

Activation of the interferon system by short-interfering RNAs

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RNA interference (RNAi) is a powerful tool used to manipulate gene expression or determine gene function^{1,2}. One technique of expressing the short double-stranded (ds) RNA intermediates required for interference in mammalian systems is the introduction of short-interfering (si) RNAs³⁻⁶. Although RNAi strategies are reliant on a high degree of specificity, little attention has been given to the potential non-specific effects that might be induced. Here, we found that transfection of siRNAs results in interferon (IFN)-mediated activation of the Jak–Stat pathway and global upregulation of IFN-stimulated genes. This effect is mediated by the dsRNA-dependent protein kinase, PKR, which is activated by 21-base-pair (bp) siRNAs and required for upregulation of IFN- β in response to siRNAs. In addition, we show by using cell lines deficient in specific components mediating IFN action that the RNAi mechanism itself is independent of the interferon system. Thus, siRNAs have broad and complicating effects beyond the selective silencing of target genes when introduced into cells. This is of critical importance, as siRNAs are currently being explored for their potential therapeutic use^{7,8}.

Interferons are cytokines that function as the host's first line of defence against viral infection. Activation of this innate immune response is triggered partly by dsRNA, a common viral replicative intermediate. The resulting signalling cascade is mediated by a variety of proteins — including the Janus family tyrosine kinases, Jak1 and Tyk2, the signal transducers and activators of transcription, Stats1 and 2, and the IRF9 transcription factor — culminating in the induction of interferon-stimulated genes (ISGs) within the nucleus^{9,10}. Here, we found that transfection of 21-bp ds siRNAs, regularly used to induce specific gene silencing, also resulted in interferon-mediated activation of the Jak–Stat signalling pathway. Transfection of an siRNA designed to target Lamin A/C resulted in a specific dose-dependent decrease in levels of lamin expression in the human glioblastoma cell line T98G (Fig. 1a). However, the presence of siRNAs also caused a dose-dependent upregulation of Stat1 expression (Fig. 1b). To ensure that this phenomenon was not exclusive to the *lamin* siRNA, we examined this trend with a

second siRNA. Transfection of *GAPDH* siRNA resulted in specific suppression of *GAPDH* mRNA and also in concomitant upregulation of *Stat1* mRNA in T98G cells, as detected by semi-quantitative RT–PCR analysis (Fig. 1c). In addition, upregulation of *Stat1* mRNA was detected in response to five other unique siRNAs, synthesized both chemically and enzymatically, at either the mRNA or protein level and in two different cell lines (see Supplementary Information, Fig. S1). These results exclude the possibility that the non-specific effect is an artefact of a subset of siRNAs. This phenomenon is dependent on interferon, as transfection of *lamin* siRNA into type-I IFN-null human glioma GRE cells suppressed lamin protein levels, but did not result in upregulation of Stat1 protein levels (Fig. 1d).

Stat1 is only one of many ISGs upregulated in response to IFNs. To investigate ISG induction in response to siRNA on a larger scale, we used a functional genomics approach. RNA from control and *GAPDH* siRNA-transfected RCC1 renal cell carcinoma cells was hybridized to a custom ISG cDNA microarray containing probes for 850 unique putative ISGs and 100 control genes. Expression of *GAPDH* was specifically suppressed tenfold, whereas 52 ISGs were induced more than twofold in at least one of the siRNA-transfected samples. This ISG induction is specific to the presence of siRNA, as the mock transfection did not induce ISG expression (Fig. 2a). Several ISGs, including oligoadenylate synthetase 2 and 3 and interferon-inducible transmembrane protein 1 and 2, were induced at all siRNA concentrations tested, whereas other ISGs were only induced in response to higher concentrations of siRNA (Fig. 2b). This suggests that although the IFN system is activated in response to all concentrations of siRNA tested, some ISGs are induced in a concentration-dependent manner. Although the ISG array used in these experiments contains 850 putative ISGs, it is not uncommon to observe induction of only a subset, as gene induction may be dependent on factors such as cell type, type of IFN and kinetics of activation. Data obtained through microarray analyses were verified by northern blot and semi-quantitative RT–PCR analysis of select ISGs (see Supplementary Information, Fig. S2).

To explore the possible mechanism of siRNA-mediated IFN induction, the dsRNA recognition protein PKR was examined. Activation of

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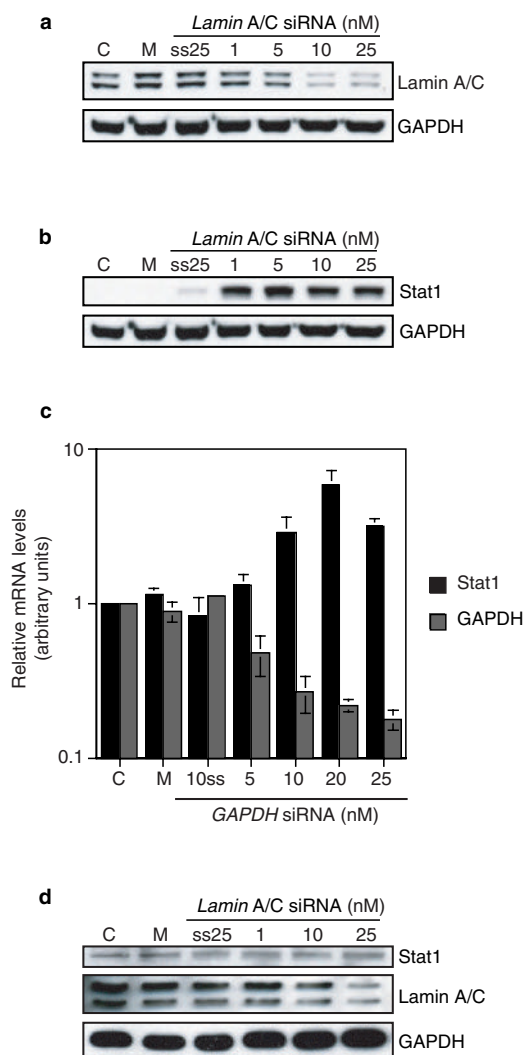


Figure 1 Intracellular effects of siRNA. (a) A western blot showing lamin levels in T98G cell lysates collected 48 h after transfection with lamin-specific siRNA. C, untreated control sample; M, mock transfection; ss, single-strand sense 21-mer. (b) A western blot of Stat1 and GAPDH from T98G cell lysates. (c) Semi-quantitative RT-PCR analysis of *Stat1* mRNA levels. The graph represents data averaged from three biological repeats \pm standard deviation. (d) A western blot showing lamin and Stat1 levels in type-I IFN-null GRE cells transfected with lamin-specific siRNA.

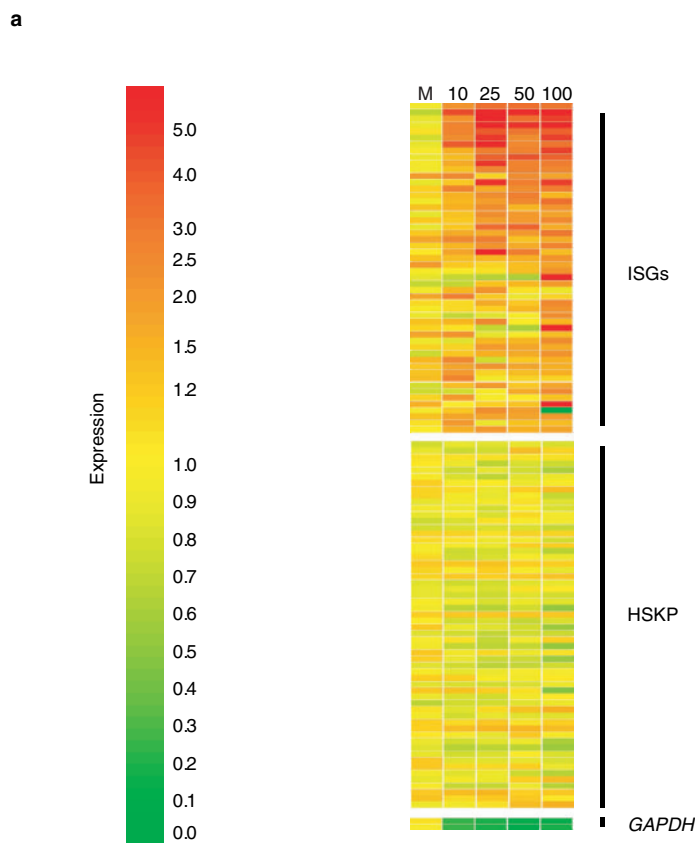
PKR by viral dsRNA results in autophosphorylation and subsequent phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) subunit¹¹, causing general inhibition of cellular protein synthesis. In addition to its role as a translational inhibitor, PKR has also been identified as a component of signal transduction pathways that regulate events such as cell growth and stress responses¹². In light of these roles as both a dsRNA recognition protein and a signal transducer, it is possible that PKR may mediate siRNA-initiated signalling events. In fact, the 21-bp siRNAs activate PKR in a concentration-dependent manner *in vitro*¹³ (Fig. 3a). The activation trend reflects the bell-shaped activation curve of PKR commonly detected with high and low concentrations of dsRNA-inhibiting PKR autophosphorylation¹⁴. To address the physiological significance of these *in vitro* findings, PKR was immunoprecipitated from siRNA-transfected T98G cells and assayed for kinase activity. Although transfection of the 21-mer sense strand did not activate PKR,

the siRNA caused an increase in kinase activity above mock-transfected levels, similar to levels obtained in the positive control (Fig. 3b). This increase in phosphorylation was also detected using an antibody specific for phosphorylated Thr 446/451 on PKR (see Supplementary Information, Fig. S3). Consistently, the PKR substrate eIF2 α is transiently phosphorylated *in vivo* in response to chemically synthesized, gel-purified HPV-E7 siRNA, but not to the single-stranded 21-mer. (Fig. 3c). It should be noted that the E7 siRNA used in the transfection is free from contaminating long dsRNAs that could be responsible for the observed effect (Fig. 3c, right). Finally, PKR-null murine embryonic fibroblasts (MEFs) failed to respond to siRNA-mediated induction of IFN- β when compared with wild-type MEFs, which respond robustly to the dsRNA challenges by upregulating IFN- β expression (Fig. 3e). These results suggest that PKR is necessary for the IFN-mediated global effects observed in response to siRNAs

It is possible that dsRNA-responsive pathways are not only activated by siRNAs, but may also mediate the specific gene-silencing effects of RNAi. Intracellular detection of dsRNA occurs through the activation of at least two major stress response pathways that exert their effects through PKR¹⁴ or Rnase L¹⁵. In addition to activation of PKR, activation of 2'-5' oligoadenylate synthetase by dsRNA produces 2'-5' oligoadenylate molecules that bind to and activate RNase L, resulting in non-specific degradation of cellular RNA¹⁵. This also results in a general repression of protein synthesis^{14,15}.

To determine whether the mammalian dsRNA response proteins facilitate the effects of RNAi, cell lines deficient in PKR and/or RNase L were tested for sequence-specific suppression of reporter luciferase activity in response to firefly (GL3) and sea pansy (RL) siRNAs³ in luciferase reporter assays. Luciferase expression for each cell line was normalized to a reporter control, which was given a value of one, and data were reported relative to this control. *Drosophila melanogaster* S2 cells were used as a positive control for RNAi, responding by sequence-specific luciferase suppression to both the 21-bp and the 500-bp dsRNA. In the PKR-null, RNase L-null, and PKR/RNase L double-knockout MEFs, transfection of GL3 siRNA resulted in a 90% sequence-specific suppression of GL3 luciferase activity. This level of specific suppression was also detected in wild-type MEFs (Fig. 4a). Cell lines containing null mutations in interferon signalling genes¹⁶ were tested in the same assay to determine whether components of this pathway are involved in the specific effects of RNAi. The cell lines tested were U2A cells deficient in IRF9, U3A cells deficient in Stat1, U4A cells deficient in Jak1 and GRE cells deficient in type-I IFN. All of the mutant cell lines clearly showed specific suppression of the targeted GL3 luciferase to approximately 13% of control levels in response to GL3 siRNA, but not to RL siRNA. The corresponding wild-type cells were also tested and the same pattern of suppression was detected. The results of these experiments are represented by the Jak1-null data (Fig. 4a and see Supplementary Information, Fig. S4)

Conversely, activation of the dsRNA-responsive pathways may prevent any specific effects of RNAi mediated by long dsRNA molecules. In RNAi model systems such as *D. melanogaster* and the nematode *Caenorhabditis elegans*, 500-bp dsRNAs can specifically suppress expression of homologous target genes. Although RNAi is observed in mammalian oocytes, embryonic stem cells and pre-implantation embryos, it is not detected in adult mammalian systems in response to these long dsRNA molecules. It has been proposed that RNAi mediated by long dsRNA molecules is prevented in adult mammalian cells because of the activated interferon pathway and associated dsRNA-responsive mechanisms. To address this question, vectors were generated that produce 500-bp inverted repeat (IR) dsRNA molecules¹⁷ complementary to GL3 or RL mRNA. Cotransfections were performed



b

Genbank	Name	Mock	10 nM	25 nM	50 nM	100 nM
R72244	2'-5'-oligoadenylate synthetase 2 (69–71 KD)	0.7	4.6	9.4	6.5	11.1
AA419251	Interferon-induced transmembrane protein 1 (9–27)	0.9	2.7	9.1	5.4	10.5
AA598817	Preferentially expressed antigen in melanoma	0.9	0.7	0.7	0.6	9.7
AI001174	Enolase 1, (α)	1.1	1.0	0.7	0.6	7.3
H79353	High-affinity Fc fragment of IgE receptor γ subunit	1.5	1.0	1.1	1.3	6.0
N63988	IFIT2	0.9	2.2	8.0	3.3	4.9
AA146773	2'-5'-oligoadenylate synthetase 1 (40–46K)	1.0	1.6	2.9	2.8	4.9
T70503	Ectonucleotide pyrophosphatase/phosphodiesterase 1	0.9	1.2	5.5	3.0	4.9
AA250771	Hypothetical protein FLJ11267	0.8	2.4	5.0	2.4	4.7
AA775616	Secreted phosphoprotein 1	0.9	1.4	3.9	4.2	3.9
AI357590	2'-5'-oligoadenylate synthetase 3	1.0	2.7	4.0	3.1	3.7
AA432030	Interferon, α -inducible protein (clone IFI-6-16)	1.0	1.5	1.9	2.6	3.2
NM_004417	Dual-specificity phosphatase 1	1.2	1.6	1.7	2.1	3.2
AA862371	Interferon-induced transmembrane protein 2 (1-8D)	0.9	2.2	3.4	3.3	3.1
AA418077	GTP-binding protein overexpressed in skeletal muscle	0.9	4.1	7.8	2.5	3.0
AA292074	Ubiquitin-conjugating enzyme E2L 6	0.9	1.7	5.0	2.7	3.0
T71879	Complement component 2	1.1	2.7	1.6	2.4	2.9
AA664040	Tryptophanyl-tRNA synthetase	1.1	1.4	2.2	1.7	2.8
AA464246	Major histocompatibility complex, class I, C	0.9	1.3	2.5	2.6	2.6
N29431	NY-REN-18 antigen	1.0	1.1	1.6	1.2	2.5

Figure 2 Global upregulation of ISGs in response to *GAPDH* siRNA in RCC1 cells. **(a)** A gene tree representing genes induced more than twofold in at least one siRNA-transfected sample (ISGs), stable expression of housekeeping genes (HSKP) and specific suppression of *GAPDH* message

levels. **(b)** The most highly induced ISGs and normalized fold change values for each sample. Fold changes greater than twofold are shown in bold. The siRNA concentration relative to final transfection volume is shown at the top. M, mock transfection.

with the 500-bp *in-vitro*-transcribed IR RNA duplexes and reporter constructs. In each cell line, GL3 luciferase activity was suppressed in a non-specific manner to the same extent in response to both GL3 and RL IR RNA (Fig. 4b). These results also show that PKR is primarily responsible for the non-specific suppression in cells treated with long

dsRNA. The level of non-specific suppression in the PKR-null cells was only 25%, compared with more than 75% detected in the wild-type and RNase L-null cells. In addition, the GL3 and RL IR RNAs suppressed GL3 luciferase activity non-specifically by approximately 95% in Jak1-null cells (Fig. 4b). This is representative of the data obtained

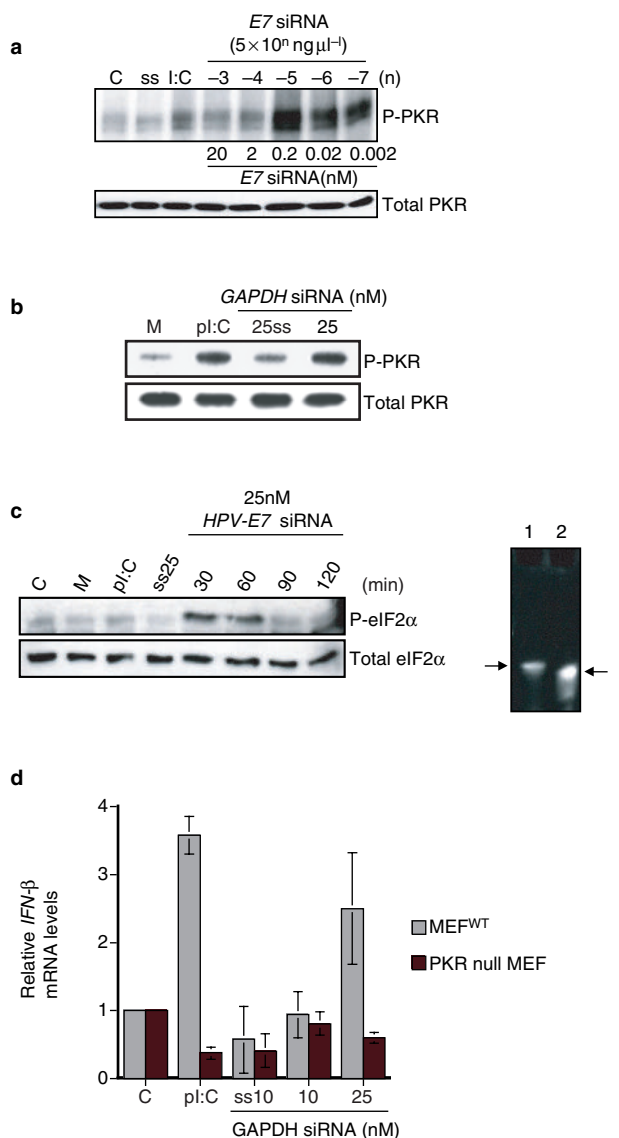


Figure 3 PKR is necessary for the non-specific siRNA-induced signalling events. **(a)** Autoradiograph of PKR autophosphorylation in response to the indicated concentrations of *HPV-E7* siRNA *in vitro* (top). Poly rI:rC (synthetic dsRNA) was used as a positive control and either buffer alone (C) or a 21-nucleotide single-stranded RNA (ss) were used as negative controls. The PKR western blot demonstrates equal loading of total protein (bottom). **(b)** Top panel represents PKR kinase activity of immunoprecipitated PKR in response to the indicated treatments. The bottom panel represents the total level of PKR. M, mock transfection; pl:C, poly rI:rC synthetic dsRNA positive control; ss, single-stranded sense 21-mer. **(c)** Western Blot of the PKR substrate, eIF2 α , from T98G cells, showing increased phosphorylation in response to transfection with *HPV-E7* siRNA (left). A polyacrylamide gel showing the purity of the *HPV-E7* siRNA (lane 2) is also shown (right). Lane 1 shows a 22-bp RNA marker. **(d)** Semi-quantitative RT-PCR analysis, showing upregulation of *IFN- β* mRNA in response to siRNA transfection in wild-type and PKR-null MEFs. The graph represents data averaged from two biological repeats performed in duplicate.

from all IFN signalling mutant cell lines examined and their parental controls (see Supplementary Information, Fig. S4). The data presented here, and the discovery of a TLR3-mediated dsRNA-responsive pathway¹⁸, clearly indicate that PKR and RNase L do not constitute the full extent of dsRNA-responsive pathways in mammalian cells. In addition,

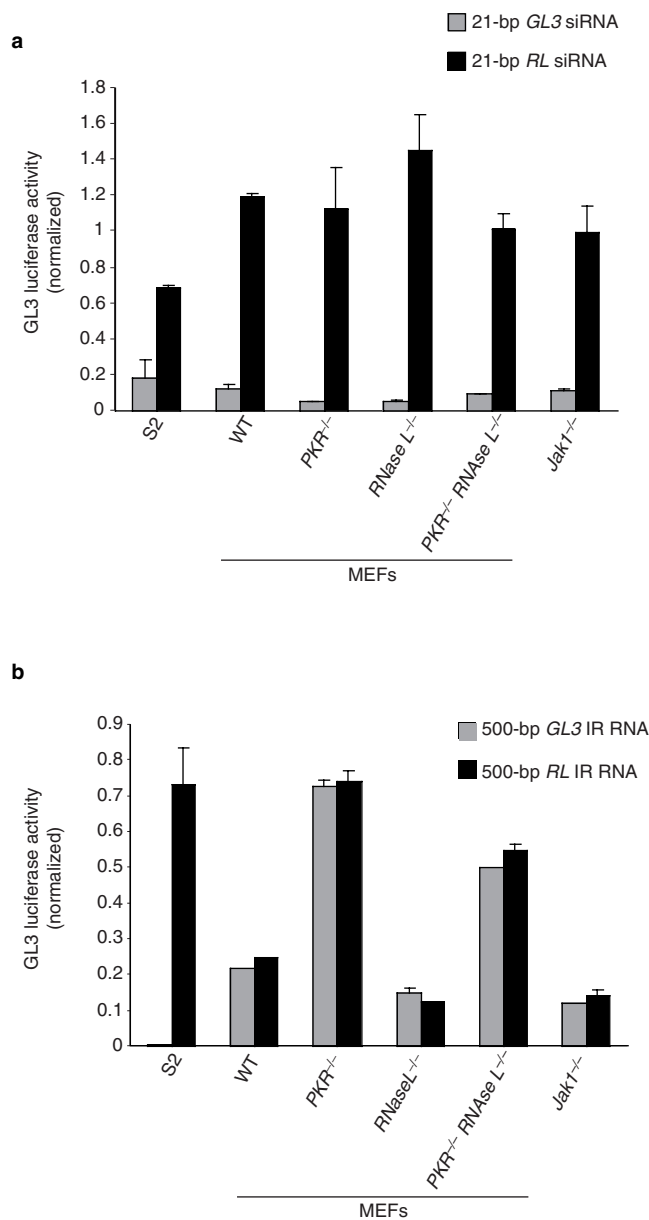


Figure 4 RNA interference of *GL3* luciferase expression by 21-bp *GL3* siRNA. The fold change of *GL3* luciferase for each cell line was normalized to its own reporter control (p*GL3*-Control and p*RL*-SV40), which was given a value of one. Graphs represent data averaged from at least three independent experiments \pm standard deviation. The *D. melanogaster* cell line S2 acts as a positive control for RNAi by responding to both the 21-bp *GL3* siRNA, as shown in **a**, and the 500-bp *GL3* IR RNA, as shown in **b**, by sequence-specific luciferase suppression. 21-bp siRNA-mediated sequence-specific silencing is shown in **a**. In **b**, 500-bp IR RNA-mediated non-specific silencing of *GL3* luciferase is shown in wild-type, PKR-null, RNase L-null and PKR/RNase L double knockout MEFs, as well as in cell lines deficient in specific components of the interferon system. The results obtained from the Jak1-null cells are representative of the results obtained from IRF9-null, Stat1-null, Jak1-null and type-I IFN-null cells.

the IFN system neither facilitates RNAi mediated by 21-bp siRNAs, nor prevents RNAi mediated by 500-bp IR RNA.

Here, we have shown that in mammalian cells, RNAi mediated by 21-bp siRNAs occurs independently of the well-characterized dsRNA-responsive proteins, PKR and RNase L, and other major components

of the interferon system (Fig. 4a). It remains possible that the dsRNA pattern recognition receptor TLR3 (refs 18, 19) is involved in cells expressing this TLR. In addition to the specific gene-silencing effects of RNAi, global ISG upregulation is detected in response to the intracellular presence of siRNAs. This phenomenon is mediated by PKR, as this kinase is activated by siRNAs and IFN- β induction in response to these siRNAs is PKR-dependent. The induction of the interferon, and possibly other, cellular signalling pathways indicates that siRNAs have broad effects beyond the selective silencing of homologous target genes when introduced into cells. Therefore, caution must be exerted in the interpretation of data from experiments using RNAi technology for suppression of specific gene expression. The side effects elicited by siRNAs are of concern not only for the use of RNAi technology as a basic research tool, but also as it moves towards therapeutic applications, such as targeting viruses to suppress infection^{7,8}. □

METHODS

Cell culture. S2 *Drosophila* Schneider cells (Invitrogen, Carlsbad, CA) were cultured at 28 °C in *Drosophila* serum-free medium (SFM) supplemented with 16.5 mM L-glutamine. Immortalized wild-type and mutant MEFs^{20–22}, IFN signalling mutants derived from parental 2fTGH, and the human glioblastoma T98G cell line were cultured at 37 °C in DMEM supplemented with 10% heat-inactivated foetal calf serum. The renal cell carcinoma cell line RCC1 was propagated under the same conditions in RPMI medium.

For luciferase assays, cells were plated in 24-well tissue-culture plates at a density of 1×10^5 cells per well and incubated for 24 h before transfection. Cotransfection of reporter plasmids and RNAs was performed with Lipofectamine Plus (Invitrogen) for mammalian cells and CellFectin (Invitrogen) in accordance with the manufacturer's instructions for *Drosophila* cells. Luciferase expression was determined with the Dual Luciferase Assay (Promega, Madison, WI) on lysates collected 20 h after transfection. At least three independent transfections were performed.

Transfections of the siRNA targeting endogenous lamin A/C and GAPDH were performed using Oligofectamine (Invitrogen) at the concentrations noted in the text, controlling for equivalent charge ratios. Total protein lysates or RNA were collected 48 h post-transfection (unless indicated otherwise) and analysed by western blotting or quantitative RT-PCR, respectively.

Vector construction. The *Photinus pyralis* luciferase gene fragment (GL3) extends from position 308–863 of the luciferase gene relative to the start of transcription. The primers used to amplify this gene fragment (Forward 5'-AAATCCAAGCTTGCCCGCAACGACATTTA and Reverse 5'-AACAAAGGGCCACCAGCAGCGACTTTGAA) added a *Hind*III site to the 5' end and an *Apa*I site to the 3' end of the fragment. The pGL3-Control vector was used as a template for PCR. The *Renilla reniformis* luciferase gene fragment (RL) extends from position 354–895 of the luciferase gene relative to the start codon of transcription. The same restriction sites were added to the RL luciferase gene fragment using the primers Forward 5'-AAATCCAAGCTTCATGATTGGGGTGCTTGTTT and Reverse 5'-AACTAAGGGCCCTATATTTCC-CATTTCATCAGGTG. The pRL-SV40 vector was used as a template for PCR amplification. These fragments were cloned into pcDNA3 (Invitrogen).

For construction of the IR vectors, the amplified fragments were digested individually with *Apa*I and then ligated to form an IR. The IR fragments were then digested with *Hind*III, and this site was used to introduce the fragments into the pcDNA3 vector. Vectors containing the IR fragments were cloned using the *Escherichia coli* SURE strain of competent cells from Stratagene (Cedar Creek, TX).

RNA preparation. 21-nucleotide synthetic RNAs were chemically synthesized to target *P. pyralis* luciferase and *R. reniformis* luciferase genes at positions 153–173 and 119–139, respectively, relative to the start codon². The synthesized oligoribonucleotides were purified on a denaturing 20% polyacrylamide (8 M urea) gel and the product bands were cut out. RNA was then extracted from the gel and concentrated by ethanol precipitation.

To produce the double-stranded luciferase siRNAs, 20 μ M single strands were incubated in annealing buffer for 1 min at 90 °C before a 1-h incubation at

37 °C (ref. 3). *In vitro* transcription was performed using the MEGascript T7 IVT kit (Ambion, Austin, TX) to generate the longer double-stranded IR RNA from the constructed vectors. Template DNA (1 μ g pcDNA3 containing either the IR-GL3 or the IR-RL fragment) linearized by *Hind*III digestion was included in the transcription reaction and incubated for 5 h. Template DNA was removed by DNase treatment. RNA was then extracted and precipitated, and concentrations were determined by spectrophotometry. Before transfection, the resulting RNAs were heated to 65 °C for 5 min and cooled on ice to promote the formation of IR structures. RNase protection assays were performed to ensure IR structures had formed.

The siRNAs targeting GAPDH and Lamin A/C were synthesized using the Silencer siRNA Construction Kit (Ambion). The siRNAs targeting HPV-E7 was chemically synthesized, annealed and gel purified by Qiagen (Valencia, CA).

Protein analyses. Transfected cells grown in 24-well plates were lysed according to the previously described protocol²³. Total protein (50 μ g) was separated on 8% polyacrylamide gels and transferred to nitrocellulose membranes. Immunostaining was performed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). The monoclonal PKR antibody was used at a 1:5000 dilution; monoclonal Stat-1 antibody (sc-464, Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:1000 dilution; monoclonal phosphorylated eIF2 α antibody (Cell Signalling, Beverly, MA) was used at 1:1000 dilution. Equivalent protein loading was determined by detection of tubulin or GAPDH.

In vitro PKR activity assays were performed using purified PKR. Purified PKR (100 ng) was incubated for 30 min at 30 °C in a kinase buffer containing ³²P- γ -ATP. Samples were treated with poly rI:rC, buffer, single-stranded GL3 siRNA or decreasing concentrations of GL3 siRNA. Proteins were resolved by SDS-PAGE and analysed by autoradiography.

For PKR immunoprecipitation, 400 μ g of total cell lysates from mock- and RNA-transfected T98G cells were prepared at 90 min after transfection by lysis in buffer according to the previously described protocol²³. Lysates were then incubated with a monoclonal PKR antibody for 2 h at 4 °C. Equilibrated protein G-Sepharose beads (25 μ l) were added to each sample and incubated overnight at 4 °C. Immunoprecipitates were washed thoroughly and used in a kinase reaction, as described above. Proteins were then separated on a 10% SDS-PAGE gel and electroblotted to an Immobilon membrane (Millipore, Bedford, MA). PKR kinase activity was determined by autoradiography. Immunoblotting of total PKR levels was performed with a polyclonal PKR primary antibody followed by anti-rabbit secondary antibody (Amersham Biosciences) and chemiluminescence (Amersham Biosciences).

Semi-quantitative RT-PCR analysis. RNA from transfected T98G cells was isolated 48 h after transfection by the TRIZOL method, according to the manufacturer's instructions (Invitrogen). First-strand cDNA was prepared using Superscript II reverse transcriptase (Invitrogen) and amplified in an ABI Prism 7700 sequence detection system. Reactions contained Stat1-specific primers and the PE Applied Biosystems SYBR Green PCR Master Mix. The internal control was 18S rRNA. Each sample was run in duplicate from three biological repeats. Data were analysed according to the comparative threshold cycle (Ct) method, where the amount of target, normalized to an endogenous reference and relative to an experimental control, is given by $2^{-\Delta\Delta Ct}$. Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The ΔCt value is determined by subtracting the average reference Ct value from the average target Ct value. The $\Delta\Delta Ct$ value involves subtraction by the ΔCt experimental control value.

Microarray analysis. Total RNA was isolated with TRIZOL reagent (Invitrogen) from untransfected control, mock-transfected RCC1 cells, and cells transfected with 10, 25, 50 or 100 nM GAPDH siRNA 48 h after transfection. RNA was fluorescently labelled by direct incorporation in a cDNA synthesis reaction. Untransfected control RNA was labelled with Cy3-dUTP (green) and RNA from mock- or siRNA-transfected cells was labelled with Cy5-dUTP (red). Samples were hybridized overnight at 55 °C in Ambion slide hyb #3. Slides were washed with 2 \times SSC/0.1% SDS at 55 °C for 5 min and 0.2 \times SSC at room temperature for 10 min before scanning with an Axon GenePix 4000 scanner. Data were analysed with GenePix Pro 4.0 and GeneSpring 4.2.1. Low-intensity values were filtered out and data were normalized on the basis of overall intensity.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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- Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
- Hannon, G. J. RNA interference. *Nature* **418**, 244–251 (2002).
- Elbashir, *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
- Donze, O. & Picard, D. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res.* **30**, e46 (2002).
- Xia, H., Mao, Q., Paulson, H. L. & Davidson B. L. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnol.* **20**, 1006–1010 (2002).
- Hutvagner G. & Zamore P. D. A cellular function for the RNA-interference enzyme Dicer in the maturation of the *let-7* small temporal RNA. *Science* **297**, 2056–2060 (2001).
- Jacque, J., Triques, K. & Stevenson, M. Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438 (2002).
- Gitlin, L., Karelsky, S. & Andino, R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**, 430–434 (2002).
- Haque, S. J. & Williams, B. R. G. Signal transduction in the interferon system. *Semin. Oncol.* **25**, 14–22 (1998).
- Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264 (1998).
- Srivastava, S. P., Kumar, K. U. & Kaufman, R. J. Phosphorylation of eukaryotic translation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J. Biol. Chem.* **273**, 2416–2423 (1998).
- Kumar, A., Haque, J., Lacoste, J., Hiscott, J. & Williams, B. R. G. Double-stranded RNA-dependent protein kinase activates transcription factor NF- κ B by phosphorylating I κ B. *Proc. Natl Acad. Sci. USA* **91**, 6288–6292 (1994).
- Williams, B. R., Gilbert, C. S. & Kerr, I. M. The respective roles of the protein kinase and pppA2'p5'A2'p5 A-activated endonucleases in the inhibition of protein synthesis by double-stranded RNA in rabbit reticulocyte lysates. *Nucleic Acids Res.* **6**, 1335–1350 (1979).
- Williams, B. R. G. PKR; a sentinel kinase for cellular stress. *Oncogene* **18**, 6112–6120 (1999).
- Silverman, R. H. in *Ribonucleases: structure and function* (eds G. D'Alessio and J. F. Riordan) Ch. 16, 515–551 (Academic Press, St Louis, 1997).
- Pellegrini, S., John, J., Shearer, M., Kerr, I. M. & Stark, G. R. Use of a selectable marker regulated by α interferon to obtain mutations in the signaling pathway. *Mol. Cell Biol.* **9**, 4605–4612 (1989).
- Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A. & Driscoll, M. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genet.* **24**, 180–183 (2000).
- Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
- Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. & Seya, T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated gene silencing. *Biochem. Biophys. Res. Commun.* **293**, 1364–1369 (2002).
- Yang, *et al.* Deficient signaling in mice devoid of the double-stranded RNA dependent protein kinase, PKR. *EMBO J.* **14**, 6095–6106 (1995).
- Zhou, A. *et al.* Interferon action and apoptosis are defective in mice devoid of 2'-5'-oligoadenylate-dependent RNase L. *EMBO J.* **16**, 3297–3304 (1997).
- Zhou, A., Paranjape, J. M., Der, S. D., Williams, B. R. G. & Silverman, R. H. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology* **258**, 435–440 (1999).
- Goh, K. C., Haque, S. J. & Williams, B. R. G. p38 MAP kinase is required for Stat1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J.* **18**, 5601–5608 (1999).

ADDENDUM

In our recent paper entitled “Human p32 protein relieves a post-transcriptional block to HIV replication in murine cells” (*Nature Cell Biol.* **5**, 611–618 (2003)), we assigned hp32 to human chromosome 11 and argued that therefore it may represent the previously described Rev-complementing activity associated with human chromosome 11 (Shukla, R. R. *et al.* *J. Virol.* **70**, 9064–9068 (1996)). We would herewith like to correct the chromosomal assignment of hp32. The coding gene is actually on human chromosome 17 (17p13.3).

ERRATUM

In Parinello, S. *et al.* (*Nature Cell Biol.* **5**, 741–747 (2003)), the far right-hand column of Table 1 was mis-labelled “Control”, when it should have read “Irradiated”. This has now been corrected online.

Four extremely well conserved pseudogenes are located on human chromosomes 4, 11, 15 and 21 (Majumdar, M. *et al.* *DNA Cell Biol.* **10**, 727–735 (2002)). Indeed, NCI BLAST searches give the pseudogene on human chromosome 11 the best score, which is why we focused our initial attention on this gene. We note that this finding does not impact the functional data presented in our manuscript or the main conclusions. This finding does mean, however, that hp32 is not the Rev-complementing activity described previously.