Supplementary information

Mini viral RNAs act as innate immune agonists during influenza virus infection

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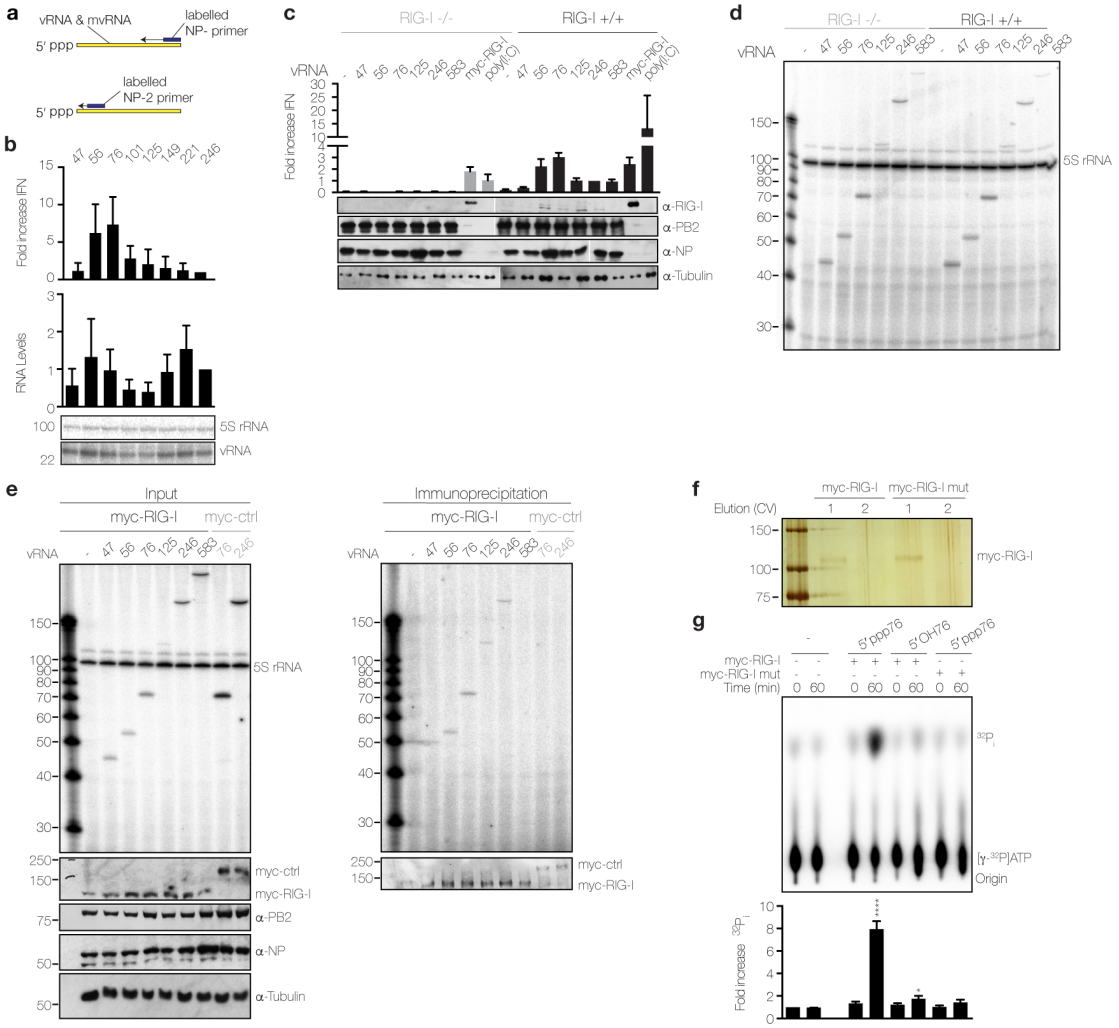

Figure S1 | Induction of IFN-β promoter activity by replication of mvRNAs and binding of mvRNAs to RIG-I. (**a**) Schematic of primer extension analysis using primers binding at the 3′ end of vRNAs (NP-) or to an internal sequence (NP-2). (**b**) IFN-β promoter activity induced by the replication of segment 5-based vRNA templates before normalisation to RNA levels measured by primer extension with primer NP-2. Normalised data is shown in Fig. 1c. (**c**) INF-β promoter activity induced by the replication of segment 5-based vRNA templates in wild-type (RIG-I +/+) or RIG knockout (RIG-I -/-) HEK 293T cells expressing luciferase from an IFN-β promoter. (**d**) Primer extension performed with primer NP- on samples from Fig. S1c. (**e**) Detection of segment 5-derived vRNAs with the NP- primer before and after myc-RIG-I or myc-EGF immunoprecipitation. Normalised data is shown in Fig. 1e. (**f**) SDS-PAGE and silverstain analysis of myc-RIG-I elutions per column volume (CV). (**g**) ATPase assay of wildtype myc-RIG-I or mutant (mut) in the presence of a triphosphorylated 76 nt mvRNA (5′ ppp76) or a dephosphorylated mvRNA (5′ OH76). In all panels, error bars indicate standard deviation. P-values were determined using ANOVA relative to the 0 min buffer control and are indicated as * $P \leq 0.05$ and **** $P \leq 0.0001$.

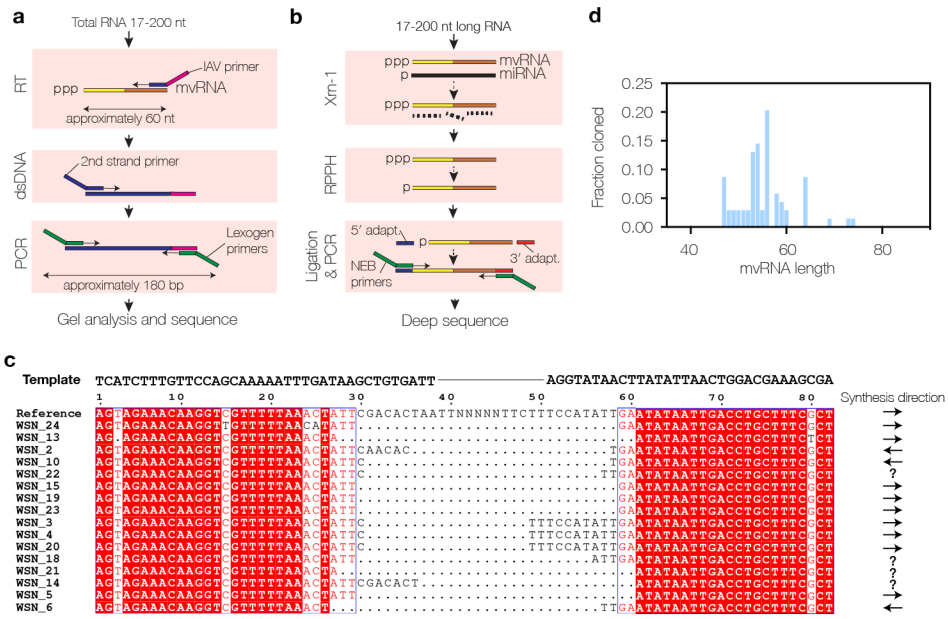

Figure S2 | Deep sequencing schematic and alignment of cloned mvRNAs. (**a**) Schematic of deep sequencing protocol using universal primers for the influenza A virus (IAV) promoter. (**b**) Deep sequencing via adapter ligation was performed on the total small RNA fraction (RNAs 17-200 nt in length) after treatment with XRN-1 and RppH. (**c**) Alignment and (**d**) size distribution of segment 1-derived mvRNAs generated during infection with influenza A/WSN/33 (H1N1) virus in the presence of overexpressed viral polymerase. Sequences were produced after gel extraction and Sanger sequencing. The possible direction of synthesis (vRNA to mcRNA or cRNA to mvRNA) via the intramolecular copy-choice model is indicated. Unknown directions of synthesis are indicated with '?'.

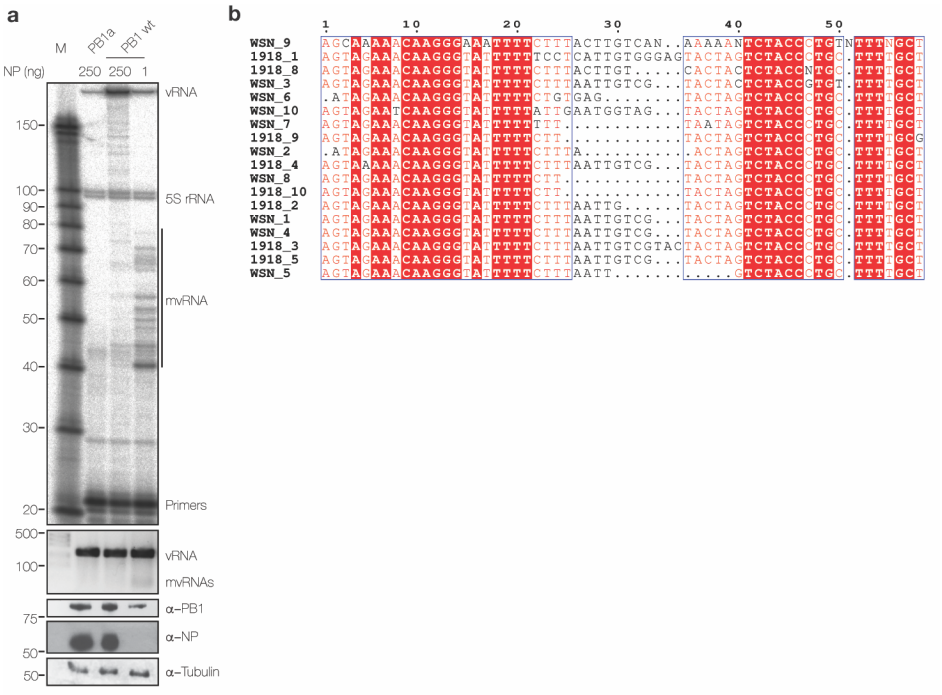

Figure S3 | **mvRNAs synthesis in replication assays with WSN polymerase in the presence of limiting NP and alignment of cloned mvRNAs.** (**a**) Analysis of steady state RNA levels in replication assays with an active site mutant WSN PB1 (PB1a) or WSN PB1 wild-type (PB1 wt) and a 246-nt vRNA that was derived from segment 5 of the influenza A virus genome, in the presence of limiting NP. The NP- primer was used for primer-extension analysis. (**b**) Alignment of mvRNAs generated in replication assays with the WSN (from panel a) and BM18 polymerases (from Fig. 3a) from segment 5-derived 246 nt vRNA template.

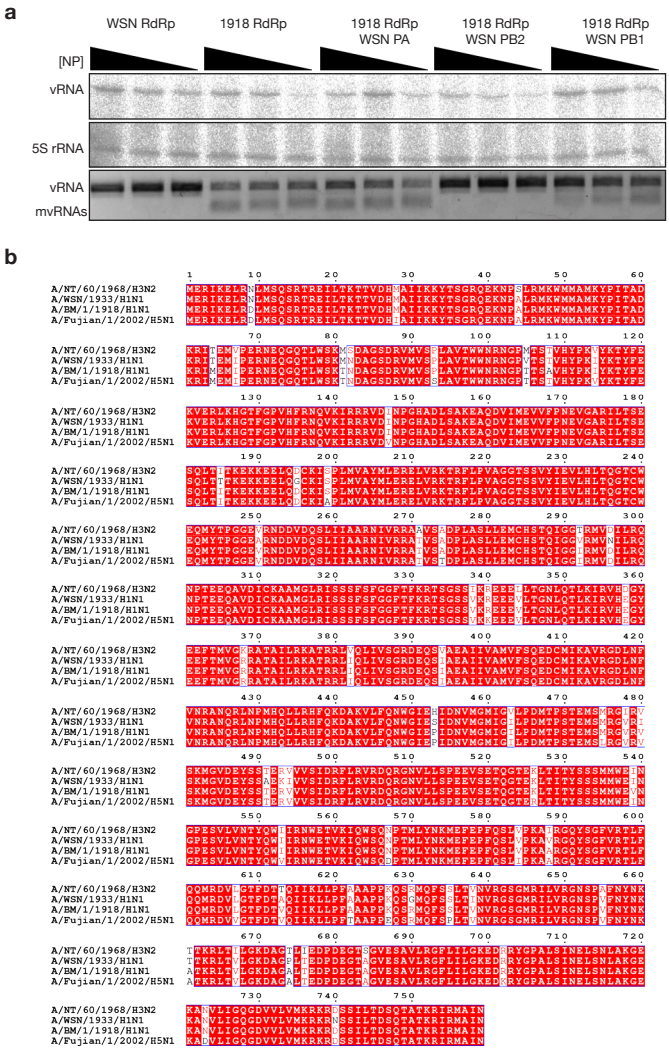

Figure S4 | mvRNA synthesis by reassorted WSN and BM18 polymerases and sequence alignments of the PB2 subunit. (**a**) Analysis of steady state RNA levels in replication assays with WSN and BM18 polymerases and their reassortants and a 246-nt vRNA that was derived from segment 5 of the influenza A virus genome, in the presence of decreasing NP. (**b**) Amino acid sequence alignment of the PB2 subunits of A/WSN/33 (H1N1) (accession number ACF54608), A/Brevig Mission/1/18 (H1N1) (accession number ABA55038), A/Northern Territory/60/68 (H3N2) (accession number AAA43613), and A/duck/Fujian/1/2002 (H5N1) (accession number AY585504) influenza viruses.

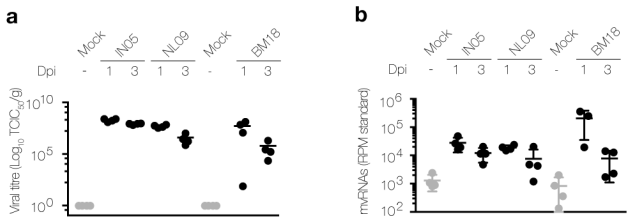

Figure S5 I **Viral titre and mvRNA levels in ferret lungs.** (**a**) Published viral titres of ferret lung tissue samples[29,30]. (**b**) mvRNA levels in lungs of ferrets one and three days after infection with IN05, NL09 or BM18 as measured using deep sequencing.

Table S1 | Primers for RdRp subunit mutagenesis

Mutant	Fw/rv	Sequence (5′ to 3′)
PB1 V43I	Fw	GGAACAGGATACACCATGGATACTATTAACAGGACACATCAGTACTCAG
	Rv	CTGAGTACTGATGTGTCCTGTTAATAGTATCCATGGTGTATCCTGTTCC
PB2 E627K	Fw	CATTTGCAGCAGCCCCACCGAAGCAGAGCAGAATGCAGTTTTCTTC
	Rv	GAAGAAAACTGCATTCTGCTCTGCTTCGGTGGGGCTGCTGCAAATG
PB2 T64M	Fw	CCAATTACAGCAGACAAGAGGATAATGGAAATGATTCCTGAGAGAAATGAGC
	Rv	GCTCATTTCTCTCAGGAATCATTTCCATTATCCTCTTGTCTGCTGTAATTGG
PB2 M81T	Fw	CAGGGACAAACTTTATGGAGTAAAACGAATGACGCCGGATCAGAC
	Rv	GTCTGATCCGGCGTCATTCGTTTTACTCCATAAAGTTTGTCCCTG
Myc-RIG-I mut	Fw	AAGTTTTGAAGCAAGAGCAGCGATATTCTGTGCCCGACAGAACTGCAGC
	Rv	GAAAACTGCGCTGGCTTGGGATGTGGTCTACTCACAAAGCATTCC

Table S2 | RNA templates

Template	Sequence (5′ to 3′)
NP47	AGUAGAAACAAGGGUAUUUUUCUUUCUCGAGCGUACUAGUCUACCCUGCUUUUGCU
NP76	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP101	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCACUAGUUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP125	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCACUAGUUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUACUAGUCUACCCUGCUUUUGCU
NP149	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAACUAGUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP246	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAAGAUCCUUCAUUACUCAUGUCAAAGGAGGGCACGAUCGGGCUCGUUGCCUUUUCGUCCGAGAGCUCGAAGACUCCCCGCCCCUGGAAAGACACUAGUCUCCAUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUACUAGUCUACCCUGCUUUUGCU
NP583	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAAGAUCCUUCAUUACUCAUGUCAAAGGAGGGCACGAUCGGGCUCGUUGCCUUUUCGUCCGAGAGCUCGAAGACUCCCCGCCCCUGGAAAGACACAUCUUCUGGUCUUGCACUUUCCAUCAGCCUUAUGAUUUCGGUUCUCAUGUCAGAUGUUCUCCCCUCUGUAUUCCCAGUGAAUGCUGCCAUAAUGGUUGGUCUGUCAAAAGGGAGAUUUCUCUGUACUGAGAACGUAGGUUGUAUGCUGAUUUGGCCCGAGGAAGCCCUCUGUUGAUUGGUGUUCCCUCCACUUCUGGUCCUUAUGGCCCAGUAUCUGCUUCUCAGUUCAAGGGUACUAGUUUAAGUUCGGUGCACAUUUGGAUGUAGAAUCGUCCAAUUCCAUCAAUCAUUUUUCCGACAGAUGCUCUGAUUUCAGUGGCAUUCUGGCGUUCUCCAUCAGUCUCCAUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
HA77	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUUCAUUUUGGUUGUUUUUAUUUUCCCCUGCUUUUGCU
HA245	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUAAUCUCAGAUGCAUAUUCUGCACUGCAAAGACCCAUUAGAACACAUCCAGAAACUGAUUGCCCCCAGGGAGACCAAAAGCACCAGCCUCCUCGCUGGCGCCGGCUGGGCAACAUUCCGAGGGGACCGUCCCCUCGGUAAUGGCGAAUGGGACUCUAGUACAAAAGCCUUCAUUUUGGUUGUUUUUAUUUUCCCCUGCUUUUGCU
NA76	AGUAGAAACAAGGAGUUUUUUGAACAAACUACUUGUCAAUUUCUGGUUUGGAUUCAUUUAAACUCCUGCUUUUGCU
NA244	AGUAGAAACAAGGAGUUUUUUGAACAAACUACUUGUCAAUGGUGAACGGGAGCUCAGCACCGUCUGGCCAAGACCAAUCUACAGUAUCACCAUUCACACCACAAAAAGAAAUGAUGCUCCCACAAUCCAUAUUGAGAUUAUAUUUCCUAUUUGCAAUAUUAGGCUAAUUAUUCCGACUACCAUACAGAUCGAUCCAAUGGUUAUUAUUUUCUGGUUUGGAUUCAUUUAAACUCCUGCUUUUGCU
49-vRNA spike	AGUAGAAACAAGGGUGUUUUUUCAGAUCUCGAGUCACCCUGCUUUUGCU
37-cRNA spike	AGCAAAAGCAGGGUAGACUAGUACCCUUGUUUCUACU

Table S3 | Primer-extension and RT-PCR primers

Primer name	Sequence (5′ to 3′)	Target
NP-	AGCAAAAGCAGGGTAGACTAGT	NP negative strand (3′ terminus)
NP-2	AAAGAAAAATACCCTTGTTTC	NP negative strand
5S100	TCCCAGGCGGTCTCCCATCC	5S rRNA (100 nt product)
NA-	GTTCAAAAAACTCCTTGTTTC	NA negative strand
HA-	GGAAAAACACCCTTGTTTCT	HA negative strand
NP 5'	AGTAGAAACAAGGGTATTTTTCT	NP positive strand (5′ terminus)
NP 3'	AGCAAAAGCAGGGTAGATAATC	NP positive strand (3′ terminus)
PB2v	AGCGAAAGCAGGTCAATTATAT	PB2 negative strand (3′ terminus)
PB2c	AGTAGAAACAAGGTCGTTTTTAAAC	PB2 positive strand (5′ terminus)
Lv3ga	GTTCAGACGTGTGCTCTTCCGATCTAGCG+AAAGCAGG	vRNA RT primer (contains LNA)
Lv3aa	GTTCAGACGTGTGCTCTTCCGATCTAGC+A+AAAGCAGG	vRNA RT primer (contains LNA)
Lv5	CACGACGCTCTTCCGATCTHNNNNNNNNAGTAGAA+A+CAAGG	vRNA forward primer (contains LNA)
Lc3	GTTCAGACGTGTGCTCTTCCGATCTAGTAGAA+A+CAAGG	cRNA RT primer (contains LNA)
Lc5a	CACGACGCTCTTCCGATCTHNNNNNNNNAGC+AAAAGCAGG	cRNA forward primer (contains LNA)
Lc5g	CACGACGCTCTTCCGATCTHNNNNNNNNAGCGAAAGCAGG	cRNA forward primer (contains LNA)
P5	AATGATACGGCGACCACCGAGATCTACACTCTTTC-CCTACACGACGCTCTTCCGATCT	vDNA and cDNA forward
i7003	CAAGCAGAAGACGGCATACGAGATACTGGTGTGA-CTGGAGTTCAGACGTGTGCTCTTCCGATCT	vDNA and cDNA reverse
Actin human fw	AGAGCTACGAGCTGCCTGAC	Actin B mRNA (forward)
Actin human rv	AGCACTGTGTTGGCGTACAG	Actin B mRNA (reverse)
Actin ferret fw	GATATTACCAGATATCTTATCAAGCTGCTGC	Actin B mRNA (forward)
Actin ferret rv	GCGGCCATCTGGGAGTGTGTAAG	Actin B mRNA (reverse)
H5N1_VN NA fw	CAATTTGGACTAGTGGGAGCAGC	NA H5N1 VN qRT-PCR (forward)
H5N1 VN NA rv	TTGAACAAACTACTTGTCAATGGTGAATG	NA H5N1 VN qRT-PCR (reverse)
NA rv	GCAACTCAGCACCGTCTGGCC	NA qRT-PCR (reverse)
NA 1918 fw	TGCTTCTGGGTTGAATTAATCAGGG	NA BM 1918 qRT-PCR (forward)
NA NL fw	GCTTCTGGGTTGAACTAATCAGAGGG	NA NL 2009 qRT-PCR (forward)
NA IND fw	GTTTCTGGGTTGAGTTGATCAGAGGG	NA Ind H5N1

+ LNA base