ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Role of RIG-I RD and RD basic residues K858 and K861 and 5'-ppp on RNA for interaction of RIG-I with IVT DI RNA. *(A)* An overview of the RNA-protein complex pull-down *(B)* Lysates from 293T cells expressing HA-tagged WT RIG-I or K858A-K861A double mutant RIG-I were incubated with 0.25 μg of RNA, RIG-I-RNA complexes were immunoprecipitated, and RNA and protein fractions were isolated. RNA was transfected into 293T-IFNβ-FF-Luc cells and 48 hrs later IFNβ promoter driven luciferase activity was measured by luciferase assay. Protein fractions were subjected to immunoblotting using HA antibody to assess pull down efficiencies. Data is representative of two independent experiments.

Figure S2. RNA binding ELISA to study RIG-I RNA interactions. *(A)* A schematic of RNA binding ELISA procedure to study the ability of NeutrAvidin immobilized biotin-RNA to pull down HA-RIG-I from cell lysates or added purified HA-RIG-I and detection of bound RIG-I by HRP activity of an HA-HRP antibody. *(B)* 0.1 μg of untreated and phosphatase treated biotin-DI RNA were immobilized on NeutrAvidin wells and incubated with lysate from HA-RIG-I expressing cells. Levels of bound HA-RIG-I were assessed by measuring HRP activity of an HA-HRP conjugate. *(C)* DI RNA was treated with phosphatase as described in *SI Methods* and 2 ng of RNA was transfected into 293T-IFNβ-FF-Luc cells and 48 hrs later IFNβ promoter driven luciferase activity was measured by luciferase assay. *(D)* Biotin-labeled and unlabeled IVT DI RNA, 5 ng and 2-fold dilutions, were transfected into 293T-IFNβ-FF-Luc cells and 24 hrs later IFNβ promoter driven luciferase activity was measured by luciferase assay. Data is representative of 3 independent experiments and error-bars show mean ± SD.

Figure S3. Analysis of purified RIG-I WT and mutant proteins. *(A)* 80 ng of biotin-DI RNA was immobilized on NeutrAvidin wells and incubated with 0.5 μg of purified indicated RIG-I proteins. Bound RIG-I was detected using mouse α-human RIG-I antibodies and secondary α-mouse HRP antibodies (SIGMA). *(B)* 1μg of indicated purified RIG-I proteins were subjected to SDS-PAGE and Coomassie Brilliant Blue staining. A major band corresponding to the expected size of indicated tagged-RIG-I is visible in each lane. *(C)* 0.5 μg of purified HA-RIG-I (WT or D372N) was incubated with 100 ng of IVT DI RNA in presence of 0.5 mM ATP and 2.5 mM Mg^{2+} at 37°C for

25 minutes. Released phosphates were measured by a colorimetric ATPase assay at absorbance 620 nm. Data is representative of 3 independent experiments and error-bars indicate mean ± SD.

Figure S4. The sequences of WT and mutant DI RNAs (*Supplementary Materials and Methods*) were used to derive predicted structures using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The colors represent base-pair probability as indicated. The animated representations of the predicted structures were generated in Adobe Illustrator.

SUPPLEMENTAL MATERIALS AND METHODS

IFNβ promoter activation reporter assay: 293T cells stably expressing firefly luciferase gene under the control of IFNβ promoter (IFNβ-FF-Luc), cultured in 24 well plates with complete DMEM (Life) with 10% fetal bovine serum (Lonza) and 1X Pen Strep solution (Life) were transfected with indicated amounts of RNAs using 2 μl of lipofectamine 2000 (Life) in Opti-MEM reduced serum medium (Life) and media was replaced 6-12 hrs later with complete DMEM (Life) with 10% fetal bovine serum (Lonza). Twenty-four hours later, firefly luciferase activity was measured using a luciferase assay system (Promega) and a luminescence plate reader (Biotek) following manufacturer's instructions.

RNAs:

In vitro generation of Sendai DI RNA and derived mutant RNAs

The DNA templates containing a modified T7 RNA polymerase promoter (TAATACGACTCACTATA), followed by Sendai DI/mutant template, HDV ribozyme and T7 RNA polymerase terminator were synthesized by Genewiz in pUC57 with flanking HindIII and NotI restriction sites:

WT DI RNA:

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGACTATCTTTATCTAT
GTCCACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAAGCAAGACTTCAATTGG
GAATAGTTTCATTATCATCCCGTGAAATCAGGAACCTGAGGGTTATCACAAAAACTTTATTAG
ACAGGTTTGAGGATATTATACATAGTATAACGTATAGATTCCTCACCAAAGAAATAAAGATTTT
GATGAAGATTTTAGGGGCAGTCAAGATGTTCGGGGCCAGGCAAAATGAATACACGACCGTG
ATTGATGATGGATCACTGGGTGATATCGAGCCATATGACAGCTCGTAATAATTAGTCCCTATC
GTGCAGAACGATCGAAGCTCCGCGGTACCTGGAAGTCTTGGACTTATCCATATGACAATAGT
AAGAAAAACTTACAAGAAGACAAGAAAATTTAAAAGGATACATATCTCTTAAACTCTTGTCTGG
TGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAAG
GGAGAAGTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGGCGGCCG
CG

5'-overhang DI RNA:

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGACTATCTTTATCTAT
GTCCACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAAGCAAGACTTCAATTGG
GAATAGTTTCATTATCATCCCGTGAAATCAGGAACCTGAGGGTTATCACAAAAACTTTATTAG
ACAGGTTTGAGGATATTATACATAGTATAACGTATAGATTCCTCACCAAAGAAATAAAGATTTT
GATGAAGATTTTAGGGGCAGTCAAGATGTTCGGGGCCAGGCAAAATGAATACACGACCGTG

ATTGATGATGGATCACTGGGTGATATCGAGCCATATGACAGCTCGTAATAATTAGTCCCTATC
GTGCAGAACGATCGAAGCTCCGCGGTACCTGGAAGTCTTGGACTTATCCATATGACAATAGT
AAGAAAAACTTACAAGAGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCT
ACTTCGGTAGGCTAAGGGAGAAGTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
GGTTTTTTGGCGGCCGCG

<u>Δ terminal base-pairing DI RNA:</u>

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGACTATCTTTATCTAT
GTCCACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAAGCAAGACTTCAATTGG
GAATAGTTTCATTATCATCCCGTGAAATCAGGAACCTGAGGGTTATCACAAAAACTTTATTAG
ACAGGTTTGAGGATATTATACATAGTATAACGTATAGATTCCTCACCAAAGAAATAAAGATTTT
GATGAAGATTTTAGGGGCAGTCAAGATGTTCGGGGCCAGGCAAAATGAATACACGACCGTG
ATTGATGATGGATCACTGGGTGATATCGAGCCATATGACAGCTCGTAATAATTAGTCCCTATC
GTGCAGAACGATCGAAGCTCCGCGGTACCTGGAAGTCTTGGACTTATCCATATGACAATAGT
AAGAAAAACTTACAAGAAGACAAGAAAATTTAAAACCTATGTATAGAGAATTTGAGAACAGAC
CAGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAA
GGGAGAAGTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGGCGGCC
GCG

<u>46 bp Stem DI RNA:</u>

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTATCTTTATCTATGTCCACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAA
GCAAGACTTCAATTGGGAATAGTTTCATTATCATCCCGTGAAATCAGGAACCTGAGGGTTATC
ACAAAAACTTTATTAGACAGGTTTGAGGATATTATACATAGTATAACGTATAGATTCCTCACCA
AAGAAATAAAGATTTTGATGAAGATTTTAGGGGCAGTCAAGATGTTCGGGGCCAGGCAAAAT
GAATACACGACCGTGATTGATGATGGATCACTGGGTGATATCGAGCCATATGACAGCTCGTA
ATAATTAGTCCCTATCGTGCAGAACGATCGAAGCTCCGCGGTACCTGGAACAAGAAAATTTA
AAAGGATACATATCTCTTAAACTCTTGTCTGGTGGGTCGGCATGGCATCTCCACCTCCTCGC
GGTCCGACCTGGGCTACTTCGGTAGGCTAAGGGAGAAGTAGCATAACCCCTTGGGGCCTCT
AAACGGGTCTTGAGGGGTTTTTTGGCGGCCGCG

<u>25 bp Stem DI RNA:</u>

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGATCTTTATCTATGTCC
ACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAAGCAAGACTTCAATTGGGAATA
GTTTCATTATCATCCCGTGAAATCAGGAACCTGAGGGTTATCACAAAAACTTTATTAGACAGG
TTTGAGGATATTATACATAGTATAACGTATAGATTCCTCACCAAAGAAATAAAGATTTTGATGA
AGATTTTAGGGGCAGTCAAGATGTTCGGGGCCAGGCAAAATGAATACACGACCGTGATTGAT
GATGGATCACTGGGTGATATCGAGCCATATGACAGCTCGTAATAATTAGTCCCTATCGTGCA
GAACGATCGAAGCTCCGCGGTACCTGGACATATCTCTTAAACTCTTGTCTGGTGGGTCGGCA
TGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAAGGGAGAAGTAG
CATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGGCGGCCGCG

<u>Half-loop DI RNA:</u>

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGACTATCTTTATCTAT
GTCCACAAGATTGGTAACTGGGTCATTCCCTGACCAGAAGTTTGAAGCAAGACTTCAATTGG
GAATAGTGGGT<u>GATATC</u>GAGCCATATGACAGCTCGTAATAATTAGTCCCTATCGTGCAGAAC
GATCGAAGCTCCGCGGTACCTGGAAGTCTTGGACTTATCCATATGACAATAGTAAGAAAAAC
TTACAAGAAGACAAGAAAATTTAAAAGGATACATATCTCTTAAACTCTTGTCTGGTGGGTCGG
CATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAAGGGAGAAGT
AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGGCGGCCGCG

<u>Short-loop DI RNA:</u>

AAGCTTTAATACGACTCACTATAACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAATTTT
CTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGACT**TTCAAGAAGA**A
GTCTTGGACTTATCCATATGACAATAGTAAGAAAAACTTACAAGAAGACAAGAAAATTTAAAA
GGATACATATCTCTTAAACTCTTGTCTGGTGGGTCGGCATGGCATCTCCACCTCCTCGCGGT

CCGACCTGGGCTACTTCGGTAGGCTAAGGGAGAAGTAGCATAACCCCTTGGGGCCTCTAAA
CGGGTCTTGAGGGGTTTTTTGGCGGCCGCG

The T7 RNA polymerase promoter was truncated (TAATACGACTCACTATA) to prevent addition of two non-templated guanines at the 5' end of DI RNA. The templates were linearized using HindIII and NotI (NEB) and 200 ng was used in *in vitro* transcription reactions using Ampliscribe T7-*Flash* Transcription kit (Epicentre-Illumina), following manufacturer's instructions. Similarly, Biotin-UTP labeled RNA was generated *in vitro* using Ampliscribe T7-Flash Biotin-RNA transcription kit (Epicentre, Illumina). RNA was purified using RNeasy columns (Qiagen) as instructed by manufacturer to remove unincorporated NTPs. Predicted structures of these RNAs are shown in Fig. S4. To remove 5'-terminal phosphates, 500 ng of WT IVT DI RNA was treated with 2 U of rAPid alkaline phosphatase (Roche) for 30 minutes at 37°C in supplied 1x phosphatase buffer. Influenza PR8 RNA was generated by infecting confluent A549 cells at high MOI and harvesting total RNA from cells at 24 hours post infection. poly I:C and synthetic 19-mer 5'-ppp dsRNA were purchased from Invivogen. To test RNAse susceptibility of dsRNA in RIG-I-RNA oligomers, 1 μg RIG-I and 0.2 μg RNA were allowed to form complexes at 37°C for 15 minutes, in presence or absence of 1 mM ATP and 2.5 mM Mg^{2+} in protein buffer, and 0, 0.01 and 0.1 units of RNase V1 (Ambion) was added directly to reactions and incubated at RT for 15 min before analyzing the complexes by native PAGE and RIG-I immunoblotting (Fig. 4I).

Co-immunoprecipitation of RIG-I: 293T cells transfected with pCAGGS plasmids expressing HA-RIG-I and eYFP-RIG-I using lipofectamine 2000 were lysed with PLB, spin-clarified, pre-cleared with protein G agarose beads, and incubated with 0.2 or 1 μg of WT DI RNA or stem deletion mutant RNAs in NT2 buffer supplemented with RNase inhibitor (Ambion) and 1 mM DTT for 1 hr at 4°C. Protein-RNA complexes were pulled down with GFP antibody (Abcam) and immunoblot was performed using GFP and HA (Abcam) antibodies.

Protein purification: 293T cells in 100 mm dishes were transfected with His-HA-RIG-I plasmid using lipofectamine 2000, and cells were collected 48 hrs later in resuspension buffer pH 8.0, 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole. Samples were sonicated briefly on ice and spun at 5000 rpm for 45 minutes at 4°C. Clarified lysates were subjected to Nickel-NTA affinity purification using Ni-NTA resin (Qiagen). Proteins were eluted with resuspension buffer with 250 mM imidazole. Eluted proteins were concentrated and buffer was exchanged with buffer containing 50 mM Tris 7.4, 100 mM NaCl, and 1 mM DTT (protein buffer) using 30 kDa Amicon ultracentrifugation filters (Millipore) following manufacturer's instructions.

Fig. S1

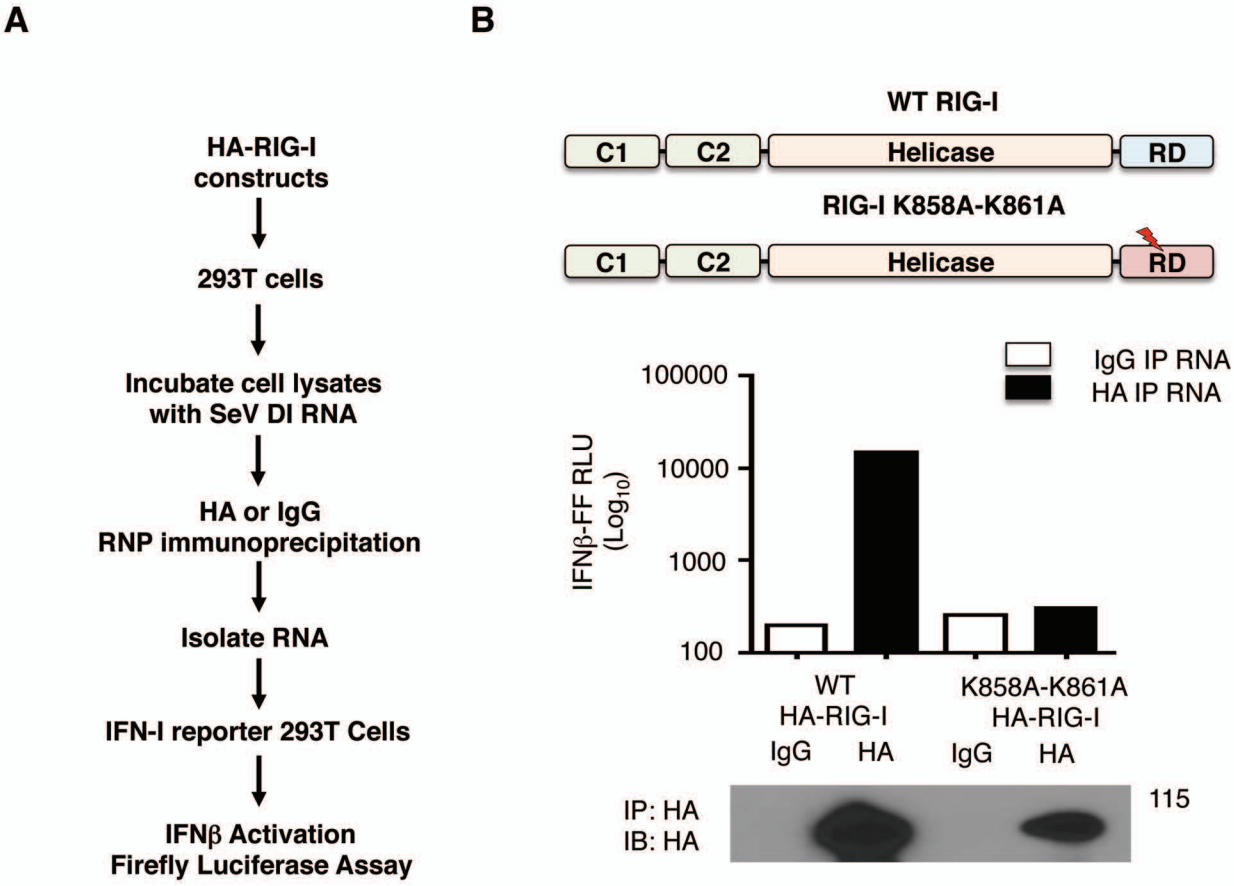

A
HA-RIG-I constructs
293T cells
Incubate cell lysates with SeV DI RNA
HA or IgG RNP immunoprecipitation
Isolate RNA
IFN-I reporter 293T Cells
IFNβ Activation Firefly Luciferase Assay
B
WT RIG-I
C1
C2
Helicase
RD
RIG-I K858A-K861A
C1
C2
Helicase
RD
IgG IP RNA
HA IP RNA
IFNβ-FF RLU (Log10)
100000
10000
1000
100
WT HA-RIG-I
K858A-K861A HA-RIG-I
IgG
HA
IgG
HA
IP: HA
IB: HA
115

Fig. S2

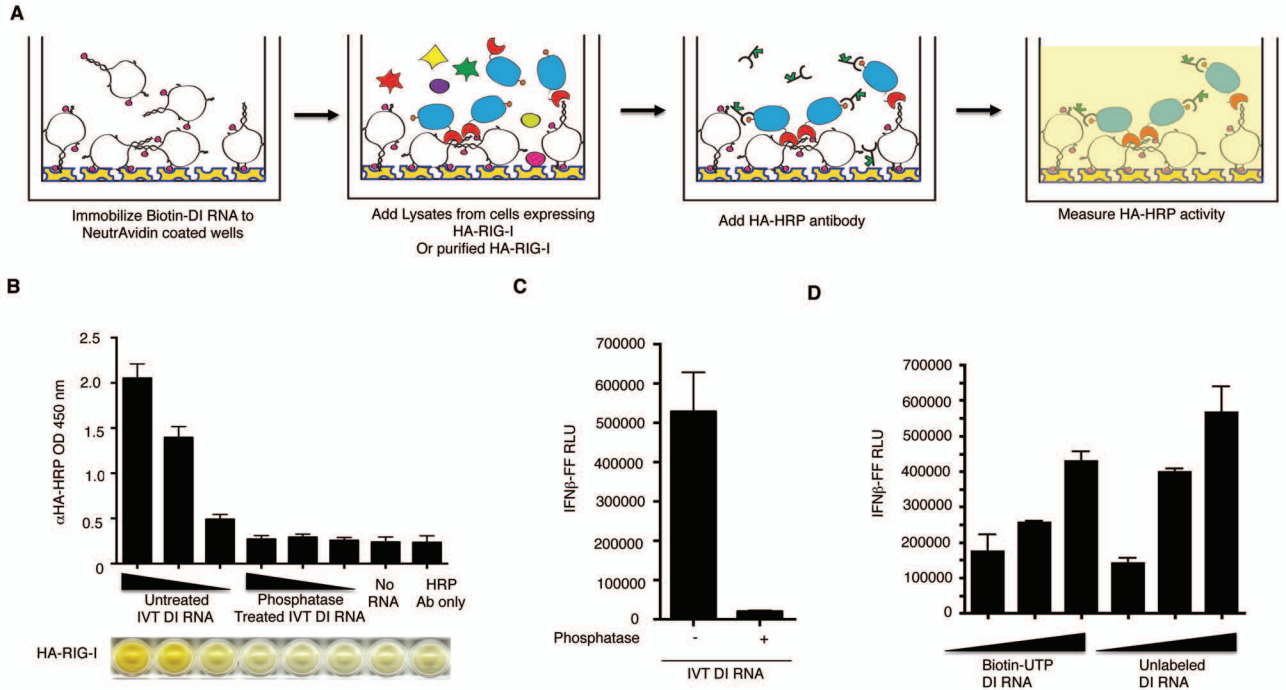

A
Immobilize Biotin-DI RNA to NeutrAvidin coated wells
Add Lysates from cells expressing HA-RIG-I Or purified HA-RIG-I
Add HA-HRP antibody
Measure HA-HRP activity
B
αHA-HRP OD 450 nm
2.5
2.0
1.5
1.0
0.5
0
Untreated IVT DI RNA
Phosphatase Treated IVT DI RNA
No RNA
HRP Ab only
HA-RIG-I
C
IFNβ-FF RLU
700000
600000
500000
400000
300000
200000
100000
0
Phosphatase
-
+
IVT DI RNA
D
IFNβ-FF RLU
700000
600000
500000
400000
300000
200000
100000
0
Biotin-UTP DI RNA
Unlabeled DI RNA

Fig. S3

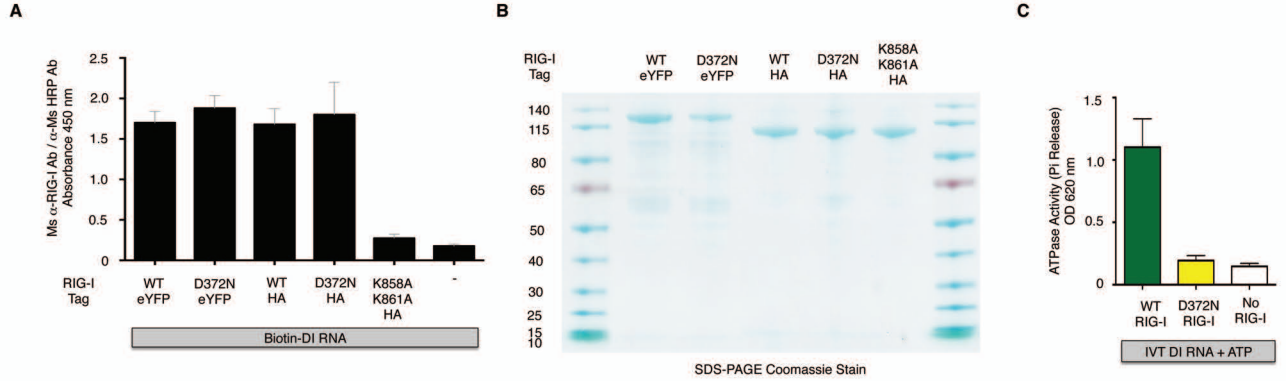
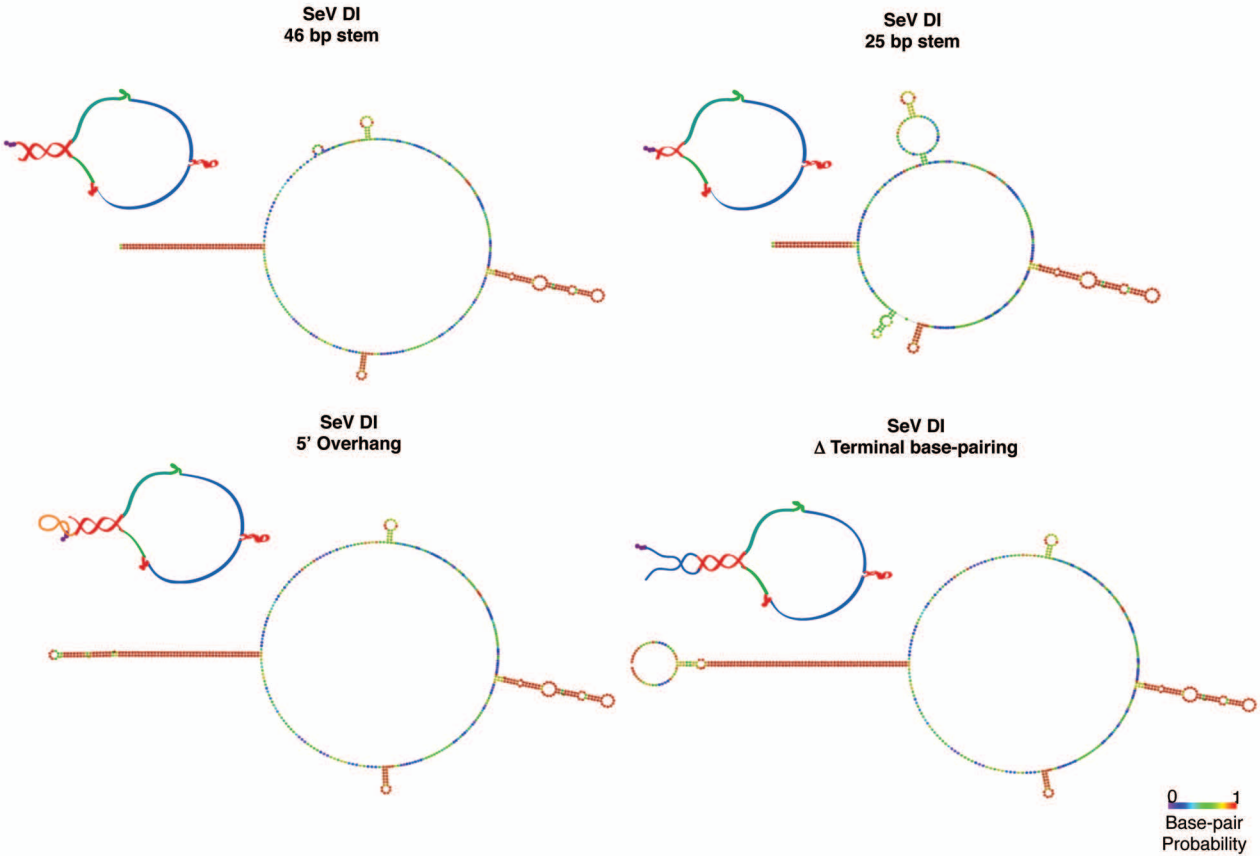

A
Ms α-RIG-I Ab / α-Ms HRP Ab
Absorbance 450 nm
2.5
2.0
1.5
1.0
0.5
0
RIG-I Tag
WT eYFP
D372N eYFP
WT HA
D372N HA
K858A K861A HA
-
Biotin-DI RNA
B
RIG-I Tag
WT eYFP
D372N eYFP
WT HA
D372N HA
K858A K861A HA
140
115
80
65
50
40
30
25
15
10
SDS-PAGE Coomassie Stain
C
ATPase Activity (Pi Release)
OD 620 nm
1.5
1.0
0.5
0
WT RIG-I
D372N RIG-I
No RIG-I
IVT DI RNA + ATP

Fig. S4


SeV DI
46 bp stem
SeV DI
25 bp stem
SeV DI
5' Overhang
SeV DI
Δ Terminal base-pairing
0
1
Base-pair
Probability