**Supplementary information legends**

**Supplementary Fig. S1.** RNase A digested RNA is recognized by IPS-1 and RIG‑I. (**A**) Wildtype (WT) and IPS-1-deficient ([1](#_ENREF_1)) (IPS-1-/-) bone marrow-derived macrophages were stimulated with 0.6 µg/ml RNA40 (TLR7-L), 0.4 µg/ml 5’ppp-RNA (5’ppp-RL) and undigested or RNase A digested HEK293 RNA. IFN-α in the supernatant was measured by ELISA. Three combined independent experiments are shown each in biological duplicates (six measurements per data point ± S.D.), \*\* p < 0.01, \* p < 0.05. (**B**) RLR-deficient Huh7.5 cells stably transfected with RIG‑I or MDA5 were stimulated with 1 µg/ml poly(I:C), 5’ppp-RNA (5’ppp-RL) and undigested or RNase A digested HEK293 RNA each. ISG56 induction was determined after 6 h by RT-PCR and plotted as % induction setting poly(I:C) (pIC) induced upregulation by RIG-I or MDA5 at 100 %, respectively. Three combined independent experiments are shown each in biological duplicates (six measurements per data point ± S.D.), \*\* p < 0.01. (**C**) Verification of MDA5 and RIG-I expression by western blotting. One individual experiment is shown.

**Supplementary Fig. S2**. Quality control of in vitro transcribed ITS2 fragment and RNase A generated eRL.(**A**) An ITS2 fragment (45S rRNA, nt 7143-7500) was in vitro transcribed and RNase A digested. The eRL was PAGE purified and subjected to NGS. Graph shows most common sequences detected by NGS. (**B**) These sequences were identified as eRL and a part of the complementary strand mapped to NR\_046235:7143-7500 Homo sapiens RNA, 45S pre-ribosomal 5 (RNA45S5).

Supplementary Fig. S3. eRL binding to RIG-I and fragment analysis. (A) HEK 293 ΔRIG-I were transfected with flag-tagged RIG I or ΔMx and stimulated with 250 ng VSV-RNA or eRL 24 h later. Immunoprecipitation of flag-tagged proteins was performed 6 h post stimulation. Western Blotting and subsequent antibody staining using anti-flag and anti-tubulin antibodies were used to verify pulldown efficiency. Western Blots from three individual experiments are shown. (B) HEK293-RIG-I-IFN-ß reporter cells were stimulated with 2 µg/ml of mock-treated, dephosphorylated (by calf intestine phosphatase or shrimp alkaline phosphatase) or RNase III-treated eRL for 16 h followed by luciferase activity measurement. Integrity of the eRL as well as digestion products were examined by denaturing PAGE and SYBRGold staining (upper panels). Fold IFN-ß induction is shown for one individual experiment (middle panel) and a diagram combining three independent experiments (lower panel) with data adjusted to % of poly(I:C) pIC induced IFN-β activation (nine measurements per data point ± S.D.), \*\*\* p < 0.001. (C) Denaturing PAGE analysis of untreated and 10 mM HCl-treated (30 min, 37°C) eRL.

Supplementary Fig. S4. Sequence and structure of ITS2 IVT constructs, predicted RNA structure and released RNA fragment. (A) DNA-sequence of unmodified ITS2 (IVT-ITS2) or ITS2 construct with disrupted G-quadruplex (IVT-ITS2\_ΔG) for *in vitro* transcription. T7 promotor sequence is underlined. (B) Sequence alignment of IVT-ITS2 and IVT-ITS2\_ΔG using clustal omega at <https://www.ebi.ac.uk/Tools/msa/clustalo/>. (C) RNA structure of IVT-ITS2 and IVT-ITS2\_ΔG predicted by RNA fold web server at <http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>. (D) Sequence alignment of eRL and predicted eRL\_ΔG after RNase A digestion.

Supplementary References

1. Meylan E*, et al.* (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437(7062):1167-1172.





