery, the RNA-induced silencing complex (RISC) (1–3). Except for preformed siRNA duplexes of ~21 bp, the RISC-loaded small RNAs are generated by a ribonuclease (RNase) III-like enzyme that is found in virtually all eukaryotic organisms. This enzyme, aptly named Dicer for its ability to cleave a variety of larger (>30 bp) dsRNA molecules into the ~21 bp dsRNA with a characteristic 3′ overhang of 2 nt, is a multidomain RNA-binding protein and itself a component of RISC. The primary sequence of the RNAs is not important in RISC formation, and RNAi can suppress virtually any target as long as rules of sequence complementarities between the small RNA and the target RNA are satisfied.

dsRNAs are also a type of pathogen-associated molecular pattern (PAMP) that are detected by cellular innate immunity sensors named Pattern Recognition Receptors (PRRs) (4). The interaction between a PAMP and a PRR triggers activation of the interferon (IFN) pathway in mammalian cells, which significantly changes the gene-expression profile in the cells and contributes to the well-documented off-target effect of RNAi. IFN induction is especially problematic in antiviral studies employing RNAi, where the antiviral effect of IFN must be distinguished from that of RNAi.

Typical IFN-inducing structure patterns include dsRNA of certain length, single-stranded RNA (ssRNA) containing 5′-triphosphates (5′-ppp), the dsRNA analogue polyinosinic-polycytidylic acid (poly I:C), and certain dsDNA molecules. These RNA patterns are generally believed to possess 'non-self' properties to allow the cell to recognize foreign (often viral) RNAs specifically. Various forms of siRNA duplexes have been reported to trigger IFN induction both *in vitro* and *in vivo* (5–9), probably through the cell surface- and/or endosome-expressed Toll-like receptors (TLRs), including TLR3 and TLR7 (6,8,9).

*To whom correspondence should be addressed. Tel: +1 850 645 2402; Fax: +1 850 645 8447; Email: tang@bio.fsu.edu

Short-hairpin RNAs (shRNAs) expressed from a DNA plasmid have also been shown to activate IFN (10). The double-stranded form of these RNAs is below the size limit of the stem-loop RNAs that can be detected by the RNA-activated protein kinase (PKR) (11) and is probably detected by other cytoplasmic PRRs. Two cytoplasmic RNA helicases, retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), signal to the IFN-β promoter when activated by specific RNA structures (12–14). Although both PRRs signal through the mitochondrial antiviral signaling protein MAVS/Cardif/VISA/IPS-1 (15–18), studies of ligand specificity suggest that RIG-I and MDA5 are parallel sensors with overlapping substrates. For example, although both PRRs are activated by poly I:C in cell culture systems (12,19–23), MDA5 appears to be more important in mediating the poly I:C response *in vivo* (13,14). In addition, RIG-I can bind and respond to ssRNAs bearing 5′-ppp, whereas MDA5 is not activated by 5′-ppp–containing RNA (24,25). Finally, several cytosolic sensors for dsDNA has been recently reported (26–31). Nevertheless, current data on what constitutes effective substrates for either PRR are incomplete and sometimes controversial. Here we report for the first time that shRNAs delivered by lentiviral transduction triggered IFN activation and that RIG-I and MAVS, but not MDA5 or TLR3, mediated the IFN activation triggered by intracellularly expressed shRNA, which could activate both IFN-α and IFN-β promoters. IFN activation depended on sequence, a 5′-ppp and correct processing of the RNA hairpin by Dicer; it was independent of promoter choice, presence of blunt ends, route of delivery and RNAi potency.

MATERIALS AND METHODS

Cells, antibodies and RNAs

GS5 and LH86 cells have been described earlier (32,33). Huh-7 and 293FT cells were maintained in DMEM supplemented with 10% FBS. We used the following antibodies: anti-CyPA (Biomol, Plymouth Meeting, PA, USA); anti-CyPB (Affinity BioReagents, Rockford, IL, USA); anti-Ku80, anti-Flag and anti-actin (Sigma-Aldrich, St Louis, MO, USA); anti-IFN stimulate gene (ISG)15 (Rockland Immunochemicals, Gilbertsville, PA, USA); anti-NS5A (Virogen, Watertown, MA, USA) and anti-NS3 (in-house). GSB1 and H801 cells have been described earlier (34). Poly I : C was purchased from Sigma-Aldrich, and synthetic hairpin RNA was purchased from Integrated DNA Technologies (Coralville, IA, USA). Synthetic siRNA was purchased from Ambion (Austin, TX, USA).

Gel electrophoresis and western blotting

Protein contents of cell lysate were quantified with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA), and an equal amount of total protein was loaded in each lane. Samples for IRF-3 dimerization assay were run on a polyacrylamide gel under non-denaturing conditions (35). Other samples were denatured and separated by sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS–PAGE). Proteins were then transferred onto a nitrocellulose membrane and stained with the appropriate antibodies with the SNAP i.d.™ system (Millipore, Worcester, MA, USA) according to the manufacturer's instructions.

Transfections

For luciferase assays, cells were seeded to a confluency of 50%, and for all other assays, cells were seeded to a confluency of 30%. The next day, transfections of DNA plasmids and synthetic RNAs were performed with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Plasmids

Plasmids pGL3-IFNA1, pGL3-IFNB, pRL-TK, pCMV-Flag-IRF-3 and pCR3.1-IRF-7A have been described earlier (36). shRNAs were expressed from a human immunodeficiency virus (HIV)-based lentiviral vector (32,37), and sh-PCAF was constructed on the basis of a previously reported sequence (38). Plasmid sh-B971/H1 was constructed by cloning of the DNA fragment encoding the sh-B971 RNA into pSilencer 3.0-H1 (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The RIG-I and TLR3 constructs have been described (39,40). The RIG-I C construct encodes Flag-tagged, C-terminal 707 aa of human RIG-I cloned into a bicistronic expression vector modified from pBICEP-CMV-1 (Sigma-Aldrich, St Louis, MO, USA), in which the CMV promoter was replaced with the elongation-factor-1 promoter. The MDA5, MDA5-C constructs were kindly provided by Fujita (12). HCV genotype 2a NS3-4A protease was expressed from the pCMV-3Tag-1a plasmid (Stratagene, La Jolla, CA, USA).

Luciferase assay for Interferon promoter activity

293FT cells were seeded in 24-well plates and were transfected 16 h later with 400 ng of a shRNA expression vector, 40 ng of pGL3-IFNA1 or pGL3-IFNB, 20 ng of pRL-TK and 50 ng of pCR3.1-IRF-7A. Cells were collected 48 h after transfection. Luciferase assays were performed with the Dual-Glo® Luciferase Assay system reagents (Promega, Madison, WI) and luminescence quantified with a Modulus Microplate reader (Turner BioSystems, Sunnyvale, CA, USA). Ratios of firefly luciferase (from the pGL3 vectors) to *Renilla* luciferase (from the pRL-TK vector) were calculated, and that of the sh-B971 sample was normalized to 100%.

Lentiviral vectors

Sequences of shRNA are shown in Table 1. Lentiviral vector production and transduction were performed as described earlier (37). Viral vectors were pelleted by ultracentrifugation at 50 000*g* at 4°C for 3 h and resuspended in a volume of PBS that was 1% of the original medium volume. The titers of the concentrated vectors were then measured with a p24 ELISA kit (ZeptoMetrix, Buffalo, NY, USA).

Real-time reverse transcription PCR

Real-time reverse transcription PCR (RT–PCR) was performed as described earlier (32). The primers used were OAS1 forward, 5′-AGG TGG TAA AGG GTG GCT CC-3′ and OAS1 reverse 5′-ACA ACC AGG TCA GCG TCA GAT-3′; RIG-I forward 5′-GAG GCA GAG GAA GAG CAA GAG G-3′ and RIG-I reverse 5′-CGC CTT CAG ACA TGG GAC GAA G-3′; GAPDH forward 5′-TCA CTG CCA CCC AGA AGA CTG-3′ and GAPDH reverse 5′-GGA TGA CCT TGC CCA CAG C-3′. The primers for HCV detection were 5′-CGC TCA ATG CCT GGA GAT TTG-3′ and 5′-GCA CTC GCA AGC ACC CTA TC-3′.

Flow cytometry

For flow cytometry, GS5 cells were fixed 48 h after treatment in a solution of 2% paraformaldehyde and analyzed with a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA). Mean GFP intensity was plotted, and that of the sh-NTC sample was normalized to 100%.

RNA extraction and northern blots

Total RNA from transiently transfected 293FT cells was extracted with RNA STAT-60 (Tel-Test, Friendswood, TX, USA) and separated on a 7.5% urea polyacrylamide gel. The transfer of RNA onto nitrocellulose membrane and hybridization were performed according to standard molecular biology protocols. The probe for detecting the expression of sh-B971 and its variants was a synthetic DNA oligomer corresponding to the bottom strand of sh-B971. Radioactive labeling of the probe was performed with an end-labeling protocol with T7 polynucleotide kinase (Ambion, Austin, TX, USA). The exposure and detection of the radioactive signal was performed with a Typhoon Imager (GE Healthcare, Piscataway, NJ, USA) with Quantity One software (Bio-Rad, Hercules, CA, USA).

RESULTS

A short-hairpin RNA directed at CyPB induces IFN production in human embryonic kidney cells

To investigate the potential role of the cyclophilins (CyPs) in HCV replication (41), we delivered several shRNAs directed at mRNAs of three CyPs into HCV replicon cells by means of a lentiviral vector, using a murine U6 promoter to drive the expression of the shRNA (Figure 1A) (37). We observed a discrepancy between two anti-CyPB shRNAs (B971 and B710) in their relative efficiency in knocking down CyPB expression and in suppressing HCV. Lentiviral vector sh-B971 was less efficient in knocking down CyPB expression but potently inhibited HCV NS5A expression in a human hepatoma cell line containing replicating HCV RNA (Figure 1B, left). Viral inhibition was independent of CyPB knockdown, as control medium from transfected 293FT cells that did not contain any lentiviral vector particles, generated by omission of the packaging plasmids during transfection, also inhibited HCV replication (Figure 1B, right) without affecting CyPB expression. The fast kinetics of viral inhibition (complete inhibition with 48 h, data not shown) was also more consistent with IFN than with RNAi-based inhibition. The presence of IFN in the lentiviral vector preparation of sh-B971 was confirmed by strong induction of 2′-5′-oligoadenylate synthetase 1 (OAS1), a classic IFN-induced gene, in both naïve Huh-7 and the HCV replicon cell line (GS5) treated with the medium (Figure 1C). In addition, HCV replication in an IFN-resistant HCV replicon cell line (H801), in contrast to that in a wild-type replicon cell line (GSB1) (34), was not inhibited by the sh-B971 medium (Figure 1D), suggesting the lack of additional viral inhibiting agents in the sh-B971 medium. Expression of sh-B971 in 293FT cells also induced dimerization of IRF-3, confirming the activation of the IFN production pathway in these transfected cells (Figure 1E). Finally, sh-B971 was able to activate both IFN-α and IFN-β promoters, although the activation of the IFN-α promoter required coexpression of IRF-7, which is normally expressed at very low levels in 293-based cells (Figure 1F). These results demonstrate that sh-B971 is a potent activator of IRF-3 and IRF-7, master regulators of IFN expression in human cells.

RIG-I mediates the IFN induction by sh-B971

We next investigated the role of the different viral/exogenous RNA sensors, RIG-I, MDA5 and TLR3, in sh-B971–triggered IFN production. Mammalian expression plasmids encoding each of these proteins, as well as the dominant negative (DN) mutants of RIG-I and MDA5, were transfected into 293FT cells with shRNAs and an IFN-β promoter reporter construct. The signaling to IFN-β promoter and the expression of the PRR proteins were then examined 48 h after transfection. In the absence of sensor proteins, the sh-B971 increased activation of the IFN-β promoter by 2.6-fold (Figure 2A). Coexpression of MDA5 or TLR3 did not increase or decrease sh-B971's ability to activate IFN-β promoter relatively to the negative control shRNA (sh-NTC), but in the presence of RIG-I coexpression, the induction of IFN-β promoter by sh-B971 was increased to ~30-fold. Moreover, ectopic expression of a DN mutant of RIG-I (RIG-I C), but not that of MDA5 (MDA5-C), completely abrogated IFN promoter activation by sh-B971. With the exception of TLR3, which required prolonged exposure of the western blot to be detected, the cytoplasmic sensors and their mutants were expressed at comparable levels (Figure 2B). Moreover, activation of IRF-3 (Figure 1E) and IFN promoters (Figure 1F) in 293FT cells, which do not contain a functional TLR3 signaling pathway (42), indicates that TLR3 plays a negligible role, if any, in IFN induction by sh-B971. The combination of sh-B971 and RIG-I produced the highest level of IFN-β promoter activity, which were confirmed by western blotting showing that endogenous ISG15 induction was only detectable in cells cotransfected with sh-B971 and wild-type RIG-I (Figure 2B). To confirm further that biologically active IFN was released from these cells, we applied the culture medium of the

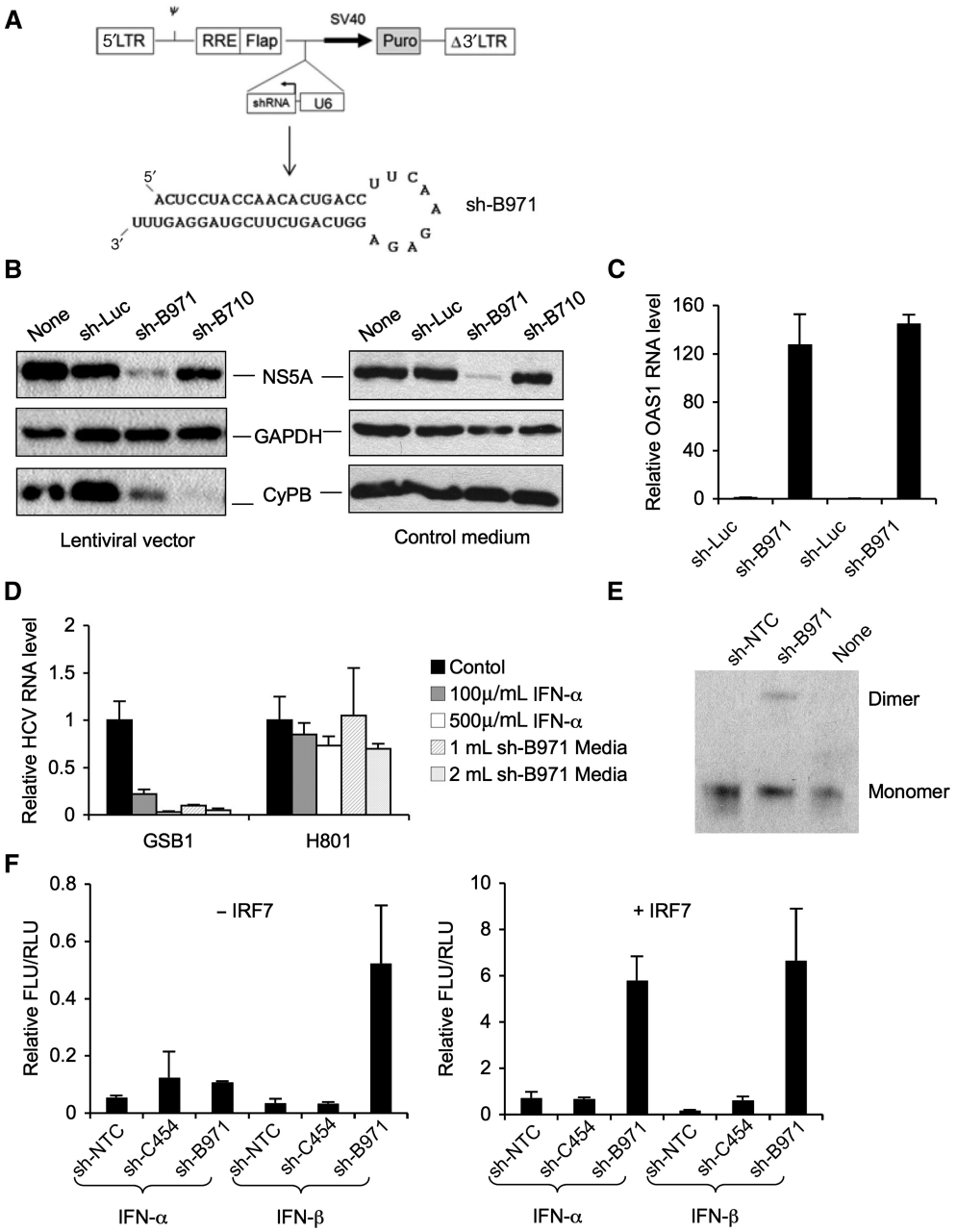

Figure 1. A small-hairpin RNA directed at CyPB induces IFN production in human embryonic kidney cells. (**A**) Sequence of sh-B971, which was expressed from a self-inactivating human immunodeficiency virus (HIV) vector with a murine U6 promoter (59). (**B**) Inhibition of HCV expression by culture media of sh-B971-transfected 293FT cells. GS5 cells were treated with culture supernatant taken from 293FT cells transfected with various shRNA plasmids with (left) or without (right) the packaging plasmids overnight. Cells were then cultured in fresh media for an additional 6 days before being lysed for western blotting. (**C**) OAS1 induction by culture supernatant from 293FT cells transfected with sh-B971. Huh 7 and GS5 cells were treated with culture supernatant from 293FT cells transfected with either sh-Luc or sh-B971 for 24 h before RNA extraction and real-time RT–PCR analysis. OAS1 RNA level was normalized to that of GAPDH RNA. (**D**) Transfected culture media failed to suppress HCV replication in an IFN-resistant cell line. HCV replicon cells were cultured as described earlier (34) and then treated with the indicated culture medium from transfected 293FT cells. HCV RNA was analyzed with real-time RT–PCR. (**E**) IRF-3 dimerization in response to sh-B971 expression. Flag-IRF-3 was cotransfected with a shRNA into 293FT cells. Cells were lysed 24 h after transfection, and total cell lysate was separated on a polyacrylamide gel under non-denaturing conditions, transferred and stained with an anti-flag antibody. (**F**) IFN-α and IFN-β promoter activation by sh-B971 expression. Sh-NTC, sh-C454 (an shRNA directed at CyPC), or sh-B971 was cotransfected along with luciferase reporter plasmids with or without IRF-7. The ratios of firefly luciferase readings to *Renilla* luciferase readings were plotted.

transfected 293FT cells to an HCV replicon cell line (GS5) in which NS5A-GFP expression is used for monitoring viral RNA replication (43). HCV replication in this cell line is extremely sensitive to IFN, and the effect of the cytokine can be readily measured as the change in the mean GFP intensity of the treated cells. As shown in Figure 2C, culture medium from sh-B971 efficiently suppressed HCV replication, resulting in a decrease in

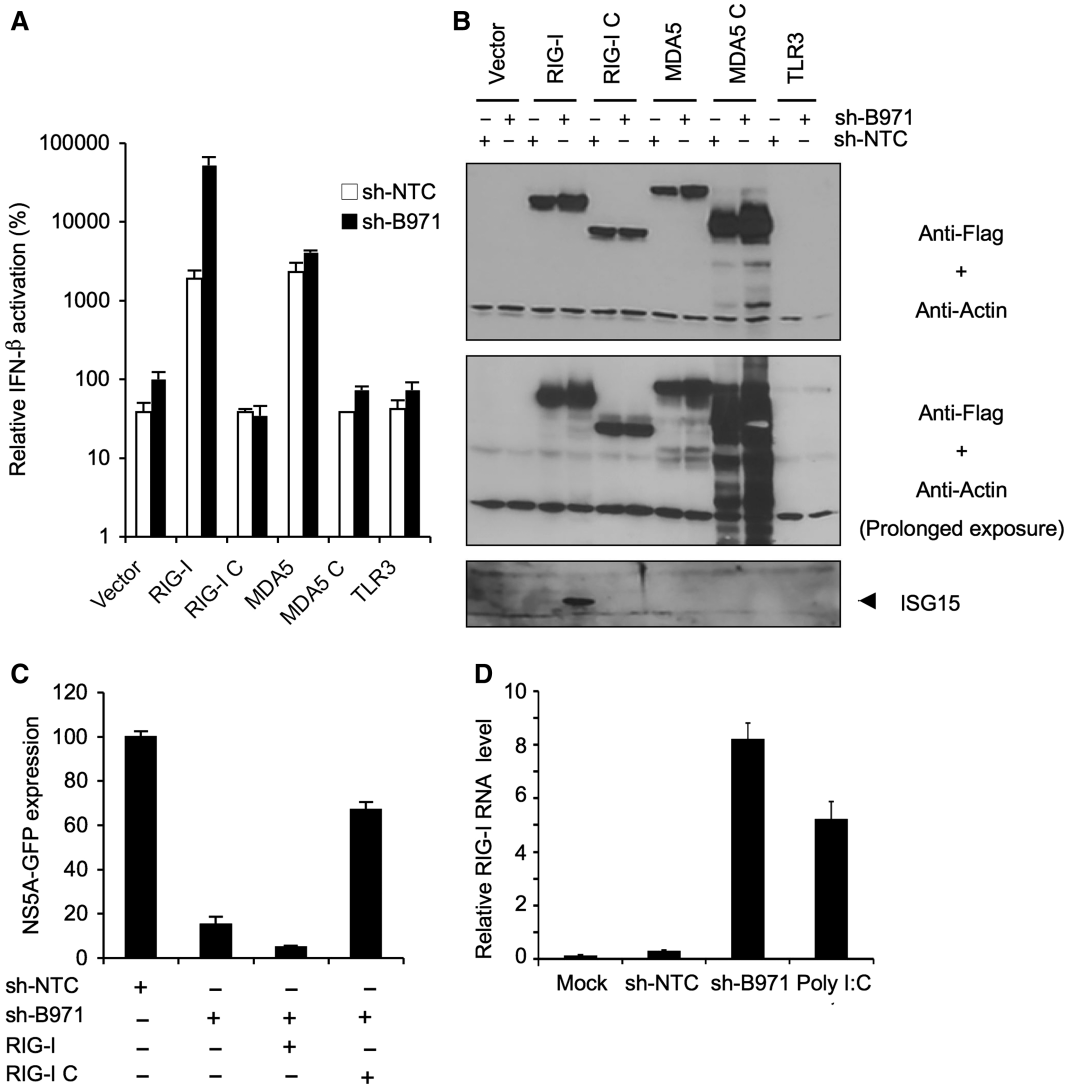

Figure 2. Sh-B971 acts through the RIG-I pathway to trigger IFN activation. (**A**) RIG-I, and not melanoma differentiation associated gene 5 (MDA5) or toll-like receptor 3 (TLR3), mediated IFN induction by sh-B971. Various PRRs and their mutant proteins were coexpressed with either sh-NTC or sh-B971 along with the luciferase reporters. The firefly luciferase readings were normalized to *Renilla* luciferase readings, and the value of sh-B971 was set to 100. (**B**) Proper expression of the transfected PRR proteins and induction of ISG 15 expression when sh-B971 was coexpressed with RIG-I. RIG-I, RIG-I C, MDA5 and MDA5 C are all tagged with two tandem copies of Flag epitope. TLR3 is tagged with single Flag epitope and required prolonged exposure for detection. (**C**) A dominant negative mutant of RIG-I blocked sh-B971-triggered IFN production. ShRNAs were cotransfected with either RIG-I or a dominant negative form of RIG-I (RIG-I C) into 293FT cells. The culture supernatant was then tested for its ability to suppress NS5A-GFP expression in GS5 cells with an overnight treatment. (**D**) Upregulation of RIG-I RNA level by sh-B971 expression. 293FT cells were transfected with sh-NTC, sh-B971 or polyinosinic-polycytidylic acid. RNA was extracted 24 h after transfection and analyzed by real-time RT PCR. The RIG-I RNA levels were normalized to those of GAPDH RNA.

the NS5A-GFP intensity within 48 h of treatment. Cotransfecting wild-type RIG-I produced a medium with stronger inhibition, whereas the RIG-C drastically suppressed the antiviral effect of the medium. Finally, real-time RT–PCR analysis revealed that sh-B971, but not the negative control shRNA, strongly activated expression of endogenous RIG-I, a well-characterized ISG whose induction requires paracrine/autocrine action of IFN (44,45). As expected, poly I:C activated RIG-I expression in the same assay (Figure 2D). These results, taken together, show that RIG-I is the cellular sensor that mediates the IFN induction by sh-B971.

Structure and sequence determinants of shRNA-mediated IFN activation

The majority of the shRNAs that we use in the lab do not activate RIG-I expression and IFN signaling despite having essentially the same structure as sh-B971, so we wanted to determine whether the sequence of sh-B971 is distinctive enough to trigger the production of IFN. We first tested a synthetic siRNA duplex with the same target sequence as sh-B971. This siRNA (si-B971-syn) should resemble the final Dicer product of sh-B971 except for the 5′-ends. The synthetic siRNA contains 5′-OH groups, whereas the Dicer products probably

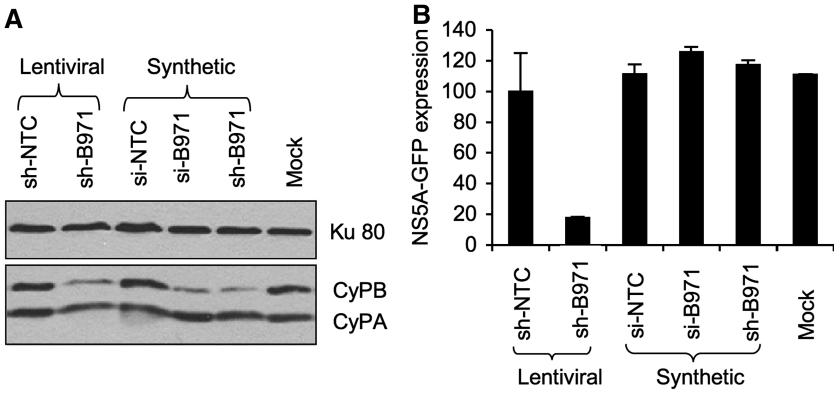

Figure 3. Structural determinants of IFN activation by sh-B971. 293FT cells were transfected with shRNA-expressing lentiviral plasmids or synthetic RNAs. (**A**) Knockdown of CyPB expression by various forms of B971 siRNA. Cells were collected 5 days after transfection and analyzed by western blot for detection of CyPB. (**B**) Lack of HCV inhibition by synthetic forms of sh-B971. Culture supernatant from transfected 293FT cells was collected 48 h after transfection and used to treat GS5 cells overnight; the GS5 cells were then analyzed by flow cytometry.

contain a 5′-monophosphate on one strand and a 5′-triphosphate on the other. Si-B971-syn knocked down CyPB expression as efficiently as sh-B971 (Figure 3A) while failing to activate IFN production, as measured by the GFP-HCV assay (Figure 3B). To determine whether the sequence of the intact hairpin RNA before Dicer cleavage is sufficient to trigger IFN, we tested a synthetic shRNA (sh-B971-syn) that had exactly the same sequence as the predicted intracellular sh-B971 transcript generated by the U6 promoter. Again, the 5′-end of the synthetic sh-B971 had a 5′-OH group instead of any phosphate. Sh-B971-syn behaved similarly to si-B971-syn in that it knocked down CyPB expression without activating IFN response (Figure 3). These results suggest that the 5′-end status of sh-B971 is important for IFN activation, consistent with the previously finding that a 5′-triphosphate is required for RIG-I activation (24,25).

To determine the contribution of the individual residues of the sh-B971 sequence, we introduced a series of point mutations into the shRNA and tested them for IFN induction. We changed the first nucleotide from A to G, C, or T while maintaining base-pairing between nucleotides +1 and +47. These mutant shRNAs lacked the ability to activate IFN production (Table 1). Changing the +1 nucleotide to G while leaving the +47 nucleotide intact also abolished IFN activation by the shRNA (A1/G), as did the reciprocal mutation U47/C. The importance of the first nucleotide was further confirmed by the inability of sh-B971+1 to activate IFN. The target of sh-B971+1 was shifted 1 nt downstream on the CyPB mRNA, producing an shRNA starting with a G at the +1 position. The presence of an A at the +1 position was not, however, sufficient to render a shRNA competent for IFN activation, as replacing the first nucleotide of the sh-NTC with an A did not generate an IFN-inducing shRNA (NTC-A and NTC+1). These results indicate that a protruding/unpaired A at the end of the hairpin or the RNA duplex, a potential result of 'breathing' at the end of the dsRNA, is not sufficient to trigger IFN induction as previously suggested (38).

Two point mutations located farther into the stem structure of the shRNA (9G9 and B18A1) also reduced its ability to induce IFN even though the base-pairing was perfectly maintained in these mutants. Finally, replacing the 9-nt hairpin loop with a 7-nt loop that had been previously shown to abolish shRNA-mediated RNAi (loop A mutant) (46) eliminated sh-B971's ability to

Table 1. Sequence of the siRNA duplexes

Name	siRNA Sequence	Interferon Induction
NTC	5′-GACUGAAGGUGUGCUGGUAUU-3′ 3′-UUCUGACUUCCACACGACCAU-5′	No
NTC-A	5′-AACUGAAGGUGUGCUGGUAUU-3′ 3′-UUUUGACUUCCACACGACCAU-5′	No
NTC+1	5′-ACUGAAGGUGUGCUGGUAGUU-3′ 3′-UUUGACUUCCACACGACCAUC-5′	No
B971	5′-ACUCCUACCAACACUGACCUU-3′ 3′-UUUGAGGAUGGUUGUGACUGG-5′	Yes
B971-G	5′-GCUCCUACCAACACUGACCUU-3′ 3′-UUCGAGGAUGGUUGUGACUGG-5′	No
B971-C	5′-CCUCCUACCAACACUGACCUU-3′ 3′-UUGGAGGAUGGUUGUGACUGG-5′	No
B971-T	5′-UCUCCUACCAACACUGACCUU-3′ 3′-UUAGAGGAUGGUUGUGACUGG-5′	No
B971+1	5′-CUCCUACCAACACUGACCAUU-3′ 3′-UUGAGGAUGGUUGUGACUGGU-5′	No
A1/G	5′-GCUCCUACCAACACUGACCUU-3′ 3′-UUUGAGGAUGGUUGUGACUGG-5′	No
U47/C	5′-ACUCCUACCAACACUGACCUU-3′ 3′-UUCGAGGAUGGUUGUGACUGG-5′	No
9G9	5′-ACUCCUACCGACACUGACCUU-3′ 3′-UUUGAGGAUGGCUGUGACUGG-5′	Intermediate
B10A9	5′-ACUCCUACCAGUGCUGGUAUU-3′ 3′-UUUGAGGAUGGUCACGACCAU-5′	No
B15A4	5′-ACUCCUACCAACACUGGUAUU-3′ 3′-UUUGAGGAUGGUUGUGACCAU-5′	No
B18A1	5′-ACUCCUACCAACACUGACAUU-3′ 3′-UUUGAGGAUGGUUGUGACUGU-5′	Intermediate
Loop A	5′-ACUCCUACCAACACUGACC(CA)G 3′-UUUGAGGAUGGUUGUGACUGG(UC)G	No
PCAF	5′-AAGAAAUUAUUCAUGGCAGACUU-3′ 3′-UUCUUUAAUAAGUACCGUCUG-5′	Yes

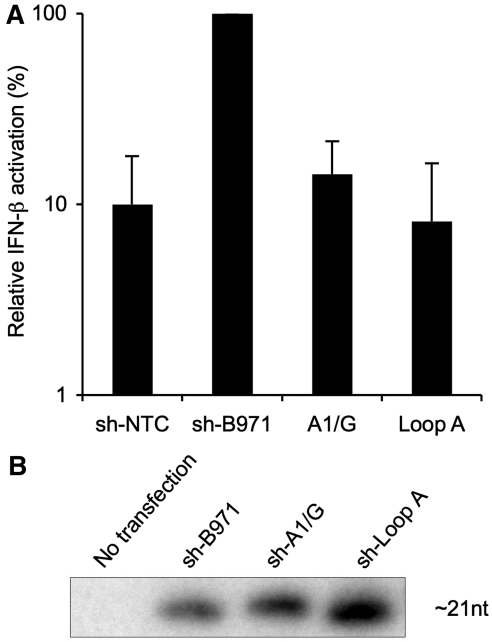

Figure 4. Comparably expressed mutant forms of sh-B971 do not induce IFN. (**A**) IFN activation by select sh-B971 mutants. The shRNA expression plasmids were transfected into 293FT cells with the luciferase reporters to measure IFN-β promoter activation. The firefly luciferase readings were normalized to *Renilla* luciferase readings, and the value of sh-B971 was set to 100. (**B**) Intracellular levels of siRNA products of sh-B971 and mutants. RNA was extracted from transfected 293FT cells and analyzed by northern blotting with a DNA oligonucleotide probe that is complementary to all three forms of sh-B971 (wt, sh-A1/G and Loop A).

induce IFN, suggesting the importance of RNA processing in the induction. To determine whether the inability of the mutant shRNAs to induce IFN was due to lower expression levels, we performed northern blotting analysis of the shRNA expression on the wild-type and two mutants. The mutants A1/G and Loop A were chosen because their final siRNA products have exactly the same sequence as that of the wild-type sh-B971 and can thus be detected with the same efficiency by the same probe. Although sh-A/G and sh-Loop A were clearly unable to activate IFN-β promoter (Figure 4A), they were both expressed at levels comparable to those of the wild-type sh-B971 product (Figure 4B). Interestingly, the final siRNA product of sh-Loop A was slightly smaller than those of sh-B971 and sh-A1/G, suggesting that cleavage did occur and perhaps occurred one or 2 nt into the stem to compensate for the shorter loop.

RIG-I-mediated IFN induction by sh-B971 is independent of a blunt end of the dsRNA

Blunt-ended siRNA has been previously reported to be stronger inducers of IFN than the siRNAs with overhangs (47). Indeed, a previously reported IFN-inducing shRNA, sh-PCAF (p300/CREB-binding protein-associated factor), contains a blunt end (38) and was more potent in activating IFN than sh-B971 (Figure 5A), which is predicted to form an overhang of 2–3 Ts at each end of the final siRNA. We therefore constructed a version of the sh-B971 that would be blunt at the end that is not processed by Dicer by adding two extra As to the 5′-end of the shRNA. This modification (Blunt sh-B971) did not increase the ability of sh-B971 to activate IFN-β promoter (Figure 5A). We confirmed, in two independent experiments, that IFN induction by sh-PCAF was also mediated by RIG-I. First, cotransfection of DN RIG-I resulted a 50- to 100-fold inhibition of IFN induction by sh-PCAF (Figure 5B), whereas wild-type RIG-I increased IFN induction by several fold in the same assay. Second, when HCV NS3-4A protease, which cleaves MAVS, thereby blocking the RIG-I pathway, was coexpressed with either sh-B971 or sh-PCAF, IFN induction by these shRNAs were severely compromised (Figure 5C), further substantiating a role of the RIG-I and MAVS pathway in mediating IFN induction by both the blunt-ended sh-PCAF and the sh-B971 with overhang. The proper expression of NS3-4A protease was confirmed by western blotting (Figure 5D).

Sh-B971 expressed from an H1 promoter triggers IFN induction

To assess the contribution of the promoter choice in IFN activation by intracellular expressed shRNA, we expressed sh-B971 from another commonly used pol III promoter, the human H1 promoter. Both the original, mU6-driven sh-B971 and the H1-driven sh-B971 activated IFN-β promoter (Figure 6A) and resulted in secretion of IFN into the transfected cell-culture media, which in turn suppressed HCV replication (Figure 6B). Proper expression of the siRNA (Figure 6C) and the subsequent knockdown of CyPB expression (Figure 6D) all appeared normal for sh-B971 expressed from the H1 promoter plasmid, which has a backbone different from that of our lentiviral vector carrying the mU6 promoter. These data suggest that IFN induction by sh-B971 is not restricted to a particular promoter or expression construct. Further supporting this conclusion was the observation that the expression cassette by itself, removed and isolated from the lentiviral plasmid by restriction digestion, could also activate IFN production in transfected 293FT cells (data not shown).

ShRNAs delivered via lentiviral transduction trigger IFN activation *in vitro*

To this point, all the IFN induction experiments were done with transient transfection of DNA vectors and it was possible that certain features of the double-stranded plasmid DNA are responsible for IFN induction. We first tried to address this point by transfecting just the shRNA-expressing cassette, generated either by PCR or restriction enzyme digestion, into 293FT cells and confirming that these fragments of ~200 bp were sufficient to trigger IFN induction (Supplementary Figure S1). To definitively rule out any contribution by dsDNA, we used a lentiviral transduction system which has been suggested to express shRNAs that can escape detection by PRRs and IFN activation (48). We produced lentiviral particles containing shRNAs from 293FT cells using standard

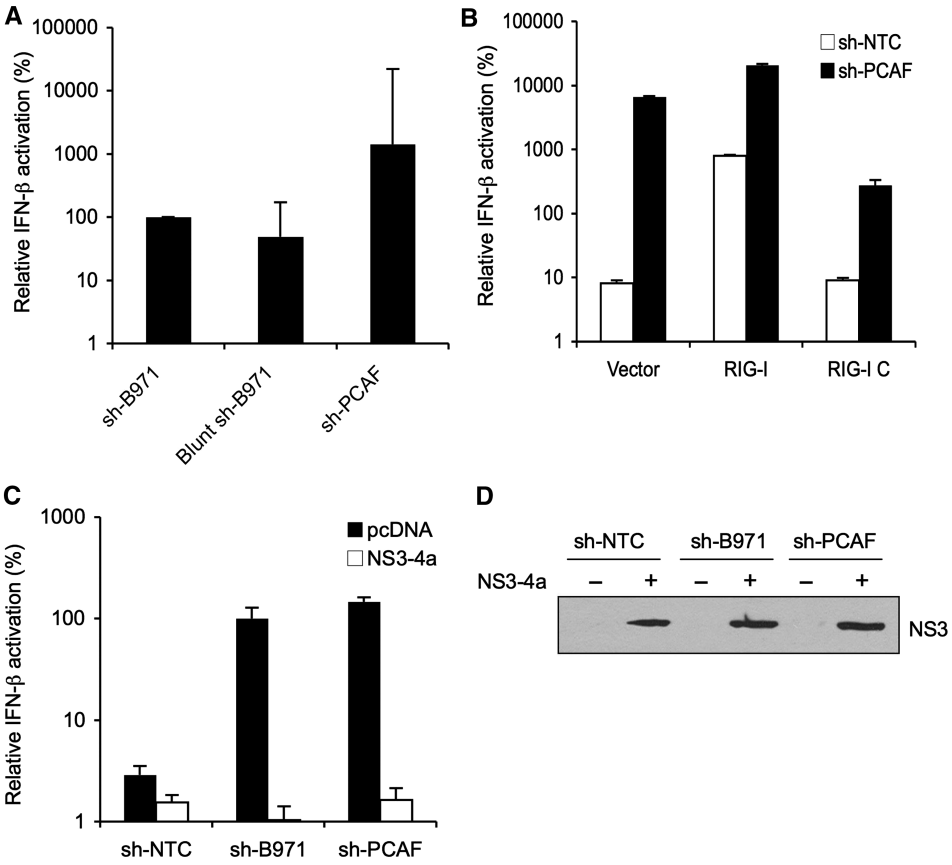

Figure 5. RIG-I mediated IFN induction by shRNA is independent of a blunt end of the dsRNA. (**A**) IFN-β activation in response to blunt-ended shRNAs. Both sh-PCAF and Blunt sh-B971 contained two extra As at the 5′-end of the shRNA, making the non-hairpin end of the shRNA blunt rather than having an overhang of two TTs. (**B**) RIG-I dependency of IFN-β activation by sh-PCAF. (**C**) Blockade of shRNAs-triggered IFN activation by HCV NS3-4A. A mammalian expression plasmid encoding the NS3-4A protease from HCV isolate JFH-1 was cotransfected with the shRNA and luciferase reporter plasmids. (**D**) Western blot showing expression of HCV NS3-4A in transfected 293FT cells. Cell lysate from (C) was separated by SDS–PAGE and probed with an anti-NS3 antibody.

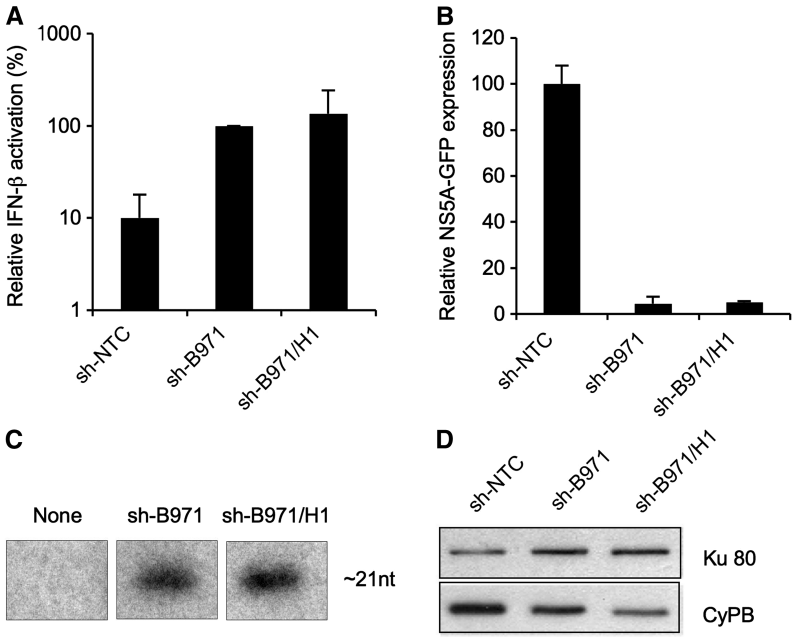

Figure 6. Sh-B971 expressed from an H1 promoter triggers IFN activation. Sh-B971 expressed from an H1 promoter was capable of (**A**) activating IFN-β promoter and (**B**) triggering IFN production to inhibit HCV replication in GS5 cells. (**C**) Intracellular levels of U6- and H1-driven sh-B971 products. RNA extraction and northern blotting were performed as described in Figure 4B. (**D**) Knockdown of CyPB expression by sh-B971 expressed from an H1 promoter.

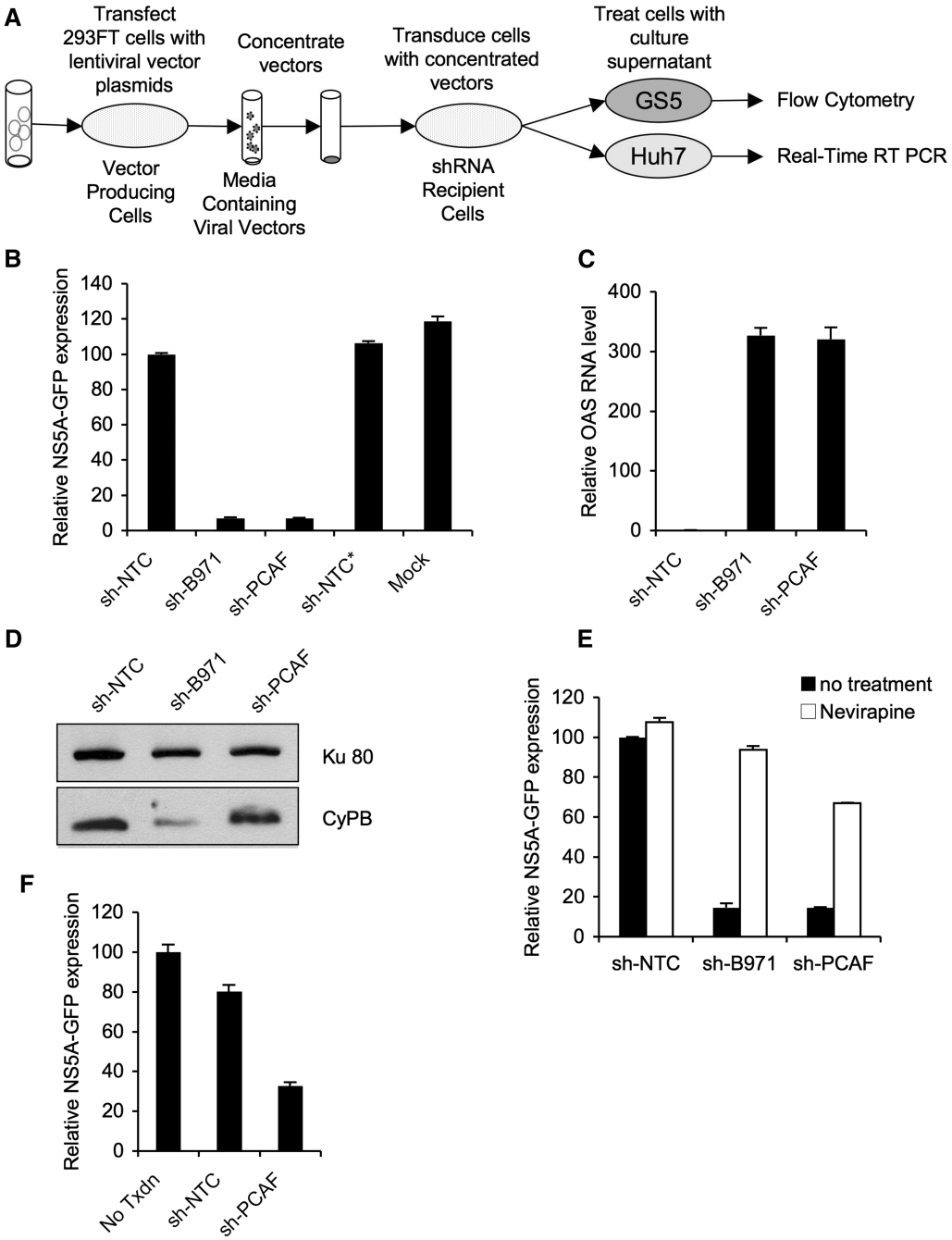

Figure 7. ShRNAs delivered by lentiviral transduction trigger IFN activation *in vitro*. (**A**) Diagram of the experimental setup. Lentiviral vectors were concentrated by ultracentrifugation for removal of soluble IFN proteins in the vectors prepared from transfected 293FT cells. (**B**) Transduction of 293FT cells with the concentrated shRNA vectors triggered IFN production. Culture supernatant was collected 48 h after transduction and was used to treat GS5 cells overnight; the GS5 cells were then analyzed 48 h after treatment by flow cytometry. (**C**) OAS induction in Huh 7 cells treated with culture supernatant from lentivirally transduced cells. Huh 7 cells were treated with culture supernatant for 24 h before RNA extraction and RT-PCR analysis. OAS1 expression level was normalized to that of GAPDH RNA. (**D**) Knockdown of CyPB expression in transduced cells, which were collected 5 days after transduction for western blotting. (**E**) Blockade of lentivirus-triggered IFN activation by a HIV reverse transcriptase inhibitor. 293FT cells were transduced in the presence of 80 nM of Nevirapine; culture supernatant was collected 48 h after transduction and used to treat GS5 cells. (**F**) Culture medium from LH86 cells transduced with sh-PCAF suppressed HCV replication. This experiment was done as depicted in (A) with the LH86 cells in the place of 293FT cells in the transduction step.

methods, centrifuged them to separate the vectors from the IFN-containing media, and then used them to infect naïve 293FT cells (Figure 7A). Both sh-B971 and sh-PCAF vectors induced IFN production when delivered as concentrated lentiviral particles, measured both by HCV suppression (Figure 7B) and by OAS induction (Figure 7C) in Huh-7 cells. To rule out the possibility that residual IFN in the concentrated viral particles was responsible for these results, we added 100 U/ml IFN to the negative control vector sample before the

concentration step. This preparation, designated sh-NTC*, was not able to trigger IFN production in naïve 293FT cells, suggesting that the concentration step effectively removed the soluble IFN from the viral particle pellet. Proper knockdown of the siRNA target of sh-B971 was confirmed by this route of shRNA delivery (Figure 7D). To prove definitively that IFN induction by the shRNAs was mediated by the lentiviral infection route, we tested the effect of an inhibitor of HIV reverse transcriptase, Nevirapine, on IFN induction by sh-B971 and sh-PCAF. As shown in Figure 7E, inclusion of Nevirapine at the time of transduction effectively blocked the ability of both shRNAs to induce IFN in the transduced cells, suggesting the importance of the reverse transcription step in the expression of the shRNAs delivered by the lentiviruses. To determine whether lentiviral vector-delivered shRNA can trigger IFN induction in cells other than 293FT cells, we transduced a human hepatoma cell line, LH86, which has been reported to produce IFN upon viral infection (33), and examined IFN induction in these cells. Culture medium from LH86 cells transduced with sh-PCAF contained biologically active IFN, which suppressed HCV replication in GS5 cells (Figure 7F), indicating that the ability of shRNAs delivered by lentivirus to induce IFN response was not limited to 293FT cells.

DISCUSSION

It has been reported that certain chemically synthesized and phage polymerase *in vitro* transcribed siRNAs can non-specifically induce IFN responses and produce off-target effect via various PRRs, including TLRs. However, the induction of IFN response by shRNAs and its underlying mechanisms have not been as well studied. The actual number of shRNAs that are capable of triggering IFN response will certainly be larger than the few that have been reported in the literature, yet very little is known about the unique characteristics of the select shRNAs and the pathway that they use to activate IFN production. The present study identifies RIG-I, but not MDA5 or TLR3, as the mediator for activation of IFN responses by two shRNAs that are distinct in sequence and structure but both capable of IFN induction in human cells. This was demonstrated by induction of IRF-3 dimerization, activation of IFN promoters, induction of endogenous ISGs (ISG15, OAS and RIG-I), and secretion of IFN, all of which depended on RIG-I and its downstream adaptor, MAVS. In addition, we show that delivery of these shRNAs via lentiviral transduction does not reduce their IFN-inducing capacity, indicating that the ability of lentiviral vector transduction to avoid IFN induction by shRNAs, as reported previously (48), may not be universally applicable to all the shRNAs.

Specific recognition of dsRNAs or ssRNAs bearing 5′-triphosphates by RIG-I is presumably determined mostly by structural features other than the nucleotide sequence of the RNA. Yet IFN activation by sh-B971 exhibited a stringent dependence on specific nucleotides at multiple positions of the shRNA. An AA dinucleotide at the beginning of the U6 transcript has previously been suggested to result in aberrant transcription, and preserving a C/G sequence at positions $-1/+1$ suggested to avert IFN induction (38). We indeed observed a strict requirement for an adenylate at the $+1$ position of sh-B971 for RIG-I recognition and IFN activation, but we observed no difference in expression levels or the apparent sizes of the sh-B971 RNAs bearing either an A or a G at the $+1$ position. Furthermore, mutations introduced elsewhere in the shRNA also abolished or diminished sh-B971's ability to activate IFN, suggesting additional sequence requirement for efficient RIG-I recognition and IFN triggering. Despite these results, because we were not successfully in cloning and sequencing the vector-expressed siRNA, we cannot exclude the possibility that the adenylate at the $+1$ position interferes with transcription and that the resultant abnormal transcript contributes to IFN induction.

Interestingly, the loop A mutant, which contains a predicted loop of 7 nt, generated a siRNA duplex inside the cells that is slightly smaller than that of the shRNAs with a wild-type hairpin loop, suggesting the processing by Dicer into the stem, perhaps fulfilling the requirement of a length of 9 nt for the hairpin loop (46). This mutant form of sh-B971 was not, however, able to trigger IFN activation.

Despite the abilities of both sh-B971 and sh-PCAF to activate the RIG-I pathway, the two shRNAs are unrelated in sequence. Two short stretches of siRNA sequences, GUCCUUCCAA and UGUGU, that have been previously defined as IFN- or cytokine-activating motifs (8,9) are not found in either sh-B971 or sh-PCAF. Any common sequence motifs of IFN-activating shRNAs, if any, remain to be defined. The two shRNAs also differ in that one is predicted to contain one blunt end and the other two ends with overhangs. These results suggest that, although blunt ends may increase siRNA's ability to be recognized by RIG-I (47), they are not required for IFN activation by an endogenously expressed shRNA. The best-characterized RNA structure motif recognized by RIG-I is the 5′-ppp, which is absent from virtually all the cellular RNAs as a result of either 5′-capping or internal cleavage before their appearance in the cytoplasm. A synthetic shRNA that has the same sequence as sh-B971 but lacks the 5′-ppp failed to induce IFN, suggesting the 5′-end status of the intracellularly expressed sh-B971 contributes to IFN activation. Whether or not the 5′-end of an shRNA is capped has not been investigated. Murine U6 RNA does not contain the trimethylguanosine cap that is present on mRNAs and other U small nuclear RNAs; instead it contains a γ-monomethyl phosphate cap at its 5′-end (49). Capping of heterologous transcripts produced from the mU6 promoter, however, requires a stem loop at the 5′-end of the transcript and an AUAUAC sequence immediately after (50). Most shRNAs, including sh-B971 and sh-PCAF, would not meet these requirements and thus should contain unmodified 5′-ppp. Similarly, no evidence of a cap structure for H1 transcripts could be found in the literature. We attempted to express sh-B971

using a miRNA expression cassette and the pol II promoter (51). The primary transcript generated with this construct would be capped at 5′-end by a trimethylguanosine cap and the final siRNA duplex would bear a monophosphate at the 5′-ends of both strands because of Drosha and Dicer cleavage. This version of the sh-B971 vector was much weaker in its ability to trigger IFN activation. Unfortunately the intracellular expression of the RNA duplex was also much weaker and barely detectable by northern blotting. In addition, no knockdown of the target CyPB mRNA was seen with this miRNA-based sh-B971 (data not shown). As a result, whether sh-B971, if expressed at higher level from this construct, could effectively activate IFN remains unclear.

So far as we know, ours is the first report of IFN activation in the target cells by shRNAs delivered by lentiviral transduction. A previous report of IFN induction by lentiviral vector-expressed shRNA only examined the IFN generated in the vector-producing cells, which then up-regulated IFN-stimulated genes in the transduced cells (10). The distinction is important as lentiviral vectors used in a gene-therapy setting will likely be purified and free of any IFN that has been generated during the vector preparation step, but IFN activation in the target cells would pose a more serious concern. Our data suggest the importance of screening shRNAs for IFN induction in the transduced cells *in vitro* before large-scale studies. An HIV reverse transcriptase inhibitor efficiently blocked IFN production by both sh-B971 and sh-PCAF when delivered by transduction, indicating the virion-encapsulated RNA was not able to trigger IFN activation. In this respect, it is interesting to note that positive-stranded RNA viruses, which produce dsRNA intermediates in the cytoplasm during replication (52–55), often replicate in membrane enclosed vesicles (56), This sequestration of viral dsRNA in membranous structures may shield the RNA from the cytoplasmic PRRs and contribute to a successful infection.

IFN-induction and RNAi by shRNAs appear to be independent functions of the same RNA (57). Our results also showed that IFN-induction by sh-B971 is independent of its ability to suppress target mRNA expression through RNAi. On the other hand, it might be possible to screen for duel functional siRNAs that confer therapeutic benefits by both RNAi and immunostimulation (58). For example, siRNAs that target either viral genomes or cellular cofactors of the viruses can be screened for their ability to trigger IFN activation in hopes of find 'super siRNAs' with increased efficacy against IFN-sensitive viruses.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Dr Andre Irsigler and Dr Jason Robotham for technical assistance and Dr Anne B. Thistle for proofreading the manuscript.

FUNDING

National Institutes of Health (AI069285 to K.L.; DE016680 to F.Z.); and the Department of Biological Science at Florida State University (to H.T.). Funding for open access charge: The American Cancer Society.

Conflict of interest statement. None declared.

REFERENCES

1. Hammond,S.M., Bernstein,E., Beach,D. and Hannon,G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, **404**, 293–296.
2. Martinez,J., Patkaniowska,A., Urlaub,H., Luhrmann,R. and Tuschl,T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, **110**, 563–574.
3. Gregory,R.I., Chendrimada,T.P., Cooch,N. and Shiekhattar,R. (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell*, **123**, 631–640.
4. Akira,S., Uematsu,S. and Takeuchi,O. (2006) Pathogen recognition and innate immunity. *Cell*, **124**, 783–801.
5. Sledz,C.A., Holko,M., de Veer,M.J., Silverman,R.H. and Williams,B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.*, **5**, 834–839.
6. Kariko,K., Bhuyan,P., Capodici,J. and Weissman,D. (2004) Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J. Immunol.*, **172**, 6545–6549.
7. Kim,D.H., Longo,M., Han,Y., Lundberg,P., Cantin,E. and Rossi,J.J. (2004) Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat. Biotechnol.*, **22**, 321–325.
8. Hornung,V., Guenthner-Biller,M., Bourquin,C., Ablasser,A., Schlee,M., Uematsu,S., Noronha,A., Manoharan,M., Akira,S., de Fougerolles,A. *et al.* (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.*, **11**, 263–270.
9. Judge,A.D., Sood,V., Shaw,J.R., Fang,D., McClintock,K. and MacLachlan,I. (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.*, **23**, 457–462.
10. Bridge,A.J., Pebernard,S., Ducraux,A., Nicoulaz,A.L. and Iggo,R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.*, **34**, 263–264.
11. Nallagatla,S.R., Hwang,J., Toroney,R., Zheng,X., Cameron,C.E. and Bevilacqua,P.C. (2007) 5′-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science*, **318**, 1455–1458.
12. Yoneyama,M., Kikuchi,M., Natsukawa,T., Shinobu,N., Imaizumi,T., Miyagishi,M., Taira,K., Akira,S. and Fujita,T. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.*, **5**, 730–737.
13. Kato,H., Takeuchi,O., Sato,S., Yoneyama,M., Yamamoto,M., Matsui,K., Uematsu,S., Jung,A., Kawai,T., Ishii,K.J. *et al.* (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*, **441**, 101–105.
14. Gitlin,L., Barchet,W., Gilfillan,S., Cella,M., Beutler,B., Flavell,R.A., Diamond,M.S. and Colonna,M. (2006) Essential role of mda-5 in type I IFN responses to polyriboinosinic : polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc. Natl Acad. Sci. USA*, **103**, 8459–8464.
15. Kawai,T., Takahashi,K., Sato,S., Coban,C., Kumar,H., Kato,H., Ishii,K.J., Takeuchi,O. and Akira,S. (2005) IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.*, **6**, 981–988.
16. Meylan,E., Curran,J., Hofmann,K., Moradpour,D., Binder,M., Bartenschlager,R. and Tschopp,J. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature*, **437**, 1167–1172.
17. Seth,R.B., Sun,L., Ea,C.K. and Chen,Z.J. (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, **122**, 669–682.

18. Xu,L.G., Wang,Y.Y., Han,K.J., Li,L.Y., Zhai,Z. and Shu,H.B. (2005) VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell*, **19**, 727–740.
19. Andrejeva,J., Childs,K.S., Young,D.F., Carlos,T.S., Stock,N., Goodbourn,S. and Randall,R.E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc. Natl Acad. Sci. USA*, **101**, 17264–17269.
20. Yoneyama,M., Kikuchi,M., Matsumoto,K., Imaizumi,T., Miyagishi,M., Taira,K., Foy,E., Loo,Y.M., Gale,M. Jr, Akira,S. *et al.* (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.*, **175**, 2851–2858.
21. Yamashita,K., Imaizumi,T., Taima,K., Fujita,T., Ishikawa,A., Yoshida,H., Oyama,C. and Satoh,K. (2005) Polyinosinic-polycytidylic acid induces the expression of GRO-alpha in BEAS-2B cells. *Inflammation*, **29**, 17–21.
22. Cardenas,W.B., Loo,Y.M., Gale,M. Jr, Hartman,A.L., Kimberlin,C.R., Martinez-Sobrido,L., Saphire,E.O. and Basler,C.F. (2006) Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J. Virol.*, **80**, 5168–5178.
23. Taima,K., Imaizumi,T., Yamashita,K., Ishikawa,A., Fujita,T., Yoshida,H., Takanashi,S., Okumura,K. and Satoh,K. (2006) Expression of IP-10/CXCL10 is upregulated by double-stranded RNA in BEAS-2B bronchial epithelial cells. *Respiration*, **73**, 360–364.
24. Hornung,V., Ellegast,J., Kim,S., Brzozka,K., Jung,A., Kato,H., Poeck,H., Akira,S., Conzelmann,K.K., Schlee,M. *et al.* (2006) 5′-Triphosphate RNA is the ligand for RIG-I. *Science*, **314**, 994–997.
25. Pichlmair,A., Schulz,O., Tan,C.P., Naslund,T.I., Liljestrom,P., Weber,F. and Reise Sousa,C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science*, **314**, 997–1001.
26. Hornung,V., Ablasser,A., Charrel-Dennis,M., Bauernfeind,F., Horvath,G., Caffrey,D.R., Latz,E. and Fitzgerald,K.A. (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature*, **458**, 514–518.
27. Fernandes-Alnemri,T., Yu,J.W., Datta,P., Wu,J. and Alnemri,E.S. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, **458**, 509–513.
28. Burckstummer,T., Baumann,C., Bluml,S., Dixit,E., Durnberger,G., Jahn,H., Planyavsky,M., Bilban,M., Colinge,J., Bennett,K.L. *et al.* (2009) An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat. Immunol.*, **10**, 266–272.
29. Roberts,T.L., Idris,A., Dunn,J.A., Kelly,G.M., Burnton,C.M., Hodgson,S., Hardy,L.L., Garceau,V., Sweet,M.J., Ross,I.L. *et al.* (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science*, **323**, 1057–1060.
30. Takaoka,A., Wang,Z., Choi,M.K., Yanai,H., Negishi,H., Ban,T., Lu,Y., Miyagishi,M., Kodama,T., Honda,K. *et al.* (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature*, **448**, 501–505.
31. Chiu,Y.H., Macmillan,J.B. and Chen,Z.J. (2009) RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell*, **138**, 576–591.
32. Robida,J.M., Nelson,H.B., Liu,Z. and Tang,H. (2007) Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro. *J. Virol.*, **81**, 5829–5840.
33. Zhu,H., Dong,H., Eksioglu,E., Hemming,A., Cao,M., Crawford,J.M., Nelson,D.R. and Liu,C. (2007) Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. *Gastroenterology*, **133**, 1649–1659.
34. Zhu,H., Nelson,D.R., Crawford,J.M. and Liu,C. (2005) Defective Jak-Stat activation in hepatoma cells is associated with hepatitis C viral IFN-alpha resistance. *J. Interferon Cytokine Res.*, **25**, 528–539.
35. Iwamura,T., Yoneyama,M., Yamaguchi,K., Suhara,W., Mori,W., Shiota,K., Okabe,Y., Namiki,H. and Fujita,T. (2001) Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells*, **6**, 375–388.
36. Zhu,F.X., King,S.M., Smith,E.J., Levy,D.E. and Yuan,Y. (2002) A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc. Natl Acad. Sci. USA*, **99**, 5573–5578.
37. Waninger,S., Kuhen,K., Hu,X., Chatterton,J.E., Wong-Staal,F. and Tang,H. (2004) Identification of cellular cofactors for human immunodeficiency virus replication via a ribozyme-based genomics approach. *J. Virol.*, **78**, 12829–12837.
38. Pebernard,S. and Iggo,R.D. (2004) Determinants of interferon-stimulated gene induction by RNAi vectors. *Differentiation*, **72**, 103–111.
39. Chen,Z., Benureau,Y., Rijnbrand,R., Yi,J., Wang,T., Warter,L., Lanford,R.E., Weinman,S.A., Lemon,S.M., Martin,A. *et al.* (2007) GB virus B disrupts RIG-I signaling by NS3/4A-mediated cleavage of the adaptor protein MAVS. *J. Virol.*, **81**, 964–976.
40. Li,K., Chen,Z., Kato,N., Gale,M. Jr. and Lemon,S.M. (2005) Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J. Biol. Chem.*, **280**, 16739–16747.
41. Yang,F., Robotham,J.M., Nelson,H.B., Irsigler,A., Kenworthy,R. and Tang,H. (2008) Cyclophilin a is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. *J. Virol.*, **82**, 5269–5278.
42. Alexopoulou,L., Holt,A.C., Medzhitov,R. and Flavell,R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, **413**, 732–738.
43. Nelson,H.B. and Tang,H. (2006) Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. *J. Virol.*, **80**, 1181–1190.
44. Akazawa,T., Ebihara,T., Okuno,M., Okuda,Y., Shingai,M., Tsujimura,K., Takahashi,T., Ikawa,M., Okabe,M., Inoue,N. *et al.* (2007) Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc. Natl Acad. Sci. USA*, **104**, 252–257.
45. Su,Z.Z., Sarkar,D., Emdad,L., Barral,P.M. and Fisher,P.B. (2007) Central role of interferon regulatory factor-1 (IRF-1) in controlling retinoic acid inducible gene-I (RIG-I) expression. *J. Cell Physiol.*, **213**, 502–510.
46. Brummelkamp,T.R., Bernards,R. and Agami,R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550–553.
47. Marques,J.T., Devosse,T., Wang,D., Zamanian-Daryoush,M., Serbinowski,P., Hartmann,R., Fujita,T., Behlke,M.A. and Williams,B.R. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol.*, **24**, 559–565.
48. Robbins,M.A., Li,M., Leung,I., Li,H., Boyer,D.V., Song,Y., Behlke,M.A. and Rossi,J.J. (2006) Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs in vitro. *Nat. Biotechnol.*, **24**, 566–571.
49. Singh,R. and Reddy,R. (1989) Gamma-monomethyl phosphate: a cap structure in spliceosomal U6 small nuclear RNA. *Proc. Natl Acad. Sci. USA*, **86**, 8280–8283.
50. Singh,R., Gupta,S. and Reddy,R. (1990) Capping of mammalian U6 small nuclear RNA in vitro is directed by a conserved stem-loop and AUAUAC sequence: conversion of a noncapped RNA into a capped RNA. *Mol. Cell Biol.*, **10**, 939–946.
51. Stegmeier,F., Hu,G., Rickles,R.J., Hannon,G.J. and Elledge,S.J. (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc. Natl Acad. Sci. USA*, **102**, 13212–13217.
52. Targett-Adams,P., Boulant,S. and McLauchlan,J. (2008) Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. *J. Virol.*, **82**, 2182–2195.
53. Weber,F., Wagner,V., Rasmussen,S.B., Hartmann,R. and Paludan,S.R. (2006) Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J. Virol.*, **80**, 5059–5064.
54. Miller,S., Sparacio,S. and Bartenschlager,R. (2006) Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. *J. Biol. Chem.*, **281**, 8854–8863.

55. Knoops,K., Kikkert,M., Worm,S.H., Zevenhoven-Dobbe,J.C., van der Meer,Y., Koster,A.J., Mommaas,A.M. and Snijder,E.J. (2008) SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol.*, **6**, e226.
56. Denison,M.R. (2008) Seeking membranes: positive-strand RNA virus replication complexes. *PLoS Biol.*, **6**, e270.
57. Schlee,M., Hornung,V. and Hartmann,G. (2006) siRNA and isRNA: two edges of one sword. *Mol. Ther.*, **14**, 463–470.
58. Poeck,H., Besch,R., Maihoefer,C., Renn,M., Tormo,D., Morskaya,S.S., Kirschnek,S., Gaffal,E., Landsberg,J., Hellmuth,J. *et al.* (2008) 5′-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. *Nat. Med.*, **14**, 1256–1263.
59. Yam,P.Y., Li,S., Wu,J., Hu,J., Zaia,J.A. and Yee,J.K. (2002) Design of HIV vectors for efficient gene delivery into human hematopoietic cells. *Mol. Ther.*, **5**, 479–484.