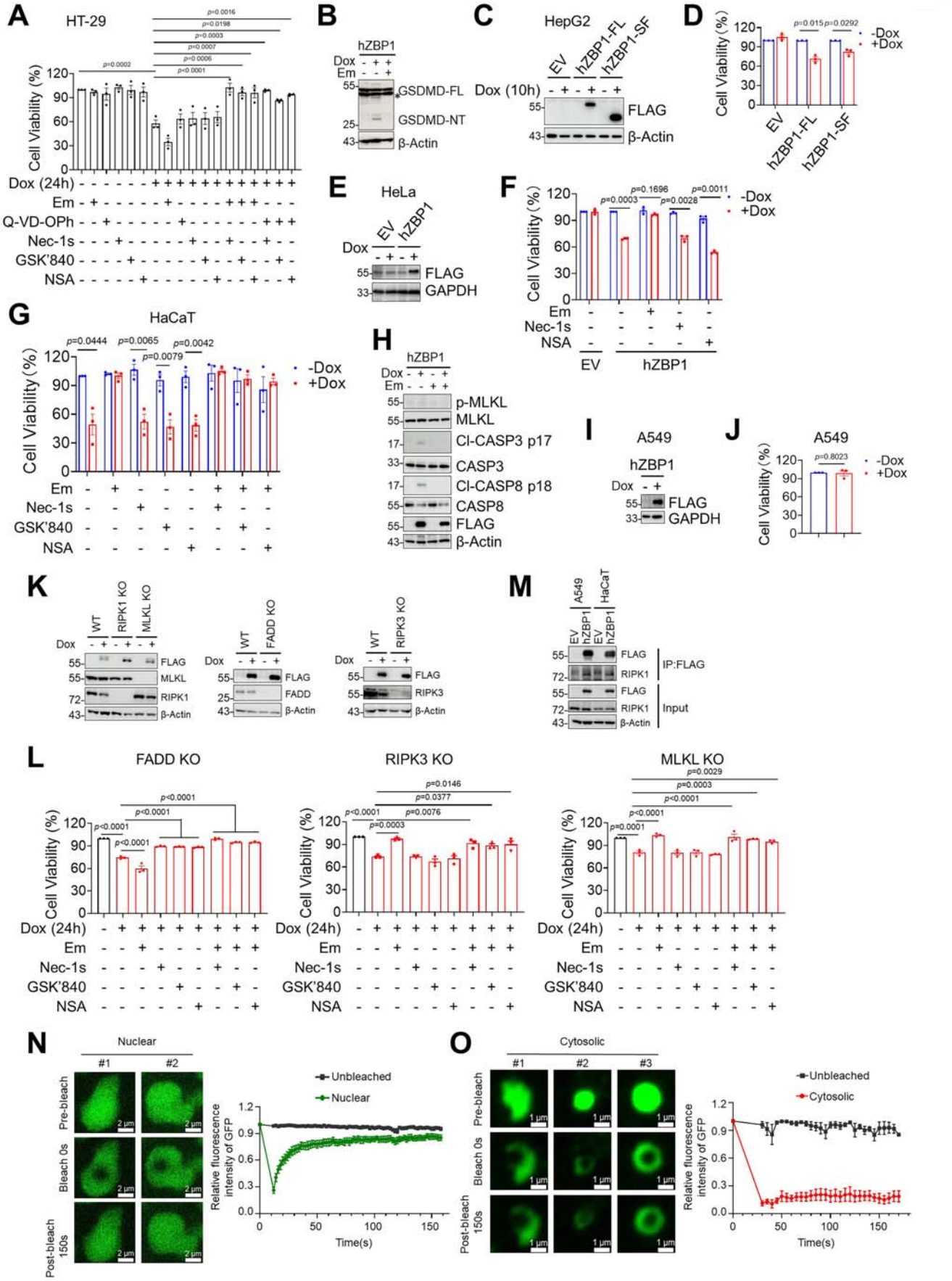
Expanded View Figures

Figure EV1. Human ZBP1 triggers spontaneous cell death via RIPK1.

(**A**) Cell viability measured by neutral red uptake in HT-29 expressing Dox-inducible FLAG-EV, FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) or combinations of Dox and emricasan (5 μM), Q-VD-OPh (10 μM), Nec-1s (10 μM), GSK'840 (3 μM), and NSA (5 μM) for 24 h. (**B**) Immunoblot analysis of total lysates in HT-29 expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) or combinations of Dox (1 μg/mL) and emricasan (5 μM) for 10 h. * nonspecific band. (**C**) Immunoblot analysis of total lysates in HepG2 expressing Dox-inducible FLAG-EV, hZBP1-FL, and hZBP1-SF stimulated with or without Dox (1 μg/mL) for 10 h. (**D**) Cell viability measured by neutral red uptake in HepG2 expressing Dox-inducible FLAG-EV, hZBP1-FL, and hZBP1-SF stimulated with or without Dox (1 μg/mL) for 24 h. (**E**) Immunoblot analysis of total lysates in HeLa expressing Dox-inducible FLAG-EV and hZBP1 stimulated with or without Dox (1 μg/mL) for 10 h. (**F**) Cell viability measured by neutral red uptake in HeLa expressing Dox-inducible FLAG-EV and FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) or combinations of Dox and emricasan (5 μM), Nec-1s (10 μM), and NSA (2 μM) for 48 h. (**G**) Cell viability measured by neutral red uptake in HaCaT expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) or combinations of Dox and emricasan (5 μM), Nec-1s (10 μM), GSK'840 (3 μM), and NSA (5 μM) for 24 h. (**H**) Immunoblot analysis of total lysates in HaCaT expressing Dox-inducible FLAG-EV and hZBP1 stimulated with Dox (1 μg/mL) or a combination of Dox (1 μg/mL) and emricasan (5 μM) for 10 h. (**I**) Immunoblot analysis of total lysates in A549 expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) for 10 h. (**J**) Cell viability measured by neutral red uptake in A549 expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) for 24 h. (**K**) Immunoblot analysis of total lysates in WT, RIPK1 KO, RIPK3 KO, MLKL KO, and FADD KO HT-29 expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL) for 10 h. (**L**) Cell viability measured by neutral red uptake in FADD KO, RIPK3 KO, and MLKL KO HT-29 expressing Dox-inducible FLAG-hZBP1 stimulated with or without Dox (1 μg/mL), or combinations of Dox and emricasan (5 μM), Nec-1s (10 μM), GSK'840 (3 μM), and NSA (5 μM) for 24 h. (**M**) Immunoblot analysis of FLAG immunoprecipitates and total lysates (Input) from A549 and HaCaT expressing Dox-inducible FLAG-EV and FLAG-hZBP1 stimulated with Dox (1 μg/mL) for 12 h. (**N**, **O**) FRAP analysis of GFP-hZBP1 assemblies formed in the nucleus (**N**) and cytosol (**O**) in HT-29 expressing Dox-inducible GFP-hZBP1 stimulated with Dox (1 μg/mL) for 10 h. Left, representative confocal images of assemblies before and after bleaching. Right, quantification of the GFP fluorescence recovery curves. Scale bars, 2 μm in (**N**) and 1 μm in (**O**). All experiments were replicated at least three times. Data with error bars are mean ± SEM. Data in panels (**A**, **D**, **F**, **G**, **J**, **L**) are pooled and analyzed from at least three independent biological experiments. *P* values were calculated by unpaired parametric *t*-test with Welch's correction (**D**, **F**, **G**, **J**) or one-way ANOVA with Tukey's post hoc test for multiple comparisons (**A**, **L**). Source data are available online for this figure.


A
HT-29
Cell Viability (%)
Dox (24h)
Em
Q-VD-OPh
Nec-1s
GSK'840
NSA
B
hZBP1
GSDMD-FL
GSDMD-NT
β-Actin
C
HepG2
EV
hZBP1-FL
hZBP1-SF
Dox (10h)
FLAG
β-Actin
D
Cell Viability (%)
-Dox
+Dox
EV
hZBP1-FL
hZBP1-SF
E
HeLa
Dox
EV
hZBP1
FLAG
GAPDH
F
Cell Viability (%)
-Dox
+Dox
Em
Nec-1s
NSA
EV
hZBP1
G
HaCaT
Cell Viability (%)
-Dox
+Dox
Em
Nec-1s
GSK'840
NSA
H
hZBP1
p-MLKL
MLKL
Cl-CASP3 p17
CASP3
Cl-CASP8 p18
CASP8
FLAG
β-Actin
I
A549
hZBP1
FLAG
GAPDH
J
A549
Cell Viability (%)
-Dox
+Dox
K
WT
RIPK1 KO
MLKL KO
FADD KO
RIPK3 KO
FLAG
MLKL
RIPK1
FADD
RIPK3
β-Actin
M
A549
HaCaT
EV
hZBP1
IP:FLAG
Input
FLAG
RIPK1
β-Actin
L
FADD KO
RIPK3 KO
MLKL KO
Cell Viability (%)
Dox (24h)
Em
Nec-1s
GSK'840
NSA
N
Nuclear
#1
#2
Pre-bleach
Bleach 0s
Post-bleach 150s
Unbleached
Nuclear
Relative fluorescence intensity of GFP
Time(s)
O
Cytosolic
#1
#2
#3
Pre-bleach
Bleach 0s
Post-bleach 150s
Unbleached
Cytosolic
Relative fluorescence intensity of GFP
Time(s)

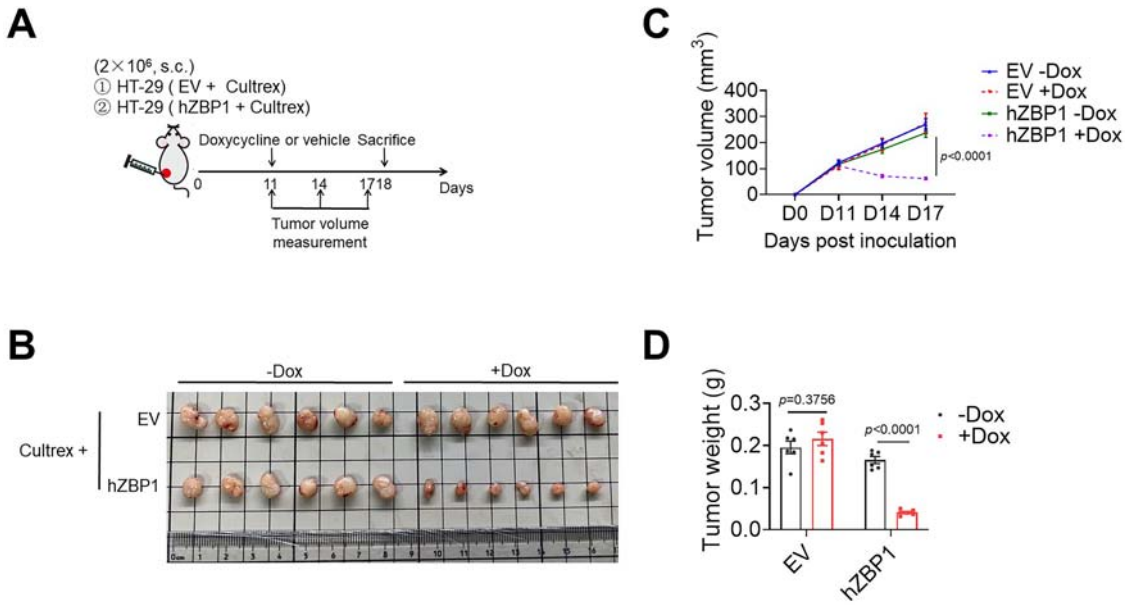


Figure EV2. Human ZBP1 exerts potent anti-tumor effects in vivo.

(**A**) Treatment schedule of nude mice bearing subcutaneous (s.c.) HT-29. Treatments were initiated at day 11 post inoculation when tumors were palpable (~100 mm^3). (**B**) Picture representation of tumors at day 18 post inoculation. (**C**) Individual tumor growth curves of Dox-treated and untreated HT-29 tumors at the indicated time points. (**D**) The tumor weight of nude mice bearing HT-29 treated with or without Dox. $n = 6$ mice for each group. Data with error bars are mean ± SEM. *P* values were calculated by unpaired parametric *t*-test with Welch's correction (**C**, **D**). Source data are available online for this figure.

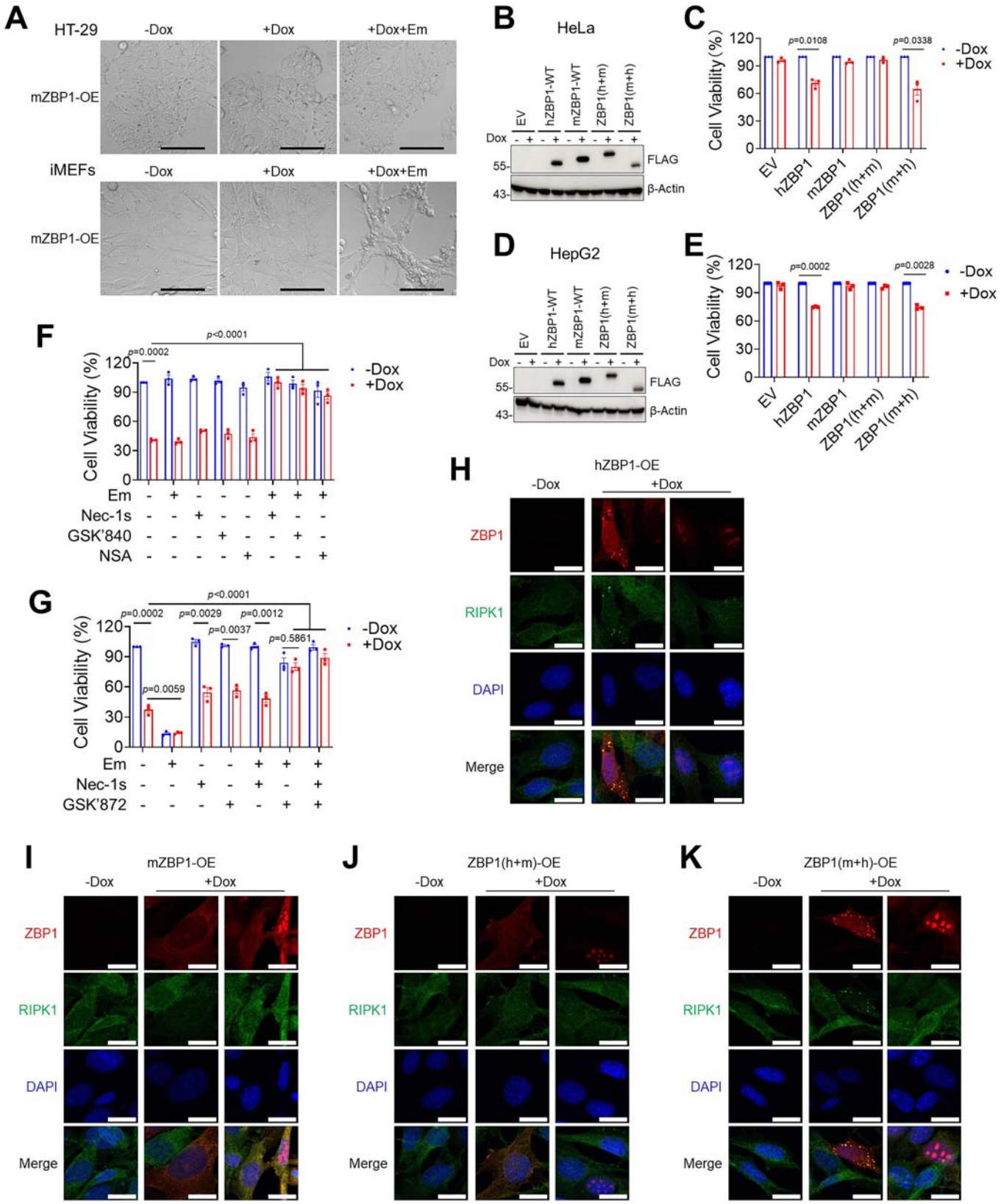

A
HT-29
-Dox
+Dox
+Dox+Em
mZBP1-OE
iMEFs
-Dox
+Dox
+Dox+Em
mZBP1-OE
B
HeLa
EV
hZBP1-WT
mZBP1-WT
ZBP1(h+m)
ZBP1(m+h)
Dox
55-
43-
FLAG
β-Actin
C
Cell Viability (%)
p=0.0108
p=0.0338
-Dox
+Dox
EV
hZBP1
mZBP1
ZBP1(h+m)
ZBP1(m+h)
D
HepG2
EV
hZBP1-WT
mZBP1-WT
ZBP1(h+m)
ZBP1(m+h)
Dox
55-
43-
FLAG
β-Actin
E
Cell Viability (%)
p=0.0002
p=0.0028
-Dox
+Dox
EV
hZBP1
mZBP1
ZBP1(h+m)
ZBP1(m+h)
F
p<0.0001
p=0.0002
Cell Viability (%)
-Dox
+Dox
Em
Nec-1s
GSK'840
NSA
G
p<0.0001
p=0.0002
p=0.0029
p=0.0012
p=0.0037
p=0.5861
p=0.0059
Cell Viability (%)
-Dox
+Dox
Em
Nec-1s
GSK'872
H
hZBP1-OE
-Dox
+Dox
ZBP1
RIPK1
DAPI
Merge
I
mZBP1-OE
-Dox
+Dox
ZBP1
RIPK1
DAPI
Merge
J
ZBP1(h+m)-OE
-Dox
+Dox
ZBP1
RIPK1
DAPI
Merge
K
ZBP1(m+h)-OE
-Dox
+Dox
ZBP1
RIPK1
DAPI
Merge

Figure EV3. C-terminal RHIM domains enable hZBP1 to induce spontaneous cell death.

(**A**) Representative cell images of HT-29 cells expressing Dox-inducible FLAG-mZBP1 (top) and immortalized MEFs expressing Dox-inducible FLAG-mZBP1 (bottom) stimulated with or without Dox (1 μg/mL) or combinations of Dox (1 μg/mL) and emricasan (5 μM) for 10 h. Scale bars, 100 μm. (**B**, **D**) Immunoblot analysis of total lysates in HeLa (**B**) and HepG2 (**D**) expressing Dox-inducible FLAG-EV, FLAG-hZBP1, FLAG-mZBP1, FLAG-ZBP1(h + m), and FLAG-ZBP1(m + h) stimulated with or without Dox (1 μg/mL) for 10 h. (**C**, **E**) Cell viability measured by neutral red uptake in HeLa (**C**) and HepG2 (**E**) expressing Dox-inducible FLAG-EV, FLAG-hZBP1, FLAG-mZBP1, FLAG-ZBP1(h + m), and FLAG-ZBP1(m + h) stimulated with or without Dox (1 μg/mL) for 24 h. (**F**) Cell viability measured by neutral red uptake in HT-29 expressing Dox-inducible FLAG-ZBP1(m + h) stimulated with or without Dox (1 μg/mL) or combinations of Dox and emricasan (5 μM), Nec-1s (10 μM), GSK'840 (3 μM), and NSA (5 μM) for 24 h. (**G**) Cell viability measured by neutral red uptake in immortalized MEFs expressing Dox-inducible FLAG-ZBP1(m + h) stimulated with or without Dox (1 μg/mL) or combinations of Dox and emricasan (5 μM), Nec-1s (10 μM), and GSK'872 (3 μM) for 24 h. (**H–K**) Representative confocal images of immortalized MEFs expressing Dox-inducible FLAG-hZBP1 (**H**), FLAG-mZBP1 (**I**), FLAG-ZBP1(h + m) (**J**), and FLAG-ZBP1(m + h) (**K**) stimulated with or without Dox (1 μg/mL) for 10 h. Scale bars, 20 μm. All experiments were replicated at least three times. Data with error bars are mean ± SEM. Data in panels (**C**, **E**, **F**, **G**) are pooled and analyzed from at least three independent biological experiments. *P* values were calculated by unpaired parametric *t*-test with Welch's correction (**C**, **E**) or one-way ANOVA with Tukey's post hoc test for multiple comparisons (**F**, **G**). Source data are available online for this figure.

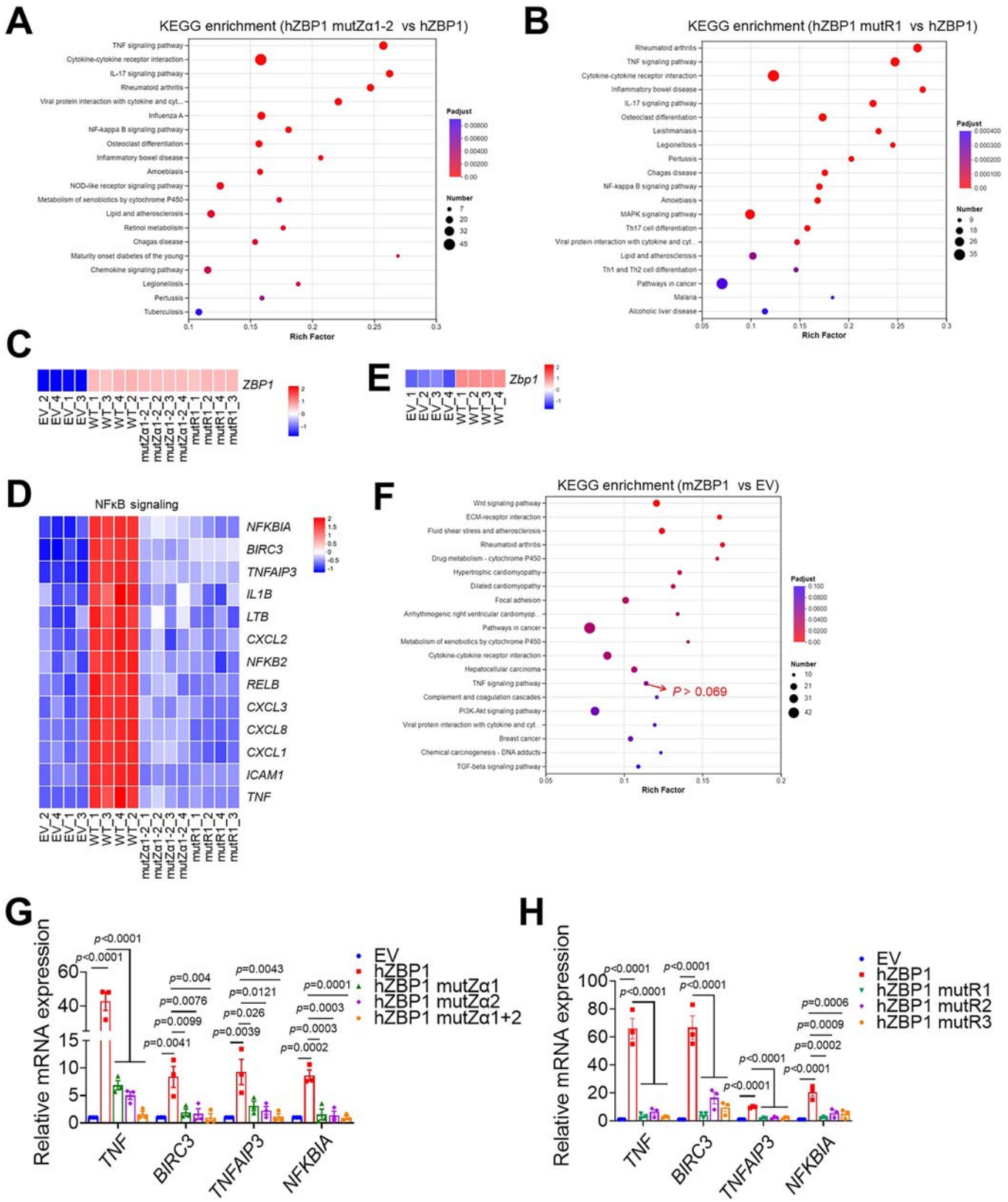

A
KEGG enrichment (hZBP1 mutZα1-2 vs hZBP1)
TNF signaling pathway
Cytokine-cytokine receptor interaction
IL-17 signaling pathway
Rheumatoid arthritis
Viral protein interaction with cytokine and cyt...
Influenza A
NF-kappa B signaling pathway
Osteoclast differentiation
Inflammatory bowel disease
Amoebiasis
NOD-like receptor signaling pathway
Metabolism of xenobiotics by cytochrome P450
Lipid and atherosclerosis
Retinol metabolism
Chagas disease
Maturity onset diabetes of the young
Chemokine signaling pathway
Legionellosis
Pertussis
Tuberculosis
Rich Factor
Padjust
Number
B
KEGG enrichment (hZBP1 mutR1 vs hZBP1)
Rheumatoid arthritis
TNF signaling pathway
Cytokine-cytokine receptor interaction
Inflammatory bowel disease
IL-17 signaling pathway
Osteoclast differentiation
Leishmaniasis
Legionellosis
Pertussis
Chagas disease
NF-kappa B signaling pathway
Amoebiasis
MAPK signaling pathway
Th17 cell differentiation
Viral protein interaction with cytokine and cyt...
Lipid and atherosclerosis
Th1 and Th2 cell differentiation
Pathways in cancer
Malaria
Alcoholic liver disease
Rich Factor
Padjust
Number
C
ZBP1
EV_2
EV_4
EV_1
EV_3
WT_1
WT_3
WT_4
WT_2
mutZα1-2_1
mutZα1-2_2
mutZα1-2_3
mutZα1-2_4
mutR1_1
mutR1_2
mutR1_4
mutR1_3
E
Zbp1
EV_1
EV_2
EV_3
EV_4
WT_1
WT_2
WT_3
WT_4
D
NFκB signaling
NFKBIA
BIRC3
TNFAIP3
IL1B
LTB
CXCL2
NFKB2
RELB
CXCL3
CXCL8
CXCL1
ICAM1
TNF
F
KEGG enrichment (mZBP1 vs EV)
Wnt signaling pathway
ECM-receptor interaction
Fluid shear stress and atherosclerosis
Rheumatoid arthritis
Drug metabolism - cytochrome P450
Hypertrophic cardiomyopathy
Dilated cardiomyopathy
Focal adhesion
Arrhythmogenic right ventricular cardiomyop...
Pathways in cancer
Metabolism of xenobiotics by cytochrome P450
Cytokine-cytokine receptor interaction
Hepatocellular carcinoma
TNF signaling pathway
Complement and coagulation cascades
PI3K-Akt signaling pathway
Viral protein interaction with cytokine and cyt...
Breast cancer
Chemical carcinogenesis - DNA adducts
TGF-beta signaling pathway
P > 0.069
Rich Factor
Padjust
Number
G
Relative mRNA expression
EV
hZBP1
hZBP1 mutZα1
hZBP1 mutZα2
hZBP1 mutZα1+2
TNF
BIRC3
TNFAIP3
NFKBIA
H
Relative mRNA expression
EV
hZBP1
hZBP1 mutR1
hZBP1 mutR2
hZBP1 mutR3
TNF
BIRC3
TNFAIP3
NFKBIA

Figure EV4. Zα and RHIM domains contribute to hZBP1-mediated inflammation.

(**A**, **B**) Dot plot of the KEGG enrichment analysis for DEGs compared between HT-29 expressing Dox-inducible FLAG-hZBP1 mutZα1-2 (**A**) and FLAG-hZBP1 mutR1 (**B**) with FLAG-hZBP1 WT. (**C**) Heatmap analysis of ZBP1 expression compared between HT-29 expressing Dox-inducible FLAG-EV, FLAG-hZBP1, FLAG-hZBP1 mutZα1-2, and FLAG-hZBP1 mutR1. (**D**) Heatmap analysis of DEGs enriched in NFκB signaling compared between HT-29 expressing Dox-inducible FLAG-EV, FLAG-hZBP1 WT, FLAG-hZBP1 mutZα1-2, and FLAG-hZBP1 mutR1. (**E**) Heatmap analysis of *Zbp1* expression compared between immortalized MEFs expressing Dox-inducible FLAG-EV and FLAG-mZBP1. (**F**) Dot plot of the KEGG enrichment analysis for DEGs compared between immortalized MEFs expressing Dox-inducible FLAG-EV and FLAG-mZBP1. (**G**, **H**) qRT-PCR analysis of mRNA expression of the indicated genes in HT-29 expressing Dox-inducible FLAG-EV, FLAG-hZBP1 WT, FLAG-hZBP1 Zα mutants (**G**), and FLAG-hZBP1 RHIM mutants (**H**) stimulated with or without Dox (1 μg/mL) for 10 h. All experiments were replicated at least three times. Data with error bars are mean ± SEM. Data in panels (**G**, **H**) are pooled and analyzed from at least three independent biological experiments. *P* values were calculated by one-way ANOVA with Tukey's post hoc test for multiple comparisons (**G**, **H**). Source data are available online for this figure.

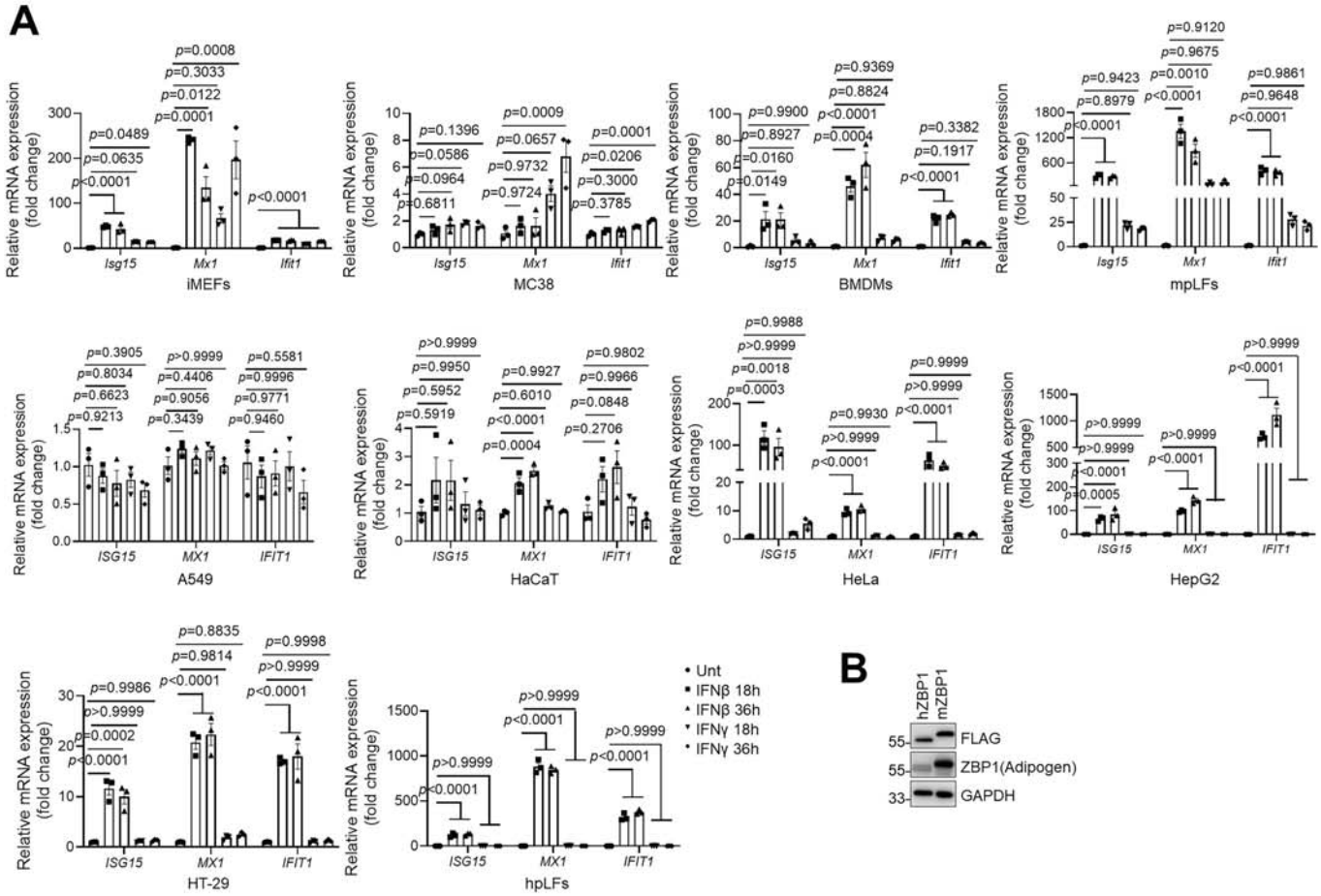

A
Relative mRNA expression (fold change)
Isg15
Mx1
Ifit1
iMEFs
MC38
BMDMs
mpLFs
ISG15
MX1
IFIT1
A549
HaCaT
HeLa
HepG2
HT-29
hpLFs
Unt
IFNβ 18h
IFNβ 36h
IFNγ 18h
IFNγ 36h
B
hZBP1
mZBP1
FLAG
ZBP1(Adipogen)
GAPDH
C
Ctrl
β 18h
β 36h
γ 18h
γ 36h
ADAR1-p150
ADAR1-p110
ACTIN
ISG15
GAPDH

Figure EV5. ISG expression in human and mouse cells upon IFN treatment.

(**A**) qRT-PCR analysis of mRNA expression of the indicated ISGs in different human and murine cells stimulated with or without IFNβ (20 ng/mL) or IFNγ (20 ng/mL) for 18 or 36 h. (**B**) Immunoblot analysis of total lysates in ZBP1 KO HeLa cells transfected with FLAG-hZBP1 and FLAG-mZBP1, and then blotted for FLAG and ZBP1 (Adipogen). (**C**) Immunoblot analysis of total lysates in different human and murine cells stimulated with or without IFNβ (20 ng/mL) or IFNγ (20 ng/mL) for 18 or 36 h, and then blotted for ADAR1-p150 and ISG15. All experiments were replicated at least three times. Data with error bars are mean ± SEM. Data from panel (**A**) are pooled and analyzed from at least three independent biological experiments. *P* values were calculated by one-way ANOVA with Tukey's post hoc test for multiple comparisons (**A**). Source data are available online for this figure.