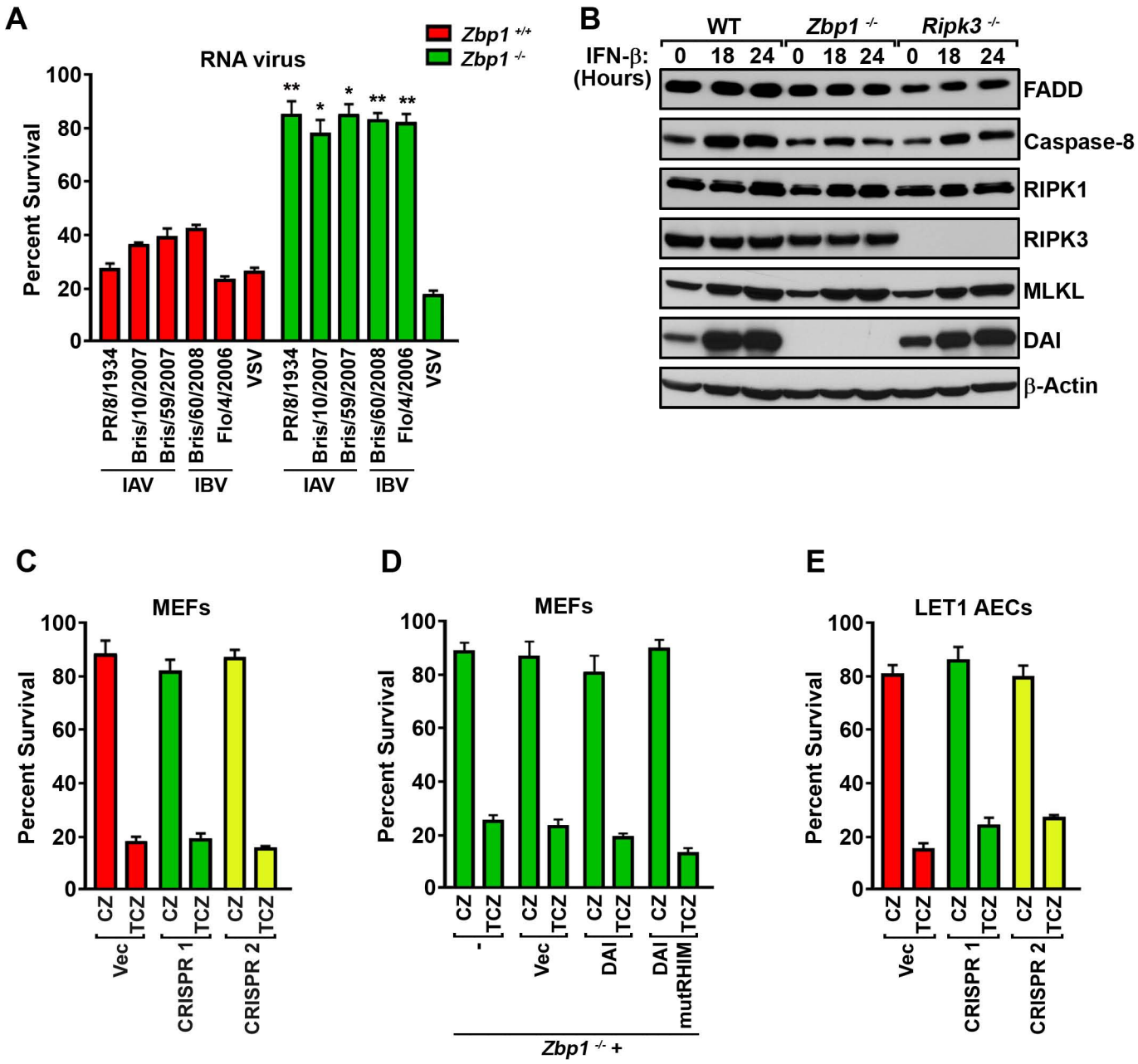

Figure S1, related to Figure 1. DAI is selectively required for IAV-and IBV- induced cell death in MEFs and airway epithelial cells.

(A) *Zbp1*$^{+/+}$ or *zbp1*$^{-/-}$ MEFs were infected with IAV strains PR8, Brisbane/10/2007, Brisbane/59/2007, IBV strains Brisbane/60/2008 and Florida/4/2006, and vesicular stomatitis virus (VSV) (MOI=2). Cell viability was measured 24 hr p.i. **(B)** Whole cell extracts from WT, *zbp1*$^{-/-}$ and *ripk3*$^{-/-}$ MEFs treated with IFN-β for the indicated times were examined for FADD, Caspase-8, RIPK1, RIPK3, MLKL and DAI. β-actin was used as a loading control. **(C)** WT MEFs expressing empty vector (Vec) or WT MEFs in which DAI expression was ablated by CRISPR/Cas9 targeting of the sequences 5'-TCTGGAGTCACACAAGAGTCCCCT-3' (CRISPR 1) or 5'-GCTCAGTACATCTACATGGACAAGTCCTG-3' (CRISPR 2) in the murine *zbp1* gene were treated with CZ or TCZ and cell viability was measured 24 hr p.i. **(D)** *Zbp1*$^{-/-}$ MEFs reconstituted with empty vector (Vec), full length murine DAI (DAI), or DAI with a mutated RHIM domain (DAI mutRHIM) were treated with CZ or TCZ and cell viability was measured 24 hr p.i. **(E)** LET1 cells in which DAI expression was ablated by CRISPR/Cas9 targeting of the murine *zbp1* gene, using sequences described in C, were treated with CZ or TCZ and cell viability was measured 24 hr p.i. Viability data shown in this figure are representative of at least three independent experiments. Error bars represent mean +/- SD. *p<0.05, **p<0.005

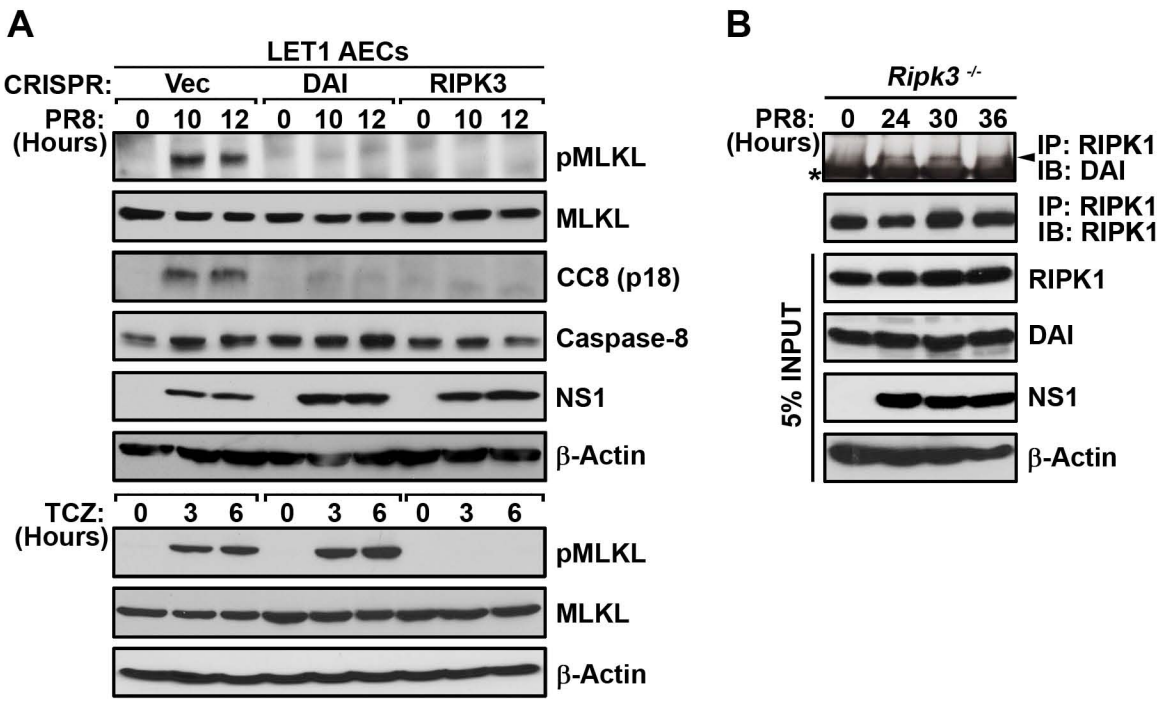

Figure S2, related to Figure 2. DAI is required for activation of RIPK3 in LET1 AECs and associates with RIPK1 in the absence of RIPK3.

(A) LET1 cells expressing empty vector (Vec), or LET1 cells in which *zbp1* or *ripk3* were ablated by CRISPR/Cas9 targeting were infected with PR8 (MOI=2, top) or treated with TCZ (bottom) and whole-cell extracts were examined for phosphorylated MLKL and cleaved caspase-8 (CC8) at the indicated times **(B)** *Ripk3*$^{-/-}$ MEFs were infected with PR8 (MOI=2) and cells were lysed at the indicated time points. Anti-RIPK1 immunoprecipitates were examined for DAI and RIPK1 as previously described (Thapa et al, 2013). Arrowhead shows DAI, and * indicates IgG heavy chain. Whole cell extract (5% input) was examined in parallel for RIPK1, DAI and IAV NS1 proteins. β-actin was used as a loading control.

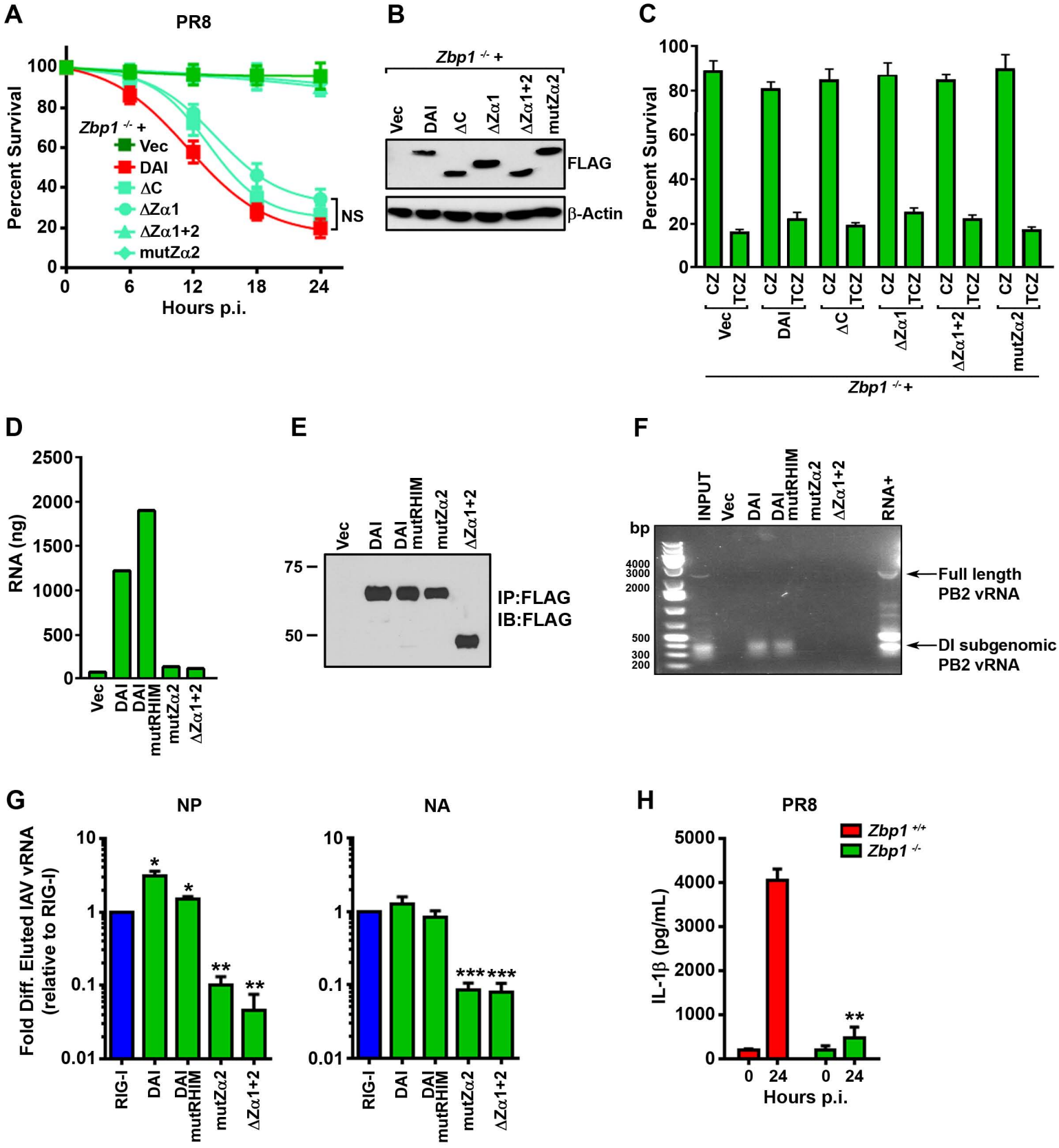
A
PR8
Percent Survival
Hours p.i.
Zbp1 -/- +
Vec
DAI
ΔC
ΔZα1
ΔZα1+2
mutZα2
NS
B
Zbp1 -/- +
Vec
DAI
ΔC
ΔZα1
ΔZα1+2
mutZα2
FLAG
β-Actin
C
Percent Survival
CZ
TCZ
Vec
DAI
ΔC
ΔZα1
ΔZα1+2
mutZα2
Zbp1 -/- +
D
RNA (ng)
Vec
DAI
DAI mutRHIM
mutZα2
ΔZα1+2
E
Vec
DAI
DAI mutRHIM
mutZα2
ΔZα1+2
75
50
IP:FLAG
IB:FLAG
F
INPUT
Vec
DAI
DAI mutRHIM
mutZα2
ΔZα1+2
RNA+
bp
4000
3000
2000
500
300
200
Full length PB2 vRNA
DI subgenomic PB2 vRNA
G
NP
NA
Fold Diff. Eluted IAV vRNA (relative to RIG-I)
RIG-I
DAI
DAI mutRHIM
mutZα2
ΔZα1+2
H
PR8
Zbp1 +/+
Zbp1 -/-
IL-1β (pg/mL)
Hours p.i.

Figure S3, related to Figures 3 and 4. DAI senses IAV RNA and activates RIPK3 in a manner requiring its Zα2 domain.

(A) Immortalized *zbp1*$^{-/-}$ MEFs reconstituted with WT DAI or its mutants were infected with PR8 (MOI=2) and cell viability was determined at the indicated times p.i. Data are representative of at least three independent experiments. **(B)** Expression levels of FLAG-tagged constructs in immortalized *zbp1*$^{-/-}$ MEFs reconstituted with wild-type (WT) DAI or its mutants. **(C)** Responsiveness to TCZ-induced necroptosis in immortalized *zbp1*$^{-/-}$ MEFs reconstituted with WT DAI or its mutants. **(D)** Quantification of RNA eluted from anti-FLAG immunoprecipitates of the indicated FLAG-DAI constructs from PR8-infected 293T cells 2 hr pi. Data are representative of three independent experiments. **(E)** Expression of FLAG-tagged DAI constructs in FLAG-immunoprecipitates from PR8-infected 293T cells, from which IAV RNA was eluted for panels D, F and G. **(F)** PCR detection of PB2 DI sub-genomic vRNA in eluted RNA from FLAG-DAI immunoprecipitates, using primers specific for the vRNA ends of the PB2 segment. RNA^{+}: A549 cells infected (MOI=3) with PR8. **(G)** RNA eluted from the indicated FLAG-construct pulldowns from IAV (12 hr)-infected 293T cells were examined by RT-qPCR using NA and NP segment-specific primers. Data were normalized to vRNA from similar FLAG-RIG-I immunoprecipitates. **(H)** *Zbp1*$^{+/+}$ or *Zbp1*$^{-/-}$ BMDMs were infected with PR8 (MOI=2) and levels of IL-1β were determined 18 hr p.i. by ELISA. Data are representative of two independent experiments. Error bars represent mean +/- SD. *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

Supplemental Experimental Procedures.

Mice, cells, viruses, and reagents. *Zbp1*$^{-/-}$ (Ishii et al., 2008), *ripk3*$^{-/-}$ (Newton et al., 2004), *mlkl*$^{-/-}$*fadd*$^{-/-}$ (Nogusa et al., 2016) and *ripk1*$^{-/-}$*ripk3*$^{-/-}$ double knock out (Dillon et al., 2014) cells and mice have been described previously. Mice were housed in SPF facilities at the Fox Chase Cancer Center and the University of Texas, Austin, and all *in vivo* experiments were conducted under protocols approved by the Committee on Use and Care of Animals at these institutions. Primary MEFs were generated from E14.5 embryos and used within five passages in experiments. For stable reconstitution studies, FLAG-DAI constructs were cloned into the pQCXIH retroviral vector (Clontech), and retrovirus production, infection, and selection of DAI-expressing *zbp1*$^{-/-}$ MEFs, immortalized by passaging on a 3T3 protocol for these studies, were performed as previously described (Upton et al., 2012). LET1 cells have been described before (Rosenberger et al., 2014). *Zbp1* was targeted for CRISPR/Cas9-mediated ablation in MEFs and LET1 cells using the sgRNA sequences 5'-TCTGGAGTCACACAAGAGTCCCCT-3' (CRISPR 1) or 5'-GCTCAGTACATCTACATGGACAAGTCCTTG-3' (CRISPR 2). Cell viability was determined by trypan blue exclusion or by the Cell Titer Glo kit (Clontech). Influenza virus A/Puerto Rico/8/34 (PR8) was generated by reverse genetics as previously described (Hoffmann et al., 2002). All IAV and IBV strains were propagated by allantoic inoculation of embryonated hen's eggs with diluted ($1:10^6$) seed virus. Stock virus titers were determined as 50% egg infectious dose (EID_{50}). Recombinant PR8-GFP virus has been described before (Manicassamy et al., 2010). Plaque-purified VSV (Indiana) was propagated in BHK-21 cells. Cell culture, *in vivo* infections, and lung virus titers were performed as described previously (Nogusa et al, 2016). For detection of IL-1β, bone marrow derived macrophages (BMDMs) plated in six-well plates were primed with *E.coli* LPS (10ng/ml; Sigma) for 24 hr. Following priming, BMDMs were infected with PR8 (MOI=2) for 18 hr, and supernatants from these cells were examined for IL-1β by ELISA (R&D Systems). Anti-murine DAI antibody (clone Zippy-1) was obtained from Millipore and Adipogen. All other biological and chemical reagents were obtained from sources described previously (Nogusa et al, 2016).

Molecular modeling. The murine DAI Zα2:Z-RNA complex was modeled based on the template of human ADAR Zα1 bound to Z-RNA (PDB code 2GXB, 33% identity, 53% similarity). DAI Zα2 was aligned with the HHpred portion of the MPI bioinformatics Toolkit (Alva et al., 2016), and a model built using MODELLER (Sali et al., 1995). Mouse DAI Zα2 dimers were superposed on the corresponding domains of the ADAR1 Zα1:Z-RNA structure using the UCSF Chimera software (Pettersen et al., 2004), and the Z-RNA co-ordinates were copied over to make the final model. Side chain rotamers were also considered using the SCWRL4 frame option (Krivov et al., 2009) to account for steric packing to RNA.

Purification and analysis of DAI-associated IAV RNA. HEK 293T cells seeded in 10 cm dishes and transfected with FLAG-tagged RIG-I or DAI constructs for 24 hr were infected with PR8 (MOI=2) for 12 hr and disrupted in 1 mL lysis buffer (50 mM HEPES, 150 mM KCl, 2 mM EDTA, 1mM NaF, 0.5% NP40, 0.5 mM DTT, protease inhibitor cocktail, 25 units RNasin). Aliquots of lysates were saved for total RNA input control and immunoblot analysis. Lysates were then incubated with 30 μL/sample anti-FLAG agarose bead slurry (Clone M2, Sigma-Aldrich) overnight with rotation at 4^oC. Beads were collected by centrifugation, washed ten times with NT2 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP40), resuspended in 250 μL of DNase digestion buffer (40 mM Tris pH 8.0, 10 mM $MgSO_4$, 1 mM $CaCl_2$) and treated with 25U RNasin (Promega) and 2U DNAse I (NEB) at 37°C for 20 min.

Beads were again collected by centrifugation, washed with NT2, and resuspended in 100 μL NT2 buffer. 10% of each sample was removed for immunoblot analysis. Samples were treated with 4 units proteinase K at 55°C for 30 minutes. 1 mL Tri-reagent (Sigma-Aldrich) was added to each sample, and RNA was harvested according to the manufacturer's instructions.

For RNA Seq analyses, RNA was prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina). Briefly, total RNA was depleted of ribosomal RNA (Ribo-Zero rRNA removal kit, Illumina), fragmented, and reverse transcribed into cDNA using reverse transcriptase and random primers. Following second strand cDNA synthesis by DNA Polymerase I in the presence of RNase H, an adenine was added to the 3'-end and specific Illumina adapters were ligated to cDNAs. The ligation products were purified and enriched by PCR to create the final cDNA library, which was loaded on the MiSeq platform (Illumina) with Illumina-provided indexed primers. Bowtie2 was used to map reads to the PR8 genome.

For PCR detection of DI particle genomes, RNA was reverse transcribed into cDNA using reverse transcriptase (SuperScript® II RT, ThermoFisher) with a universal genomic viral RNA (vRNA) primer (5-AGCAAAAGCAGG-3), per manufacturer's instructions. PCR was then performed using the following primers PB2: 5'-ATGGAAAGAATAAAAGAACTAAG-3', and 5'CTAATTGATGGCCATCCGAATTC-3'. For detection of individual vRNA segments by RT-qPCR, RNA was reverse-transcribed into cDNA using the iScript Select cDNA Synthesis kit (Bio-Rad) and the following vRNA specific-primers: NP: 5' - GGCCGTCATGGTGGCGAATGAATGGACGAAAAACAAGAATTGC - 3' and NA: 5' - GCCGTCATGGTGGCGAATACTATAATGACTGATGGCCC - 3') (Kawakami et al., 2011). For qPCR, the following segment-specific primers were used: NP: 5'-GGCCGTCATGGTGGCGAAT-3', 5'- CTCAATATGAGTGCAGACCGTGCT-3'. NA: 5'-GCCGTCATGGTGGCGAAT-3', 5'- ACATCACTTTGCCGGTATCAGGGT-3'. Fold difference in segment-specific vRNA levels relative to RIG-I were determined using the following equation: $2^{-\Delta\Delta Ct} = [(Ct_{\text{sample pulldown}} - Ct_{\text{sample input}})] - [(Ct_{\text{RIG-I pulldown}} - Ct_{\text{RIG-I input}})]$ (Schmittgen and Livak, 2008).

Statistics. Statistical significance was determined by use of either Student's *t*-test or ANOVA. Significance of *in vivo* survival data was determined by the log-rank (Mantel-Cox) test. *P*-values of 0.05 or lower were considered significant. Graphs were generated using GraphPad Prism 6.0 software.

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