Figure 3 | The exonuclease activity of NP is important for blocking the IFN induction. Results shown are the average (n=3) with error bars indicating the standard deviations. a, The NP catalytic mutants were expressed at similar levels to the WT in mammalian cells and had similar transcriptional activities in the LASV minigenome assay. b, The NP catalytic mutants were defective in suppressing the Sendai virus-induced IFN induction by a LUC-based IFNbeta promoter assay. c, The NP catalytic mutants were defective in suppressing the IFN production induced by the immunostimulatory RNAs, poly(I:C) and Pichinde virion-associated RNAs.

Figure 4 | The cap-binding residues and their roles in viral RNA transcription. Results shown are the average (n=3) with error bars indicating the standard deviations. a, A cap analog dTTP is bound within the deep cavity of the N domain of LASV NP. Original Fo-Fc map for the dTTP in blue contoured 2.5 σ. The F176 and W164 or L172 (L120) residues form a typical cap-binding sandwich structure. The middle cavity binds the triphosphate moiety and the hydrophobic cavity entrance can accommodate the second base of the cap structure. b, The NP mutants were expressed at similar levels as the WT at 15 to 30 ng plasmid (WT-15, WT-30) in the transfected mammalian cells. c, Mutational analyses of the residues within the cap-binding cavity for the transcriptional activity using the LASV minigenome assay.

METHODS

Protein expression and purification

The full-length LASV NP gene (Josiah strain) was cloned into the pMAL-c2X-derived pLou3 plasmid, downstream of the TEV cleavage site following the MBP gene. This construct, encoding the N-terminal MBP tagged NP protein, was transformed into Rosetta cells (Novagen). After IPTG induction at a final concentration of 0.03 mM overnight at 20°C, the cells were harvested by centrifugation at 8000 rpm for 20 min and suspended in TEN buffer (20 mM Tris, pH7.5; 0.2 M NaCl, 10% glycerol, 1 mM EDTA) with protease inhibitors (Roche), 1 uM DNase (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma). After cells were lysed by a cell disruptor (Constant System Ltd), the cell lysates were collected by centrifugation at 20,000 rpm for 30 min and applied on an amylose column. The column was washed with >10-column volumes of the sample buffer. The MBP-NP fusion protein was eluted with the TEN buffer containing 10 mM Maltose. The MBP-
NP fusion protein was then cleaved by Tev proteinase. The MBP portion was
removed through two amylose columns, and the NP protein was purified to
homogeneity by gel filtration column. Trypsin digestion coupled with mass
spectroscopy confirmed that the purified LASV NP protein was homogenous (data
not shown), with a final concentration of 7 mg per ml.

Crystallization and data collection

A Cartesian robot (Genomic solutions) was used to screen for optimal crystallization
conditions. The native crystals were obtained in 0.2 M LiCl and 20% PEG3350 in
one week at 20°C. To obtain the NP complex with m7GpppG, m7GTP, or m7GDP,
the NP protein was incubated with individual compound at a concentration of 2 mM
for 30 min on ice and the crystallization conditions were screened. The NP complex
with other triphosphorylated, diphosphorylated or monophosphorylated nucleotides
were formed by incubating the NP protein with 50 mM of the respective compounds
for 30 min on ice and the crystallization conditions were screened. The crystallization
conditions were optimized until the data’s resolution was better than 2.5 angstrom.
All crystals grew in 0.2 M KCl or 0.2 M LiCl and 14 - 22% PEG3350. The NP
complexed with manganese ion was obtained by crystallizing the NP protein in 0.2 M
MnCl₂, 25% PEG3350 followed by soaking the crystals in 0.2 M NaCl₂, 20%
PEG3350 and 15% glycerol three times for 15 min each. The presence of the
manganese and the zinc ions was confirmed in all the crystals by fluorescence
scanning at the Diamond light sources UK. All the crystals were protected by
cryoprotectants that contain 15% to 20% glycerol in the crystallization conditions
before data collection in IO2 or IO3 at the Diamond light sources UK. The Samarium
derivative crystals were obtained by soaking the crystals overnight in 100 mM
Samarium acetate, 0.2 M LiCl, and 16% PEG3350, and was protected in a cryoprotectant of 0.2 M LiCl, 16% PEG3350, and 20% glycerol. The Samarium derivative MAD data were collected at a wavelength of 1.83 Angstrom for peak data, 1.84 Angstrom for inflection data and 1.45 Angstrom for remote data from a single crystal. All the data were indexed, integrated and scaled by HKL2000 or Mosflm and Scale.

Structure determination
The crystals were heavily twined with a twining fraction of 0.43. The initial phases were obtained from a space group of P321 using the MAD data and SOLVE. The initial model was built using RESOLVE, Buccaneer and Coot. It was found that the true space group of the crystals was P3 during the structure refinement. The structures were refined using REFMAC5, and the water molecules were added into the structure by ARP/wARP. The fo-fc maps for ligands (dTTP, UTP, zinc and manganese) were calculated before any ligand was added into the structures. The structures were detwinned at last using REFMAC5, and the structures were evaluated using Molprobity.

In vitro RNA synthesis
The 30-nt cRNA (sense) sequence 5’ CUGGGCUUACCUAUUCUCAGCUGAUGACCC 3’ was derived from the LASV NP (Josiah strain) S segment (nt 2186 -2215 in antigenomic orientation) and chemically synthesized by Eurogentec. The 30-nt vRNA (in genomic orientation) sequence 5’ GGGUCAUCAGCUGAGAAUAGGUAAGCCCAG3’ was complementary to the cRNA. The cRNA (30 nt) was used as one of the three substrates for 3’-5’ exoribonuclease assay. To obtain the blunted dsRNA, both cRNA
and vRNA oligos were dissolved into 0.1 M NaCl, 1 mM EDTA and 0.1 M Tris pH8.0 at the final concentration of 200 mM, and an equal amount of the two oligos was mixed together and annealed in a thermocycler as follows: 95°C for 3 min, 68°C for 1 min and then 4°C.

The 5’ triphosphorylated vRNA was generated by in vitro transcription of the partial dsDNA template formed by the T7 promoter sequence 5’
AATTTAATACGACTCAGCTATAGG3’ and the reverse complement of the T7 promoter sequence and of the LASV (Josiah strain) S segment (nt 2186-2215)
5’CTGGGCTTACCTATTCTCAGCTGATGACCCTATAGTGAGTCGTATTAAAT
T 3’ using the T7 MEGAshortscript kit following the manufacturer’s instructions (Ambion). Similar strategy was use to generate the 32-nt triphosphorylated cRNA with the T7 primer and LASV (Josiah strain) S segment (nt 2186-2213) 5’
GGGTACATCAGCTGAGAAATGGTAAGCCAGCCTATAGTGAGTCGTATTAA
ATT3’. Similar strategy was used to generate the 60-nt vRNA corresponding to LASV (Josiah strain) S segment (nt 2186-2213), using the partial dsDNA template formed by 5’ AATTTAATACGACTCAGCTATAGG3’ and 5’
GTAAATCCCTGCAGTCGGCAGGGTTTACCCTACGCTGATGACCCTATAGTGAGTCGTATTAAATT 3’ as a template. To generate the doubly 5’ triphosphorylated dsRNA, equal amounts of the triphosphorylated 5’ppp-vRNA and 5’ppp-cRNA of equal amounts were annealed in vitro. To make the singly 5’ triphosphorylated dsRNA, equal amounts of the in vitro synthesized 32-nt 5’ ppp-vRNA and the chemically synthesized 30-nt unphosphorylated cRNA were annealed in vitro. The human 18S rRNA fragment (128 nt) was generated by a T7 RNA polymerase-directed in vitro RNA synthesis reaction, using the pTRI-RNA 18S control plasmid (Ambion), following the manufacture’s instruction.
To synthesize the capped viral mRNA transcripts corresponding to nt 992-1117 of the LASV NP gene, the DNA template was PCR amplified from the NP expression plasmid with a forward primer 5’

AATTTAATACGACTCACTATAGGGAAAAACACTGTCGTTGATCTGGAATC 3’
(underlined are T7 promoter sequences) and a reverse primer 5’
GGGTCACTCAGCTGAGAATAGGTAAGCCACCGG 3’, and subjected to in vitro RNA synthesis using mMESSAGE mMACHINE T7 Ultra kit (Ambion) following the manufacturer’s instruction, except that no polyA tail was added.

A plasmid phRL-CMV that encodes the T7 promoter (T7p)-directed human beta-globin gene was kindly provided by Drs. Richard Elliott and Gjon Blakqori. The T7p-globin DNA fragment was purified by agarose electrophoresis after digestion of the phRL-CMV plasmid with HindIII and Smal. The capped human globin mRNA transcripts were generated using the T7p-globin fragment as a template and the mMESSAGE mMACHINE T7 Ultra kit from Ambion, and the poly A tail was added following the manufacturer’s instruction.

The ssRNA markers (perfect RNA markers, 0.1 - 1 kb) were purchased from Novagen. The low molecular weight ssRNA marker (10-100 nt) was purchased from USB. The dsRNA ladder (21-500 bp) was purchased from New England Biolabs.

**In vitro 3’-5’ exoribonuclease assays**

The in vitro 3’-5’ exoribonuclease assays were carried out in 10 μl of the reaction solution containing 0.3 M NaCl, 10% glycerol, 20 mM Tris pH7.5, 10 mM MnCl$_2$, 7 μg of either WT or mutant NP proteins, and 8 units of the RNaseIN inhibitor (Promega), in the presence of various substrate(s), at 37 °C for 60 to 100 min. The control reactions included all but MnCl$_2$, which was substituted by 20 mM EDTA. All
the reactions, each in triplicates, were stopped by the addition of EDTA to a final concentration of 20 mM. The samples were mixed with equal volumes of RNA loading buffer (Ambion), heated at 95 °C for 3 min, cooled on ice for 5 min, and separated in 15% or 6% urea-polyacrylamide gel, or 2% agarose gel. The gels were stained in 0.05% ethidium bromide for 25 min, visualized using the 2UV transilluminator (UVP).

**The luciferase-based assay to quantify virus-induced and immunostimulatory RNAs-induced interferon-beta activation**

The Sendai virus-induced IFN-beta activation assay was conducted as described previously. In brief, 293T cells were cotransfected using calcium phosphate with 100 ng of a vector that expresses the firefly luciferase (Fluc) reporter gene from a known functional promoter sequence of the IFN-beta gene (pIFNbeta-LUC), variable amounts of either WT or mutant LASV NP vectors, and 50 ng of a β-gal-expressing plasmid for transfection normalization. At 24 h post-transfection, cells were infected with Sendai virus (at moi = 1) in order to induce IFN-β expression. At 24 hpi, cell lysates were prepared for luciferase and β-gal assays. Fluc activities were normalized by the β-gal values. Each transfection was conducted in triplicates and repeated in at least two independent experiments.

To determine whether NP can suppress the immunostimulatory RNAs-induced IFN production, HEK293 cells were transfected with pIFNbeta-LUC, variable amounts of either WT or mutant LASV NP vectors, and a beta-gal-expressing plasmid for transfection normalization. 18 h later, cells were transfected with either 1 ug of Poly(I:C) or 250 ng of Pichinde virion RNAs by lipofectamine 2000. Luciferase
activity was determined at 18 h after the immunostimulatory RNA transfection and normalized by the beta-gal activity.

**Pichinde virion RNA preparation**

Pichinde viruses were purified by 20% sucrose gradient ultracentrifugation at 50,000Xg for 2 h. Virus RNA was extracted with RNABee (Tel Test) according to the manufacturer’s protocol.

**LASV minigenome (MG) transcription assay**

The full-length LASV L and NP genes (Josiah strain) were cloned into the pCAGGS vector for expression in mammalian cells. LASV MG construct contains the T7 promoter-directed LASV S segment-like sequences that include all the important cis-acting elements required for viral RNA synthesis (5’ UTR, intergenic region, and 3’ UTR) and encode a renilla luciferase (RLuc) gene in place of the viral NP coding sequence. This LASV-based LUC-encoding mini-genome (MG) RNA was transcribed in vitro by the T7 MEGAScript kit (Ambion) and transfected into 293T cells, together with the LASV L expression plasmid, and WT or mutant NP expression plasmid. A beta-gal expression vector was included in each transfection to normalize for cell transfection efficiency. LUC activity was determined at 24 h post-transfection, normalized by beta-gal activity, and shown as fold increase over a control sample that lacked the L expression plasmid. Each reaction was conducted in triplicates and in at least two independent experiments.

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