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 γ³²P[ATP] (see Supplementary methods) and analyzed on a denaturing polyacrylamide gel. DNA markers (Lambda HindIII/ΦX174 HaeIII) 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.
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\textsuperscript{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)\textsuperscript{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 \(^1\) |
| IP-10        | Chemokine (C-X-C motif) ligand \(^3\) |
| IRF7         | Interferon regulatory factor \(^4\) |
| IRF9         | Interferon regulatory factor \(^5\) |
| MDA-5        | Melanoma-differentiation-associated gene \(^6\) |
| MX1          | Interferon-induced myxovirus (influenza virus) resistance protein \(^7\) |
| OAS1         | 2',5'-oligoadenylate synthetase \(^8\) |
| 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 \(^1\) \(^{12}\) |
| 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                                     |                         |
| IL6    | 2.05        | Interleukin 6                                          | x                       |
| IL8    | 2.04        | Interleukin8                                           | x                       |
| HERC5  | 2.02        | Hect domain and RLD 5                                  |                         |
| 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                     |                         |
| 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 factor                            | 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                       |
| PIRIC285| 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      | x                       |
| 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 | x                       |
| 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                         |                         |
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).

| Gene   | Fold Change | Description                                                                 |
|--------|-------------|-----------------------------------------------------------------------------|
| 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                          |
| NUAK2  | 1.50        | NUAK family, SNF1-like kinase, 2                                             |

x
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\textsuperscript{13}. Genes were classified according to Pathway, Molecular Function and Biological Process (P ≤ 0.05).

| Biological Process                                                                 | 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) |
|-----------------------------------------------------------------------------------|------------------------------------------|------------------|-------------------------|--------------------------|-----------------------|
| 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              |
## Supplementary Table 4: Primer pairs used for qPCR

| Primer     | Forward                      | Reverse                      |
|------------|------------------------------|------------------------------|
| β-actin    | 5'-GTTGCCTGTTACACCTTTTC     | 5'-GCCATGCCCAATCTGATTTT     |
| eIF4G2     | 5'-CAGAGGCAGTCTATTGCAAGGAC | 5'-ACCGCAACAACCATAAATGCAAG  |
| G3BP2      | 5'-GCCCTGCCATCCATGAAA       | 5'-GCTGAATGCGCTTTTGCTCTACT  |
| GapDH      | 5'-TGCAACCACCACCCTGTTAGC   | 5'-GGCATGACTGTGGCTCATGAG     |
| MacF1      | 5'-ATGATCCCTGGGAGCAGCA     | 5'-GAAGATGTTGGGACCTTCG       |
| RPS24      | 5'-TCCAATCTCCAGCTACTTTTT   | 5'-GCCCTGATGAGGAAGAAAGACC   |
| MRFAP1L1   | 5'-TCAAGAGCGATGGGATGAAAAAT | 5'-CTCAACCAAGTAGCGGCTAGTTGT |
| PPP2R2D    | 5'-GGGTCTCTTAAAACACTCTTCTCA| 5'-ATCCCTCCGGGCTGCTCTCT     |
| TBC1D22B   | 5'-CTCCCTCAACCGGACTTAAA   | 5'-AACCAACCACTTGGGACACAC     |
| CCL20      | 5'-TCCTGGGCTTGGCTTTGATG    | 5'-GTTGCTTGGCTCTTGATT       |
| CFB        | 5'-AGTCTCTGTTGGCATGTTTT    | 5'-TTGCTTGGTGGTAATGCGGTA    |
| GADD34     | 5'-TCCTGACTACAGGTGTCTTT    | 5'-GTCCCTCCCTGGCTGATA       |
| HERC-5     | 5'-TCCTGAAAGTTGGGAATGAAAGAG| 5'-GGAGGAAGAGGACACTGAAA     |
| IFI6       | 5'-TACCTGCTCTTCCTTCA       | 5'-TCCTTACCCGGATTCCTCA      |
| IFI27      | 5'-CTGCATATTGCGAGGTTCTACT  | 5'-CCTGGCATGTTCTCTTCTCT     |
| IFI44      | 5'-CAAGCTAGAGGAAGTCAAAG    | 5'-CCACGGAGATGTCAAGAAG      |
| IFIT1      | 5'-CTCTCTTGGGTTTGCTCTTA   | 5'-ACTCCAGGCTTCTACCTCA      |
| IFIT2      | 5'-CCTCGACTGTTCTACTATCA   | 5'-CGAAGGCTTGGAGTTCTTA      |
| IFIT3      | 5'-AGGCCCAACATGATGCTGAT    | 5'-GGAATGGGTGGTTATATTGTAAGCT|
| Gene   | 5' Primer | 3' Primer                      |
|--------|-----------|--------------------------------|
| IFITM1 | 5'-AACCTTCACTCAACACTTCTTC | 5'-TCCTCCTTGTGACATCTTC         |
| IL-6   | 5'-TGGCGATTCTCTTCTTCTG | 5'-GTGTCCTAAACGCTCATAC          |
| IL-8   | 5'-TGCCGGGAGAATATACAA | 5'-CAAAACCATTTCAATTCTCTGA       |
| IL-29  | 5'-CGATGGGAACCTGTGCTCTT | 5'-GGGTCAGGCGGATAAATAAG         |
| IP-10  | 5'-GCTCTACTGAGTGCTATTTCTT | 5'-CCCTTGGGAAAGATGGGAAGG         |
| IRF7   | 5'-AGGGCCACAGCCTCTTGA    | 5'-CAGCTTTCTGGAGATTTTGA         |
| IRF9   | 5'-TCATCTGTAAGGGAAGGAA   | 5'-AGGTCAAGGAAAGGGGAA           |
| ISG20  | 5'-AACAGCCTGCTGGGACAC   | 5'-CGGATTCTCTGGGAGATTGAT         |
| KLF6   | 5'-ATGCCCTTGACACCTTA     | 5'-GCTATGCCCTTCTTACA            |
| MDA-5  | 5'-CCACAGTGCGAAGAAAGA    | 5'-CGGAGCCATAACGGGAAACA         |
| MX1    | 5'-TAGCTCGTCTCTCTTATCC   | 5'-ACTGCTCTCACAGCTTAC           |
| NFKBIA | 5'-CCTGCTGTCCTGCTGTCTT  | 5'-GTGAGCTGTTAGGGAGAATA         |
| OAS1   | 5'-CTGACTCTGCCCTTCTATG   | 5'-GGGCTGTTAGAATGTTATCTATG      |
| OAS2   | 5'-TTCCTTTGTCAGTCTTCTATC | 5'-GGGTTTTGCACTTCTGGATT         |
| PRIC285| 5'-TCAGAACACAGGAGGAAAC   | 5'-CACAGACACACATCCAG            |
| PKR    | 5'-TGCAAGAAGCTATGGGAAGG  | 5'-AGCAGTGTCAAATGAAAGG          |
| STAT1  | 5'-GATGGGTTCGAGCGACTTCT | 5'-TGATGATAATATAGTTGCTGTA       |
| TRAIL  | 5'-TCACACCTGTAAATCCCAAAC | 5'-GATTCGCTGATCTACCCA          |
| UBE2L6 | 5'-TCCACGGATGATGTCACAAT  | 5'-CAGGAACTGGCAATCTAACA         |
| XAF1   | 5'-GCACGCTATGACATTTCTGAGG | 5'-AAGCTAAACCGCCATTTTCTC       |
| ZC3HAV1| 5'-TCAGACAAACATAGCTTCCAA | 5'-TCTGCTGACATACACT             |
| ZNFX1  | 5'-CTGCACTTTGGGATGGA    | 5'-GGATCACACTTTGGGATGGA         |
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 \((2 \times 10^5)\) 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\(^\circledR\) (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 \(^\circ\)C \((\text{total volume } 20\, \mu\text{l})\). 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 \(\mu\text{l}\) using \(\gamma^{32}\text{P}[\text{ATP}]\) and T4 polynucleotide kinase (NEB) \((37\, \text{C for } 1\)h\). Labeled RNAs were purified using a G-50 column and subsequently analyzed using denaturing polyacrylamide gels \((6\% \text{ (w/v))}\). DNA markers (Lambda HindIII/ΦX174 HaeIII) were used to determine the size of the labeled mRNA.
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