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Supplementary Information for

HSC70 regulates cold-induced caspase-1 hyperactivation by an autoinflammation-causing mutant of cytoplasmic immune receptor NLRC4

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Supplementary text

Materials and Methods

Antibodies and Chemicals- Antibodies against GFP (cat no. SC-9996), caspase-1 (SC-56036), myc (SC-40; SC-789), HSC70 (SC-7298), HSP70 (SC-66048), Haemagglutinin (HA; SC-805), IL-1β (SC-7884) and caspase-1 (SC-515) were obtained from Santa Cruz Biotechnology, USA. Antibodies against NLRC4 (CST#12421) was from Cell Signaling Technology. Actin (MAB1501) and GAPDH antibodies (MAB-374) were from Millipore. Agarose conjugated Myc antibody (MycTrap; yta-20), GFP antibody (GFPTrap; gta-20) and control agarose beads (bab-20) used for immunoprecipitation were purchased from ChromoTek, Germany. HRP-conjugated mouse and rabbit secondary antibodies were obtained from GE Healthcare. FemtoLUCENT™ PLUS HRP Kit (786-10) and protease inhibitor cocktail were procured from GBiosciences. Cy3 (Indocarbcyanine) conjugated mouse or rabbit secondary antibodies were purchased from EMD Millipore. Alexa633- / Alexa488- conjugated secondary antibodies were procured from Thermo Fisher. NEM (cat no. E3876) was procured from Sigma-Aldrich. Blotto (sc-2324), apoptozole (sc-221257) and siRNAs targeting HSC70 (sc-29349) and HSP70 (sc-29352) were from Santa Cruz Biotechnology, USA.

Expression vectors- pEGFP-C1-NLRC4 and its deletion constructs, pCB6-WT-Caspase-1 and pcDNA3-IL-1β, which express corresponding human proteins, have been described previously (1). Disease associated mutants GFP tagged NLRC4-H443P, NLRC4-V341A and NLRC4-T337S were generated by CAC-CCC, GTG-GCG and ACC-TCC nucleotide substitutions, respectively. Dr. Barbara Kazmierczak, Yale University kindly provided pCruzMycB-NLRC4 and NLRC4-V341A expression vectors. Myc tagged NLRC4-H443Pand NLRC4-T337S mutants were generated using site directed mutagenesis. cDNAs coding for human HSC70 and HSC70-SBD were cloned in BamHI and XhoI sites of pGEX-5X-2 bacterial expression vector (GE Healthcare). Sequences of all the mutants were confirmed by automated DNA sequencing.

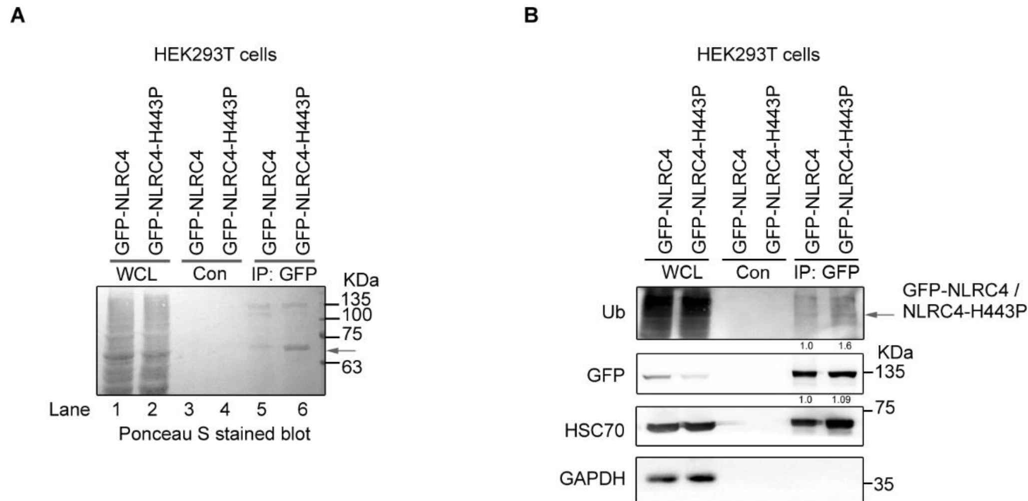
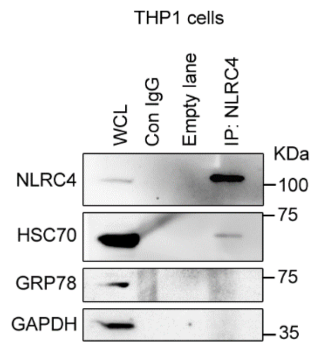


Fig. S1. HSC70 is present in molecular complexes formed by NLRC4. (A) HEK293T cells were transfected with GFP-NLRC4 or GFP-NLRC4-H443P and immunoprecipitation was carried out using either GFP antibody or control antibody (Con). A Ponceau S stained blot of immunoprecipitates and whole cell lysates (WCL) is shown. Arrow indicates the prominent band of approximately 70KDa. **(B)** Blot shown in (A) was probed with antibodies as indicated. NLRC4-H443P shows enhanced interaction with HSC70 and enhanced ubiquitination. Arrow indicates position of GFP-NLRC4 or GFP-NLRC4-H443P in the blot probed with ubiquitin antibody (Ub).

A



B

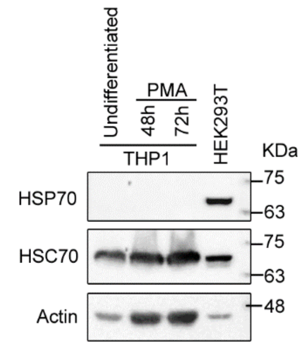


Fig. S2. GRP78 does not show detectable interaction with endogenous NLRC4. (A) THP1 cells were differentiated with 10nM PMA for 72h. Whole cell lysate was subjected to immunoprecipitation using NLRC4 antibody or control antibody (Con IgG) and immunoprecipitates analysed by western blotting. HSC70, but not GRP78 was co-immunoprecipitated with NLRC4. GAPDH was used as a control. **(B)** Expression of HSP70 is not detectable in human THP1 cells. THP1 cells were treated with 10nM PMA and allowed to differentiate into macrophage-like cells for 48h or 72h. Lysates of undifferentiated and differentiated THP1 cells were tested for expression of HSP70 protein. Lysate of HEK293T cells was used as positive control. Actin was probed as loading control.

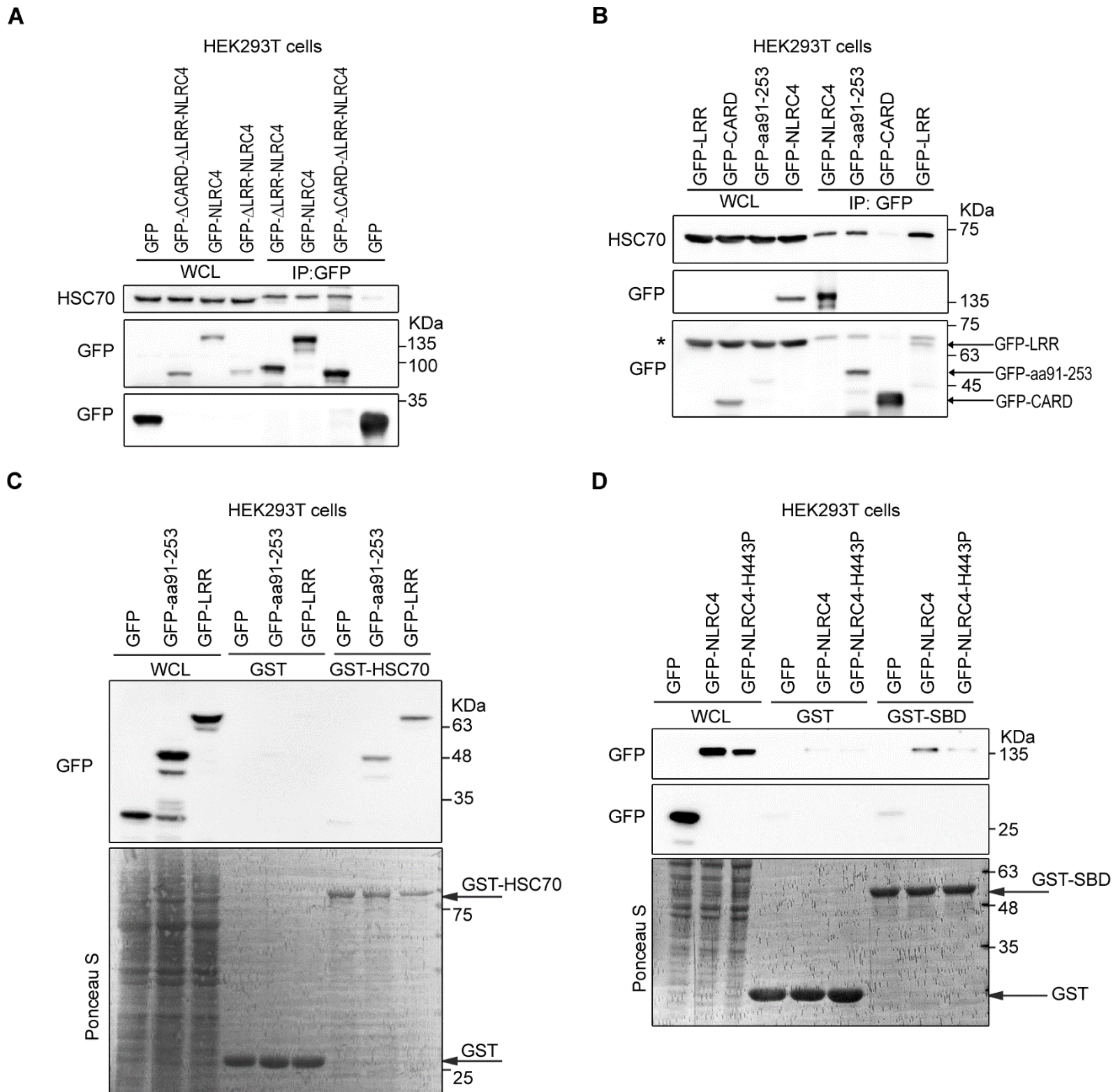


Fig. S3. Domains of NLRC4 and HSC70 required for complex formation. (A and B) NBD (aa91-253) and LRR (aa658-1024) domains of NLRC4 can independently bind to HSC70. GFP-tagged NLRC4 or its deletion constructs were transiently transfected in HEK293T cells for 16h. Whole cell lysates were subjected to immunoprecipitation using GFP antibody and analysed by western blotting for presence of endogenous HSC70. The blot in (B) was probed with HSC70 antibody followed by reprobing with GFP antibody; * indicates residual signal from bands of endogenous HSC70; arrows indicate bands corresponding to the indicated NLRC4 proteins. **(C)** GST pulldown assay showing binding of GST-HSC70 with GFP-aa91-253 and GFP-LRR. Purified GST or GST-HSC70 were incubated with lysates of HEK293T cells expressing GFP, GFP-aa91-253 or GFP-LRR and bound proteins analysed by western blotting. Arrows point to positions of GST or GST-HSC70 in the Ponceau S stained blot. **(D)** Substrate binding domain of HSC70 (SBD) is sufficient to bind with NLRC4 or NLRC4-H443P. GST or GST-SBD was incubated with lysates of HEK293T cells expressing WT-NLRC4 or NLRC4-H443P and bound proteins analysed by western blotting. Arrows indicate expression of the recombinant proteins.

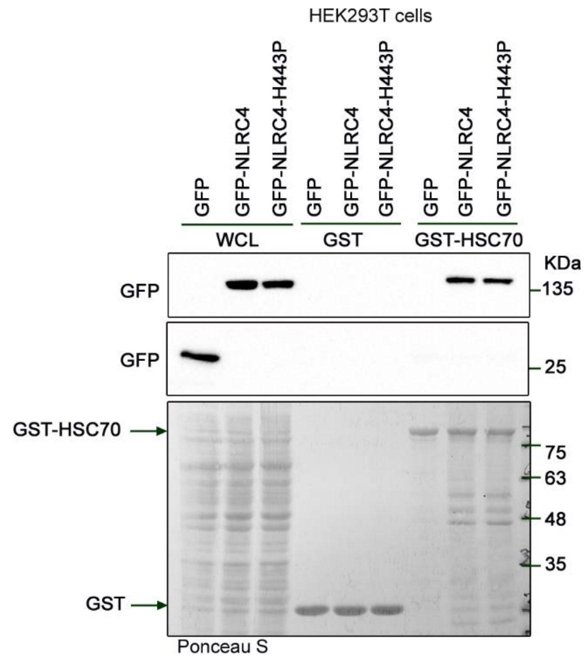


Fig. S4. Lysates of HEK293T cells expressing GFP, GFP-NLRC4 or GFP-NLRC4-H443P were prepared in buffer without N-ethylmaleimide and incubated with purified GST or GST-HSC70 for 30 minutes at 37°C and bound proteins analysed by western blotting. GST-HSC70 did not show increased binding with NLRC4-H443P compared to WT-NLRC4.

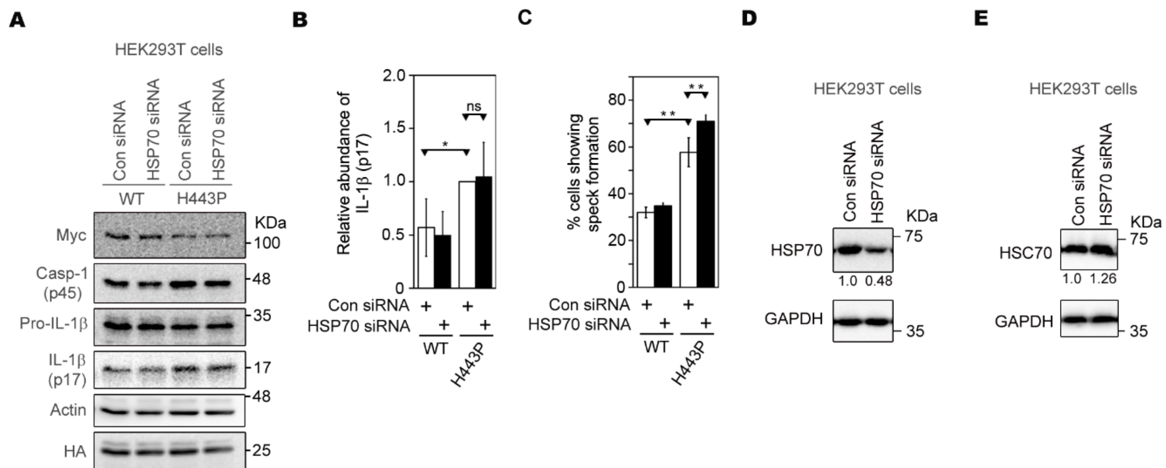


Fig. S5. Effect of HSP70 knockdown on IL-1β maturation and ASC-mediated speck formation by NLRC4-H443P. (A) HEK293T cells were transfected with control siRNA or HSP70 siRNA along with caspase-1, pro-IL-1β, HA-tagged ASC and Myc-tagged NLRC4 or NLRC4-H443P. Whole cell lysates were analysed by western blotting for presence of mature IL-1β (p17). Actin was probed as loading control. **(B)** Bar diagram shows quantitation of relative abundance of IL-1β (p17) in cells expressing NLRC4 or NLRC4-H443P normalized with pro-IL-1β (p32) signal; n=3. * $p<0.05$; ns= not significant. **(C)** Quantitation of effect of siRNA mediated knockdown of HSP70 on ASC-mediated speck formation by WT-NLRC4 or NLRC4-H443P mutant. Cells co-expressing HA-ASC and Myc-NLRC4 or Myc-NLRC4-H443P were scored for presence or absence of specks. n=4. ** $p<0.005$. **(D)** Western blot shows efficacy of HSP70 knockdown by siRNA. **(E)** HSP70 siRNA does not affect cellular HSC70 indicating target specificity. GAPDH was used as loading control.

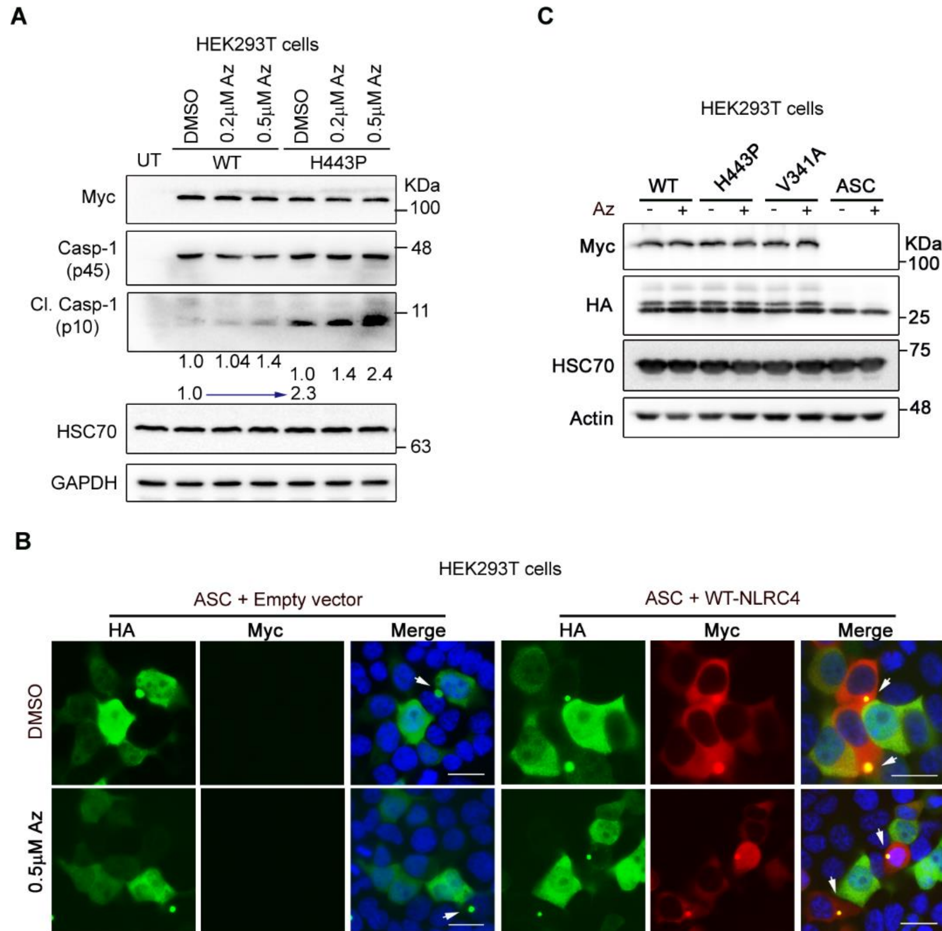


Fig. S6. Effect of apoptozole treatment on caspase-1 activation by NLRC4-H443P. (A) HEK293T cells expressing Myc-tagged NLRC4 or NLRC4-H443P along with caspase-1 were treated with Az or DMSO for 6h in concentrations as indicated. Lysates analysed by western blotting for abundance of cleaved caspase-1 (p10). GAPDH was used as loading control. **(B)** Effect of apoptozole treatment on ASC-mediated speck formation. Representative immunofluorescence images showing effect of apoptozole treatment on ASC-speck formation by Myc-NLRC4. White arrows indicate specks. DAPI was used to stain nucleus. Scale bars, 20µm. **(C)** HEK293T cells were transfected with HA-ASC along with Myc tagged NLRC4, H443P or V341A mutant. The cells were treated (+) with 0.5µM apoptozole (Az) or DMSO (-) for 6h. Expression levels of indicated proteins were checked by western blotting.

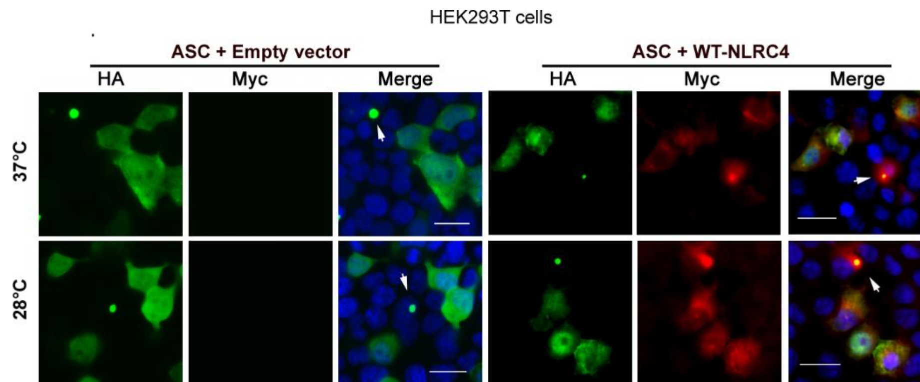


Fig. S7. Exposure to subnormal temperature does not increase ASC-mediated speck formation by WT-NLRC4. Representative immunofluorescence images show effect of exposure to subnormal temperature (28°C for 6h) on ASC-speck formation in cells expressing HA-ASC along with Myc-NLRC4. White arrows indicate specks. DAPI was used to stain nucleus. Scale bars, 20μm.

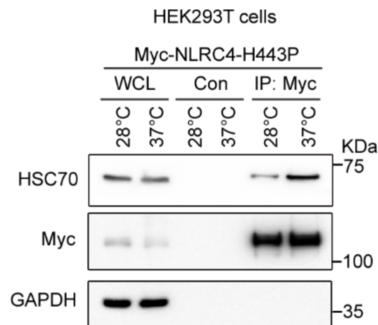


Fig. S8. NLRC4-H443P shows reduced interaction with HSC70 upon exposure of cells to subnormal temperature. HEK293T cells expressing Myc-NLRC4-H443P were grown at 37°C for 24h or exposed to 28°C for 6h after 18h of expression. Lysates were subjected to immunoprecipitation using agarose conjugated control antibody (Con) or Myc antibody and immunoprecipitates analysed by western blotting. GAPDH was used as a control.

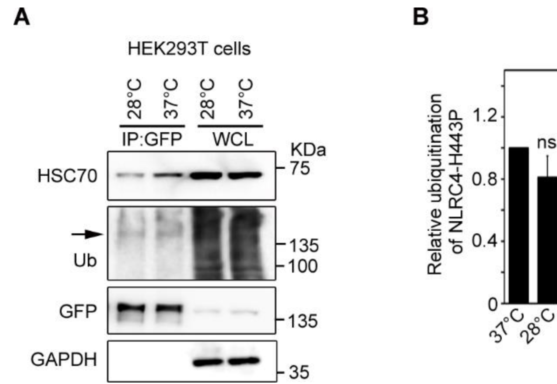


Fig. S9. Effect of exposure to subnormal temperature on ubiquitination of NLRC4-H443P. (A) HEK293T cells expressing GFP-NLRC4-H443P were grown at 37°C for 16h or exposed to 28°C for 4h after 12h of expression. Lysates were prepared in buffer containing 10mM NEM to prevent deubiquitination and subjected to immunoprecipitation using agarose conjugated GFP antibody. Immunoprecipitates were analysed by western blotting. Arrow indicates bands corresponding to GFP-NLRC4-H443P in the blot probed with ubiquitin antibody. GAPDH was used as a control. **(B)** Bar diagram shows quantitation of effect of exposure to subnormal temperature on NLRC4-H443P ubiquitination. Levels of ubiquitinated NLRC4-H443P was normalized against corresponding GFP-signal. The change in ubiquitination levels is not significant (ns). n=3.

SI Appendix Reference:

1. Kumar Y, Radha V, & Swarup G (2010) Interaction with Sug1 enables Ipaf ubiquitination leading to caspase 8 activation and cell death. *Biochem J* 427(1):91-104.