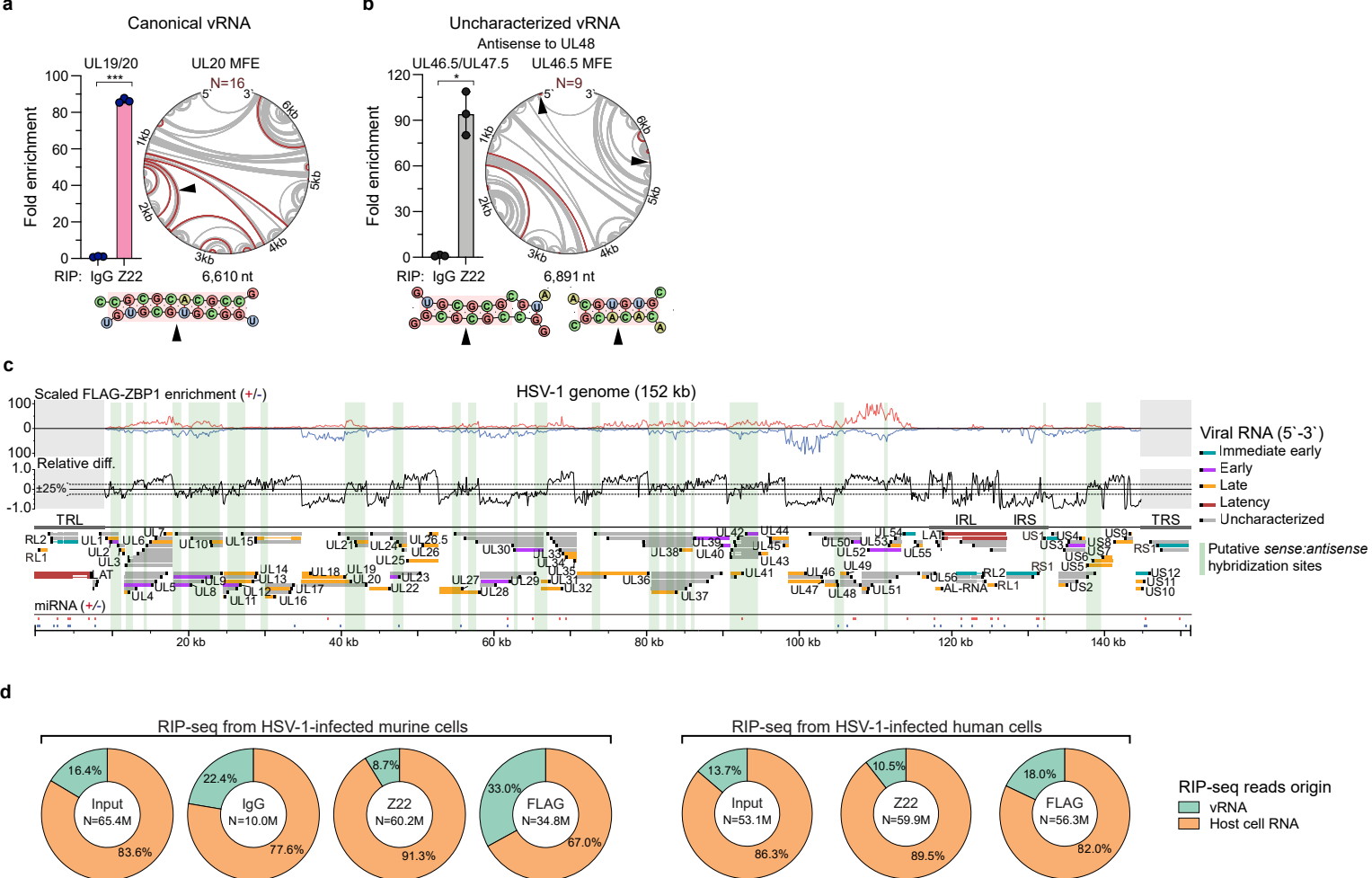
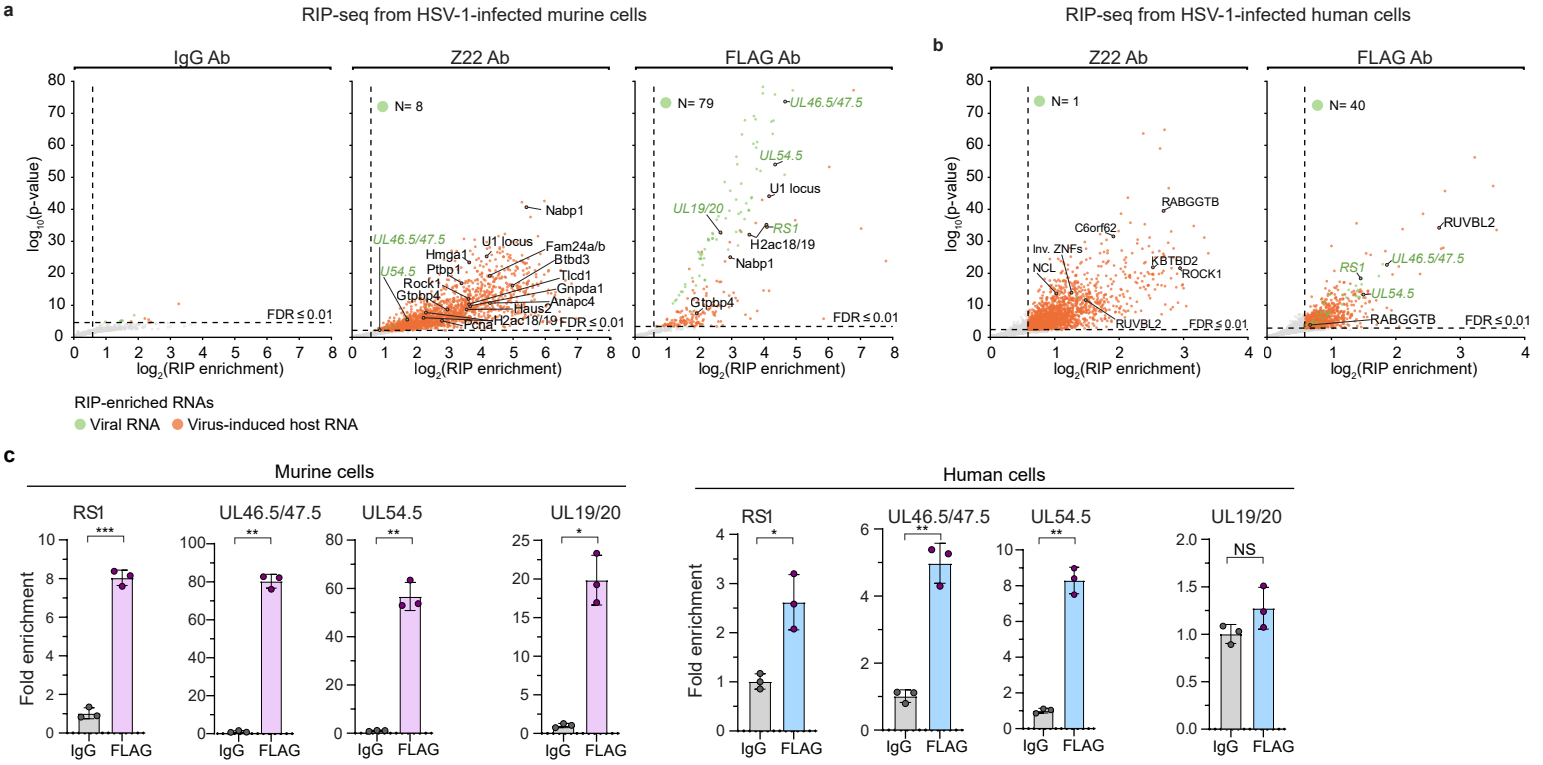

Supplementary information

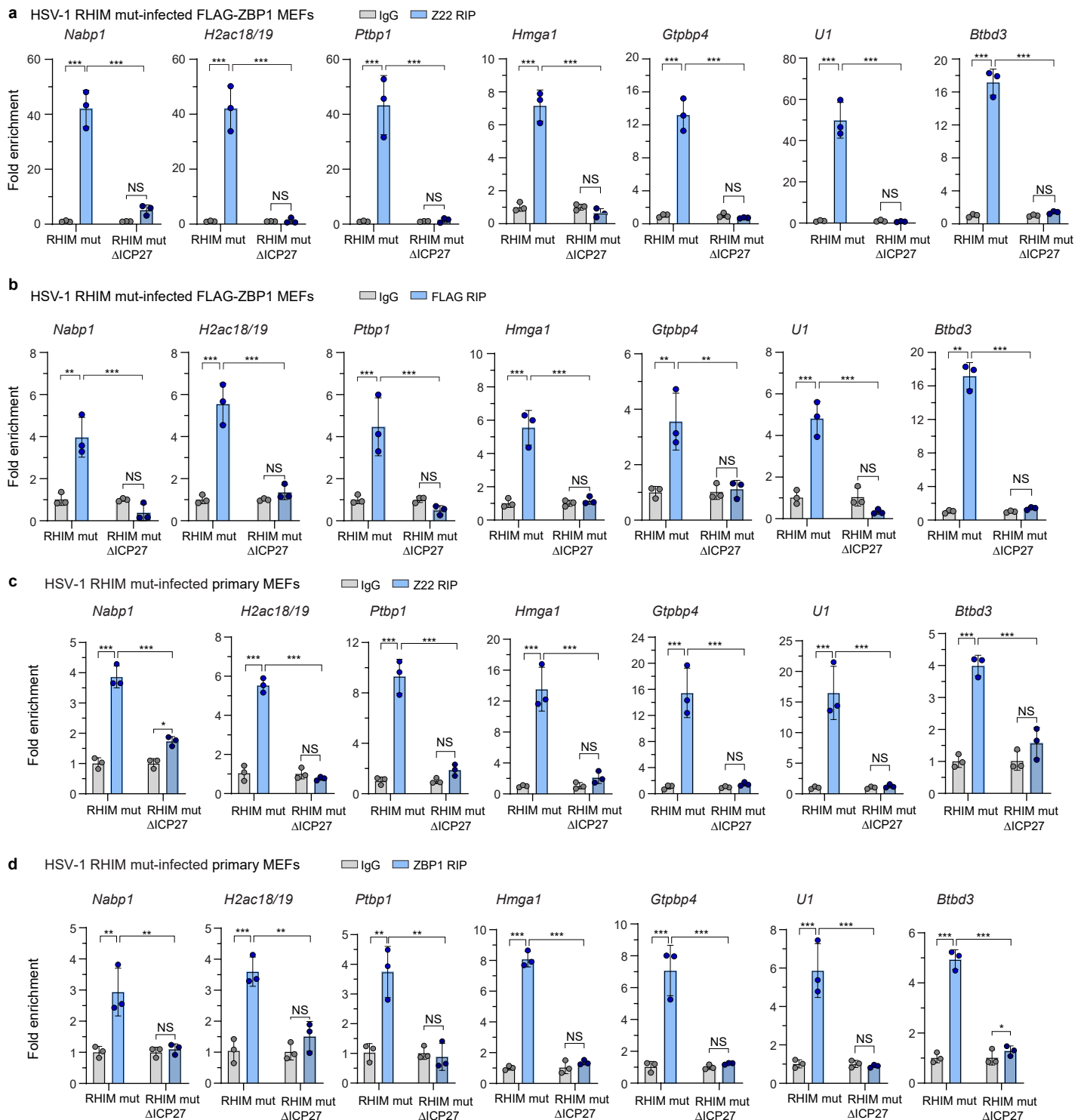
Host cell Z-RNAs activate ZBP1 during virus infections

In the format provided by the
authors and unedited





Supplementary Figure 2. (Related to Fig. 2.) Characterization of HSV-1 generated Z-RNAs.
a, b, Enrichment of viral (green) and virus-induced endogenous (orange) RNAs in RIP-seq experiments from HSV-1-infected FLAG-ZBP1-expressing MEFs (**a**) or HT-29 cells (**b**). Viral or host RNAs assessed by qPCR are labeled. The number of enriched viral hits in these pull-downs are indicated within each scatterplot.
c, qPCR validation of indicated RNAs significantly enriched in both mouse and human FLAG-ZBP1 RIP-seq experiments (RS1, UL46.5/47.5, UL54.5), or enriched only in mouse FLAG-ZBP1 RIP-seq (UL19/20). Data were normalized to Input.
 Data are mean ± s.d. (n=3 biologically independent samples per group in c). Two-tailed unpaired t-test with Welch's correction (c).
 *P < 0.05, **P < 0.005, ***P < 0.0005. NS, no significance.

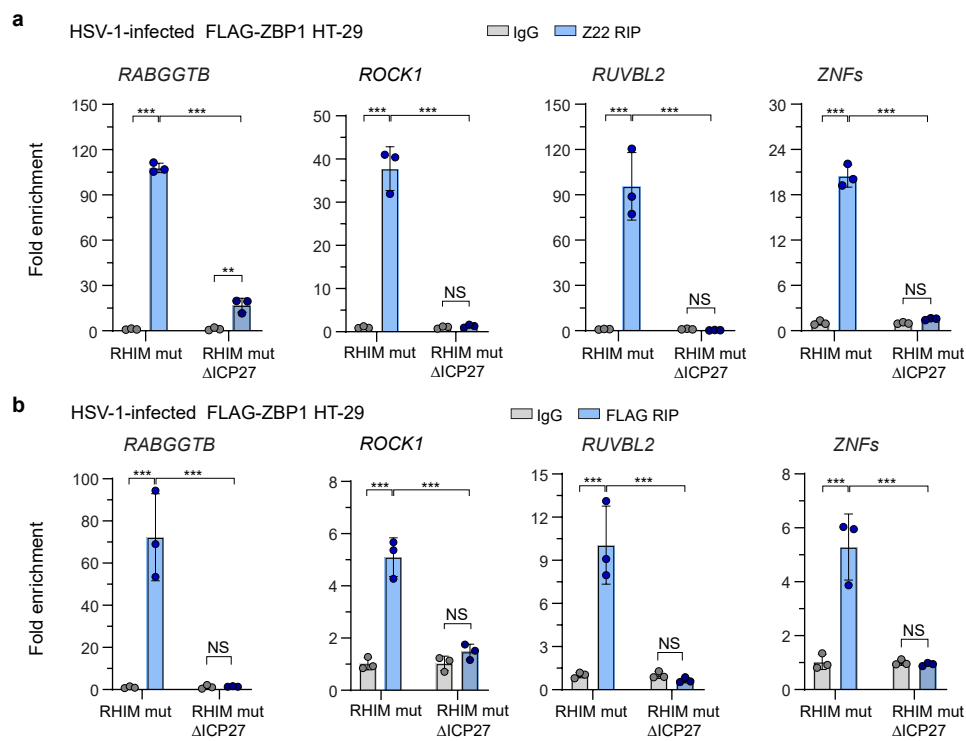


Supplementary Figure 3. (Related to Figure 3.) Deleting ICP27 attenuates HSV-1-induced host cell Z-RNA formation in MEFs.

a, b, RNA eluted from Z22- (**a**), anti-FLAG antibody- (**b**) or control IgG antibody pulldowns from HSV-1 RHIM mut (MOI=5) or HSV-1 RHIM mut Δ ICP27 (MOI=15)-infected MEFs were examined by qPCR for Z-RNAs formed within aberrant transcripts produced from *Nabp1*, *H2ac18/19*, *Ptbp1*, *Hmga1*, *Gtpbp4*, *U1* or *Btbd3* loci. Data were normalized to Input.

c, d, RNA eluted from Z22- (**c**), anti-ZBP1 antibody- (**d**) or control IgG antibody pulldowns from HSV-1 RHIM mut (MOI=5) and HSV-1 RHIM mut Δ ICP27 (MOI=15)-infected primary MEFs were examined by qPCR for Z-RNAs formed within aberrant transcripts produced from *Nabp1*, *H2ac18/19*, *Ptbp1*, *Hmga1*, *Gtpbp4*, *U1* or *Btbd3* loci. Data were normalized to Input.

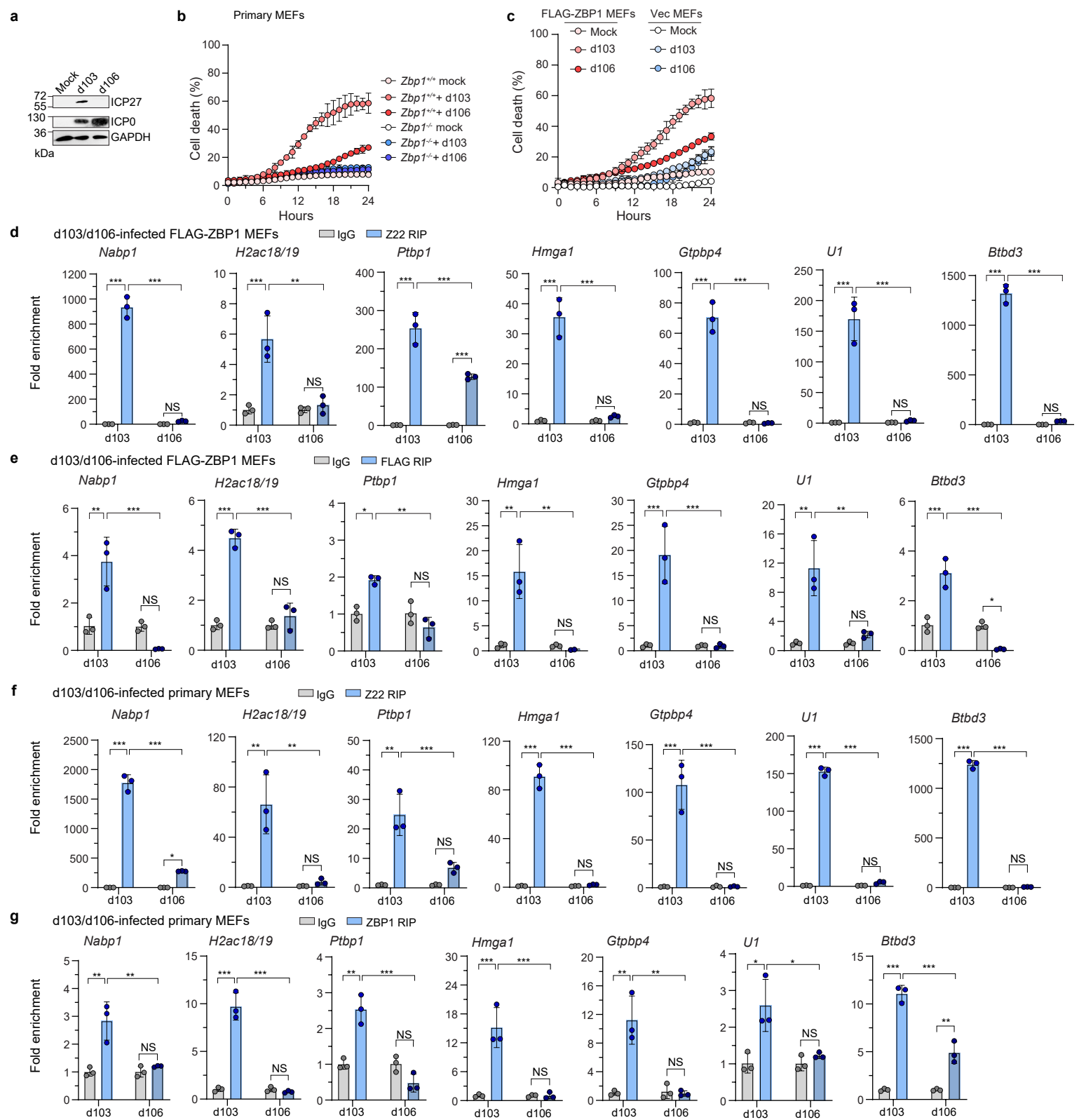
Data are mean \pm s.d. (n = 3 biologically independent samples in **a-d**). Two-way ANOVA with Tukey's multiple comparisons test (**a-d**). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. NS, no significance.



Supplementary Figure 4. (Related to Figure 3.) Deleting ICP27 attenuates HSV-1-induced host cell Z-RNA formation in HT-29 cells.

a, b, RNA eluted from Z22- (**a**), anti-FLAG antibody- (**b**) or control IgG antibody pulldowns from HSV-1 RHIM mut (MOI=5) and HSV-1 RHIM mut Δ ICP27 (MOI=15)-infected FLAG-ZBP1 HT-29 cells were examined by qPCR for Z-RNAs formed within aberrant transcripts generated from *RABGGTB*, *ROCK1*, *RUVBL2*, or *ZNFs* loci. Data were normalized to Input.

Data are mean \pm s.d. (n = 3 biologically independent samples in **a, b**). Two-way ANOVA with Tukey's multiple comparisons test (**a, b**). ** $P < 0.005$, *** $P < 0.0005$. NS, no significance.



Supplementary Figure 5. (Related to Figure 3.) Deleting ICP27 attenuates HSV-1-induced host cell Z-RNA formation in MEFs.

a, Levels of ICP27 and ICP0 upon infection of MEFs with either of the two HSV-1 mutant viruses d103 (MOI=10) and d106 (MOI=10) at 6 h post infection.

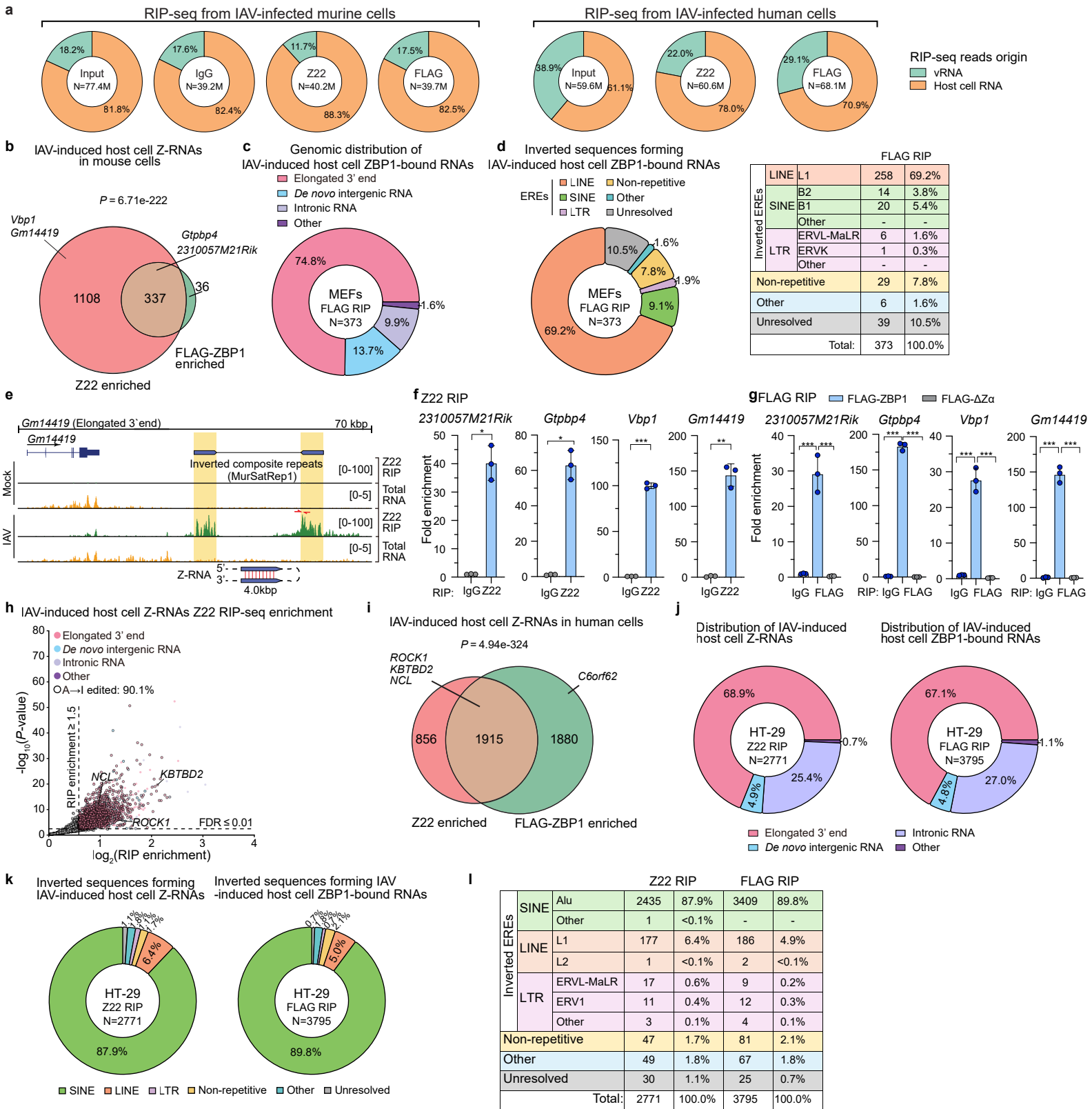
b, Kinetics of cell death in IFN β pretreated (100 ng/mL, 16 h) primary *Zbp1*^{+/+} or *Zbp1*^{-/-} MEFs infected with d103 (MOI=10) and d106 (MOI=10).

c, Kinetics of cell death in Vec or FLAG-ZBP1 MEFs infected with d103 (MOI=10) and d106 (MOI=10).

d, **e**, RNA eluted from Z22- (**d**), anti-FLAG antibody- (**e**), or control IgG antibody pulldowns from d103 (MOI=10) or d106 (MOI=10)-infected FLAG-ZBP1 MEFs were examined by qPCR for Z-RNAs formed within aberrant transcripts produced from *Nabp1*, *H2ac18/19*, *Ptbp1*, *Hmga1*, *Gtpbp4*, *U1*, or *Btbd3* loci. Data were normalized to Input.

f, **g**, RNA eluted from Z22- (**f**), anti-ZBP1- (**g**), or control IgG antibody pulldowns from d103 (MOI=10) or d106 (MOI=10)-infected primary MEFs were examined by qPCR for Z-RNAs formed in aberrant transcripts produced from *Nabp1*, *H2ac18/19*, *Ptbp1*, *Hmga1*, *Gtpbp4*, *U1*, and *Btbd3* loci. Data were normalized to Input.

Data are mean \pm s.d. (n = 4 in **b,c**, n = 3 biologically independent samples in **d-g**). Two-way ANOVA with Tukey's multiple comparisons test (**d-g**). * P < 0.05, ** P < 0.005, *** P < 0.0005. NS, no significance. Data are representative of at least two independent experiments (**a**) or three independent experiments (**b,c**).



Supplementary Figure 6. (Related to Figure 4.) Characterization of IAV-induced host cell Z-RNAs and ZBP1-bound RNAs.

a, Overall proportion of viral and host RNA fragments across input and RIP-seq samples from FLAG-ZBP1 expressing MEFs (left) or HT-29 cells (right) infected with IAV (PR8, MOI=2, 8h post-infection). Data are from RIP experiments with anti- FLAG, Z22, or IgG antibodies, or from matched input samples. Mean total mapped reads (in millions) for each experiment are indicated within each chart.

b, Overlap of virus-induced host Z-RNAs (Z22 enriched) or ZBP1-bound (FLAG-ZBP1 enriched) RNAs from FLAG-ZBP1 MEFs infected with IAV. Hypergeometric p-value, indicative of the odds that the overlap between the two pull-downs arises from chance alone, is shown above the Venn diagram.

c, Genomic distribution of virus-induced host cell Z-RNAs enriched in FLAG RIP-seq from FLAG-ZBP1 MEFs infected with IAV.

d, Source of inverted reverse-complement sequences (left) and detailed distribution of inverted EREs (right) within virus-induced ZBP1-bound host cell RNAs from FLAG-ZBP1 MEFs infected with IAV. The 'Unresolved' category includes Z-forming RNAs whose secondary structures could not be readily solved.

e, Coverage tracks for Z-RNAs formed within 3'-extended transcripts originating from *Gm14419* showing Z22 RIP (green), input total RNA (orange), A→I editing sites (red ticks, top), positions of qPCR primers (red arrows), and a schematic of the putative Z-RNA structure (bottom). Coverage exceeding indicated limits is denoted by thick black caps.

f, RNA eluted from Z22- or control IgG antibody pull-downs from IAV-infected MEFs were examined by qPCR for Z-RNAs formed within 3'-extended transcripts originating from *2310057M21Rik*, *Gtpbp4*, *Vbp1*, and *Gm14419* loci. Data were normalized to Input.

g, RNA eluted from anti-FLAG antibody pull-downs from either FLAG-ZBP1 or FLAG-ZBP1 $\Delta Z\alpha$ mutant MEFs infected with IAV were examined by qPCR for Z-RNAs formed within 3'-extended transcripts originating from *2310057M21Rik*, *Gtpbp4*, *Vbp1*, and *Gm14419* loci. Data were normalized to Input.

h, Scatterplot showing enrichment of virus-induced host cell Z-RNAs in Z22 RIP-seq from FLAG-ZBP1 HT-29 infected with IAV. Z-RNAs with evidence of A→I editing (i.e., from this study, or overlapping REDportal-annotated sites) are circled in black. Statistical significance was assessed using a one-sided Wald test in DESeq2. Only RNAs significantly induced by infection (adj. p-value ≤ 0.1 , fold change ≥ 1.5) are shown, as determined by separate two-sided Wald tests comparing RIP/RIP or input/input between infected and matched mock cells (see Methods). P values were adjusted for multiple testing using the Benjamini-Hochberg procedure.

i, Global overlap of virus-induced host cell Z-RNAs (Z22 enriched) and ZBP1-bound (FLAG-ZBP1 enriched) RNAs from FLAG-ZBP1 HT-29 cells infected with IAV. Hypergeometric p-value, indicative of the odds that the overlap between the two pull-downs arises from chance alone, is shown above the Venn diagram.

j, Genomic distribution of IAV-induced host cell Z-RNAs enriched in Z22 (left) or FLAG (right) RIP-seq from FLAG-ZBP1 HT-29 cells infected with IAV.

k, Source of inverted reverse complement sequences within virus-induced host cell Z-RNAs (left) or ZBP1-bound RNAs (right) from FLAG-ZBP1 HT-29 cells infected with IAV. The 'Unresolved' category includes Z-forming RNAs whose secondary structures could not be readily solved.

l, Detailed distribution of inverted EREs in virus-induced host cell Z-RNAs or ZBP1-bound RNAs from FLAG-ZBP1 HT-29 cells infected with IAV Rico/8/1934 (MOI=2, 8h post-infection). Data are mean \pm s.d. (n=3 biologically independent samples per group in f, g). Two-tailed unpaired t-test with Welch's correction (f). One-way ANOVA with Dunnett's multiple comparisons test (g). *P < 0.05. **P < 0.005, ***P < 0.0005.

Supplementary Figure 7. Uncropped immunoblots used to prepare main and extended data figures.

Figure 3e

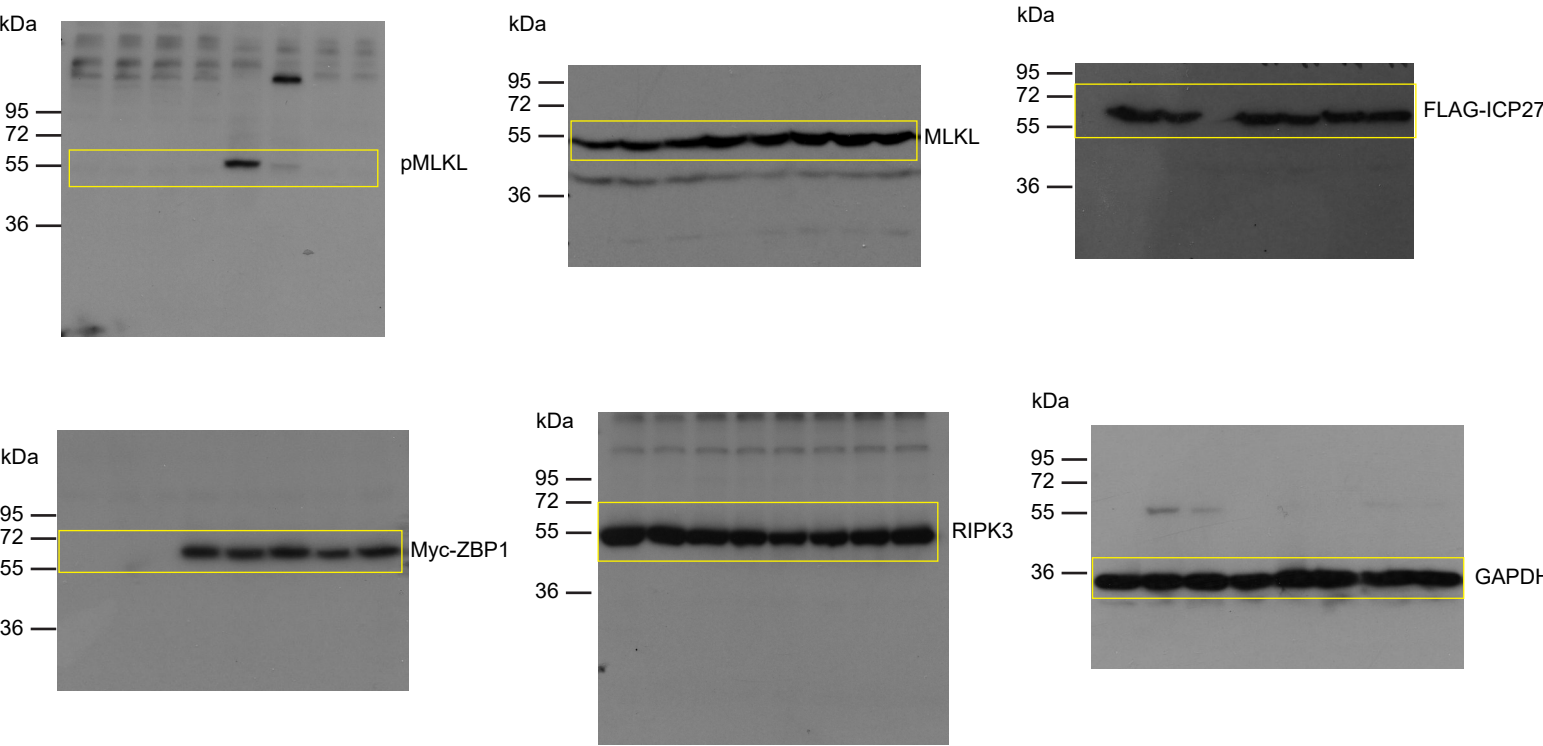
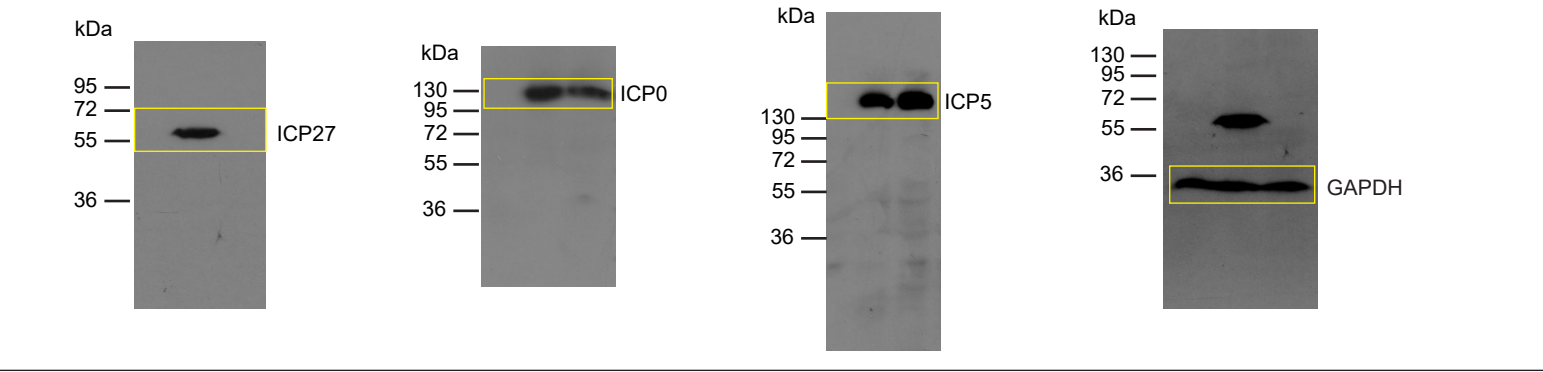
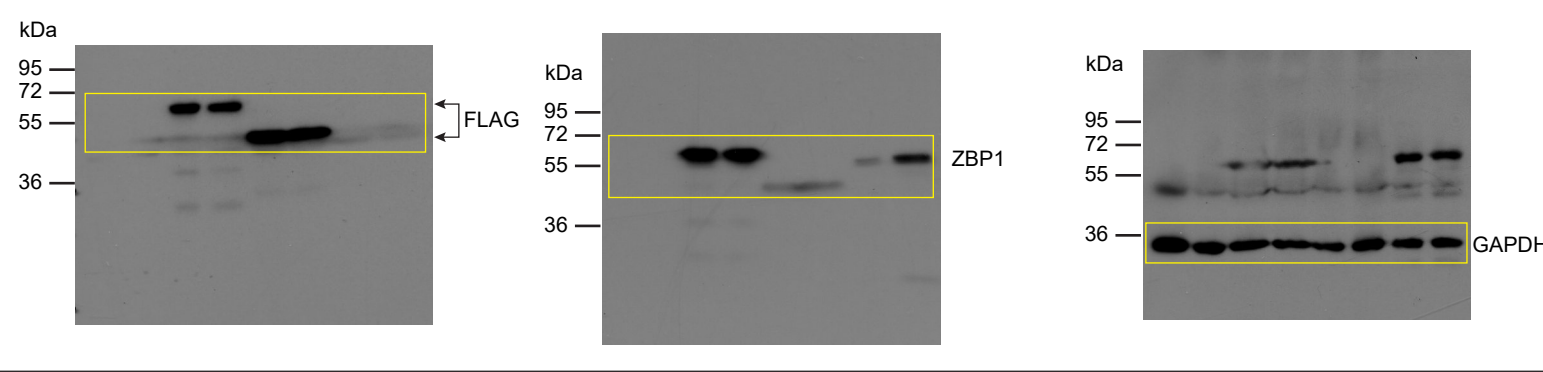


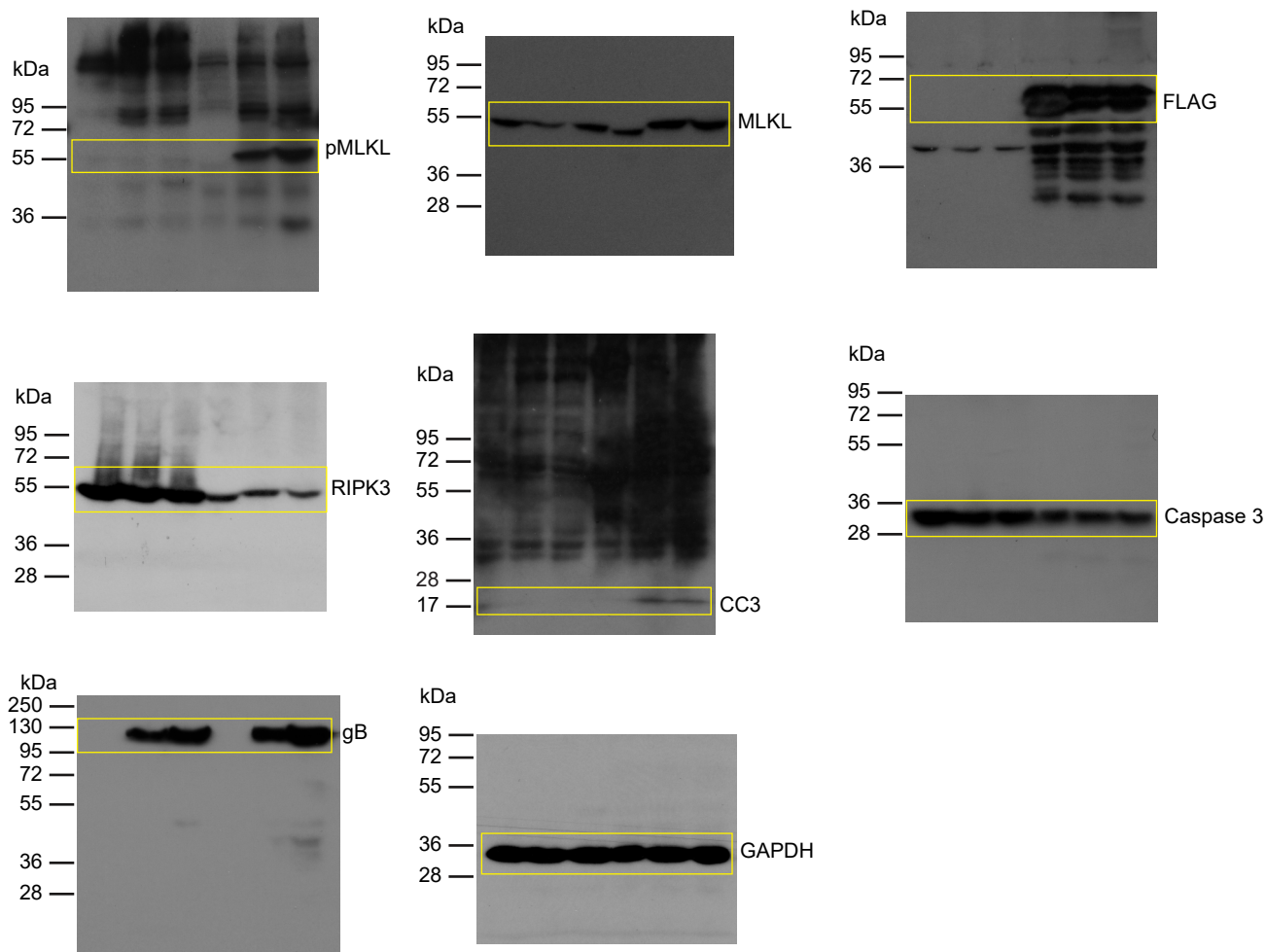
Figure 3g



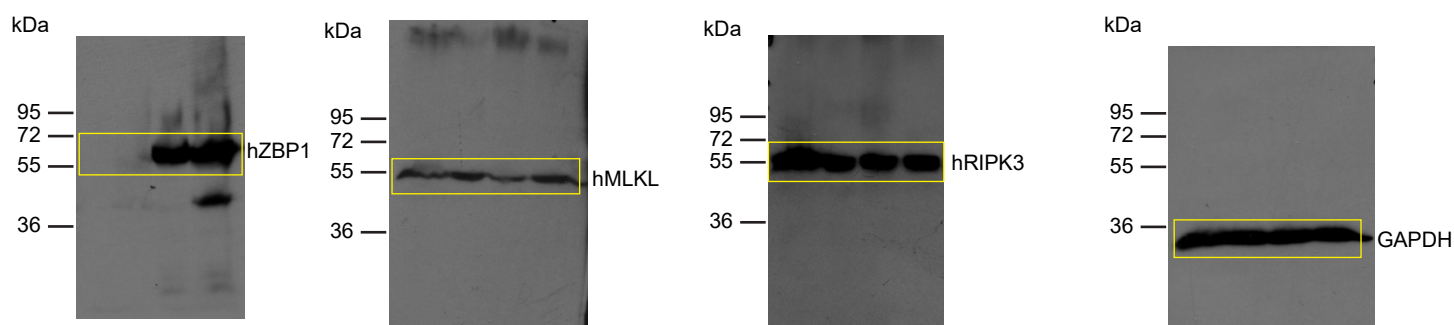
Extended Data Fig. 1g



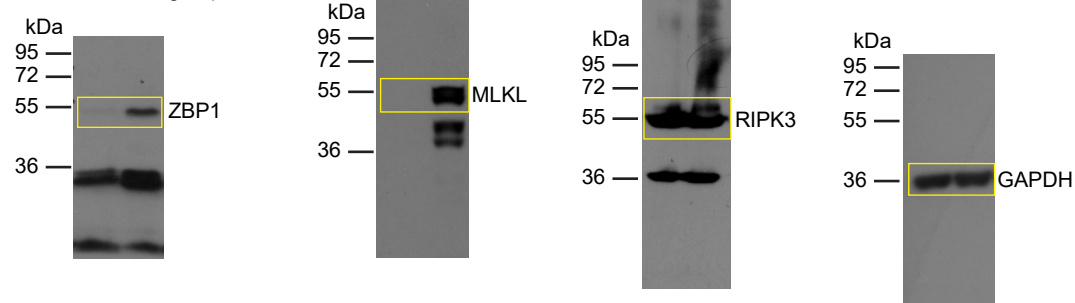
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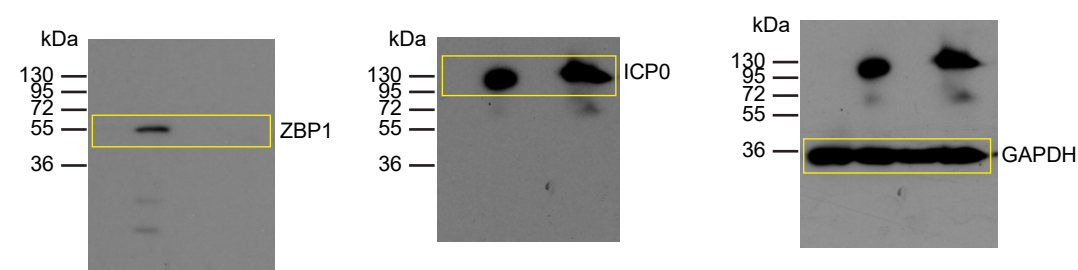
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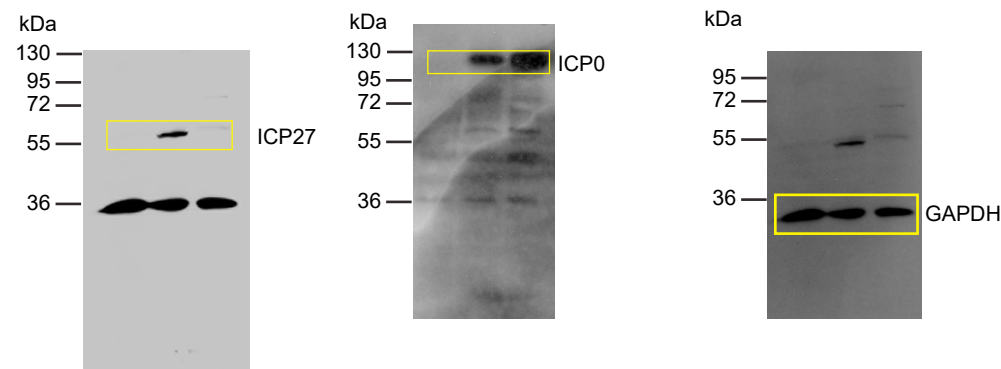
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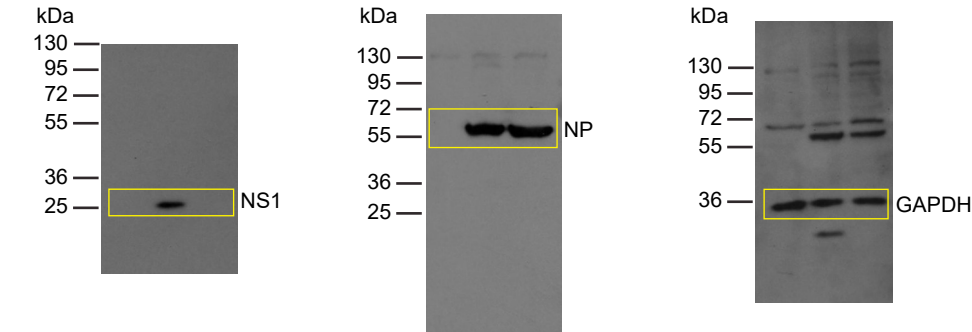
Extended Data Fig. 5t



Supplementary Fig. 5a



Extended Data Fig. 8e



Extended Data Fig. 10h

