PHYSIOLOGY

# Z-DNA binding protein 1 promotes heatstroke-induced cell death

Fangfang Yuan[1,2], Jizhen Cai[1,2], Jianfeng Wu[3,4], Yiting Tang[5], Kai Zhao[1,2], Fang Liang[1,2], Fanglin Li[1,2], Xinyu Yang[6], Zhihui He[1,2], Timothy R. Billiar[7], Haichao Wang[8], Lei Su[9], Ben Lu[1,2]*

Heatstroke is a heat stress–induced, life-threatening condition associated with circulatory failure and multiple organ dysfunctions. If global warming continues, heatstroke might become a more prominent cause of mortality worldwide, but its pathogenic mechanism is not well understood. We found that Z-DNA binding protein 1 (ZBP1), a Z–nucleic acid receptor, mediated heatstroke by triggering receptor-interacting protein kinase 3 (RIPK3)–dependent cell death. Heat stress increased the expression of ZBP1 through heat shock transcription factor 1 (HSF1) and activated ZBP1 through a mechanism independent of the nucleic acid sensing action. Deletion of ZBP1, RIPK3, or both mixed lineage kinase domain-like (MLKL) and caspase-8 decreased heat stress–induced circulatory failure, organ injury, and lethality. Thus, ZBP1 appears to have a second function that orchestrates host responses to heat stress.

Heatstroke is estimated to soon become a prominent cause of mortality worldwide (*1*, *2*). Clinically, heatstroke is characterized by extreme hyperthermia, systemic inflammatory responses, circulatory failure, bleeding and blood clotting disorder, and multiple organ dysfunctions (*1*–*3*) that result from a complex interplay between heat-related cytotoxicity, inflammation, and disseminated intravascular coagulation (DIC) (*1*, *3*). The molecular mechanisms that underlie the pathogenesis of this acute critical illness still remain largely unknown (*1*).

In *Caenorhabditis elegans*, heat stress causes pervasive necrotic cell death through calreticulin and the protease calpain—the depletion of these proteins limits heat stress–induced lethality of these nematode worms (*4*). In vertebrates, programmed necrosis is mediated by mixed lineage kinase domain-like (MLKL) or gasdermin family proteins (e.g., gasdermin D) that form pores on cytoplasmic or intracellular membranes upon activation (*5*–*9*). MLKL is activated by receptor-interacting protein kinase 3 (RIPK3)–dependent phosphorylation and executes a type of programmed necrosis, termed necroptosis (*6*, *7*). Activation of gasdermin D (GSDMD) triggers another type of programmed necrosis, termed pyroptosis (*8*, *9*). Excessive activation of GSDMD causes DIC and multiple organ dysfunctions in sepsis (*10*, *11*), an infection-induced critical illness that clinically resembles heatstroke (*1*, *10*, *11*). We investigated the possible role of programmed cell death in the pathogenesis of heatstroke and found that Z-DNA binding protein 1 (ZBP1), a Z–nucleic acid sensor (*12*–*14*), mediates the pathologic features of heatstroke by triggering RIPK3-induced activation of MLKL-dependent necroptosis and, to a lesser extent, caspase-8 (casp8)–dependent cell death.

[1]Department of Critical Care Medicine and Hematology, The 3rd Xiangya Hospital, Central South University, Changsha 410000, P.R. China. [2]Key Laboratory of Sepsis Translational Medicine of Hunan, Central South University, Changsha 410000, P.R. China. [3]Laboratory Animal Research Center, Xiamen University, Xiamen 361102, P.R. China. [4]State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signaling Network, School of Life Sciences, Xiamen University, Xiamen 361000, P.R. China. [5]Department of Physiology, School of Basic Medical Science, Central South University, Changsha 410000, P.R. China. [6]Department of Hematology, Xiangya Hospital, Central South University, Changsha 410000, P.R. China. [7]Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA. [8]The Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY 11030, USA. [9]Department of Intensive Care Unit, General Hospital of Guangzhou Command, Guangzhou 510000, P.R. China.
*Corresponding author. Email: xybenlu@csu.edu.cn

## RIPK3 mediates heat stress–induced cell death and features of heatstroke

To investigate the roles of programmed cell death in the pathogenesis of heatstroke, we placed *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, and *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ mice or corresponding wild-type (WT) control mice in a temperature-controlled environmental chamber conditioned at 39°C with a relative humidity of 60 ± 5% to induce heatstroke. Markers of programmed cell death were measured over time in various tissues. The heat stress increased animals' core temperature to 43°C; induced phosphorylation of RIPK3 and MLKL in the liver, lung, and intestine within 2 hours; and increased serum concentrations of RIPK3 (Fig. 1, A and B, and fig. S1A). Heat stress also induced the cleavage of pro-casp8, pro-casp3, GSDME, and GSDMD (Fig. 1A and fig. S1A). In *Ripk3*$^{-/-}$ mice, heat stress–induced activation of programmed cell death pathways was diminished (Fig. 1A and fig. S1A). Heat stress increased serum concentrations of lactate and proinflammatory cytokines, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF), and caused severe electrolyte disturbances, all of which were prevented by genetic deletion of RIPK3 (Fig. 1C and fig. S1, B and C). Heat stress also induced intravascular thrombin generation, platelet aggregation, fibrin deposition, occlusion of the microcirculation, and increase in circulating DIC markers, which were all attenuated by *Ripk3* deficiency (Fig. 1, D and E, and fig. S1, D and E). DIC promotes multiple organ injury and lethality in critical illness (*3*). In line with such clinical observations, deletion of RIPK3 attenuated heat stress–induced damage in the liver, lung, and intestine; prevented renal and pulmonary dysfunction; and prevented lethality (Fig. 1, F and G, and fig. S1, F to J). These findings indicated that RIPK3 mediates the pathologic features of heatstroke.

RIPK3 mediates necroptosis through MLKL in a kinase-dependent manner and induces apoptosis and pyroptosis through casp8 in a kinase-independent manner (*15*). To study the scaffolding function of RIPK3 in heat stress, we used *Ripk3*$^{\Delta/\Delta}$ mice that express a catalytically inactive RIPK3 resulting from the deletion of four amino acids (QWDF) in the RIPK3 kinase domain (*16*). Loss of the kinase activity of RIPK3 abrogated heat stress–induced phosphorylation of MLKL but did not affect the cleavage of pro-casp8, pro-casp3, or GSDME (Fig. 1H and fig. S2A). Inactivation of the kinase domain of RIPK1 (*17*) did not affect levels of death markers after heat stress (Fig. 1H and fig. S2A). Furthermore, deletion of both casp8 and MLKL blocked the cleavage of pro-casp3 and GSMDE and decreased the cleavage of GSDMD (Fig. 1A).

Next, we determined the kinase-dependent and kinase-independent roles of RIPK3 in heat stress–associated lethality. Deletion of RIPK3 achieved almost full protection, whereas loss of the kinase activity of RIPK3 rescued 70% of mice after lethal heat stress (Fig. 1I). In line with these observations, deletion of MLKL attenuated organ injury and rescued 75% of mice, whereas deletion of both MLKL and casp8 allowed all mice to survive heat stress (Fig. 1I). Deficiency of GSDMD conferred relatively minor protection (fig. S2B). Heat stress increased the core temperature of *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ mice to 43°C as it did in WT mice (fig. S2C), excluding the possibility that the increased survival might be the result of lower body temperature. Thus, these findings support a role for RIPK3-dependent cell death in heat stress.

## Heat stress triggers cell death through activating RIPK3 in vitro

We examined whether heat stress alone could activate RIPK3-dependent cell death. Exposure of cultured L929 mouse fibroblasts to 43°C for 2 hours or 42°C for 6 hours induced the phosphorylation of RIPK3 and MLKL within 2 hours and the cleavage of pro-casp8, pro-casp3, and GSDME by 6 hours after heat stress exposure (Fig. 2A). Deletion of RIPK3 using CRISPR-CAS9 blocked heat stress–induced phosphorylation of RIPK3 and MLKL and the

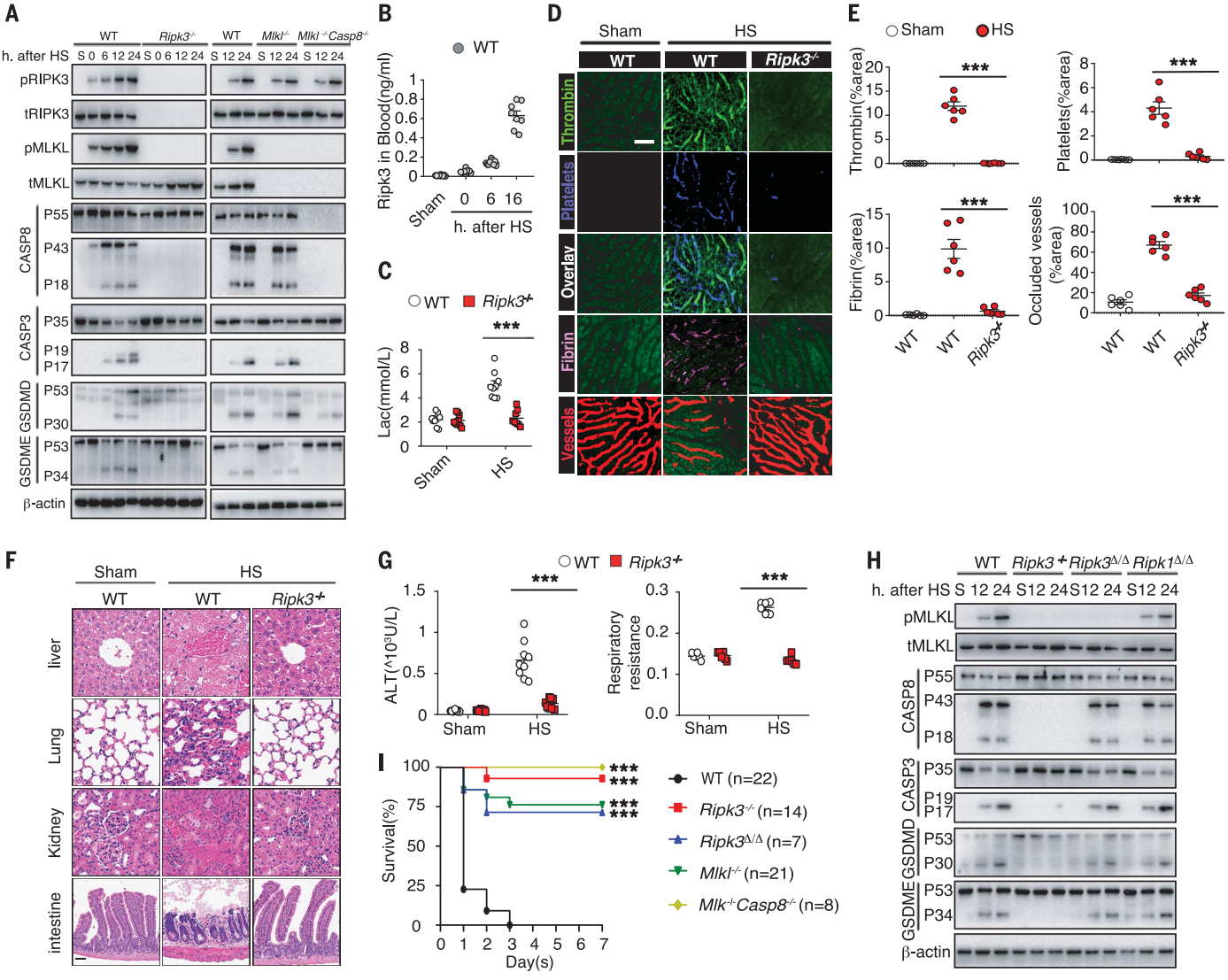
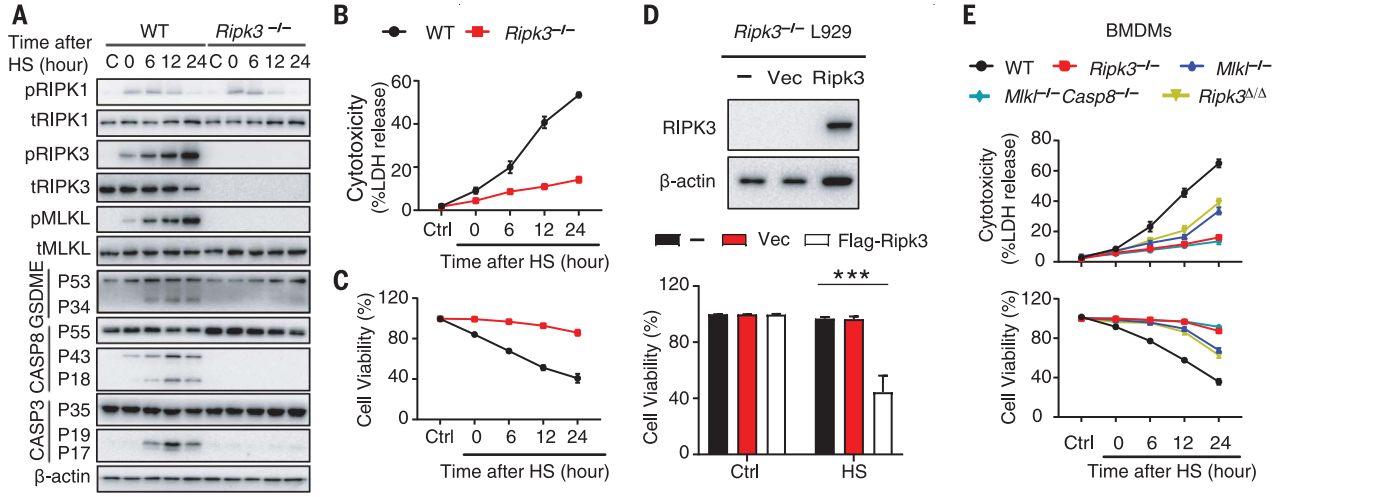



**Fig. 1. RIPK3 mediates heat stress–induced cell death and features of heatstroke.** (**A**) Western-blot analysis of the quantity of phosphorylated (p) RIPK3 and MLKL, total RIPK3 (tRIPK3), total MLKL (tMLKL), pro- (P55) and cleaved (P18) CASP8, pro- (P35) and cleaved (P19 and P17) CASP3, pro- (P53) and activated (P30) GSDMD, and pro- (P53) and activated (P34) GSDME in the livers of mice of indicated genotypes at the indicated time points after heat stress (HS). $n = 3$ independent biological repeats. h., hours. (**B** and **C**) Plasma concentrations of RIPK3 (B) and lactate (Lac) (C) in mice 16 hours after heat stress. (**D**) Representative images were acquired in the liver microvasculature of mice by spinning disk confocal intravital microscopy (SD-IVM) 12 hours after heat stress. Thrombin activation (green), platelet adhesion (blue), fibrin deposition (dark red), and circulating albumin (red) are shown. $n = 6$ repeats per genotype. Scale bar, 50 μm. (**E**) The fluorescence intensity was quantified by ImageJ software. (**F**) Representative images of hematoxylin and eosin (H&E) staining of different organs from WT or *Ripk3*$^{-/-}$ mice 16 hours after heat stress. Original magnification: H&E 400X. $n = 4$ repeats per genotype. (**G**) Serum alanine aminotransferase (ALT) activity and respiratory resistance were measured in WT or *Ripk3*$^{-/-}$ mice 16 hours after heat stress. (**H**) Quantity of indicated proteins in the livers of mice of indicated genotypes at 12 or 24 hours after heat stress. $n = 3$ independent biological repeats. (**I**) Survival analysis of genetic background–, age-, and sex-matched mice of indicated genotypes subjected to heat stress. Data were pooled from at least two independent experiments. Circles represent individual mice. Error bars indicate ±SEMs. ***$P < 0.001$. Statistics are by one-way analysis of variance (ANOVA) (B), two-way ANOVA test [(C), (E), and (G)], or survival curve comparison [log-rank (Mantel-Cox) test] (I).

cleavage of pro-casp8, pro-casp3, and GSDME (Fig. 2A). These events were associated with a reduction of cell death as judged by lactate dehydrogenase (LDH) release, loss of cell membrane integrity, and increased cell membrane permeability (Fig. 2, B and C, and fig. S3, A to C). Heat stress promoted the interaction between RIPK3 and MLKL in WT but not *Ripk3*-deficient L929 cells (fig. S3, D and E). Depletion of RIPK3 with short hairpin RNA (shRNA) inhibited heat stress–induced cell death (fig. S3F), whereas reconstitution of the expression of RIPK3 restored the capacity of *Ripk3*-deficient L929 cells to undergo cell death in response to heat stress (Fig. 2D). Similar observations were made using bone marrow–derived macrophages (BMDMs) and peritoneal macrophages (PMs) from WT or *Ripk3*$^{-/-}$ mice (Fig. 2, E and F, and fig. S4, A to E). Although MLKL deficiency protected cells from heat stress–induced necroptosis within 12 hours, delayed cell death occurred 24 hours after heat stress exposure in *Mlkl*$^{-/-}$ but not *Ripk3*$^{-/-}$ BMDMs (Fig. 2E). Because RIPK3 can mediate cell death through its scaffolding function and casp8 (*15*), we thought heat stress might induce RIPK3-dependent cell death through both MLKL and casp8.

To test this, *WT*, *Ripk3*$^{\Delta/\Delta}$, *Mlkl*$^{-/-}$, and *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ BMDMs were exposed to heat stress.



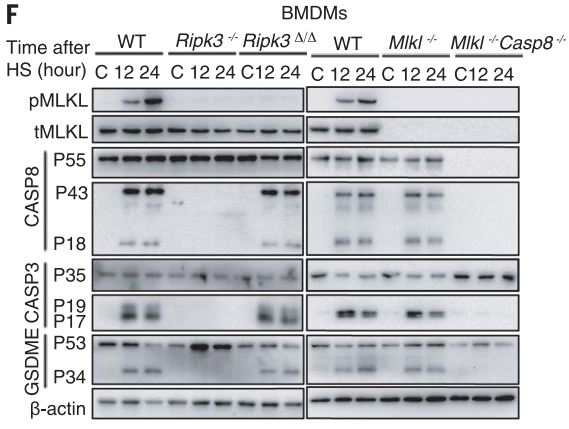




**Fig. 2. Heat stress triggers RIPK3-dependent cell death.** (**A**) Western-blot analysis of the quantity of indicated proteins in WT or *Ripk3*$^{-/-}$ L929 cells at the indicated time points after heat stress (43°C, 2 hours). *n* = 3 independent biological repeats. (**B** and **C**) LDH release (B) and cell viability (C) of WT or *Ripk3*$^{-/-}$ L929 cells at the indicated time points after heat stress. (**D**) *Ripk3*$^{-/-}$ L929 cells were infected with lentivirus containing blank vector (Vec) or vector encoding RIPK3. Cell viability was determined at 6 hours after heat stress. (**E**) LDH release and cell viability of BMDMs of indicated genotypes at the indicated time points after heat stress. (**F**) Quantity of indicated proteins in BMDMs of indicated genotypes after heat stress. *n* = 3 independent biological repeats. Error bars indicate ±SEMs of three independent experiments. ***$P < 0.001$. Statistics by two-way ANOVA test.

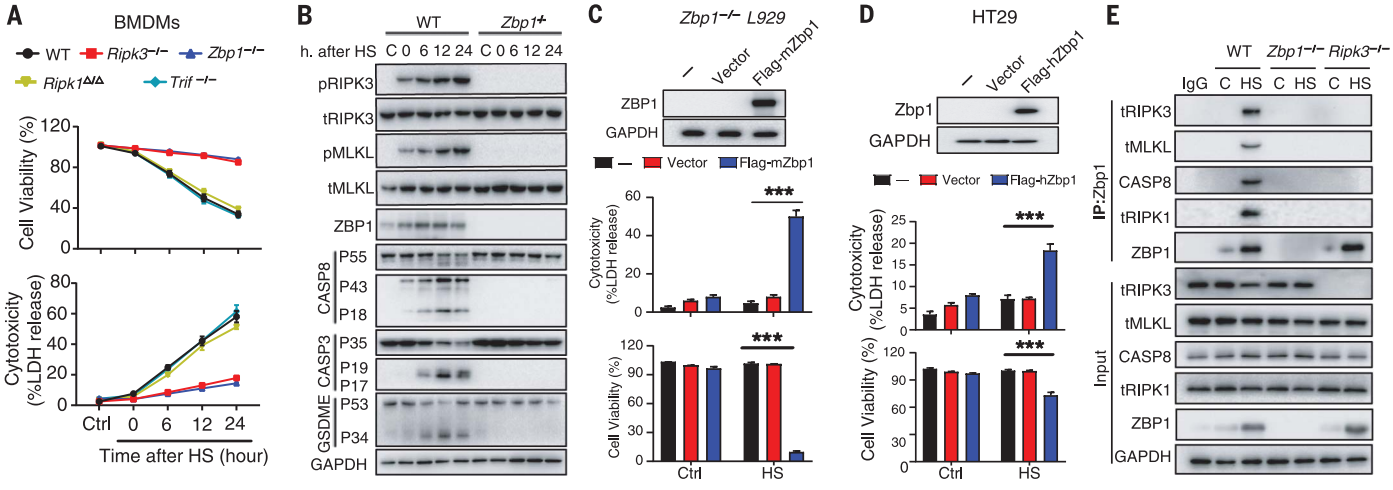
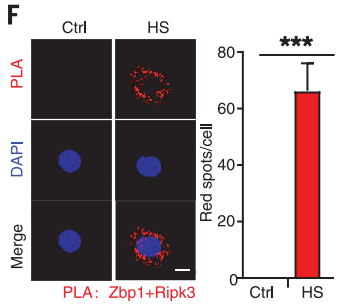



**Fig. 3. Heat stress activates RIPK3 through ZBP1.** (**A**) LDH release and cell viability of WT and *Zbp1*$^{-/-}$ BMDMs at the indicated time points after heat stress. (**B**) Western-blot analysis of the quantity of indicated proteins in WT and *Zbp1*$^{-/-}$ BMDMs at the indicated time points after heat stress. *n* = 3 independent biological repeats. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**C** and **D**) *Zbp1*$^{-/-}$ L929 (mouse) (C) or HT29 cells (human) (D) were infected with lentivirus containing blank vector or vector encoding *Zbp1* for 36 hours. Cell viability and LDH release were measured at 6 hours after heat stress. (**E**) ZBP1 immunoprecipitates (IPs) in WT, *Ripk3*$^{-/-}$, or *Zbp1*$^{-/-}$ L929 cells at 6 hours after heat stress and probed for the complex. *n* = 3 independent biological repeats. IgG, immunoglobulin G. (**F**) The physical associations between ZBP1 and RIPK3 were detected as red spots using proximity ligation assay (PLA) in WT L929 cells at 6 hours after heat stress. Scale bar, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole. Error bars indicate ±SEMs of three independent experiments. ***$P < 0.001$. Statistics by two-way ANOVA test [(A), (C), and (D)] or two-tailed *t* test (F).



The loss of RIPK3 kinase activity prevented the phosphorylation of MLKL but not the cleavage of casp8, casp3, and GSDME (Fig. 2F). Deletion of both MLKL and casp8 almost completely blocked the cleavage of casp3 and GSDME (Fig. 2F). Although ablation of the kinase activity of RIPK3 or deletion of MLKL prevented LDH release and cell death at 6 hours after heat stress, BMDMs from *Ripk3*$^{\Delta/\Delta}$ or *Mlkl*$^{-/-}$ mice released LDH and underwent cell death 24 hours after heat stress exposure (Fig. 2E). Delayed cell death and LDH release were prevented by deletion of RIPK3 or both MLKL and casp8 (Fig. 2E). Depletion of casp8 with shRNA or pharmacological inhibition of casp8 by Z-IEDT-FMK reduced heat stress–induced cell death in *Mlkl*-deficient L929 cells (fig. S5, A to C). Further, heat stress transiently (within 12 hours) increased the expression of cellular FLICE-like inhibitory protein (cFLIP) (fig. S5D), which inhibits casp8 activation and associated cell death (*18*). Thus, heat stress triggers cell death through RIPK3-dependent activation of MLKL and casp8.

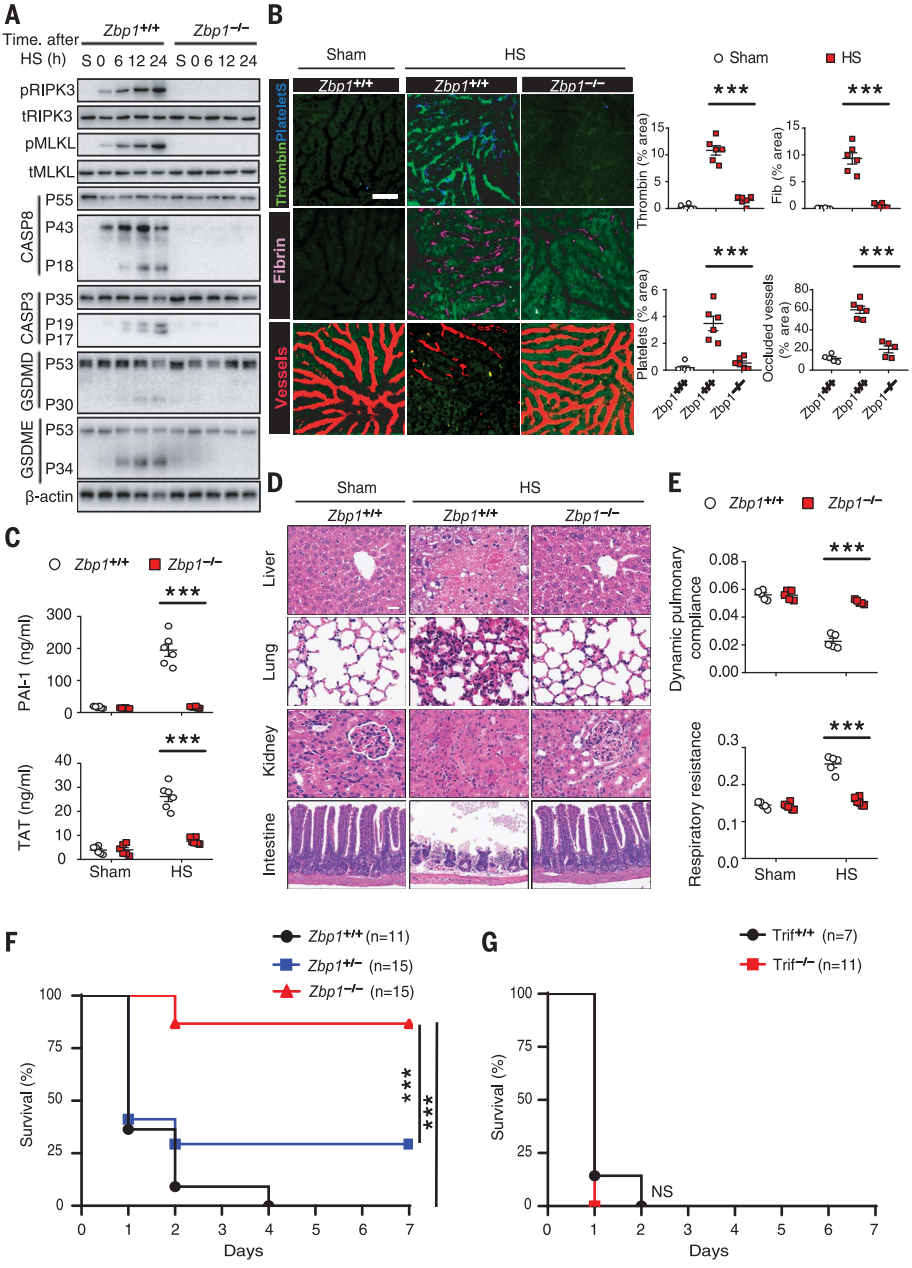



**Fig. 4. ZBP1 mediates heat stress–induced cell death and features of heatstroke.** (**A**) Western-blot analysis of the quantity of indicated proteins in the livers of *Zbp1*$^{+/+}$ and *Zbp1*$^{-/-}$ mice at the indicated time points after heat stress. *n* = 2 independent biological repeats. (**B**) Representative images were acquired in the liver microvasculature by SD-IVM 12 hours after heat stress. Scale bar, 50 μm. The fluorescence intensity was quantified by ImageJ software. Circles represent individual mice. Fib, fibrin. (**C**) Plasma concentrations of PAI-1 and TAT complex in *Zbp1*$^{+/+}$ and *Zbp1*$^{-/-}$ mice 16 hours after heat stress. (**D**) Representative images of H&E staining different organs from mice of indicated genotypes 16 hours after heat stress. *n* = 6 repeats per genotype. (**E**) Respiratory function in *Zbp1*$^{+/+}$ and *Zbp1*$^{-/-}$ mice at 16 hours after heat stress. (**F** and **G**) Survival analysis of mice of indicated genotypes subjected to heat stress. Data were pooled from at least two independent experiments. Circles represent individual mice. Error bars indicate ±SEMs. NS, not significant ($P \geq 0.05$); ***$P <$ 0.001. Statistics by one-way ANOVA (B), two-way ANOVA test [(C) and (E)], and survival curve comparison [log-rank (Mantel-Cox) test] [(F) and (G)].

## Heat stress activates RIPK3 through ZBP1

RIPK1, Toll/interleukin-1 receptor domain–containing adapter-inducing interferon-β (TRIF), and ZBP1 are RIP homotypic interaction motif (RHIM)–containing proteins that can interact with and activate RIPK3 (*5*, *19*, *20*). We therefore investigated whether these RIPK3-interacting proteins are required for heat stress–induced cell death. Deletion of TRIF, mutation of the kinase domain of RIPK1 (*RIPK1*$^{\Delta/\Delta}$), or inhibition of RIPK1 by necrostain-1 did not affect heat stress–induced cell death in BMDMs (Fig. 3A and fig. S6, A and B). RIPK1 deficiency did not significantly inhibit heat stress–induced cell death in L929 cells (fig. S6A). By contrast, deletion of ZBP1 abrogated the phosphorylation of RIPK3 and MLKL; the cleavage of casp8, casp3, and GSDME; and cell death after heat stress (Fig. 3, A and B). These findings were confirmed in L929 cells (fig. S6, C and D). Restoration of ZBP1 expression restored the capacity of *Zbp1*-deficient cells to undergo cell death after heat stress (Fig. 3C). Human HT-29, a colon cancer cell line that express RIPK3 and RIPK1 but not ZBP1, also failed to undergo heat stress–induced cell death (Fig. 3D). Expression of exogenous human ZBP1 rendered HT-29 cells susceptible to heat stress–induced cell death (Fig. 3D). Furthermore, heat stress induced the interaction between ZBP1 and RIPK3 (Fig. 3, E and F). These data demonstrate that heat stress activates RIPK3 through ZBP1.

## ZBP1 mediates the pathologic features of heatstroke

We determined whether ZBP1 activates RIPK3 and mediates heatstroke after heat stress by exposing *Zbp1*$^{-/-}$ mice and their WT littermates to heat stress. Deletion of ZBP1 blocked the heat stress–induced phosphorylation of RIPK3 and MLKL as well as the cleavage of CASP8, CASP3, GSDMD, and GSDME in the liver and intestine (Fig. 4A and fig. S7A). Loss of ZBP1 prevented heat stress–induced DIC, systemic inflammatory responses, circulatory failure, multiple organ injury, and lethality,

as similarly observed with RIPK3 deficiency (Fig. 4, B to F, and fig. S7, B to F). By contrast, deletion of TRIF or mutation of the RIPK1 kinase domain failed to protect mice against lethal heat stress (Fig. 4G and fig. S7G). Heat stress increased the core temperature of *Zbp1*$^{-/-}$ mice to 43°C as it did in WT mice (fig. S7H), excluding the possibility that the increased survival of *Zbp1*$^{-/-}$ mice might be a result of a lower body temperature. These data demonstrate that ZBP1 mediates heat stress–induced RIPK3 activation and the pathologic features of heatstroke.

### Heat stress increases the expression of ZBP1 through HSF1

ZBP1 is an interferon-inducible factor (*21*), and heat stress up-regulated the expression of ZBP1 in L929 cells and mouse macrophages in a manner similar to that of type 1 interferon (Fig. 5, A to D, and fig. S8, A and B). Furthermore, heat stress stimulated ZBP1 transcription in the lung, liver, kidney, and intestine (Fig. 5E and fig. S8C). We used the bioinformatics tool Jaspar to analyze the promoter region of ZBP1 and identified a predicted binding site for heat shock transcription factor 1 (HSF1) (Fig. 5F), which is activated by heat stress (*22*). Deletion of the putative HSF1 binding site prevented the increase in transcriptional activation through the ZBP1 promoter after heat stress (Fig. 5F). Heat stress enhanced HSF1 activation and occupancy at the HSF1 binding site in the ZBP1 promoter (Fig. 5G and fig. S8, D to F). Deletion of HSF1 inhibited a heat stress–induced increase in ZBP1 expression and cell death (Fig. 5H, fig. S8G, and fig. S9A). In response to heat stress, HSF1 regulated the expression of heat shock proteins (HSPs) (fig. S9B) (*22*). HSP90 enhances TNF-induced necroptosis through the promotion of MLKL oligomerization and activation (*23*, *24*). However, depletion of HSP90 did not affect heat stress–induced phosphorylation of RIPK3 or MLKL and cell death (fig. S9, C and D). This discrepancy might be because of the different stimuli used (TNF versus heat stress). These data establish that heat stress increases the expression of ZBP1 through HSF1.

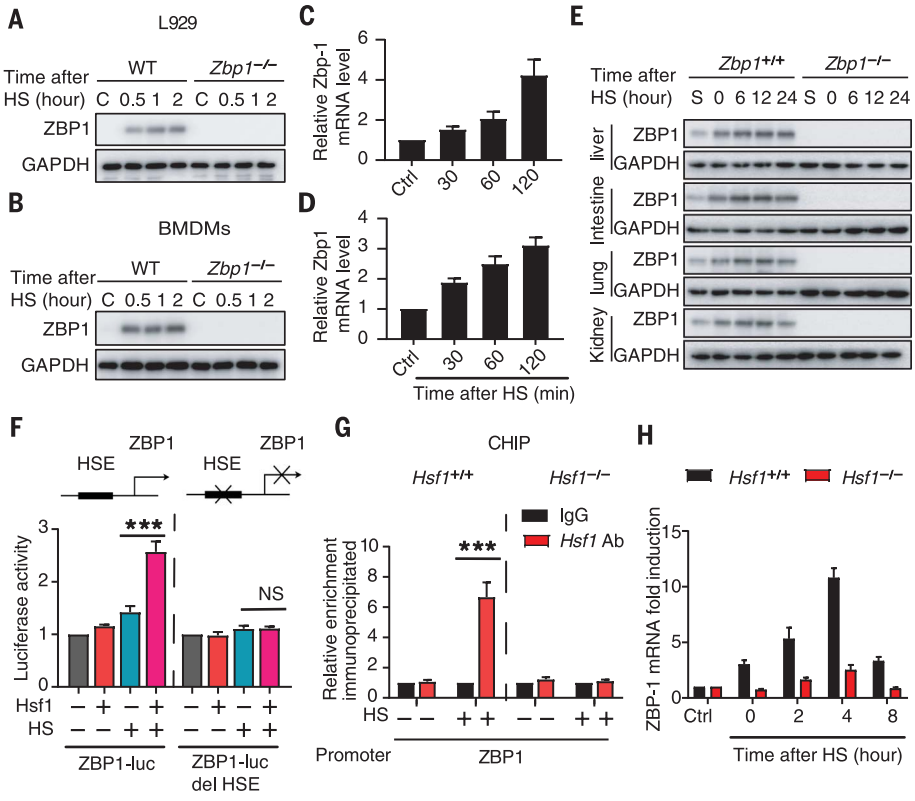



**Fig. 5. Heat stress increases the expression of ZBP1 through HSF1.** (**A** and **B**) Western-blot analysis of the ZBP1 expression in L929 cells (A) or BMDMs (B) subjected to heat stress for the indicated time. $n = 3$ independent biological repeats. (**C** and **D**) Quantitative real-time PCR (qRT–PCR) analysis of ZBP1 mRNA in L929 cells (C) or BMDMs (D) subjected to heat stress for the indicated time, presented relative to the quantity of GAPDH mRNA. (**E**) Western-blot analysis of ZBP1 expression in multiple organs of *Zbp1*$^{+/+}$ or *Zbp1*$^{-/-}$ mice at the indicated time points after heat stress. $n = 3$ independent biological repeats. (**F**) The transcriptional activity of WT ZBP1 promoter (ZBP1-luc) or ZBP1 promoter with a deletion of the putative heat shock element (HSE) site (ZBP1-luc del HSE) in 293T cells overexpressing HSF1 after heat stress was determined by the luciferase activity in cell lysates. (**G**) Chromatin immunoprecipitation (ChIP) assay in PMs to assess HSF1 binding at the putative HSE site in the ZBP1 promoter after heat stress. (**H**) qRT–PCR analysis of ZBP1 mRNA expression in *Hsf1*$^{+/+}$ or *Hsf1*$^{-/-}$ BMDMs at the indicated time points after heat stress. Error bars indicate ±SEMs of three independent experiments. NS ($P \geq 0.05$); ***$P < 0.001$. Statistics by two-way ANOVA test.

### Z–nucleic acid sensing is dispensable for heat stress–induced ZBP1 activation

Because expression of ZBP1 by itself is insufficient to induce cell death (Fig. 3, C and D), we next investigated the mechanisms by which heat stress promotes ZBP1 activation. ZBP1 is activated by virus-derived or endogenous Z–nucleic acids during development, viral infection, and in other diseases through its Zα domain (*12*–*14*, *19*–*21*). We generated genetically modified L929 cells expressing intact ZBP1 or ZBP1 mutants that either lacked the Zα, Zα1, or Zα2 domain (ΔZα, ΔZα1, or ΔZα2) or that contained a point mutation within the Zα2 domain (Zα2 mut) that prevents Z–nucleic acid sensing (Fig. 6A). However, heat stress still triggered a ZBP1-RIPK3 interaction, RIPK3 and MLKL phosphorylation, and cell death in L929 cells expressing these ZBP1 mutants (Fig. 6, B to E). ZBP1 contains a C-terminal domain and an RHIM domain (*21*). The RHIM but not C-terminal domain was indispensable for heat stress–induced ZBP1-RIPK3 interaction, RIPK3 and MLKL phosphorylation, and cell death (Fig. 6, B to E). These findings indicate that heat stress activated ZBP1 through its RHIM domain independent of Z–nucleic acid sensing.

### Heat stress promotes aggregation of ZBP1 fusion proteins

To further study how heat stress activates ZBP1, we generated HEK 293T cells transfected with plasmids that express green fluorescent protein (GFP)–tagged ZBP1. Exposure of cells to 43°C led to ZBP1-GFP aggregation into puncta within the cytosol (fig. S10A). The Zα domain was dispensable for heat stress–induced ZBP1-GFP aggregation (fig. S10A). Lack of the Zα2 domain or point mutation within the Zα2 domain did not affect ZBP1 aggregation during heat stress (fig. S10A). Immunoblots under nonreducing conditions revealed that heat stress induced the aggregation of endogenous ZBP1 (fig. S10B). Using cells expressing Flag- or Myc-tagged ZBP1 or ZBP mutants, we confirmed that exposure of cells to heat stress increased the aggregation of ZBP1 (fig. S10, C and D). The Zα domain, the C-terminal domain, and the Z–nucleic acid sensing were dispensable for ZBP1 aggregation and cell death (fig. S10, C to G).

ZBP1 contains two RHIM domains, referred to as RHIM-A and RHIM-B (*21*). Point mutation within the RHIM-A domain prevented

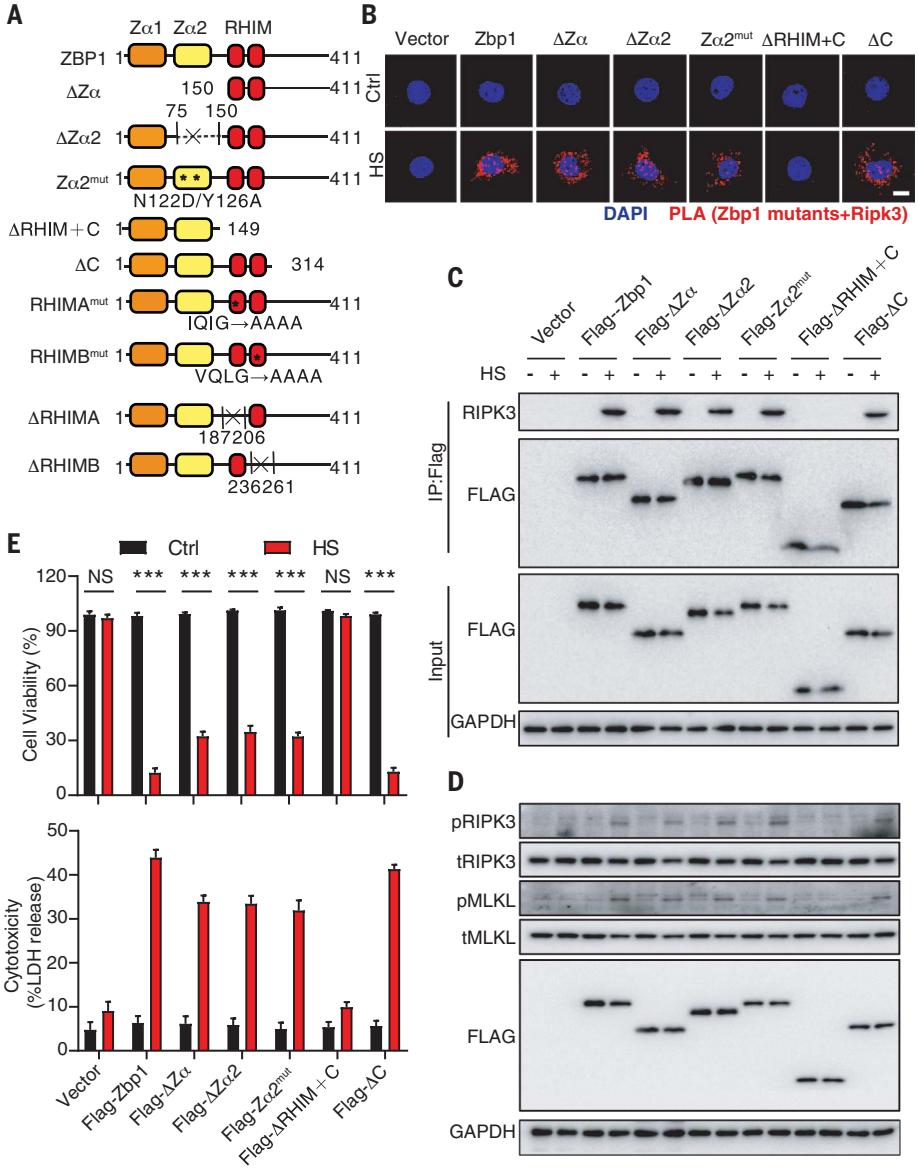



**Fig. 6. Z–nucleic acid sensing is dispensable for heat stress–induced ZBP1 activation.** (**A**) Schematic representation of full-length ZBP1 or indicated mutants. (B to E) *Zbp1*$^{-/-}$ L929 cells were infected with lentivirus expressing flag-tagged ZBP1 and its truncation mutants and then were subjected to heat stress or not (ctrl) at 36 hours after infection. $n$ = 3 independent biological repeats. (**B**) The physical association between RIPK3 and ZBP1 or ZBP1 mutants was detected by PLA. (**C**) Coimmunoprecipitation assay to assess the interactions between RIPK3 and ZBP1 or its truncation mutants. Whole-cell extract (5% input) was examined in parallel for Flag-tagged ZBP1 or its mutants. (**D**) Western-blot analysis of the quantity of pRIPK3 and pMLKL at 6 hours after heat stress. (**E**) LDH release and cell viability at 6 hours after heat stress. Error bars indicate ±SEMs of three independent experiments. NS ($P \geq 0.05$); ***$P < 0.001$. Statistics by two-way ANOVA test.

heat stress–induced aggregation of ZBP1 fusion proteins (fig. S10, A to F). Reconstitution of *Zbp1*$^{-/-}$ L929 cells with WT ZBP1 and mutant ZBP1 with RHIM-B deletion or mutation, but not mutant ZBP1 with RHIM-A deletion or mutation, induced the aggregation of ZBP1 fusion proteins, the phosphorylation of MLKL, the cleavage of casp8, and cell death after heat stress (fig. S10, E to G). Aggregation of ZBP1 fusion proteins occurred as early as 30 min after heat stress, which was followed by RIPK3 recruitment, phosphorylation of MLKL, and cleavage of casp8 (fig. S10F). These events were all blocked by deletion or point mutation of the RHIM-A domain (fig. S10F). Using L929 cells expressing hormone-binding domain G521R mutant (HBD*) fused ZBP1 proteins, we observed that treatment of such cells with the HBD dimerizer 4-hydroxytamxifen (4-OHT) caused the aggregation of HBD-ZBP1 independent of heat stress (fig. S10H) and triggered cell death (fig. S10I). Thus, heat stress may promote cell death through ZBP1 aggregation.

## Discussion

This study identifies a role of ZBP1 in promoting the pathologic features of heatstroke through RIPK3-dependent cell death. Heat stress increases the expression of ZBP1, which acts in a mechanism independent of Z–nucleic acid sensing. ZBP1 binds virus-derived or endogenous retrovirus-derived Z–nucleic acids during viral infection, embryonic development, and the pathogenesis of diseases such as psoriasis and inflammatory bowel disease (*12*–*14*, *19*–*21*, *25*). In these scenarios, the activation of ZBP1 requires both the Zα domain that binds Z–nucleic acids and the RHIM domain that activates RIPK3. We found that the Zα domain is dispensable for the ZBP1 activation upon heat stress. RHIM-A domain–dependent aggregation of ZBP1 may contribute to heat stress–induced cell death. Endogenous Z–nucleic acids might enhance heat stress–induced ZBP1 activation because deletion or mutation of the Zα or Zα2 domain slightly inhibits heat stress–induced cell death. Because ZBP1 expression by itself is insufficient to cause cell death, it is likely that other factors may induce ZBP1 aggregation and activation in response to heat stress.

The finding that ZBP1 promotes cell death in response to both Z–nucleic acids and heat stress could provide insight into how the host combats invading pathogens. Programmed cell death is an important strategy for the host to eliminate intracellular pathogens, such as viruses (*13*, *21*, *26*). By passively releasing pro-inflammatory damage-associated molecular patterns, dead cells can alert the immune system to augment inflammatory responses (*5*, *27*–*29*). Considering that infections can cause high fever, it is conceivable that hyperthermia might promote pathogen clearance and inflammation by activating ZBP1. However, extreme hyperthermia resulting from persistent environmental heat exposure could cause excessive activation of ZBP1-dependent cell death, leading to circulatory failure, DIC, multiple organ dysfunction, and even death.

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**Author contributions:** B.L. conceived the project, supervised the research, and wrote the manuscript; B.L. and F.Y. designed the experiments; F.Y., J.C., J.W., K.Z., F.Li, F.Lia., and X.Y. performed the experiments; T.R.B., H.W., Y.T., Z.H., and L.S. commented on and edited the manuscript; and F.Y. and J.C. analyzed the data and made the figures. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data are available in the main text or the supplementary materials. Correspondence and requests for materials should be addressed to B.L.

## SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

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Science

# Z-DNA binding protein 1 promotes heatstroke-induced cell death

Fangfang YuanJizhen CaiJianfeng WuYiting TangKai ZhaoFang LiangFanglin LiXinyu YangZhihui HeTimothy R. BilliarHaichao WangLei SuBen Lu

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**Understanding heat-induced necrosis**

Z-DNA binding protein 1 (ZBP1), a sensor for Z–nucleic acids, is implicated in necrosis caused by heat stress in the worm *Caenorhabditis elegans*. Yuan *et al*. examined the roles of proteins that regulate necrosis in worms undergoing heat stroke. The protein kinase receptor-interacting protein kinase 3 (RIPK3) leads to activation of mixed lineage kinase domain-like (MLKL) and Caspase-8, and all were required for heat-induced death. ZBP1 is a RIPK3 and necrosis activator and it appeared to contribute to heat-induced necrosis in the worms. This response did not require the nucleic acid–sensing domain of ZBP1. Transcription of ZBP1 was enhanced by heat shock transcription factor 1 (HSF1). —LBR







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