Table S1. Summary of data collection, structure determination and refinement statistics. Related to Figure 1.

Ligands	**2-5A + AMP-PNP**	**2-5A**
Space group	$P2_1$	$P2_1$
Unit cell parameters (Å; °)		
a	59.20	59.78
b	267.4	268.48
c	110.21	111.89
β	90.04	90.04
Molecules/asu	4	4
Resolution	59.2 – 2.50 (2.66 – 2.5)	49.7 – 3.25 (3.37 – 3.25)
Unique reflections	106347 (15940)	51209 (5016)
Redundancy	3.0 (3.2)	2.4 (2.3)
Completeness (%)	95.7 (88.4)	94.1 (92.9)
Wilson *B* (Å^2)	52.7	88.3
$I/\sigma I$	6.8 (1.6)	8.2 (1.2)
$CC_{1/2}$ (%)	69.1	43.9
R_{meas}	0.104 (0.526)	0.131 (0.980)
R_{work}/R_{free}	0.196/0.231	0.230/0.289
RMSD from ideal geometry		
Bonds (Å)	0.007	0.010
Angles (°)	1.231	1.394
Average *B*-factors (Å^2)		
Protein	52.9	129.5
2-5A	41.1	115.0
AMP-PNP:Mg^{2+}	37.7	-
Ramachandran plot		
Favored regions (%)	97.54	96.26
Disallowed regions (%)	0	0

Table S2. Small-angle X-ray scattering data collection and analysis. Related to Figure 7.

Ligands	**2-5A**	**2-5A +ADP**
Data-collection parameters		
Instrument	BioSAXS-1000	BioSAXS-1000
Beam geometry (mm)	0.5	0.5
Wavelength (Å)	1.5418	1.5418
q range ($Å^{-1}$)	0.008-0.400	0.008-0.4000
Exposure time (hr)	0.5	2
Concentration (μM)	14	3.9
Temperature (°C)	10	10
Structural parameters		
I_0 (cm^{-1}) [from Guinier]	1.774 ± 0.030	0.1946 ± 0.0136
R_g (Å) [from Guinier]	57.50 ± 1.16	35.7 ± 3.04
Fidelity of Guinier	0.937	0.998
I_0 (cm^{-1}) [from P(r)]	1.642 ± 0.010	0.1975 ± 0.003
R_g (Å) [from P(r)]	55.19 ± 0.256	37.38 ± 0.388
D_{max} (Å)	175	115
Total Quality Estimate [from P(r)]	0.891	0.917
Chi^2 of *Ab Initio* Model	1.17-1.18	0.99

Supplenmentary Experimental Procedures

2-5A synthesis and purification.
2-5A was synthesized as described previously with modifications (Jha et al., 2011). Enzymatic synthesis of 2–5A from ATP was done with recombinant porcine 2'–5' oligoadenylate synthetase (pOAS1) (a gift from Rune Hartmann, Aarhus, Denmark) activated with poly(rI):poly(rC) (pIC) conjugated to CL6B agarose (Ag-pIC). Briefly, pIC was conjugated with CL6B agarose beads (Amersham Biosciences) using per-iodate conjugation chemistry, and Ag-pIC was incubated with 0.4 mg/mL of purified pOAS1 at 25°C in 10 mM HEPES pH 7.5 containing 1.5 mM magnesium acetate, 10% glycerol, 50 mM KCl, and 7 mM β-mercaptoethanol for 1 h. pOAS1 immobilized on Ag-pIC beads was washed three times with buffer and resuspended in the reaction mixture (10 mM ATP, 10 mM magnesium acetate, 20% glycerol, 50 mM KCl, and 7 mM β-mercaptoethanol) for 20 h at 37^{o}C with constant shaking. The unfractionated 2–5A mixture was harvested by centrifugation at 3,000 g for 15 min and then separated from pOAS1with a Centriprep (Millipore) molecular weight cutoff of 3000 Da. The reaction mixture was analyzed on a Dionex PA100 (4 mm x 250 mm) analytical column (Dionex Inc.) interfaced with a System gold HPLC under the control of a 32 Karat work station using $NH_4HCO_3/(NH_4)_2CO_3$ pH 8.0 as mobile phase. The preparative purification of (2'-5')p_3A_3 was done by HPLC on Dionex PA100 (22 mm X250 mm) preparative column by using gradient of 10 mM – 800 mM $NH_4HCO_3/(NH_4)_2CO_3$ pH 8.0 gradient in 70 minutes. The peak fractions were collected and lyophilized (2X in DEPC treated water). The characterization of (2'-5')p_3A_3 (>95% purity) was done by analytical HPLC and ES-MS by direct infusion. The molecular ion was observed at MH_3^+ 1164.23, MH_3^{2+} 581.76 and MH_3^{3+} 387.61. The theoretical molecular weight of (2'-5')p_3A_3 is 1161 Da.

Protein Expression and Purification
Cloning and description of the cDNA for porcine RNase L was described previously (Rios et al., 2007; Zhou et al., 1993)**.** Wild type and mutant *Sus scrofa* RNase L proteins **(**residue 21 to 732 for crystallization studies and residues 1 to 743 for mutational, SAXS and biochemical studies) were recombinantly expressed from a modified pGEX-2T (Pharmacia) plasmid in *E. coli* BL21 cells as TEV protease-cleavable GST-tagged fusions. Bacterial cell pellets were suspended in lysis buffer (30 mM HEPES, pH 7.8, 400 mM NaCl, 2 mM DTT), lysed by homogenization using a cell homogenizer (Avestin Inc.) and centrifuged at 30,000 g for 40 minutes to remove cell debris. Clarified lysate was bound to glutathione Sepharose resin (GE Healthcare) and eluted by TEV protease treatment. Eluate was then purified by anion exchange chromatography (Q-Sepharose^{TM} – GE Healthcare) and finally by size exclusion chromatography using a Superdex^{TM} 200 column (GE Healthcare) equilibrated in 30 mM HEPES (pH 7.8), 100 mM NaCl and 2 mM DTT. Peak protein fractions were pooled and concentrated to 10 to 16 mg/ml (125 to 200 μM) and then flash frozen in liquid nitrogen for long-term storage. RNase L mutants were made using the QuikChange protocol (Agilent) on WT plasmid and verified by DNA sequencing. We note that omission of the ion exchange chromatography step yielded RNase L protein with only a partial dependency of RNase function on nucleotide addition.

Crystallization, Data Collection, and Structure Determination

RNase L protein was labeled with seleno-methionine in BL21 CodonPlus(DE3)-RIL cells (Stratagene) using a published protocol (Van Duyne et al., 1993). Crystals were obtained in 4 µl hanging drops containing an equal volume of protein solution with RNase L (10-14 mg/ml, 125-165 µM), +/- AMP-PNP (300 µM), (2'-5')p_3A_3 (400 µM) and of well solution with 5 mM $MgCl_2$, 5 mM DTT, 18% PEG2000, 100 mM NaCl, and 100 mM SPG buffer, pH 7; $MgCl_2$ was not added for the crystals lacking AMP-PNP. Harvested crystals were cryoprotected with lower well solution. Anomalous dispersion experiment was performed on an AMP-PNP containing crystal on beamline 24-ID (Advanced Photon Source, Argonne, IL). Data was processed with XDS (Kabsch, 2010). An initial electron density map was obtained using a combination of molecular replacement and SAD with Phaser (McCoy et al., 2007) using the human RNase L ANK domain (PDBID 4G8K) as a search model. Density modification was performed using Parrot in CCP4 (1994). The structure was auto-built with Buccaneer (Cowtan, 2006) followed by manual adjustment using COOT (Emsley and Cowtan, 2004). Refinement was performed using REFMAC5 (Murshudov et al., 2011). The structure of crystals grown lacking AMP-PNP was solved by molecular replacement using the AMP-PNP bound model.

Structure analysis and sequence alignments

Multiple sequence alignments were performed using MUSCLE (Edgar, 2004) and displayed, edited and annotated using ALINE (Bond and Schuttelkopf, 2009). Secondary structure was analyzed using DSSP (Kabsch and Sander, 1983) and buried surface area and residue contacts were calculated using the programs AREAIMOL and CONTACT from the CCP4 suite (1994). Structure alignments and structure representations were performed using the PyMOL Molecular Graphics System, Schrödinger, LLC.

Analytical ultracentrifugation

Sedimentation velocity analytical ultracentrifugation was performed with a Beckman ProteomeLab XL-I at 42,000 rpm with either refractive index or fluorescence (Aviv Biomedical Inc.) detector system. Data was obtained over 8 h of centrifugation at 20°C using refractive index detection. Concentrations of RNase L, ranging from 0.5 µM to 10 µM, were analysed in AUC buffer (15 mM Hepes pH 8, 250 mM NaCl, 3 mM DTT, 2.5 mM $MgCl_2$) in the presence or absence of 40 µM 2-5A, 250 µM AMP-PNP, or 250 µM AMP-PNP and 40 µM 2-5A. Concentrations of fluorescein labelled RNase L, ranging from 3 nM to 30 nM, were analysed in AUC buffer in the presence or absence of 2 µM 2-5A, or 12.5 µM AMP-PNP and 2 µM 2-5A. The raw data was analyzed by Sedfit (Schuck et al., 2002) and transformed into a c(s) plot.

Fluorescein labeling of RNase L

A 5 ml reaction volume containing 24 µM purified full length RNase L and 48 µM 2-5A was mixed with 21 µM 5-(and-6)-Carboxyfluorescein, Succinimidyl Ester (5(6)-FAM, SE) (Life Technologies™) in a buffer containing 20 mM HEPES (pH 7.8), 100 mM NaCl, 1 mM DTT and 0.1% DMSO. After incubation at 20 °C for 3 hours shielded from light, labeled protein was separated from free 5(6)-FAM, SE by size exclusion

chromatography. The efficiency of labeling determined by absorbance (ε protein 280 nm = 77810 cm^{-1} M^{-1} ; ε fluorescein 494 nm = 71000 cm^{-1} M^{-1}) was 23%.

Preparation of Conjugate of 2-5A with Biotin

2',5'-tetraadenylate 5'-triphosphate [(2'-5')p_3A_4] was synthesized and purified as described for (2',5')p3A3. The 6-(6-hydrazidohexyl) amidohexyl D-biotinamide was purchased from Dojindo Co. (Kumamoto, Japan). All other reagents were of analytical grade from Sigma-Aldrich (St Louis, Mo USA) and used without any further purification. Hydrazidohexyl-D-biotinamide was linked to (2'-5')p_3A_4 by using periodate conjugation chemistry. Briefly, 5 mM aqueous solution of (2'-5')p_3A_4 was treated with 20 mM $NaIO_4$ on an ice bath for 45 min followed by addition of 50 mM potassium iodide. The resulting precipitate was removed by centrifugation. 6-(6-hydrazidohexyl)amidohexyl D-biotinamide dissolved in dimethylformamide was added to a final concentration of 10 mM to the oxidized (2'-5')p_3A_4 in 20 mM sodium acetate buffer (pH 6.5). The reaction mixture was allowed to proceed on an ice bath with continuous stirring for 60 min. Finally, sodium cyanoborohydride (50 mM final concentration) was added to the reaction mixture and incubated for 90 minutes at 0°C. Formation of the conjugate of (2'-5')p_3A_4 with biotin was validated by HPLC on a Xbridge™ BEH C-18 2.5 µm (2.1 mm x 50 mm) analytical columns (Waters) interfaced with system gold HPLC (Beckmann Coulter Inc). The biotin labelled (2'-5')p_3A_4 was purified by HPLC on a semi preparative Xbridge™ BEH C-18 2.5 µm (4.6 mm x 50 mm) column using mobile phase of (A) (15 mM triethylamin and 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol and (B) methanol gradient (10%-30% in 40min). The conjugate, which eluted at 36 min, was collected and lyophilized, resulting in the purified product (16.4 OD_{260}, yield 40%). The formation of the conjugate was confirmed by ES MS on Quattro Ultima (Micromass) triple quadrupole tandem mass spectrometer. The (2'-5')p_3A_4 -biotin was tested for functional activity in a FRET based RNase L activation assay and used for surface plasmon resonance (SPR) binding analyses.

2-5A binding analysis using surface plasmon resonance (SPR)

SPR studies were performed using a Biacore3000 (Piscataway, NJ). Biotin-labeled 2-5A (10 nM) in buffer A [20 mM Hepes buffer (pH 7.4), 5% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 7 mM 2-mercaptaethanol with 0.005% surfactant P20] was immobilized on streptavidin chips (Biacore SA BR-1003-98) at a flow rate of 10 µl/min for 4 minutes, yeilding 150-160 response units (RU) in the sample cell with buffer A in reference cell. Various concentration of porcine RNase L (0.01 to 5 µM in buffer A either in the absence or presence of 20 µM ATP) was used for binding to chips containing immobilized (2'-5')p_3A_4-Biotin at a flow rate of 20 µl/min for 2.4 min, with a 10 minute stabilization time prior to injection followed by 5 min dissociation in buffer alone. Each concentration was injected in duplicate over all surfaces. Sensograms were analyzed with the BIAevaluation version 3.0. Surface regeneration of chips was achieved by using two pulses of 3 M guanidine-HCl in binding buffer at a flow rate of 50 µl/min for 20 seconds. Non-specific interaction of the analyte and the streptavidin chips were blocked with 50 nM free biotin on both the control as well as the experimental flow channels.

In vitro **RNA cleavage assays**

Methods are essentially as described previously (Thakur et al., 2005) with the following deviations. Reaction volumes were decreased to 30 μL to allow measurement in 384 well micro plates. 2-5A activator, FRET RNA substrate and RNase L proteins were employed at 5 nM, 135 nM, and 1.6 nM respectively and nucleotides were employed at concentrations of 0, 2 or 50μM. Results are representative of minimally two independent experiments performed in duplicate.

Cell Based RNA cleavage assay

The full length (FL) porcine RNase L DNA in pET-M30-2(HTa) was subcloned into pENTR 2B Dual Selection Vector (LifeTechnologies) with BamHI at 5' terminus and XhoI at 3' terminus. The RNase L (FL) cDNA in pENTR 2B was then subcloned into pCS2 (Addgene) mammalian expression vector containing a CMV promoter by LR recombination. Mutants were made with QuikChange II Site-Directed Mutagenesis Kit (Agilent) on WT porcine RNase L and verified by DNA sequencing. The cell based assay for RNase L activity was performed as described previously with modifications (Carpten et al., 2002). HeLa-M cells, deficient in endogenous RNase L, were grown in DMEM with 10% FBS were cultured in 12 well plates ($1x10^5$ per well) for 16 hrs. Cells were transfected with 0.7 μg of the different porcine RNase L cDNAs using Lipofectamine 2000 (LifeTechnologies) for 20 hrs. Subsequently, culture media was replaced for 4 hrs and transfected with 1 μg/well of poly(rI):poly(rC) (Calbiochem) for 3 hrs. Media was removed and total RNA was isolated using Trizol reagent (LifeTechologies) and quantitated by measuring absorbance at 260 nm. RNA, about 0.2 ng per sample, was separated on RNA chips with an Agilent Bioanalyzer 2000. Western blot assays on the transfected cells was determined with monoclonal anti-FLAG antibody and anti-β-actin (Sigma), anti-mouse IgG HRP-linked antibody (Cell signaling) and Western Bright ECL chemiluminescent substrate (Advansta).

SAXS analysis

RNase L (200 μM) was incubated with natural 2-5A ligand (300 μM) in the absence or presence of ADP (300 μM) in 30 mM Hepes pH 8.0, 100 mM NaCl, 2 mM DTT and 5 mM $MgCl_2$, as well as 200 μM ADP for the ADP-bound complex. The RNase L and RNase L-ADP complexes were subsequently resolved over a Superdex-200 (GE Healthcare) size exclusion chromatography column and the homogeneity of the samples was confirmed by dynamic light scattering. The scattering data were collected on a BioSAXS-1000 configured on the right port of a MicroMax-007HF X-ray generator using a 45 μL sample volume. Consecutive scans of 10, 30 and 120 minutes were collected at 10°C over a range of RNase L (1.3-14 μM) and RNase L-ADP (0.7-7.8 μM) concentrations. SAXSLab 3.0.0r1 software (Rigaku) was used to subtract buffer scattering from the protein scattering to generate the sample scattering curves. SAXS profiles were analyzed using the ATSAS program suite (Konarev, 2006). Data quality for different protein concentrations and exposure times was assessed with Kratky plots and screened for aggregation using Guinier Plots. Guinier regions and radius of gyration estimates were derived by the Guinier approximation using the AutoRg function of

Primus (Konarev, 2003). The highest quality estimate as determined by AutoRg and AutoGNOM functions was used to select the samples of RNaseL and RNaseL-ADP that were processed further. Data collection and scattering-derived parameters for the highest quality samples are summarized in **Table S2**. *Ab initio* models were generated using DAMMIF (Franke, 2009) and an averaged model from ten independently generated *ab initio* models was calculated using DAMAVER (Volkov, 2003).

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