dsRNAs containing multiple IU pairs are sufficient to suppress interferon induction and apoptosis

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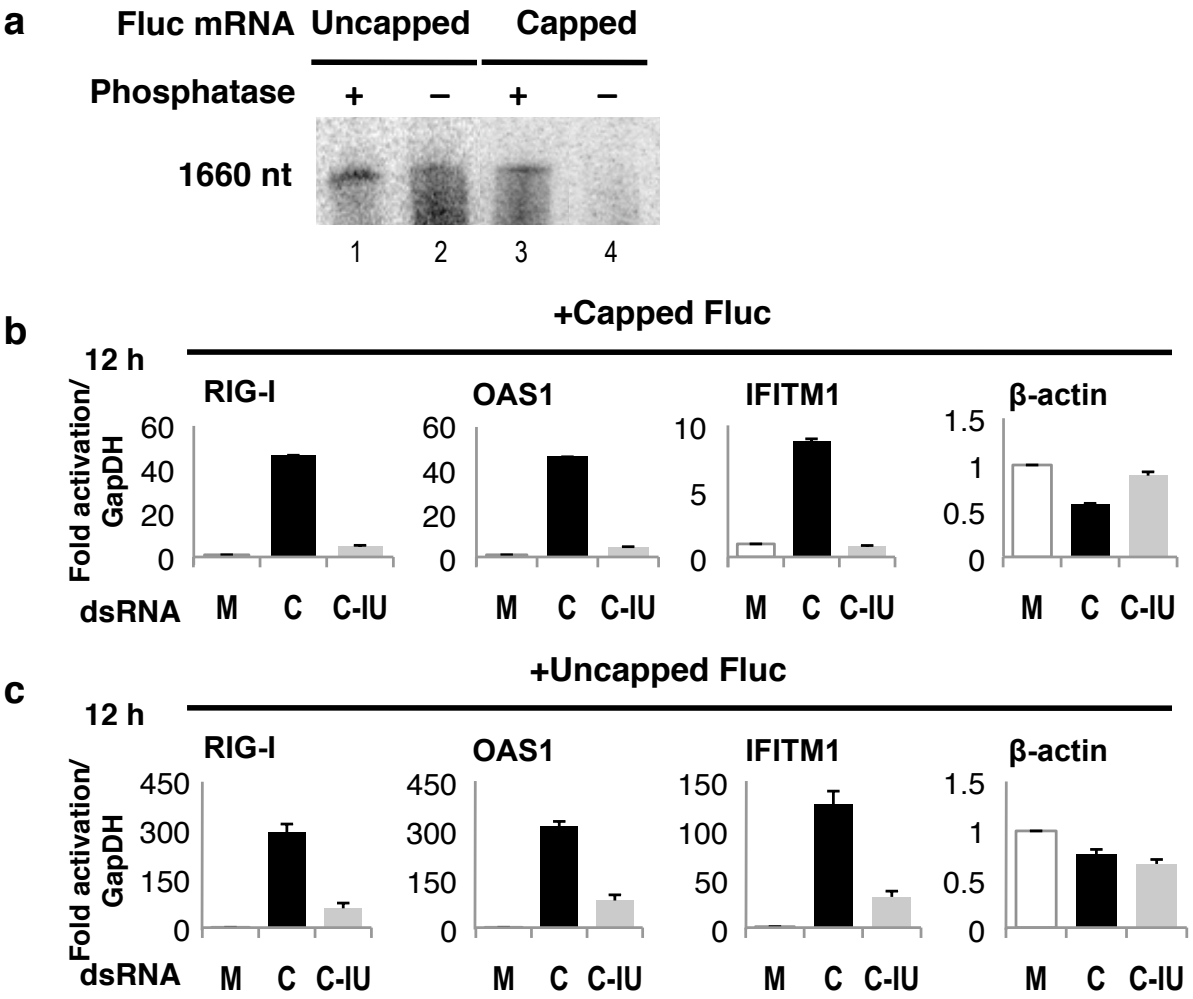
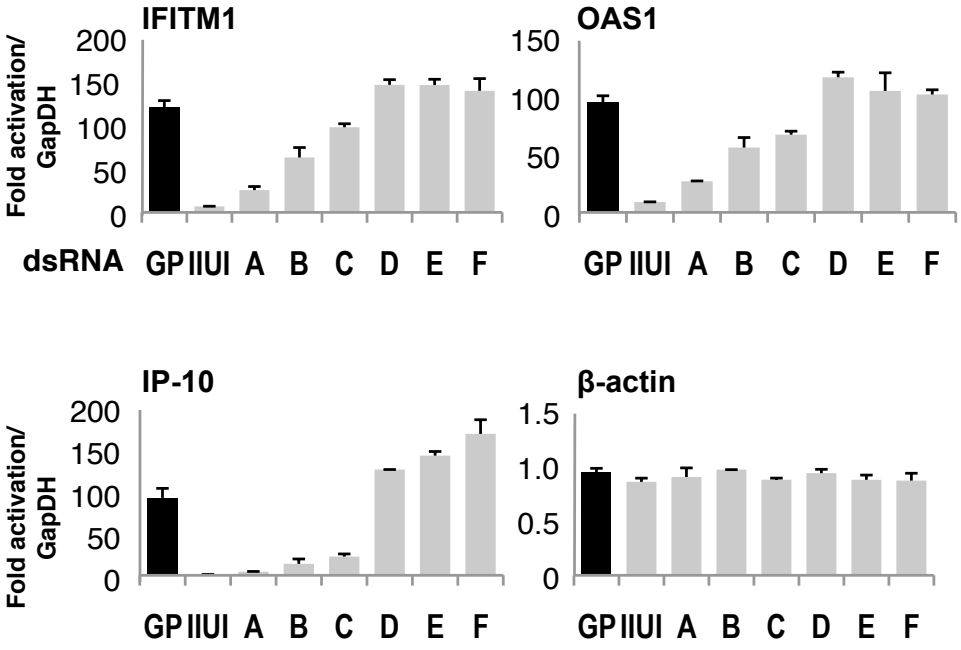


Supplementary Figure 1: (**a**) ***In vitro* transcription gives rise to incompletely capped *Fluc* mRNA**. Capped or uncapped *Fluc* mRNA were treated ±alkaline phosphatase, radiolabeled using γ^{32}P[ATP] (see Supplementary methods) and analyzed on a denaturing polyacrylamide gel. DNA markers (Lambda *Hin*dIII/ΦX174 *Hae*III) were used. (**b,c**) **IU-dsRNA suppresses induction of ISGs by both capped and uncapped *Fluc* mRNA**. HeLa cells were mock transfected (M) or co-transfected with 250 ng capped (**b**) or uncapped (**c**) *Fluc* mRNA and C or C-IU dsRNAs. RT/qPCR was used to quantify expression of ISGs (*RIG-I*, *OAS1*, *IFITM1*) or *β-actin* after 12h (n=4). Fold-change in mRNA levels were calculated relative to mock-transfected cells, and normalized to *GapDH*. Error bars are mean ± s.d.

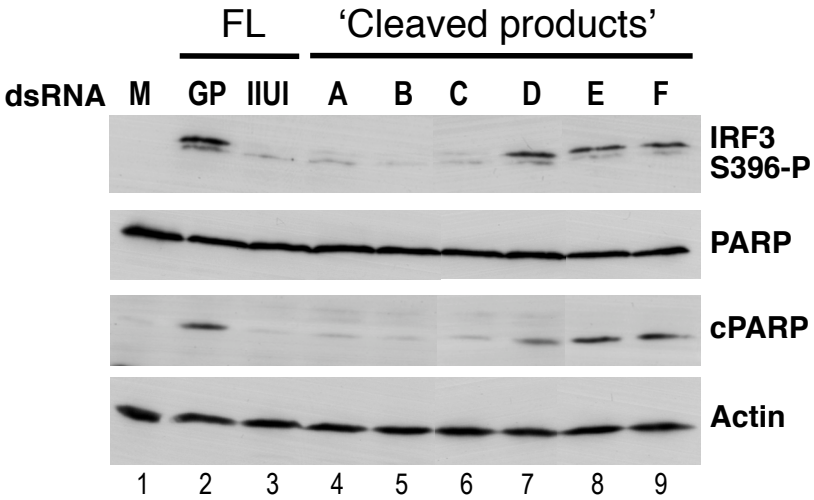
a

	dsRNA	Sequence
Full-length dsRNAs	GP	ACUGGACA**GGUG**CUCCGAGG UGACCUGU**CCAC**GAGGCUCC
	IIUI	ACUGGACA**IIUI**CUCCGAGG UGACCUGU**UUIU**GAGGCUCC
Cleaved dsRNAs	A	ACUGGACA**IIUI**CUCCGAGG **UIU**GAGGCUCC
	B	ACUGGACA**II** UGACCUGU**UUIU**GAGGCUCC
	C	ACUGGACA**IIUI**CUCCGAGG UGACCUGU**U**
	D	**UI**CUCCGAGG UGACCUGU**UUIU**GAGGCUCC
	E	ACUGGACA**II** UGACCUGU**U**
	F	**UI**CUCCGAGG **UIU**GAGGCUCC

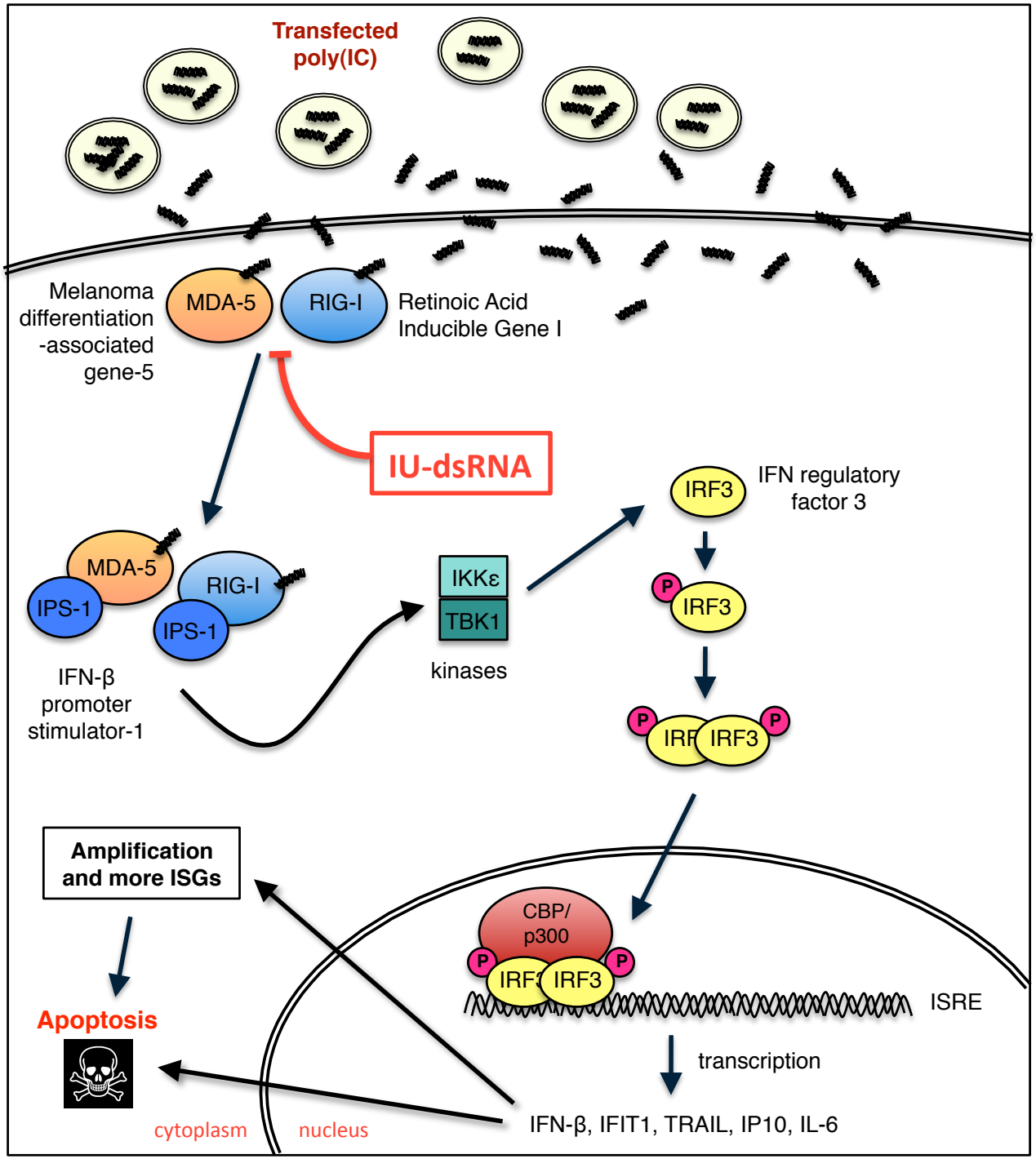
b



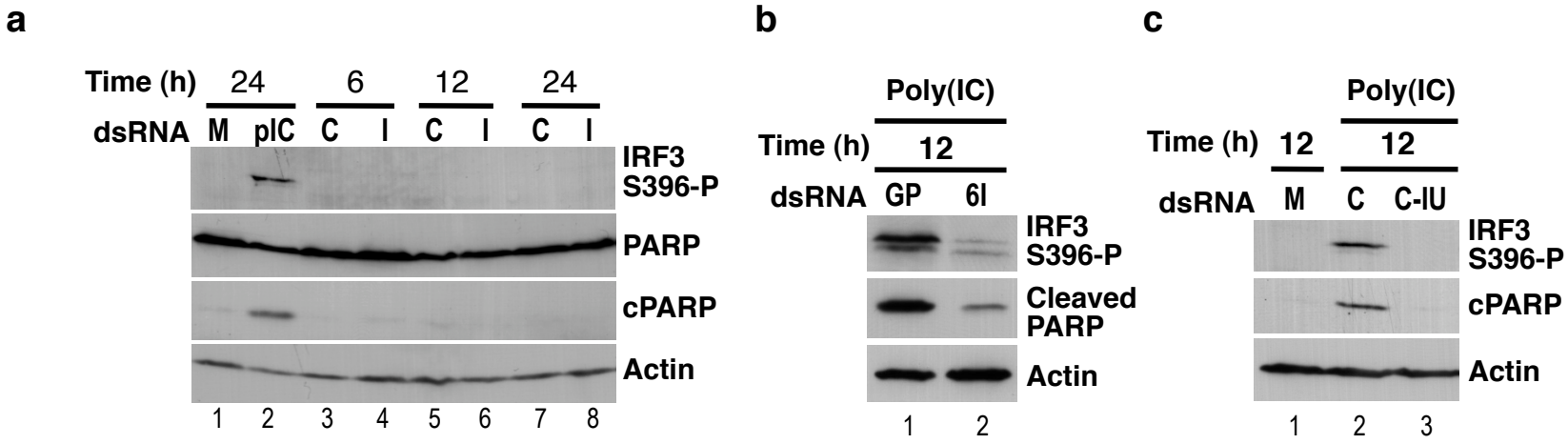
c



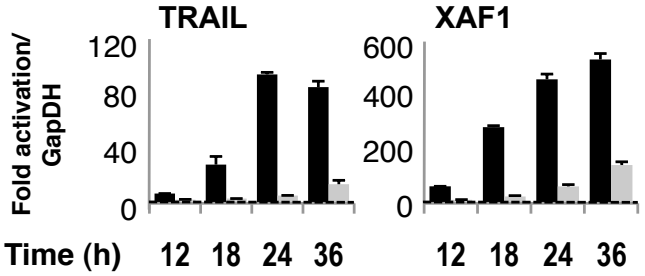
Supplementary Figure 2: ISGs are suppressed by 'cleaved' dsRNAs containing multiple inosines. (**a**) Full-length' dsRNAs correspond to GP and IIUI. Base pairs that differ between the pair of dsRNAs are in bold. 'Cleaved dsRNAs' correspond to duplexes A–F. These duplexes correspond to potential products arising from cleavage of IIUI dsRNA[14,15]. (**b**) HeLa cells were co-transfected with poly(IC) and either full-length (control dsRNA (GP) or IU-dsRNA (IIUI)) or 'cleaved' dsRNAs (Duplexes A–F). RT/qPCR was used to quantify expression of *β-actin* or ISGs (*IFITM1*, *OAS1*, *IP-10*)after 12h (n=4). Fold-change in mRNA was calculated relative to that at 6h with GP, and normalized to *GapDH*. Error bars are mean ± s.d. (**c**) Following co-transfection of HeLa cells with poly(IC) and either full-length or 'cleaved' dsRNAs, immunoblotting was used to analyze activation of IRF3 (IRF3 S396-P). Apoptosis was detected by analyzing PARP cleavage (cPARP). Uncleaved PARP was also detected (PARP). Actin was a loading control.



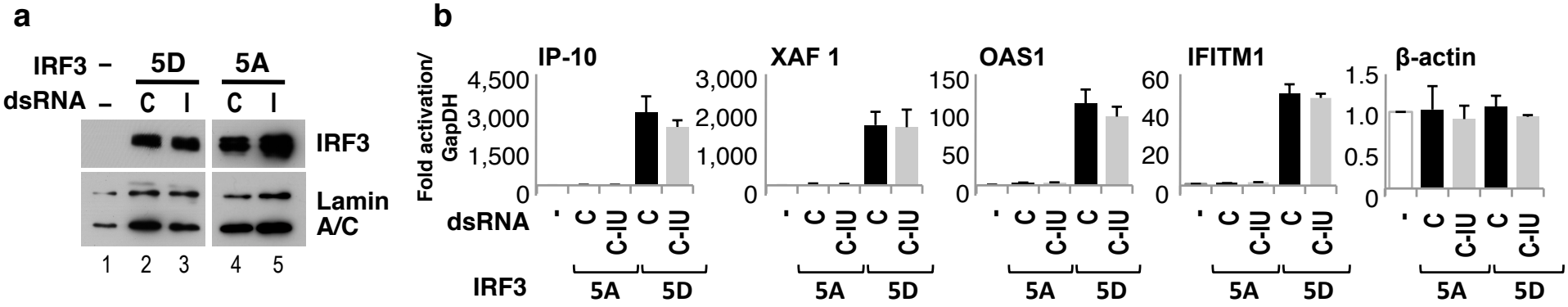
Supplementary Figure 3: IRF3 is activated by transfected poly(IC). A simplified view of IRF3 activation following transfection of cells with poly(IC)[14,15]. Transfected poly(IC) interacts with the cytosolic receptors MDA-5 or RIG-I, which triggers a cascade resulting in phosphorylation, dimerization and nuclear translocation of IRF3. Activated IRF3 interacts with CBP to initiate transcription of genes containing specific IRF3 binding sites (ISREs). We propose that IU-dsRNA suppresses this pathway by preventing activation of MDA-5 or RIG-I, as indicated.



Supplementary Figure 4: **poly(IC)-induced activation of IRF3 and apoptosis is inhibited by IU-dsRNA**. (**a**) HeLa cells were mock transfected (M), or transfected with poly(IC) (pIC) or C or C-IU dsRNAs, and lysates prepared after 6–24h. Immunoblotting was used to detect activation of IRF3 (IRF3 S396-P), and apoptosis (cPARP). Uncleaved PARP was also seen (PARP). Actin was a loading control. (**b**) HeLa cells were co-transfected with poly(IC) (pIC) and GP or 6I dsRNAs. Immunoblotting was used to detect apoptosis after 12h by analyzing PARP cleavage. IRF3 S396-P was used to detect IRF3 activation. Actin was a loading control. (**c**) Human placental choriocarcinoma (JEG-3) cells were co-transfected with poly(IC) and C or C-IU dsRNAs. Immunoblotting was used to detect apoptosis after 12h by analyzing PARP cleavage. IRF3 S396-P detected IRF3 activation. Actin was a loading control.



Supplementary Figure 5: poly(IC) induces expression of the ISGs *XAF1* and *TRAIL*. HeLa cells were co-transfected with poly(IC) and C or C-IU (I), and lysates prepared after 2–36h. RT/qPCR was used to quantify expression of *XAF1* and *TRAIL* after 12–36h (n=4). Fold-change in mRNA was relative to that at 2h with C, and normalized to *GapDH*. Error bars are mean ± s.d.



Supplementary Figure 6: C-IU dsRNA inhibits IRF3 activation. (**a**) Immunoblotting was used to verify equal expression of IRF3 5A and 5D with C or C-IU dsRNAs (see Supplementary methods). (**b**) Cells were mock transfected, or co-transfected with IRF3 5A or IRF3 5D, along with C or C-IU. RT/qPCR was used to quantify expression of ISGs (*IP10*, *XAF1*, *OAS1*, *IFITM1*) or *β-actin* after 12h (n=4). Fold-change in mRNA was relative to the mock, and normalized to *GapDH*. Error bars are mean ± s.d.

Supplementary Table 1: Interferon Stimulated Genes

Abbreviation	Full Name
IFITM1	Interferon-inducible trans-membrane protein 1 (9-27)[1]
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1[2]
IP-10	Chemokine (C-X-C motif) ligand 10[3]
IRF7	Interferon regulatory factor 7[4]
IRF9	Interferon regulatory factor 9[5]
MDA-5	Melanoma-differentiation-associated gene 5[6]
MX1	Interferon-induced myxovirus (influenza virus) resistance protein[7]
OAS1	2',5'-oligoadenylate synthetase 1[8]
PKR	Interferon-induced serine/threonine protein kinase[9]
STAT1	Signal transducer and activator of transcription 1[10]
TRAIL	TNF-related apoptosis inducing ligand[11]
XAF1	X-linked inhibitor of apoptosis -associated factor 1[12]

Supplementary Table 2: Genes identified using microarrays

Gene ID	Fold change	Description	Validated using RT/qPCR
IP-10	3.34	Chemokine (C-X-C motif) ligand 10	x
IFIT2	3.05	Interferon-induced protein with tetratricopeptide repeats 2	x
IFIT3	2.92	Interferon-induced protein with tetratricopeptide repeats 3	x
OASL	2.43	2'-5'-oligoadenylate synthetase-like	
ISG15	2.27	ISG15 ubiquitin-like modifier	
CFB	2.15	Complement factor B	x
IL6	2.05	Interleukin 6	x
IL8	2.04	Interleukin8	x
HERC5	2.02	Hect domain and RLD 5	x
IFITM1	1.99	Interferon induced transmembrane protein 1	x
IFI27	1.98	Interferon, alpha-inducible protein 27	x
IFI6	1.94	Interferon, alpha-inducible protein 6	x
CCL20	1.93	Chemokine (C-C motif) ligand 20	x
ZC3HAV1	1.91	Zinc finger CCCH-type, antiviral 1	x
IFIT1	1.90	Interferon-induced protein with tetratricopeptide repeats 1	x
CCL5	1.89	Chemokine (C-C motif) ligand 5	x
IRF1	1.89	Interferon regulatory factor1	x
OAS2	1.88	2',5'-oligoadenylate synthetase 2	x
UBE2L6	1.87	Ubiquitin-conjugating enzyme E2L 6	x
OAS1	1.85	2',5'-oligoadenylate synthetase 1	x
CXCL11	1.83	Chemokine (C-C motif) ligand 11	x
PRIC285	1.82	Peroxisomal proliferator-activated receptor A interacting complex 285	x
XAF1	1.80	XIAP associated factor 1	x
IL29	1.78	Interleukin 29 (interferon, lambda 1)	x
IFI44	1.73	Interferon-induced protein 44	x
GADD34	1.72	Growth arrest and DNA damage-inducible protein GADD34	x
RIG-I	1.71	Retinoic acid-inducible gene 1	
IRF7	1.71	Interferon regulatory factor 7	x
KLF6	1.70	Kruppel-like factor 6	x
NFKBIA	1.69	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	x
ZNFX1	1.69	Zinc finger, NFX1-type containing 1	x
GBP1	1.69	Guanylate binding protein 2, interferon-inducible	
TNFAIP3	1.69	Tumor necrosis factor, alpha-induced protein 3	
DDX58	1.66	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	
PLAUR	1.65	Plasminogen activator, urokinase receptor	
ISG20	1.65	Interferon stimulated exonuclease gene 20kDa	x
EFNA1	1.63	Ephrin-A1	
SLC25A28	1.62	Solute carrier family 25, member 28	
MDA-5	1.61	Melanoma-differentiation-associated gene 5	x
NFKBIZ	1.60	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	
OAS3	1.60	2',5'-oligoadenylate synthetase 3	
ATF3	1.60	Activating transcription factor 3	
CEBPD	1.59	CCAAT/enhancer binding protein (C/EBP), delta	
MX2	1.58	Myxovirus (influenza virus) resistance 2 (mouse)	
ZFP36	1.56	Zinc finger protein 36, C3H type, homolog (mouse)	
TRIM26	1.55	Tripartite motif-containing 26	
MT2A	1.55	Metallothionein 2A	
IFI35	1.55	Interferon-induced protein 35	
G1P3	1.54	Interferon inducible gene 6-16	

RPPH1	1.54	Ribonuclease P RNA component H1	
CCL2	1.54	Chemokine (C-C motif) ligand 2	
CENTA1	1.53	ArfGAP with dual PH domains	
SAMD9	1.53	Sterile alpha motif domain containing 9	
PARP14	1.53	Poly (ADP-ribose) polymerase family, member 14	
SLC15A3	1.52	Solute carrier family 15, member 3	
TRIM21	1.52	Tripartite motif-containing 21	
CITED4	1.52	CREB binding protein	
WARS	1.52	Tryptophanyl-tRNA synthetase	
TAP1	1.52	Transporter 1, ATP-binding cassette, sub-family B	
STAT1	1.51	Signal transducer and activator of transcription 1	**x**
NUAK2	1.50	NUAK family, SNF1-like kinase, 2	

HeLa cells were co-transfected with poly(IC) and C or C-IU dsRNAs, and RNA harvested after 12h. Microarrays were subsequently used to analyze gene expression in the presence of C or C-IU dsRNAs, where fold-change in mRNA with C was calculated relative to that with C-IU (n=3). Expression of 59 genes was ≥1.5-fold greater with C dsRNA than with C-IU after 12h. Genes validated using RT/qPCR are indicated (**x**).

Supplementary Table 3: Gene Ontology analyses

	NCBI: H. sapiens genes - REFLIST (25431)	C/C-IU array (59)	C/C-IU array (expected)	C/C-IU array (over/under)	C/C-IU array (*P* value)
Biological Process					
Interferon-mediated immunity	63	13	0.15	+	**6.99E–20**
Immunity and defense	1318	27	3.06	+	**5.72E–18**
Biological process unclassified	11321	10	26.26	–	**2.28E–04**
Nucleoside, nucleotide and nucleic acid metabolism	3343	21	7.76	+	**3.18E–04**
Cytokine and chemokine mediated signaling pathway	252	7	0.58	+	**4.04E–04**
Cytokine/chemokine mediated immunity	125	5	0.29	+	**1.67E–03**
Apoptosis	531	7	1.23	+	**7.07E–03**
Ligand-mediated signalling	421	7	0.98	+	**1.08E–02**
Macrophage-mediated immunity	140	4	0.32	+	**4.76E–02**
Pathway					
Inflammation mediated by chemokine and cytokine signaling pathway	315	7	0.73	+	**1.44E–03**
Molecular Function					
Nucleic acid binding	2850	21	6.61	+	**2.29E–05**
Chemokine	54	5	0.13	+	**3.16E–05**
Synthetase	96	5	0.22	+	**5.21E–04**
Nucleotidyltransferase	70	4	0.16	+	**3.73E–03**
Synthase and synthetase	213	5	0.49	+	**4.11E–03**
Molecular function unclassified	10934	12	25.37	–	**6.40E–03**
Helicase	173	5	0.4	+	**8.65E–03**
Defense/Immunity protein	369	5	0.86	+	**4.87E–02**

Cells were co-transfected with poly(IC) and either C or C-IU dsRNAs (n=3). Fold-change in gene expression at 12h with C, relative to C-IU, was determined using microarrays. Genes enriched ≥1.5-fold in the presence of C relative to C-IU were subject to gene ontology analyses using PANTHER[13]. Genes were classified according to Pathway, Molecular Function and Biological Process ($P \leq 0.05$).

Supplementary Table 4: Primer pairs used for qPCR

Primer	Forward Reverse
β-actin	5’-GTTGCGTTACACCCTTTC 5’-GCCATGCCAATCTCATCTT
eIF4G2	5’- CAGAGGCAGTCTATTGCAAGGAC 5’- ACGGCAACAACCATCAATTACAG
G3BP2	5’- GCCCTGCCATCCATGAAA 5’- GCTGAATGGCTCTTTGCTCTACT
GapDH	5’-TGCACCACCACCTGCTTAGC 5’-GGCATGGACTGTGGTCATGAG
MacF1	5’-ATGATCCCTGCCGAGCAC 5’-GAAGATGGTTTGGACCTTCG
RPS24	5’-TCCAATCTCCAGCTCACTTTT 5’-GCCTGTATGAGAAGAAAAAGACC
MRFAP1L1	5’-TCAAGACGTGGGATCAGAAT 5’-CTCAACCAAGTAGCGTAGTGT
PPP2R2D	5’-GGGTCCTATAACAACTTCTTCA 5’-ATCCCTCCGCGTGTCTCT
TBC1D22B	5’-CTCCCTCAACCGGACTAA 5’-AACCACCACTTGGGAACAC
CCL20	5’-TCCTGGCTGCTTTGATGT 5’-GTTGCTTGCTGCTTCTGATT
CFB	5’-AGTCTCTGTGGCATGGTTT 5’-TTGCTTGTGGTAATCGGTA
GADD34	5’-TCCTGCTACAGGTGTCTT 5’-GTCCTCTCCTGGCTGATA
HERC-5	5’-TCCTGAAAGTTGGAATGAAAGAG 5’-GGAGGAAGAGGACACTGAAA
IFI6	5’-TACCTGCTGCTCTTCACT 5’-TCCTTACCCGCATTCTCA
IFI27	5’-CTGTCATTGCGAGGTTCTACT 5’-CCTGGCATGGTTCTCTTCT
IFI44	5’-CAAGCTAGAGGAAGTCCAAAG 5’-CCACCGAGATGTCAGAAAG
IFIT1	5’-CCTCCTTGGGTTCGTCTA 5’-ACTCCAGGGCTTCATTCA
IFIT2	5’-CCTCGACTGGTCTACTATCA 5’-CGAAGCCCTGGACTCTTA
IFIT3	5’-AGGCCCACATGATGCTGAT 5’-GGAATGGTGGTTATATTGTGAACT

IFITM1	5’-AACCTTCACTCAACACTTCCTTC
	5’-TCCTCCTTGTGCATCTTC
IL-6	5’-TGGGCATTCCTTCTTCTG
	5’-GTGTCCTAACGCTCATAC
IL-8	5’-TGCACGGGAGAATATACAA
	5’-CAAACCCATTCAATTCCTGA
IL-29	5’-CGATGGGAACCTGTGTCT
	5’-GGGCTCAGCGCATAAATAAG
IP-10	5’-GCTCTACTGAGGTGCTATGTTC
	5’-CCCTTGGAAGATGGGAAAGGT
IRF7	5’-AGCGCCAACAGCCTCTAT
	5’-CAGCTTTCTGGAGTTCTCATTA
IRF9	5’-TCATCTGTAAGGGACTAGGAAA
	5’-AGGTCAGGGAAGAGGGAA
ISG20	5’-AACAGCCTGCTTGGACAC
	5’-CGGATTCTCTGGGAGATTTGAT
KLF6	5’-ATGCCGTTCTGCACCCTA
	5’-GCTATGCCGCTTCTTACA
MDA-5	5’-CCACAGTGGCAGAAGAAG
	5’-CGAGACCATAACGGATAACA
MX1	5’-TAGTCCGTCTCTGCTTATCC
	5’-ACTGCTCTCACAGCTTCCT
NFKBIA	5’-CCTGGTGTCACTCCTGTT
	5’-GTGAGCTGGTAGGGAGAATA
OAS1	5’-CTGACTCCTGGCCTTCTATG
	5’-GGCTGTGGAGAATGTTATCTATG
OAS2	5’-TTCCCTTGATGGTCCCTATTC
	5’-GGGTTTGCAGTCTTGATTGATT
PRIC285	5’-TCAGAACCAGGAAGGAAAC
	5’-CACAGACACCATCCAGAG
PKR	5’-TCGCAAGACTATGGAAAGG
	5’-AGCAGTGTCACATACATGAAGA
STAT1	5’-GATGGGTTTGACAAGGTTCT
	5’-TGCATGATAATATAGTTGTGGTA
TRAIL	5’-TCACACCTGTAATCCCAAC
	5’-GATCTCGTGATCTACCCA
UBE2L6	5’-TCCACGGATGAGTCACAAT
	5’-CAGGAACTGGCAATCTAACA
XAF1	5’-GCAGCCTATGACATTCTGAGGAG
	5’-AAGCTAACCACCGGCATTTCTC
ZC3HAV1	5’-TCAGACAAACATAGCTTCCAAA
	5’-TCTGCTGCACATACCACT
ZNFX1	5’-CTGCACTTTGGGATGGTA
	5’-GGATCACACTTGGGATAGG

The sequences of all primer pairs (forward and reverse) used in this study. Primers were designed using RealTimeDesign Software (http://www.biosearchtech.com/).

Supplementary Methods

Transfections with IRF3 5A and 5D. HeLa cells (2×10^5) were initially transfected with 100 ng IRF3 5A and 5D plasmids[14]. After 24h, cells were co-transfected with 120 pmol dsRNA (80 pmol specific and 40 pmol C) using Lipofectamine-2000 (Invitrogen). RNA was isolated using TRIzol® (Invitrogen), and lysates were prepared using RIPA buffer. Protein concentrations were determined by Bradford assays.

Radiolabeling *Fluc* mRNA. Capped or uncapped *Fluc* mRNA (1–10 pmol) was treated with alkaline phosphatase (CIP; NEB) for 1h at 37 °C (total volume 20 µl). RNA was extracted with phenol/chloroform, and precipitated with ethanol. After resuspending in water, the RNA was 5′ end-labeled in a total volume of 20 µl using γ^{32}P[ATP] and T4 polynucleotide kinase (NEB) (37 °C for 1h). Labeled RNAs were purified using a G-50 column and subsequently analyzed using denaturing polyacrylamide gels (6% (w/v)). DNA markers (Lambda *Hin*dIII/ΦX174 *Hae*III) were used to determine the size of the labeled mRNA.

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