Cell

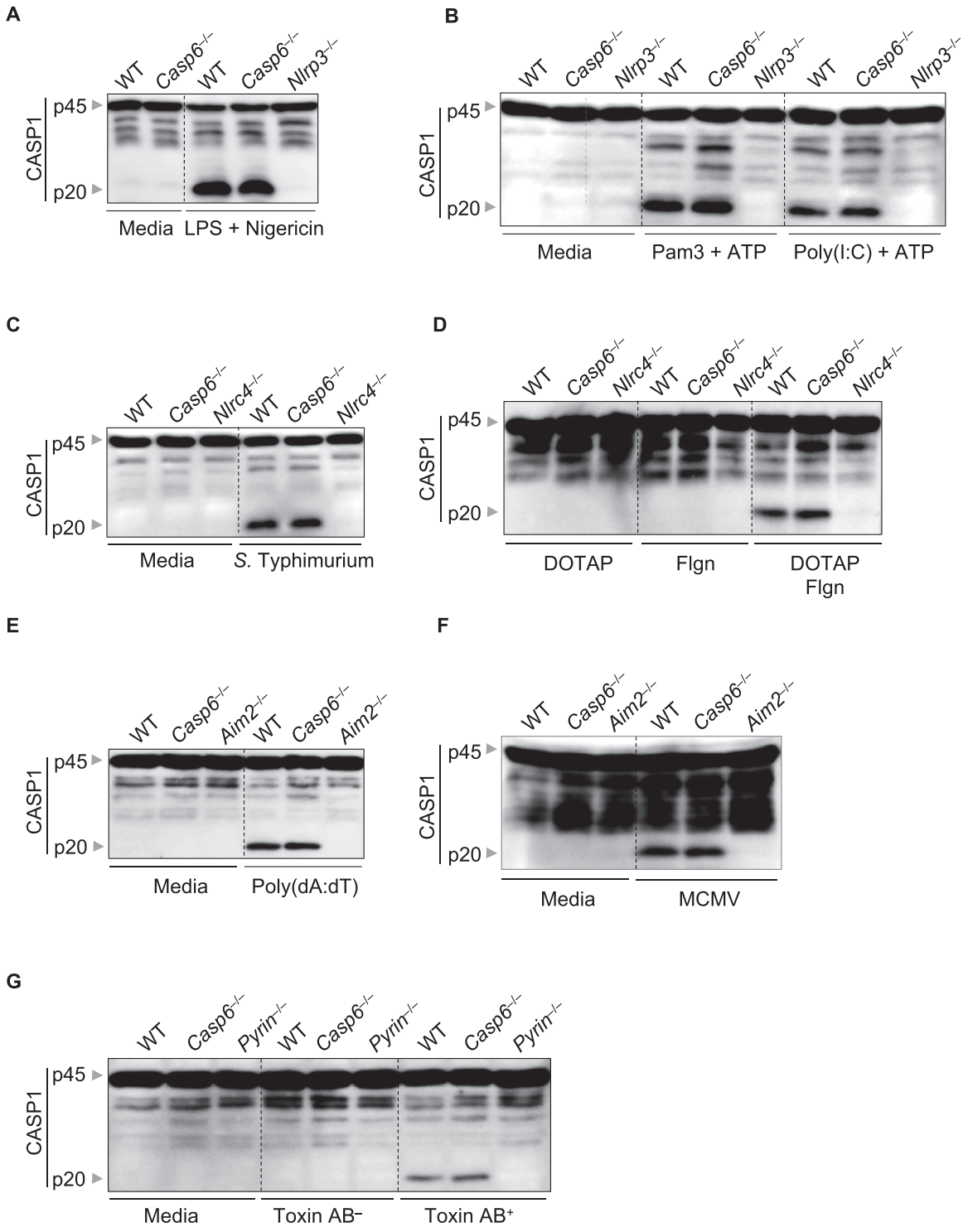


Figure S1. Caspase-6 Is Dispensable for Canonical-NLRP3, NLRC4, AIM2, and PYRIN Inflammasome Activation, Related to Figure 1

(A–G) Immunoblot analysis of pro- and cleaved caspase-1 (CASP1) in bone marrow-derived macrophages (BMDMs) after LPS plus nigericin stimulation (A), Pam3CSK4 (Pam3) plus ATP and poly(I:C) plus ATP treatment (B), *S.* Typhimurium infection (MOI, 2) for 2–3 h (C), flagellin (Flgn) transfection (D), poly(dA:dT) transfection (E), murine cytomegalovirus (MCMV) infection (MOI, 10) for 16 h (F), or toxin stimulation from *C. difficile* AB− and AB+ (G). Data are representative of three independent experiments.

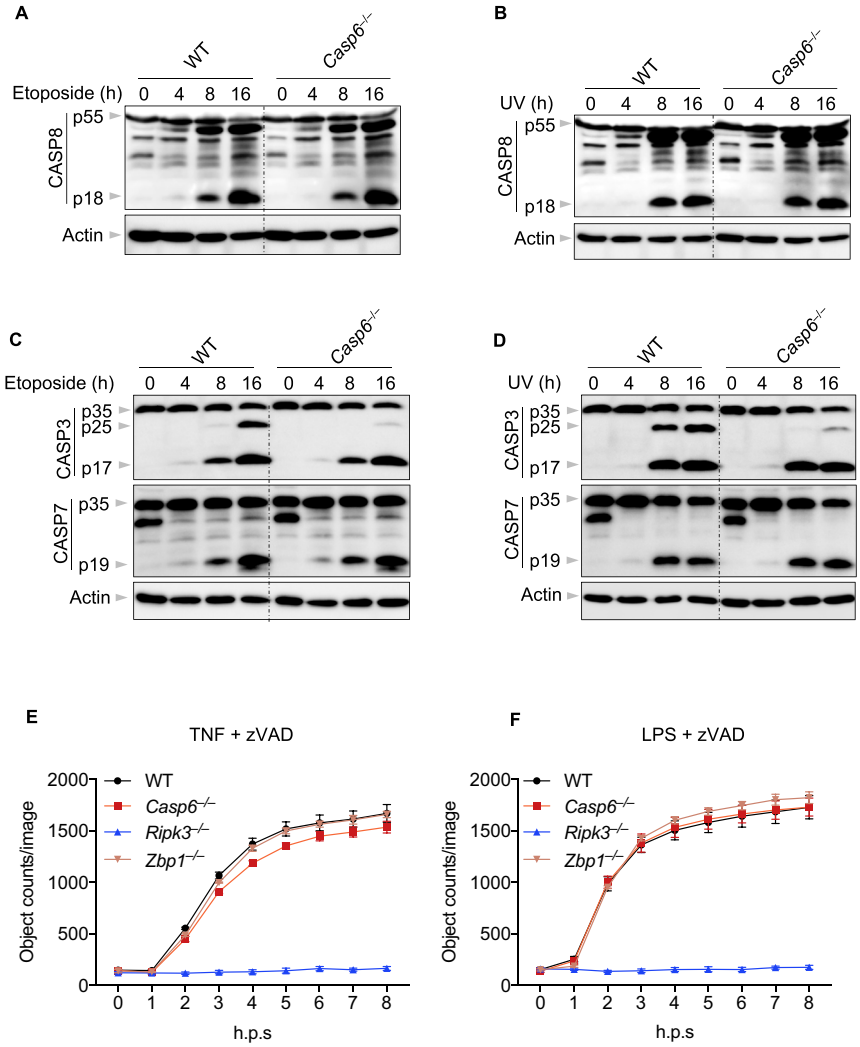


Figure S2. Caspase-6 Is Dispensable for Apoptosis Induced by Etoposide and Ultraviolet and Necroptosis Induced by TNF or LPS Plus zVAD, Related to Figure 3

(A and B) Immunoblot analysis of pro- and cleaved caspase-8 (CASP8) in bone marrow-derived macrophages (BMDMs) after etoposide stimulation (A) or ultraviolet (UV) treatment (B) at the indicated time points. Actin is used as the internal control. (C and D) Immunoblot analysis of pro- and cleaved caspase-3 (CASP3) and caspase-7 (CASP7) in BMDMs after etoposide stimulation (C) or UV treatment (D) at the indicated time points. Actin is used as the internal control. (E and F) Real-time analysis of cell death in BMDMs after stimulation with TNF plus zVAD (E) or LPS plus zVAD (F). Data are shown as mean ± SEM (E, F). Data are representative of three independent experiments.

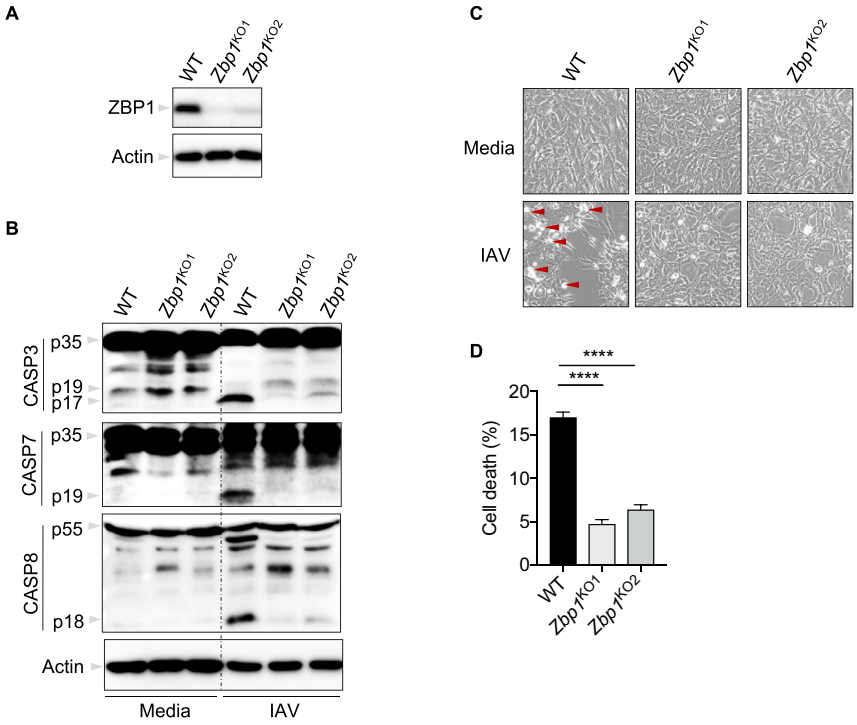


Figure S3. ZBP1 Is Required for IAV-Induced Cell Death in MEFs, Related to Figure 5

(A) Immunoblot analysis of Z-DNA binding protein 1 (ZBP1) in mouse embryonic fibroblasts (MEFs) following CRISPR-directed deletion. (B) Immunoblot analysis of pro- and cleaved caspase-3 (CASP3), caspase-7 (CASP7), and caspase-8 (CASP8) in MEFs after influenza A virus (IAV) infection for 24 h. Actin is used as the internal control. (C) Microscopic analysis of cell death in MEFs infected with IAV for 30 h (original magnification × 10). (D) Quantification of the cell death observed in (C). ****$p < 0.0001$ (one-way ANOVA). Data are shown as mean ± SEM (D). Data are representative of three independent experiments.

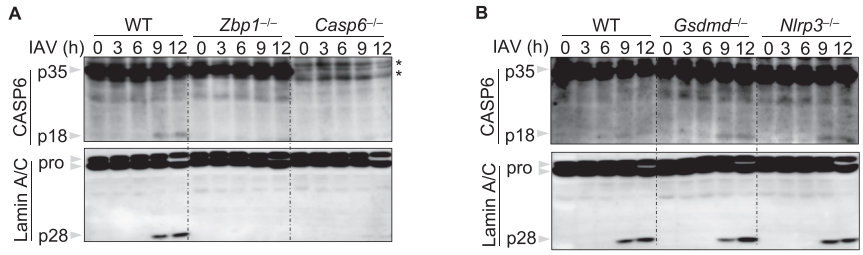
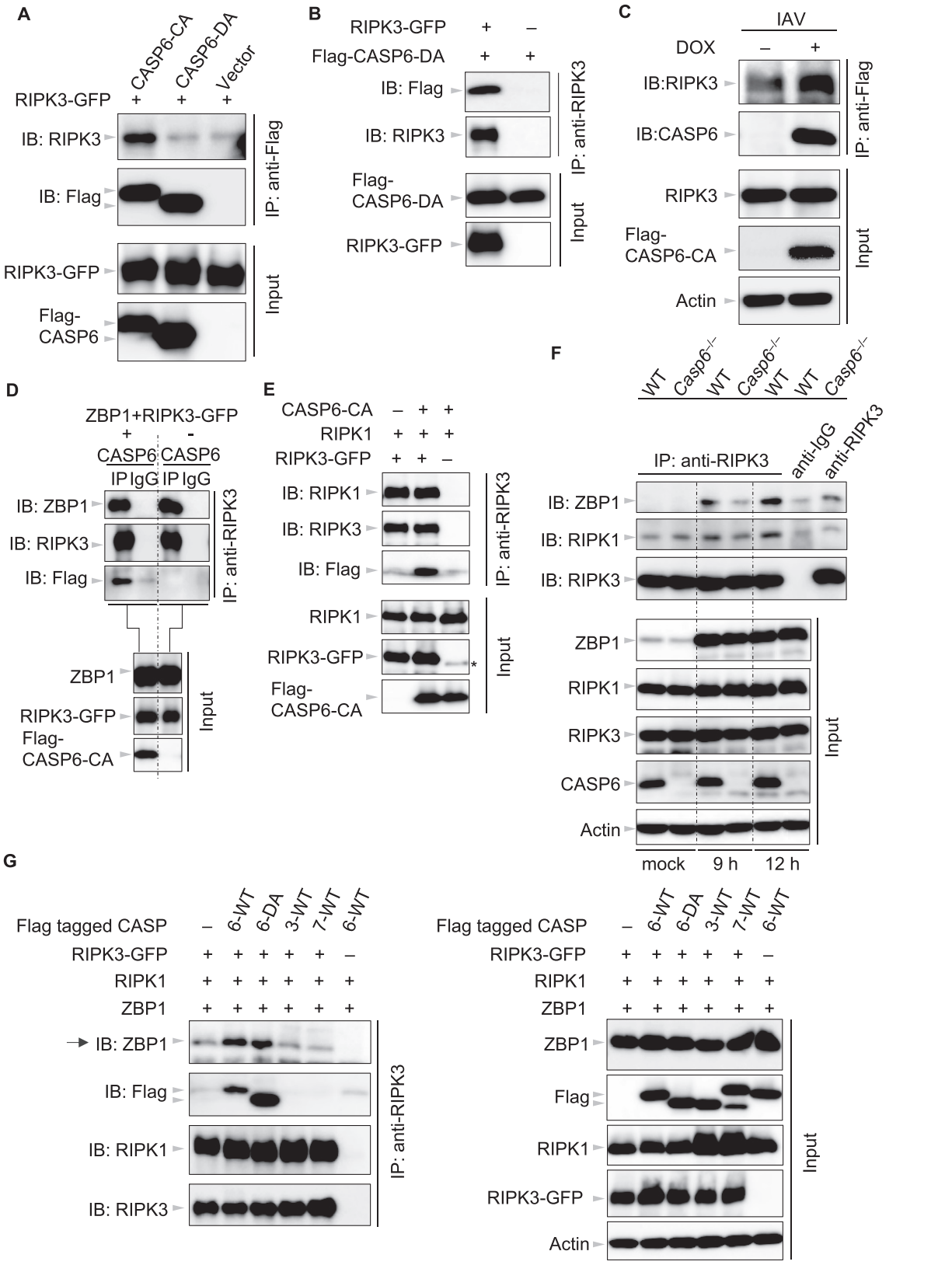


Figure S4. Caspase-6 Activation in Bone-Marrow-Derived Macrophages after IAV Infection Is Independent of NLRP3 Inflammasome Assembly, Related to Figure 5

(A, B) Immunoblot analysis of pro- and cleaved caspase-6 (CASP6) and lamin A/C in bone marrow-derived macrophages (BMDMs) from wild-type (WT) or various knockout mice after influenza A virus (IAV) infection at the indicated time points. The star designates a nonspecific band. Data are representative of four (A) or two (B) independent experiments.



(legend on next page)

Figure S5. Caspase-6 Is Dispensable for the Binding of RIPK3 to ZBP1 or RIPK1 in the Absence of RIPK1 or ZBP1, Respectively, but Is the Only Executioner Caspase to Enhance the Interaction between RIPK3 and ZBP1, Related to Figure 5

(A) Immunoprecipitates and total lysates from 293T cells after co-transfection of GFP tagged receptor-interacting protein kinase (RIPK) 3 (RIKP3-GFP) with a catalytically dead mutant of caspase-6 (CASP6-CA), an uncleavable mutant of CASP6 (CASP6-DA), or empty vector for 30 h. CA, CASP6-C146A; DA, CASP6-D5A/D162A/D175A. (B) Immunoprecipitates and total lysates from 293T cells after co-transfection of CASP6-DA with or without RIPK3-GFP for 30 h. (C) Immunoprecipitates and total lysates from *Casp6*$^{-/-}$ immortalized BMDMs (iBMDMs) reconstituted with a FLAG tagged-catalytically dead mutant of caspase-6 (Flag-CASP6-CA) whose expression is induced by doxycycline (Dox) after influenza A virus (IAV) infection for 9 h. (D) Immunoprecipitates and total lysates from 293T cells after co-transfection of RIPK3-GFP and Z-DNA binding protein 1 (ZBP1) in the absence or presence of CASP6 for 30 h. IgG, immunoglobulin G. (E) Immunoprecipitates and total lysates from 293T cells after co-transfection of RIPK3-GFP and RIPK1 in the absence or presence of CASP6 for 30 h. (F) Immunoprecipitates and total lysates from BMDMs without or with IAV infection for the indicated time points. The star designates a nonspecific band. (G) Immunoprecipitates and total lysates from 293T cells after co-transfection with RIPK1, RIPK3-GFP, and ZBP1 in the absence or presence of the indicated caspase (CASP) proteins for 30 h. WT, wild-type. Data are representative of three independent experiments.

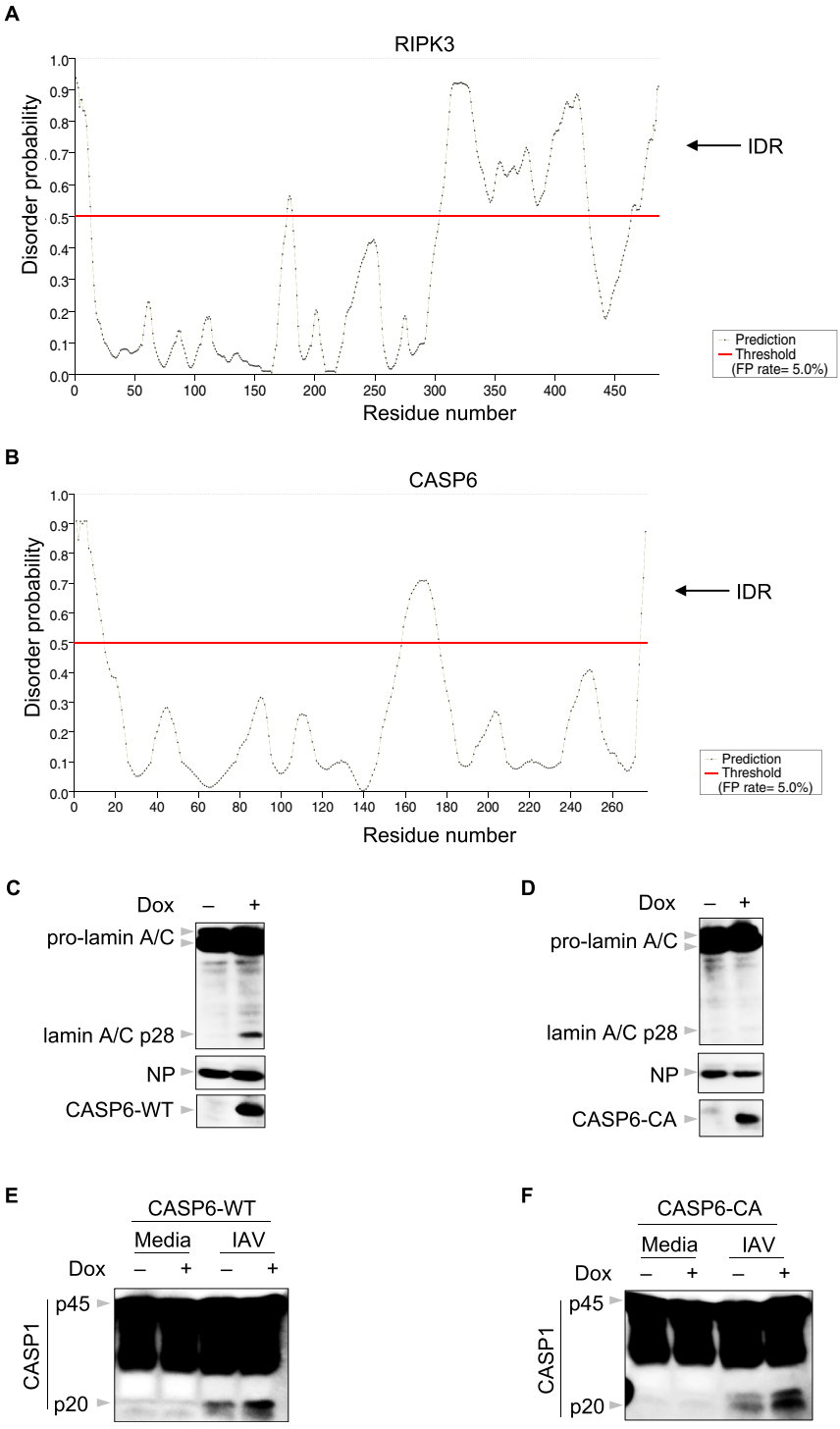


Figure S6. Intrinsically Disordered Region (IDR) Predication of RIPK3 and Caspase-6, and Protease Activity of Caspase-6 Is Not Required for IAV-Induced Inflammasome Activation, Related to Figure 6

(A and B) Prediction of the IDRs in receptor-interacting protein kinase 3 (RIPK3) (A) or caspase-6 (CASP6) (B) using the Protein DisOrder prediction System (PrDOS). (C and D) Immunoblot analysis of pro- and cleaved lamin A/C and viral nucleoprotein (NP) in *Casp6*$^{-/-}$ immortalized bone marrow-derived macrophages (iBMDMs) reconstituted with wild-type (C) or catalytically dead (D) caspase-6 (CASP6-WT or CASP6-CA, respectively) after influenza A virus (IAV) infection at an MOI of 20 for 9 h. Dox, doxycycline. (E and F) Immunoblot analysis of pro- and cleaved caspase-1 (CASP1) in *Casp6*$^{-/-}$ iBMDMs reconstituted with CASP6-WT (E) or CASP6-CA (F). Data are representative of three independent experiments.