Supporting Information

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SI Methods

Solid-Phase Synthesis of 5′-Triphosphate RNA. Oligonucleotides were synthesized on the 1.0 μmol scale using standard phosphoramidite solid phase synthesis. The 5′-terminal dimethoxytrityl (DMT) group was removed after synthesis of the full-length RNA backbone. The synthesis column was dried under vacuum (<200 mtorr) for 2 h, and subsequently flushed by an argon stream and kept under argon atmosphere during further reaction steps. The CpG support was covered with 300 μL of 1:3 pyridine:dioxane (vol/vol). Subsequently, 25 μL of salicyl phosphorochloridite (0.5 M) in dioxane were added and vigorously shaken for 15 min at room temperature. After removal of the reagent, 200 μL of a solution of tributylammonium pyrophosphate (0.5 M) in dimethylformamide (DMF) were added followed quickly by 63 μL of tri-*n*-butylamine. The reaction mixture was then shaken for 15 min. After sedimentation of the CPG, the supernatant was removed and replaced by 500 μL of 1% iodine in tetrahydrofuran:pyridine:water (80:10:10, vol/vol/v), and the mixture shaken for 20 min. The supernatant was removed after CpG sedimentation, and the CpG was washed 3 times with 1 mL of acetonitrile, then 3 times with 1 mL of ethanol. After drying the CpG, the oligonucleotide was cleaved from the support, deprotected according to standard procedures for either DNA or RNA, and dried. The product was purified by HPLC using a RP18 column, and verified by MALDI-TOF MS.

A

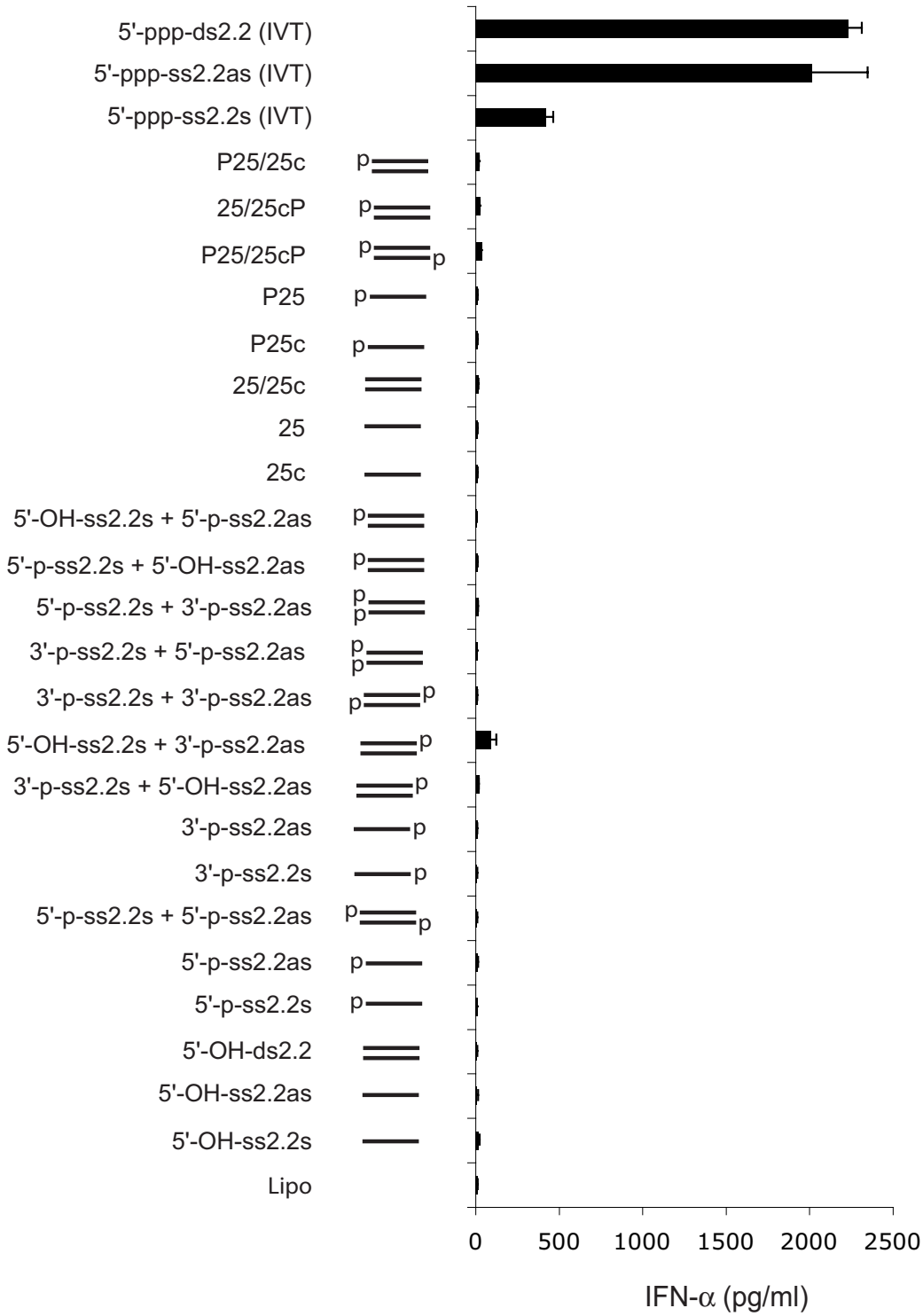

Fig. S1. Single-stranded (ss) or double-stranded (ds) 5′- and 3′-monophosphorylated RNAs do not induce significant amounts of IFN in primary human monocytes, but in peripheral blood mononuclear cells (PBMCs). (*A*) Human monocytes were transfected with the indicated RNAs. IFN-α levels in the cell culture supernatant were measured after 36 h by ELISA. All RNAs used in this experiment were chemically synthesized except those labeled as IVT (in vitro transcription). For sequences of the used RNAs, see Table S1. Schematic representations of the RNAs and their modifications are shown alongside the data graph.

B

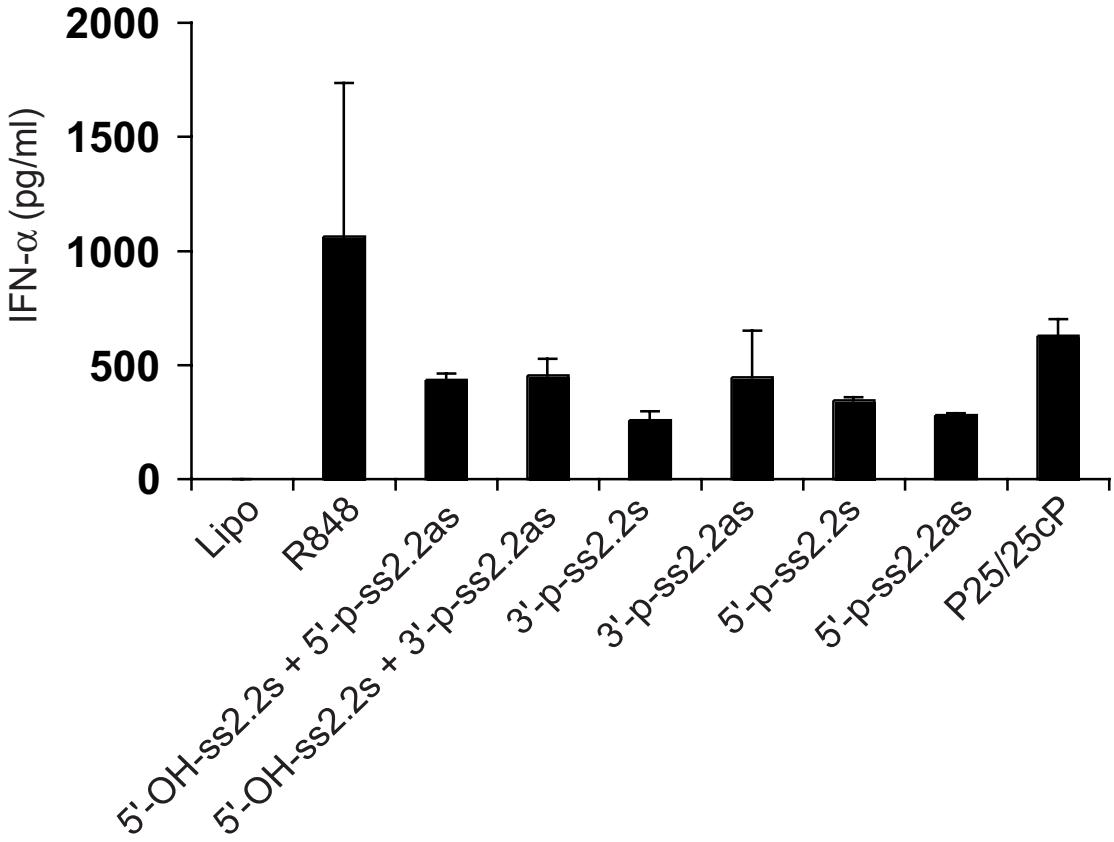

Fig. S1. (*B*) PBMCs containing plasmacytoid dendritic cells were stimulated with the indicated RNAs or with the toll-like receptor agonist R848 (10 μM); 36 h after stimulation, IFN-α secretion into the culture supernatant was assessed by ELISA.

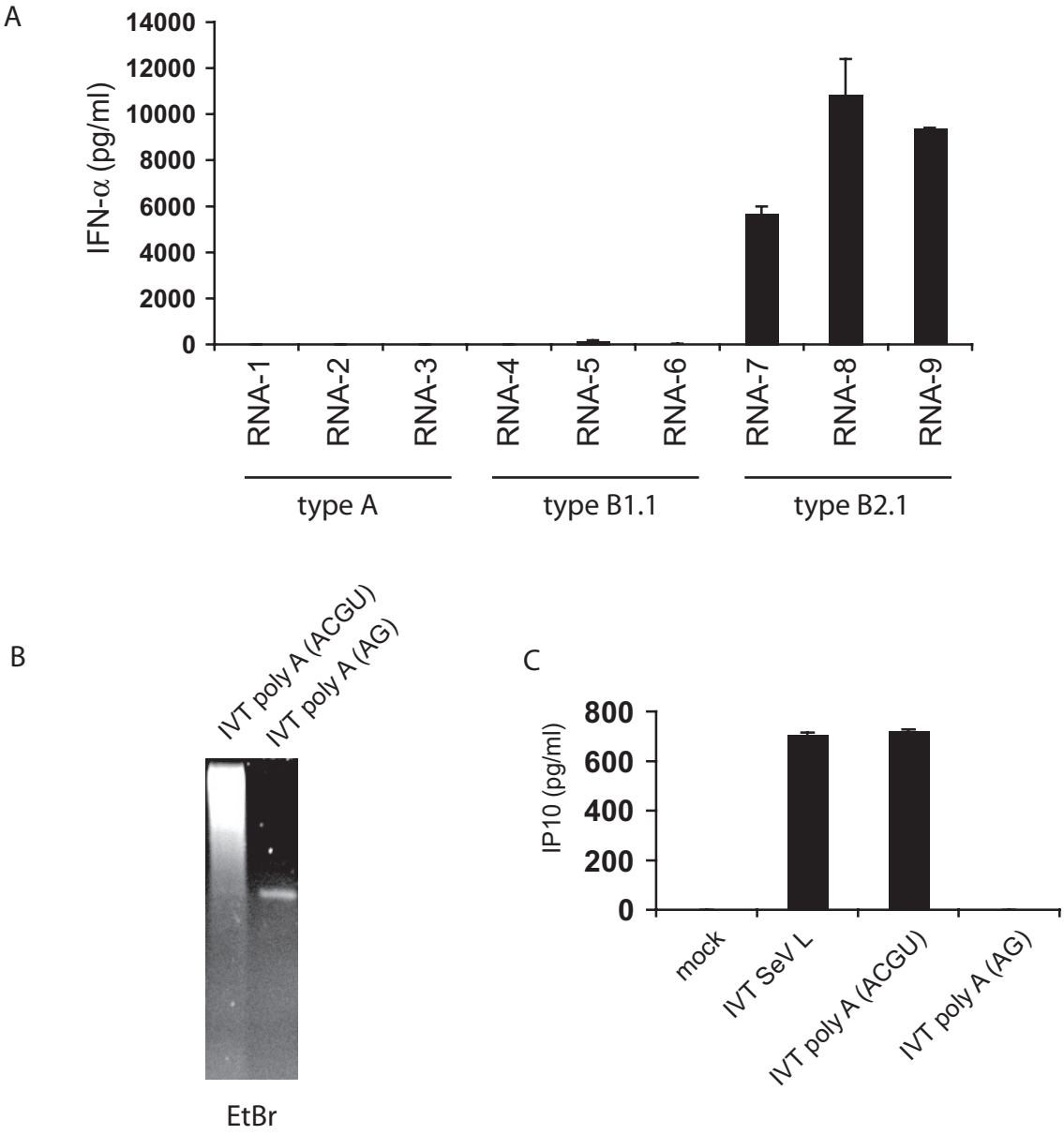

Fig. S2. In vitro transcribed RNAs prone to form self-complementary structures are potent IFN inducers. (*A*) Human monocytes were transfected with different in vitro transcribed RNAs derived from templates taken from Nacheva and Berzal-Herranz [Nacheva GA, Berzal-Herranz A (2003) Preventing nondesired RNA-primed RNA extension catalyzed by T7 RNA polymerase. *Eur J Biochem* 270:1458–1465]. These 17-nt RNAs fall into 3 groups: type A templates (RNA 1-3) are predicted to yield only expected product; type B1.1 templates (RNA 4-6) are expected to yield 2% extended products; type B2.1 templates (RNA 7-9) are expected to yield up to 90% extended products; 36 h after stimulation, IFN-α secretion into the culture supernatant was assessed by ELISA. (*B*) Polyacrylamide gel analysis of IVT poly A RNAs derived from a template as published by Saito et al. [Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M (2008) Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 454:523–527] generating a 100-mer poly A with three initial Gs. For IVT poly A (ACGU) all nucleotides were included in the synthesis reaction using the Megascript kit from Ambion; for IVT poly A (AG) only the nonpairing nucleotides A and G were included. (*C*) IVT RNAs from *B* and the Sendai virus leader sequence (SeVL) were transfected into human 1205Lu melanoma cells and tested for their ability to induce IP10 by ELSIA 18 hours after stimulation.

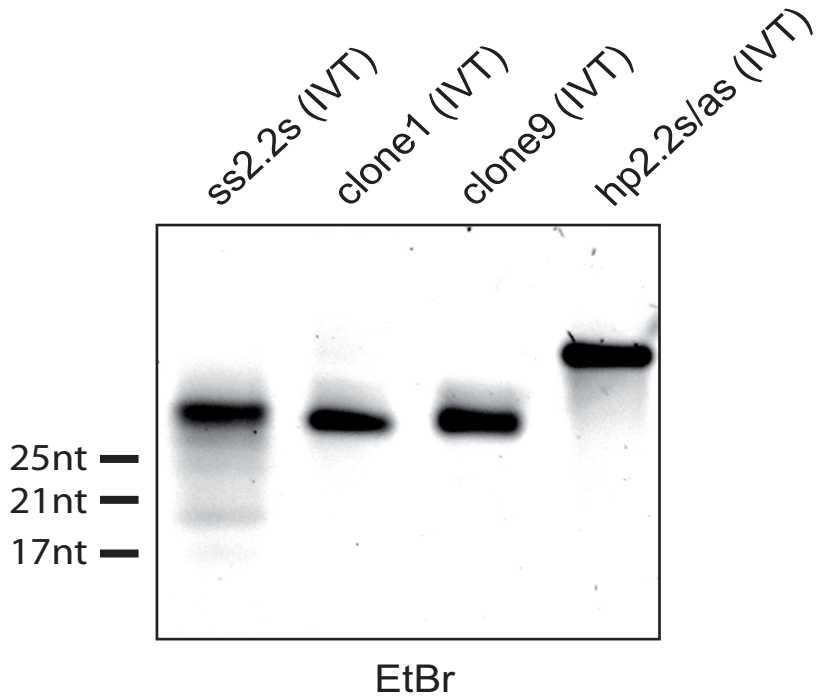

Fig. S3. Denaturing urea gel electrophoresis of cloned hairpin RNAs. The sequences identified by cloning and sequencing ss2.2 sense (s) IVT unexpected products and a designed hairpin sequence served as templates for in vitro transcription reactions. The products used for stimulation and ATPase assays were analyzed side by side with ss2.2s IVT on denaturing urea gels.

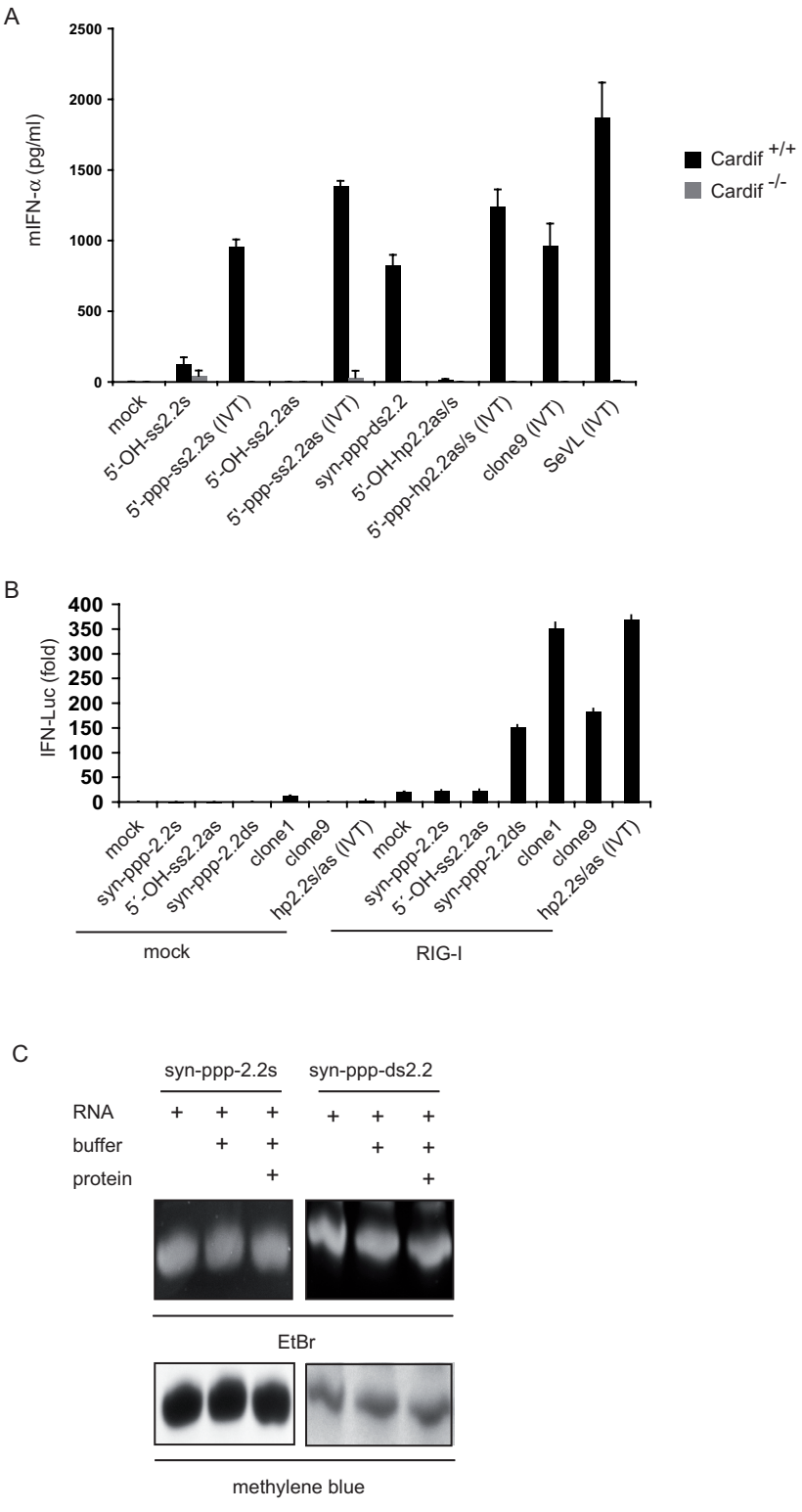

Fig. S4. Short synthetic or in vitro transcribed RNAs are recognized via the RIG-I pathway. (*A*) Different 2.2-based RNAs and Sendai virus leader RNA (SeVL) were transfected into murine bone-marrow dendritic cells (bmDCs) derived from wild-type mice or mice homozygously deleted for the Cardif gene. IFN levels in the supernatant were determined 36 h after stimulation by ELISA. (*B*) HEK 293 cells were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase, and RIG-I or the empty vector (mock), and subjected to luciferase assay. Cells were treated with chemically synthesized RNAs (5′-OH or syn-ppp) and IVT RNAs. (*C*) syn-ppp-2.2s RNA or syn-ppp-ds2.2 RNA was incubated with ATPase assay buffer in the presence or absence of recombinant RIG-I. RNAs were analyzed for signs of degradation on polyacrylamide gels stained with ethidium bromide or methylene blue.

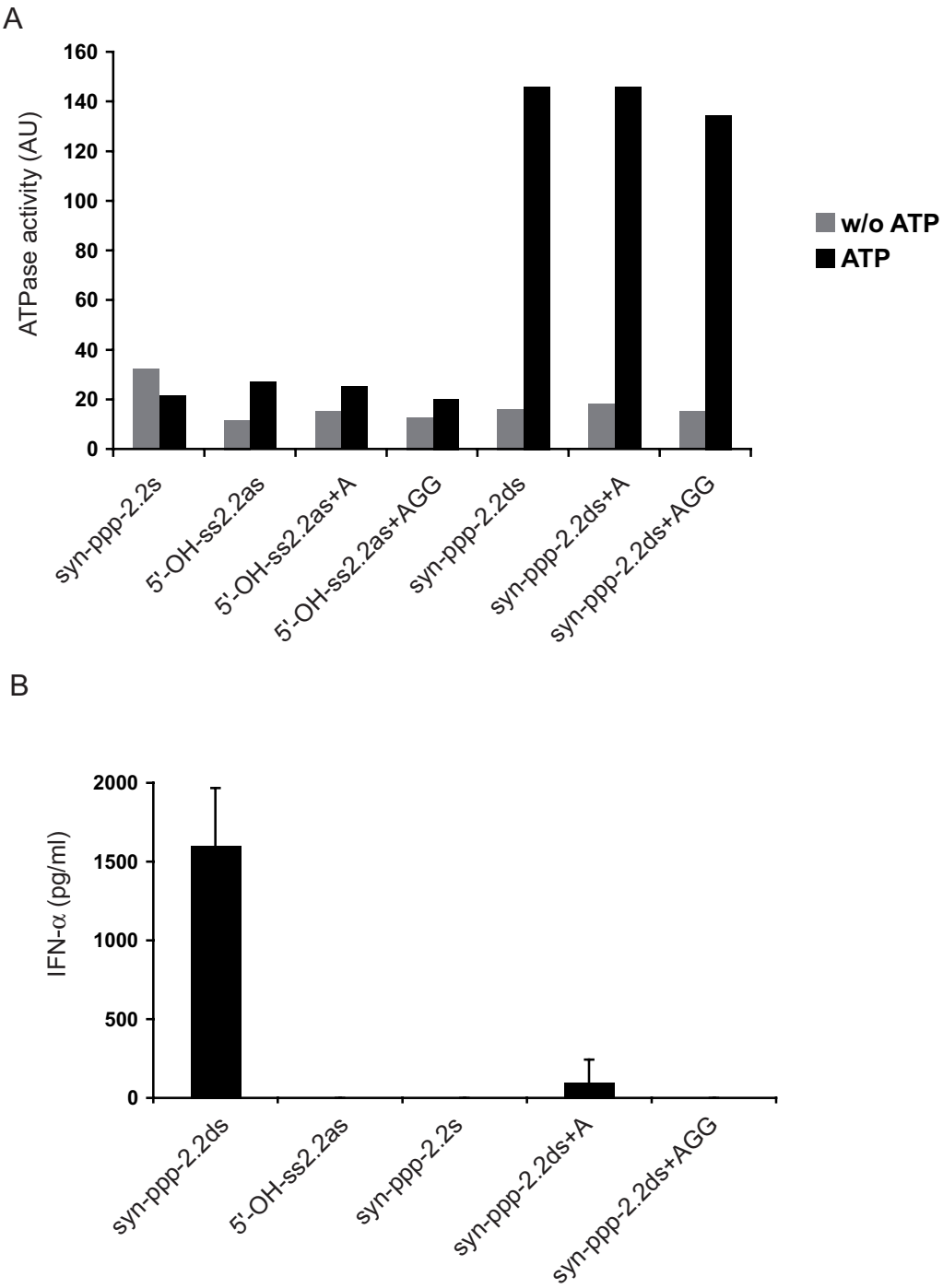

Fig. S5. Base-paired synthetic RNAs bearing a short 3′-extension adjacent to the 5′-triphosphate do not induce IFN, but still activate ATPase activity of RIG-I. (*A*) syn-ppp-2.2s RNA was tested for induction of RIG-I ATPase activity either alone or annealed to different RNA oligos producing a 1-nt (+A) or 3-nt (+AGG) 3′-overhang in proximity to the 5′-triphosphate end. (*B*) Human monocytes were transfected with the RNAs described in *A* and examined for IFN production by ELISA of the cell culture supernatant.

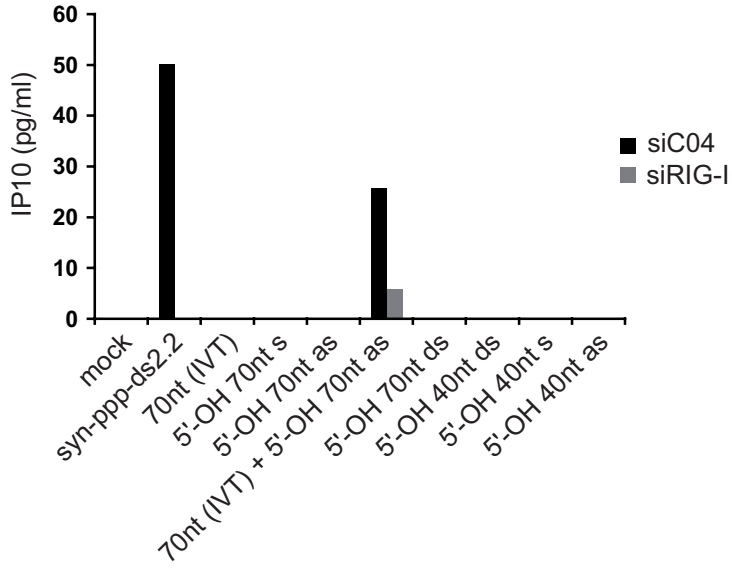

Fig. S6. Long RNAs require a 5′-triphosphate end to trigger RIG-I dependent signaling. Based-paired short synthetic 5′-triphosphate RNA and long unstructured synthetic (5′-OH 70/40-nt s and 5′-OH 70/40-nt as) or IVT (70 nt) RNAs were transfected into 1205Lu human melanoma cells that were treated with either control (siCO4) or RIG-I (siRIG-I) siRNAs either alone or as dsRNAs (70-nt ds, 40-nt ds, and 70-nt IVT + 5′-OH 70-nt as). Notably, to avoid the production of extended RNA-products in case of the 70-nt RNA, the DNA template was designed to code for only 1 uridine nucleotide at the end of the sequence that could be used as a terminator by addition of 3′-deoxy-UTP to the IVT reaction. This method facilitated the production of defined 70-nt 5′-triphosphate RNA that does not contain extended by-products. Cytokine production in the supernatant was measured by ELISA.

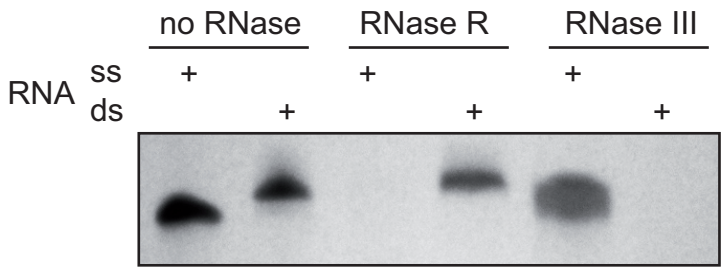

Fig. S7. RNase III and RNase R specifically digest dsRNA and ssRNA. Synthetic ss- or dsRNA was incubated with equal activities of RNase III and RNase R similar to the viral RNA preparations in Fig. 3*F*. RNAs were analyzed for degradation by denaturing urea PAGE and staining with methylene blue.

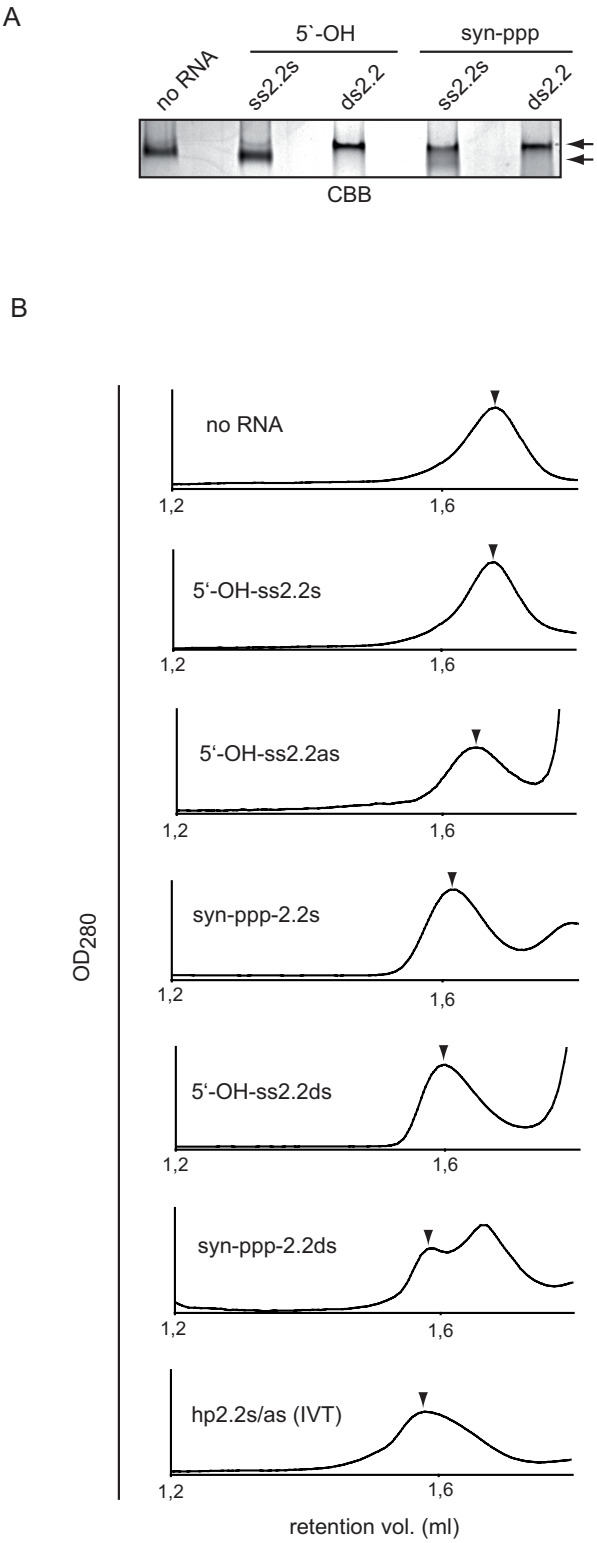

Fig. S8. Both features of the PAMP can mediate binding to RIG-I and cooperate for the structural transition that accompanies RIG-I activation. (*A*) Recombinant RIG-I protein was incubated with the indicated RNA oligonucleotides or without RNA and loaded onto a native polyacrylamide gel. Protein-RNA complex formation was examined by gel-shift after staining with Coomassie brilliant blue (CBB). (*B*) The absorbance traces recorded at 280-nm wavelength of the gel-filtration experiments described in Fig. 4*A* are shown. Peaks of the protein-RNA complex elution at the respective volumes are indicated by arrowheads.

Table S1. RNA sequences used for stimulation

RNA type	Modification	Name	Sequence
syn	5′-OH	2.2s	GCAUGCGACCUCUGUUUGA
		2.2as	UCAAACAGAGGUCGCAUGC
		2.2as + A	UCAAACAGAGGUCGCAUGCA
		2.2as + AGG	UCAAACAGAGGUCGCAUGCAGG
		2.2 5-mer	UCAAA
		2.2 10-mer	UCAAACAGAG
		2.2 15-mer	UCAAACAGAGGUCGC
		2.2 18-mer	UCAAACAGAGGUCGCAUG
		2.2 5-mer′	CAUGC
		2.2 10-mer′	GGUCGCAUGC
		2.2 15-mer′	ACAGAGGUCGCAUGC
		2.2as/s hp	GUCAAACAGAGGUCGCAUGCGGCAUGCGACCUCUGUUUGA
		″25c″	CACUUUCACUUCUCCCUUUCAGUUU
		″25″	AAACUGAAAGGGAGAAGUGAAAGUG
		40-nt s	GAACUAUGAAGAGAAGACCAGACGCGUAAUUGUAAAGAAC
		40-nt as	GUUCUUUACAAUUACGCGUCUGGUCUUCUCUUCAUAGUUC
		70-nt s	GAACCGAAGAGACACGACAAGACGCCGAGACACACAAGACACACGCAGAGACCACACCCGAACACACGCU
		70-nt as	AGCGUGUGUUCGGGUGUGGUCUCUGCGUGUGUCUUGUGUGUCUCGGCGUCUUGUCGUGUCUCUUCGGUUC
	3′-p	2.2s	GCAUGCGACCUCUGUUUGA
		2.2as	GUCAAACAGAGGUCGCAUGC
	5′-p	2.2s	GCAUGCGACCUCUGUUUGA
		2.2as	UCAAACAGAGGUCGCAUGC
		″P25c″	CACUUUCACUUCUCCCUUUCAGUUU
		″P25″	AAACUGAAAGGGAGAAGUGAAAGUG
IVT	5′-ppp	2.2s	GGCAUGCGACCUCUGUUUGA
		2.2as	GUCAAACAGAGGUCGCAUGC
		2.2s/as hp	GGCAUGCGACCUCUGUUUGAUCAAACAGAGGUCGCAUGCC
		2.2as/s hp	GUCAAACAGAGGUCGCAUGCGGCAUGCGACCUCUGUUUGAC
		RNA 1	GCGUGACAGCGCUGUUU
		RNA 2	GCGUGACCGCGCUGUUU
		RNA 3	GCGUGACGGCGCUGUUU
		RNA 4	GCGUGACCGGCCUGUUU
		RNA 5	GCGUGACUGGCCUGUUU
		RNA 6	GCGUGACUGGUCUGUUU
		RNA 7	GCGUGACGGAACUGUUU
		RNA 8	GCGUGACUGAACUGUUU
		RNA 9	GCGUGACCGAACUGUUU
		Clone1	GGCAUGCGACCUCUGUUUGAUCAGAGGU
		Clone9	GGCAUGCGACCUCUGUUUGAGGUCGCAUG
		70-nt s	GAACCGAAGAGACACGACAAGACGCCGAGACACACAAGACACACGCAGAGACCACACCCGAACACACGCU
		SeVL	ACCAAACAAGAGAAGAAACAUGUAUGGAAUAUAUAAUGAAGUUAGACAGGAA
syn3p	5′-ppp	2.2s	GCAUGCGACCUCUGUUUGA
		2.2as/s hp	GUCAAACAGAGGUCGCAUGCGGCAUGCGACCUCUGUUUGAC

Table S2. Templates and primers for in vitro transcription

Name	Sequence
Ambion T7 promotor primer	TAATACGACTCACTATA
Template 2.2 s	TCAAACAGAGGTCGCATGCCTATAGTGAGTCGTA
Template 2.2as	GCATGCGACCTCTGTTTGACTATAGTGAGTCGTA
Template 2.2 s/as hp	GGCATGCGACCTCTGTTTGATCAAACAGAGGTCGCATGCCTATAGTGAGTCGTATTAGAATTCGC
Template 2.2as/s hp	GTCAAACAGAGGTCGCATGCCGCATGCGACCTCTGTTTGACTATATAGTGAGTCGTATTAGAATTCGC
Template clone1	ACCTCTGATCAAACAGAGGTCGCATGCCTATAGTGAGTCGTATTAGAATTCGC
Template clone9	CATGCGACCTCAAACAGAGGTCGCATGCCTATAGTGAGTCGTATTAGAATTCGC
Template 70-nt s	AAAGCGTGTGTTCGGGTGTGGTCTCTGCGTGTGTCTTGTGTGTCTCGGCGTCTTGTCGTGTCTCTTCGGTTCTATAG TGAGTCGTATTAGAATTCGC
Template SeVL	TTCCTGTCTAACTTCATTATATATTCCATACATGTTTCTTCTCTTGTTTGGTTATAGTGAGTCGTATTAGAATTCGC

Table S3. Small interfering RNA sequences

Target protein	Sequence
RIG-I	ATCACGGATTAGCGACAAA
MDA-5	GTATCGTGTTATTGGATTA
PKR	GAAGGCAGTTAGTCCTTTA
Cardif	CCACCTTGATGCCTGTGAA
CO4 (control siRNA)	GCGCAUUCCAGCUUACGUA

The sequence depicted is the 19-nt portion in the s strand of the targeted mRNA. 3′ overhangs were dTdT.