Supplementary Information

A Phenolic Small Molecule Inhibitor of RNase L Prevents Cell Death from ADAR1 Deficiency

SHORT TITLE: Regulation of RNase L with small molecules

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids, protein expression and purification

pGEX-2T- *Sus scrofa* RNase L WT was previously described (1). pGEX-2T-*Sus scrofa* RNase L H680N and pGEX-2T-*Sus scrofa* RNase L H680A were generated by site directed mutagenesis using KOD Hot Start DNA Polymerase (CAT# 71086-4, Millipore-Sigma) in pGEX-2T-*Sus scrofa* RNase L WT. AviTagged RNase L WT for SPR studies was generated by introducing a C-terminal-AviTag into the pGEX-2T-*Sus scrofa* RNase L WT construct by site directed mutagenesis using KOD Hot Start DNA Polymerase.

WT and mutant *Sus scrofa* RNase L proteins (residues 1–743 for biochemical studies, residues 21-732 for NMR studies), and human RNase L proteins (residues 21-719 for biochemical studies) (2) were expressed in *E. coli* as TEV-cleavable GST fusions and purified by glutathione affinity, TEV cleavage, anion exchange, and finally size exclusion chromatography (SEC). Briefly, bacterial cell pellets were resuspended 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 4B resin (CAT# 17-0756-04, GE Healthcare) and eluted by TEV protease treatment. Eluate was then purified by anion exchange chromatography (HiTrap™ Q XL, CAT# 17-5159-01, GE Healthcare) and finally by size exclusion chromatography using a Superdex™ 200, 10/300 GL column (CAT# 17-5175-01, 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 WT-AviTag was expressed and purified as described above with the following modification. After elution from the anion exchange column, protein fractions were pooled, and concentrated to 8 mg/mL for *in vitro* biotinylation reactions.

The full length human E2 enzyme UBE2S gene and Shigella flexneri E3 enzyme IpaH9.8 gene were subcloned into a modified pET28a expression vector with a TEV protease cleavage site. Proteins were expressed in *E. coli* and purified by His-tag affinity, TEV cleavage, anion exchange, and finally size exclusion chromatography.
Briefly, bacterial cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM 2-Mercaptoethanol), lysed by sonication on ice and centrifuged at 30,000 g for 40 minutes to remove cell debris. The UBE2S and IpaH9.8 proteins were first purified using Ni-affinity column (HisTrap™ HP, CAT# 17-5248-02, GE Healthcare). The rest of the purification was the same as for RNase L, except for the use of 50 mM Tris-HCl pH 7.5, 1 mM DTT, and 100 mM NaCl as the SEC buffer. Lysozyme and BSA proteins were purchased from Sangon Biotech, China (CAT# A610308) and Solarbio, China (CAT# A8020), respectively. The pET21a-BirA bacterial expression plasmid for 6X-His-BirA was acquired from Addgene (# 20857) (3) and the expression and purification of 6X-His-BirA protein was performed as previously described (4).

**In vitro biotinylation of AviTagged RNase L**

_In vitro_ biotinylation reactions were performed by mixing 1 mg of 1 mg/mL RNase L WT-AviTag with 0.2 mg of 12 mg/mL His-BirA WT in a 1 ml reaction volume containing 50 mM Tris-HCl pH 8.6, 100 mM ATP, 50 mM D-Biotin and 100 mM MgCl₂. Reactions were incubated at 4 °C overnight. Size exclusion chromatography was performed as described above to separate biotinylated RNase L from His-BirA and free biotin. Peak protein fractions were pooled and concentrated to 1.5 mg/mL and then flash frozen in liquid nitrogen or used fresh for Surface Plasmon Resonance (SPR) experiments.

**In vitro endoribonuclease and fluorescence polarization assays**

_RNase L endoribonuclease assay_: Fluorescence based endoribonuclease cleavage assays (1) were performed with 1.6 nM RNase L protein and 135 nM ssRNA substrate (5’-UUA-UCA-AAU-UCU-UUG-CCC-CAU-UUU-UUU-GGU-UUA-3’), labelled on the 5’ and 3’ termini with 6-FAM and black hole quencher, respectively (Integrated DNA Technologies Inc.) unless stated otherwise. RNase L protein was incubated with 5 nM 2-5A p2A3 (synthesized in house (5)) co-activator for 5 minutes on ice. Reaction buffer consisted of 25 mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl₂, 0.01% Brij-35, and 50 µM ADP. Compounds were pre-incubated in reaction buffer with RNase L proteins for 30 minutes. Upon addition of RNA substrate, cleavage reactions were monitored in real time by fluorescence intensity (λ$_{excite}$ = 495 nm, λ$_{emission}$ = 517nm) at two-minute intervals.
using a Molecular Dimensions Analyst HT microplate reader. Graphs were generated using Graphpad Prism. Orthogonal, gel based RNA cleavage assays were performed as above to rule out fluorescence artefacts using a FAM labeled RNA substrate lacking the black hole quencher. Upon addition of RNA substrate, the cleavage reactions were analysed at 30 minutes in TBE acrylamide gel. The gel was then imaged using a Typhoon FLA 9500 Variable mode Imager (GE Healthcare).

**IRE1 endoribonuclease assay:** Fluorescence based endoribonuclease cleavage assays were performed with 10 nM IRE1 protein and 100 nM of a single hairpin RNA substrate (5’-CAU GUC CGC AGC GCA UG-3’), labeled on the 5’ and 3’ termini with Alexa Fluor 647 fluorophore and black hole quencher, respectively unless stated otherwise. Reaction buffer consisted of 50 mM Tris pH 7.0, 0.5 mM MgCl₂, 10 mM KCl, 0.025% Tween-20, 0.063 mg/mL tRNA, and 2 mM DTT. Compounds were pre-incubated in reaction buffer with IRE1 proteins for 1 hour at room temperature. Upon addition of RNA substrate (diluted in 10 mM Tris pH 7.0, 0.1 mM EDTA), cleavage reactions were monitored in real time by fluorescence intensity (λ<sub>excite</sub>= 651 nm, λ<sub>emission</sub>= 672 nm) at two-minute intervals using a Molecular Dimensions Analyst HT microplate reader.

ATP and compound binding was determined by fluorescence polarization using 2 μM RNase L, 25 nM BODIPY™ FL ATP-γ-S (Invitrogen Molecular Probes™, CAT# A22184), 25 μM 2-5A and titrating in cold ATP and compound in a buffer containing 25 mM HEPES, 50 mM NaCl, 1 mM DTT, 0.01% Brij 35, and 10 mM MgCl₂, in a total reaction volume of 25 μL. Polarization was measured on an Analyst HT (ex:em, 485 nm:535 nm, Molecular Devices) and results analyzed in Graphpad Prism.

**Ribonuclease activity inhibitor screen**
A 500 compound library (OICR-L100, Medicinal Chemistry Platform at the Ontario Institute for Cancer Research) assembled from previously reported protein kinase inhibitors and close analogues was screened using a fluorescence based RNA cleavage assay (described above) in 384 well format using a Biomek FX liquid handler.
at the SMART screening facility at LTRI. Each well contained 1.6 nM porcine RNase L, 135 nM ssRNA substrate, 5 nM 2-5A, 50 μM ADP and 6.6 μM library compound in 25 mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl₂, 0.01 % Brij-35, and 0.66% DMSO in a final volume of 30 μL per well. 0.2 μL of each library compound from a 1 mM stock in DMSO was added to a final concentration of 6.6 μM, mixed and incubated at room temperature for 30 minutes. Samples were excited at 485 nm and emission was read at 535 nm on an MD Analyst plate reader (Molecular Devices Corp., Sunnyvale CA). High controls contained the complete reaction components including 0.2 μL of DMSO, and low controls contained the same with the exception of 2-5A activator. The Z score was calculated as the individual sample value minus the mean of the sample values, divided by the standard deviation of the sample values (Z = (X - μ) / σ). Dose response analyses on the top initial hits were carried out using the same conditions as above, and only ellagic acid reproduced as a hit.

**Inhibitor binding analysis using Nuclear Magnetic Resonance (NMR)**

All NMR samples were prepared in buffered solutions containing 20 mM sodium phosphate, 100 mM NaCl, pH 7.5 and 5% d₆-DMSO, 95% D₂O. For the detection of VAL binding to RNase L, NMR samples contained 400 μM VAL (CAT# 60202-70-2, BOCSCI Inc.) and 0 μM or 2.0 μM or 5.0 μM RNase L (residues 21-732). For control experiments, NMR samples contained 400 μM VAL and 0 μM or 2.0 μM or 5.0 μM of the control proteins BSA, lysozyme, Ube2S and IpaH9.8. Where indicated, NMR samples were prepared with 20 μM 2-5A or 20 μM / 100 μM ATP/MgCl₂, or a mixture of 20 μM 2-5A and 20 μM / 100 μM ATP / MgCl₂. NMR data was acquired on a Bruker Avance III-600 Mz spectrometer equipped with a cryogenically cooled probe at 25 °C. The relaxation time for CMPG experiments was 80 ms and the saturation time for STD experiments was 2 s.

**Inhibitor binding analysis using Surface Plasmon Resonance (SPR)**

Biotinylated C-terminal AviTagged RNase L alone or in a preformed complex with 10 μM 2-5A or with 10 μM 2-5A and 10 μM / 5 mM ATP/MgCl₂ was captured on a streptavidin surface (Series S Sensor Chip SA; GE Healthcare) using a Biacore S200
instrument (GE Healthcare). Varied concentrations of VAL or Sunitinib were flowed over the surface at a rate of 30 mL/sec as solutions in 10 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol and 1% DMSO. The surface plasmon resonance binding signal, measured in resonance units (RU), was recorded in real time and simultaneously monitored both directly and as a difference signal against a mock streptavidin surface. Dissociation constants (Kds) were calculated using Biacore T200 Evaluation software from the equilibrium steady state sensorgram signal assuming a 1:1 interaction model. All kinetic runs were performed in triplicate and the reported Kds represent the average values calculated using Prism GraphPad.

**Inhibitor-binding analysis using Differential Scanning Fluorimetry**

Differential scanning fluorimetry (thermal melt) was performed with 10 μM porcine RNase L H680N protein and 20X SYPRO Orange Protein Gel Stain (5000X) (CAT#S6650, ThermoFisher) in an optimized buffer containing 50 mM Tris pH7.5, 150 mM NaCl, 1 mM DTT, in a total reaction volume of 20 μL. Where indicated, thermal melts were performed in the presence of 10 μM of 2-5A or 10 mM / 100 μM of ATP/MgCl₂, or 100 μM of VAL or a combination of 10 μM of 2-5A, 100 μM / 10 mM of ATP/MgCl₂, or a combination of 10 μM of 2-5A, 100 μM / 10 mM of ATP/ MgCl₂, 100 μM of VAL. The reaction mixtures were incubated at 20 °C for 30 minutes. The Roche LightCycler 480 was used to determine protein melting points between 20 °C to 85 °C with a 0.1 °C incremental ramp over 1 hour.

**In vitro inhibition of CK2α by VAL or EA**

Inhibitor activity of VAL and EA against CK2α was determined by Eurofins. In brief, Human CK2α was incubated with 20 mM HEPES pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 0.1% Triton X-100, 165 μM RRRDDDSDDD substrate peptide, 10 mM Magnesium acetate and [9-33P]-ATP (specific activity and concentration as required). The reaction was initiated by the addition of the ATP/MgCl₂ mix. After incubation for 40 minutes at room temperature, the reaction was stopped by the addition of phosphoric acid to a concentration of 0.5%. 10 μL of the reaction was then spotted onto a P30
filtermat and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting.

**Cellular thermal shift assay (CETSA)**

CETSA experiments were performed as described (6). Six 10 cm dishes of 293T cells were transiently transfected with pCMV-Flag-RNase L using polyethylenimine (PEI) (CAT#23966-1, from Polysciences Inc.). Three days later, the cells were harvested and pelleted by centrifugation. Cells from three dishes were each lysed in 400 µl of buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM DTT, 1 mM PMSF and protease inhibitors cocktails (Sigma-Aldrich) and centrifugated 30 min at 14000rpm. The supernatants were collected and treated with either 100 µM of VAL (4 µl) or 4 µl of DMSO for 30 min at room temperature. Then, ten 25 µl aliquots of the treated lysate were distributed into 0.2 ml PCR tubes. Using a Veriti 96-well thermal cycler, individual PCR tubes were heated to one of the following temperatures for 3 minutes (45 °C, 47 °C, 49 °C, 51 °C, 53 °C, 55 °C 59 °C, 63 °C, 65 °C and 67 °C). The tubes were then incubated at room temperature for 3 min followed by snap-freezing in liquid nitrogen.

For RNase L stabilization analysis, the treated lysates were thawed, briefly vortexed and centrifugated at 14000 rpm for 45 min at 4°C. The supernatants were collected without disturbing the pelleted debris and transferred to new tubes. The soluble fraction was then analysed by western blot and RNase L levels were monitored using Flag (M2) antibody.

**Monitoring specific rRNA cleavage products as an index of RNase L activity in intact cells**

The cell-based assay for rRNA cleavages in intact cells was performed as we described previously (1, 7).

**Cell Culture**

Human lung epithelial cells A549 were grown in RPMI1640 (GIBCO). Mouse L929 cells and human embryonic kidney HEK293T (293T) cells (ATCC, CRL-3216) were grown in
Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL streptomycin and 1 % L-glutamine. A549 cells were authenticated by STR analysis by ATCC (compared to ATCC CCL-185), whereas L929 cells were confirmed to be of mouse origin with no human cell cross-contamination by DNA marker analysis through Genetica Cell Line Testing, Co. The construction of NS2-ADAR1 WT and NS2-ADAR1-KO cells was described previously (8). 293T cells were authenticated by ATCC and regularly tested negative for mycoplasma contamination as assessed by DAPI staining. Proliferation rates and proper cell morphology of all the cell lines were continuously monitored.

**Antibodies for Western Blot**
Rabbit anti-ADAR1 D7E2M antibody (1:1000) was from Cell Signaling Technology (#14175S). Mouse monoclonal anti-ß-actin (1:50,000, A1978) and mouse monoclonal anti-Flag (M2) (1:2000, F3165) were from Sigma-Aldrich. Mouse monoclonal anti-HSP90α/β (F-8) (1:2000, sc-13119) was from Santa Cruz. The secondary antibodies were anti-rabbit (1:5000, Cell Signaling Technology, #7074S), goat anti-mouse antibody (1:5000, Santa Cruz, #7076S) and rabbit anti-mouse (1:10000, Abcam, #ab97046) conjugated with horseradish peroxidase, HRP. For western blot analysis, total cell extracts were used for SDS-PAGE and immunoblotting was performed as previously reported (9).

**Cell death and cell survival assays**
Cells were incubated with 250 nM Sytox-Green dye (CAT# S7020, ThermoFisher), a nucleic acid stain that is an indicator of dead cells and which is impermeant to live cells, and 250 nM of cell permeable dye Syto 60-Red (CAT# S11342, ThermoFisher), which allows quantification of the total number of cells present in each field, using an IncuCyte Live-Cell Imaging System and software (Essen Instruments 2016B) as described previously (8). Cell death was measured by counting the green objects per field (dead cells, green) and then normalizing to the total number of cells field (red objects) at each time point using IncuCyte software.
**PolyI:polyC (pIC) transfections**

A549 cells (1 × 10^5) were seeded in 24-well plates and after 12 hours were transfected with Poly(I:C) (high molecular weight (HMW); 1.5–8 kb, CAT# 31852-29-6, InvivoGen) with Lipofectamine 2000 (CAT# 11668027, ThermoFisher), at the concentrations indicated in the figure legends, according to the manufacturer's protocol.

Bone marrow-derived macrophages (BMM) were prepared from the femurs of C57Bl6 mice as described previously (10). Briefly, the bone marrow cells were differentiated in culture for 7 days in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum (FBS), 20% L929-cell conditioned medium (as the source of murine macrophage colony-stimulating factor), with penicillin and streptomycin (10 units and 10 µg/mL, respectively). The differentiated BMM (1X10^6) were pre-incubated with 10 µM of VAL for 3 hours and then were transfected with 1 µg/mL Poly(I:C) (high molecular weight (HMW); 1.5–8 kb, InvivoGen) with Lipofectamine 2000 (ThermoFisher), according to the manufacturer's protocol, for 5 hours or mock transfected without removing VAL. The total RNA was isolated using RNA-isolation kit (CAT# BS88583, Bio Basic) and separated in RNA chips.

**siRNA transfections**

siRNA against ADAR1 (sc-37657) and corresponding control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology Inc. SiRNAs against NS2 (Integrated DNA Technologies) and GFP (Santa Cruz Biotechnology Inc.) were described previously and were shown to be effective in depleting cells of NS2 protein (8). A549 cells (5 x10^4) were seeded in 24 well plates. After 12 hours the cells were incubated without or with VAL (10µM) for 2 hours and transfected with siRNAs (30 nM) using Dharmafect 1 according to the manufacturer's protocol (Dharmacon).

**ADAR1 KO cell-based assays**

Cells were plated onto 24-well plates and transfected with 100 pM of siRNA NS2-1 (Integrated DNA Technologies) or siRNA NS2-2 (Integrated DNA Technologies) or siRNA GFP (Santa Cruz) using lipofectamine 2000 (8). At the time of transfection, cells
were treated with 20μM of VAL or mock treated and at 96 hours post transfection, cells were fixed with 4% paraformaldehyde and stained with crystal violet. For comparison of the effects of VAL, EA (CAT# E2250, Sigma-Aldrich) and Sunitinib Malate (SUTENT®) (Pfizer) on cell survival, WT A549 cells were treated with different concentrations of the compounds and cell survival was monitored by IncuCyte assays as a function of time.
Supplementary Fig. 1 (related to Fig. 1):
Dose response inhibition profile of ellagic acid against RNase L using a gel-based cleavage assay with FAM labeled RNA substrate.
Supplementary Fig. 2 (related to Fig. 3):
Inhibition of CK2 kinase activity by EA (A) and VAL (B). Experiments were performed in singlicate at Eurofins.
A

STD-NMR

5.0 μM RNase L + VAL (400 μM)

B

5.0 μM RNase L + 2-5A (20 μM) + VAL (400 μM)

C

5.0 μM RNase L + ATP/MgCl2 (20/100 μM) + VAL (400 μM)

D

5.0 μM RNase L + 2-5A + ATP/MgCl2 (20/100 μM) + VAL (400 μM)

E

Immobilized AviTagged-Biotinylated-RNase L

[VAL]
0.010 μM
0.025 μM
0.040 μM
0.060 μM
0.125 μM
0.250 μM
0.500 μM
1.000 μM
2.000 μM
4.000 μM
6.000 μM
8.000 μM
10.000 μM

F

Immobilized AviTagged-Biotinylated-RNase L + 2-5A

[VAL]
0.010 μM
0.025 μM
0.040 μM
0.060 μM
0.125 μM
0.250 μM
0.500 μM
1.000 μM
2.000 μM
4.000 μM
6.000 μM
8.000 μM
10.000 μM

G

Immobilized AviTagged-Biotinylated-RNase L + 2-5A + ATP-MgCl2

[VAL]
0.010 μM
0.025 μM
0.040 μM
0.060 μM
0.125 μM
0.250 μM
0.500 μM
1.000 μM
2.000 μM
4.000 μM
6.000 μM
8.000 μM
10.000 μM
Supplementary Fig. 3 (related to Fig.4):
Binding analysis of VAL to porcine RNase L using NMR and SPR.  
(A-D) The saturation transfer difference-NMR (STD-NMR) spectra for VAL recorded in the presence of the indicated concentrations of RNase L, 2-5A and ATP/MgCl2.  
(E-G) SPR dose dependent binding of VAL to immobilized RNase L WT. Representative sensograms shown for one of three replicate experiments. See Figure 4 E-G for extrapolated steady state binding profiles and binding constants (Kds).
A

CPMG-NMR

0.0 μM RNase L + VAL (400 μM)
2.0 μM RNase L + VAL (400 μM)
5.0 μM RNase L + VAL (400 μM)

STD-NMR

5.0 μM RNase L + VAL (400 μM)

B

CPMG-NMR

0.0 μM BSA + VAL (400 μM)
2.0 μM BSA + VAL (400 μM)
5.0 μM BSA + VAL (400 μM)

STD-NMR

5.0 μM BSA + VAL (400 μM)

0.0 μM Lysozyme + VAL (400 μM)
2.0 μM Lysozyme + VAL (400 μM)
5.0 μM Lysozyme + VAL (400 μM)

STD-NMR

5.0 μM Lysozyme + VAL (400 μM)

0.0 μM UBE2S + VAL (400 μM)
2.0 μM UBE2S + VAL (400 μM)
5.0 μM UBE2S + VAL (400 μM)

STD-NMR

5.0 μM UBE2S + VAL (400 μM)

0.0 μM IpaH 9.8 + VAL (400 μM)
2.0 μM IpaH 9.8 + VAL (400 μM)
5.0 μM IpaH 9.8 + VAL (400 μM)

STD-NMR

5.0 μM IpaH 9.8 + VAL (400 μM)
Supplementary Fig. 4 (related to Fig. 4):

(A) Detection of VAL binding to RNase L by NMR. **Left panels:** The dose-dependent CPMG-NMR spectra for VAL recorded in the presence of the indicated concentrations of RNase L, 2-5A and ATP/MgCl2. **Right panels:** The saturation transfer difference-NMR spectrum for VAL recorded in the presence of the indicated concentrations of RNase L, 2-5A and ATP/MgCl2. (B) Detection of VAL binding to four negative control proteins by NMR. **Left panels:** The dose-dependent CPMG-NMR spectra for VAL recorded in the presence of the indicated concentrations of bovine serum albumin (BSA), lysozyme, UBE2S, or IpaH 9.8. **Right panels:** The saturation transfer difference-NMR spectrum for VAL recorded in the presence of the indicated concentrations of bovine serum albumin (BSA), lysozyme, UBE2S, or IpaH 9.8.
A

**Immersed AviTagged-Biotinylated-RNase L**

![Graph showing RU response vs time for Immobilized AviTagged-Biotinylated-RNase L.]

B

**Immersed AviTagged-Biotinylated-RNase L + 2-5A**

![Graph showing RU response vs time for Immobilized AviTagged-Biotinylated-RNase L + 2-5A.]

C

**Immersed AviTagged-Biotinylated-RNase L + 2-5A + ATP-MgCl2**

![Graph showing RU response vs time for Immobilized AviTagged-Biotinylated-RNase L + 2-5A + ATP-MgCl2.]

Kd = 2.53 ± 0.09 µM

Kd = 2.06 ± 0.09 µM
Supplementary Fig. 5 (related to Fig.4):
Binding of SU to immobilized porcine RNase L using SPR.
(A-C) Dose dependent sensograms of the binding of SU to immobilized RNase L WT (left panels). Representative profiles shown for one of three replicate experiments. Steady state plot of SU binding to RNase L (right panels). Representative plot shown for one of three replicate experiments. Kd represents the average for n = 3 ± standard deviation.
Supplementary Fig. 6 (related to Fig. 4)
CETSA analysis of RNase L stabilization by VAL. Cell lysates from 293T cells overexpressing Flag-RNase L were treated with either 100 μM of VAL or DMSO carrier control solution and then subjected to heat treatment at the temperatures indicated. Flag-RNase L thermal stabilization was monitored by western blot using anti-Flag (M2) antibody. HSP90 was used as a loading control. A, B and C represent results from three independent biological replicates.
A

- **RNase L**
  - Tm = 33.9 ± 1.3 °C

- **RNase L + 2-5 A**
  - Tm = 34.2 ± 0.3 °C
  - ΔTm = 0.3 °C

- **RNase L + ATP/MgCl2**
  - Tm = 39.5 ± 0.07 °C
  - ΔTm = 5.6 °C

- **RNase L + 2-5 A + ATP/MgCl2**
  - Tm = 33.9 ± 1.3 °C
  - ΔTm = 0.3 °C

- **RNase L + VAL**
  - Tm = 34.2 ± 0.21 °C
  - ΔTm = 0.3 °C

- **RNase L + 2-5 A + ATP/MgCl2 + VAL**
  - Tm = 39.9 ± 0.2 °C
  - ΔTm = 6 °C

B

- **RNase L + RNA**
  - Tm = 33.9 ± 1.3 °C
  - ΔTm = -1.4 °C

- **RNase L + 2-5 A + ATP/MgCl2 + RNA**
  - Tm = 39.5 ± 0.2 °C

- **RNase L + 2-5 A + ATP/MgCl2 + RNA + VAL**
  - Tm = 38.9 ± 0.3 °C
Supplementary Fig. 7 (related to Fig.4):
Binding of ligands to porcine RNase L assessed using a fluorometric thermal denaturization assay.
(A) Binding analysis of 2-5A, ATP/MgCl2, and VAL (as indicated) to the ribonuclease dead RNase L mutant H680N assessed using a fluorometric thermal denaturization assay. Concentrations of the indicated ligands are as follows: 10 µM RNase L H680N, 10 µM 2-5A, 100 µM/5 mM ATP/MgCl2, and 100 µM VAL. Tm values corresponding to the midpoint of thermal denaturation transition represent the average for n = 3 ± standard deviation.
(B) Binding analysis of RNA substrate, 2-5A, ATP/MgCl2, and VAL (as indicated) to the ribonuclease dead RNase L mutant H680N assessed using a fluorometric thermal denaturization assay. Concentrations of ligands are as follows: 100 µM RNA substrate (other ligands as in A). Tm values corresponding to the midpoint of thermal denaturation transition represent the average for n = 3 ± standard deviation.
Supplementary Fig. 8 (related to Fig. 6)
Cross-species reactivity analysis of VAL on the ribonuclease activities of porcine and human RNase L in vitro. 1.6 nM of porcine RNase L and 2.0 nM of human RNase L were used. IC50 values represents mean ± SEM of three inhibition profiles.
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