Science

The heme-regulated inhibitor is a cytosolic sensor of protein misfolding that controls innate immune signaling

Mena Abdel-Nour, Leticia A. M. Carneiro, Jeffrey Downey, Jessica Tsalikis, Ahmed Outlioua, Dave Prescott, Leandro Silva Da Costa, Elise S. Hovingh, Armin Farahvash, Ryan G. Gaudet, Raphael Molinaro, Rob van Dalen, Charles C. Y. Lau, Farshad C. Azimi, Nichole K. Escalante, Aaron Trotman-Grant, Jeffrey E. Lee, Scott D. Gray-Owen, Maziar Divangahi, Jane-Jane Chen, Dana J. Philpott, Damien Arnoult and Stephen E. Girardin

Science **365** (6448), eaaw4144.
DOI: 10.1126/science.aaw4144

Linking protein misfolding and innate immunity

Multiple innate immune sensors undergo rapid assembly into large complexes known as signalosomes. This is an essential step during cellular responses to microbes and danger signals. How this process is regulated to avoid accumulation of potentially toxic protein aggregates remains poorly understood. Abdel-Nour *et al.* identified a pathway, dependent on heme-regulated inhibitor, eukaryotic initiation factor 2 α, activating transcription factor 4, and heat shock protein B8, which controls the folding and scaffolding of innate immune sensors, allowing optimal proinflammatory signaling (see the Perspective by Pierre). The pathway appears to mirror the endoplasmic reticulum unfolded protein response (UPR), and so was named the cytosolic UPR (cUPR). The cUPR may represent a general mechanism to control protein misfolding in cells.

Science, this issue p. eaaw4144; see also p. 28

Supplementary Materials for

The heme-regulated inhibitor is a cytosolic sensor of protein misfolding that controls innate immune signaling

Mena Abdel-Nour, Leticia A. M. Carneiro, Jeffrey Downey, Jessica Tsalikis, Ahmed Outlioua, Dave Prescott, Leandro Silva Da Costa, Elise S. Hovingh, Armin Farahvash, Ryan G. Gaudet, Raphael Molinaro, Rob van Dalen, Charles C. Y. Lau, Farshad C. Azimi, Nichole K. Escalante, Aaron Trotman-Grant, Jeffrey E. Lee, Scott D. Gray-Owen, Maziar Divangahi, Jane-Jane Chen, Dana J. Philpott, Damien Arnoult*, Stephen E. Girardin*

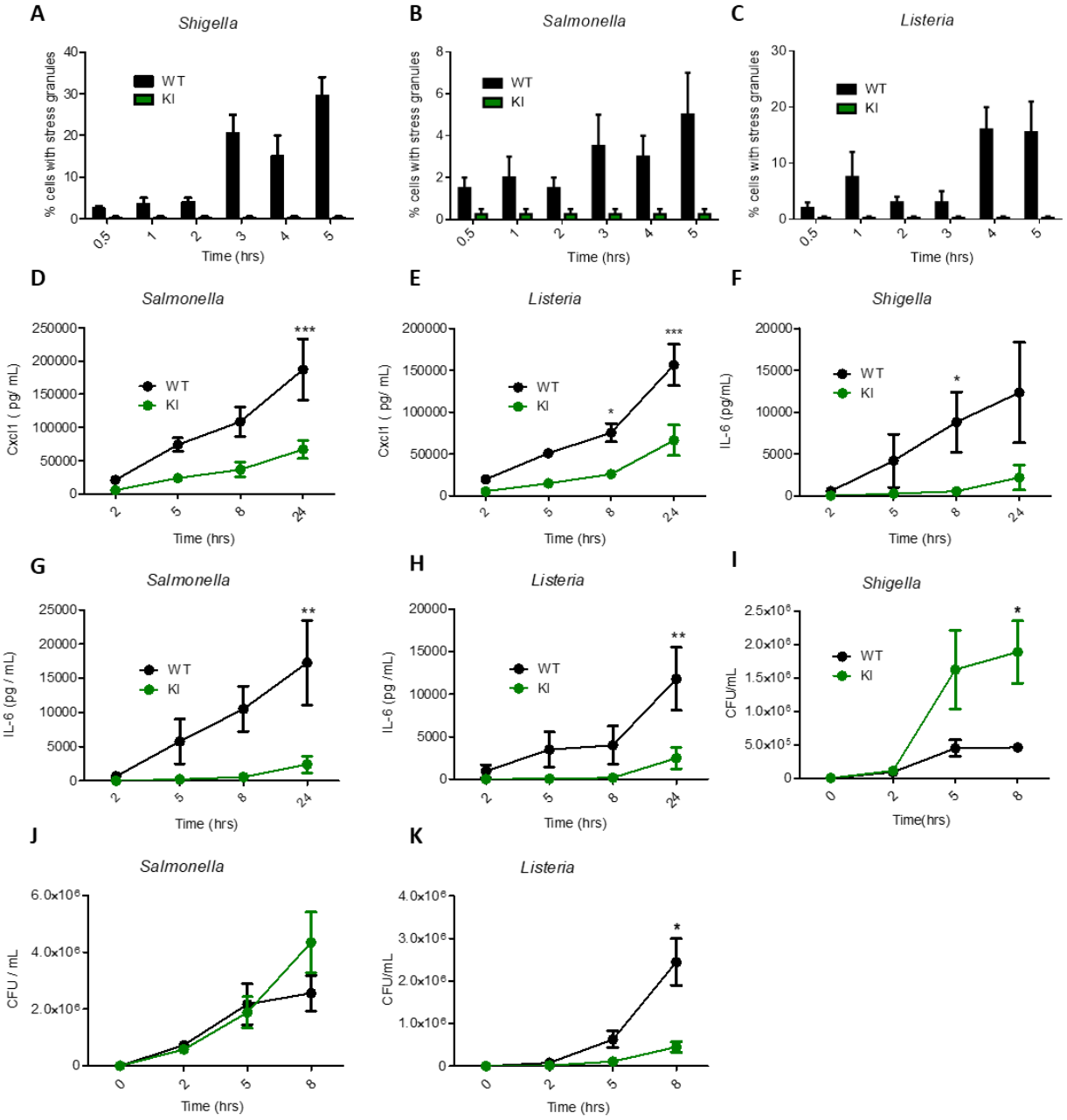
*Corresponding author. Email: stephen.girardin@utoronto.ca (S.E.G.); damien.arnoult@inserm.fr (D.A.)

Published 5 July 2019, *Science* **365**, eaaw4144 (2019)
DOI: 10.1126/science.aaw4144

This PDF file includes:

Figs. S1 to S35
Tables S1 to S5

Fig. S1. eIF2*α* phosphorylation is required for inflammatory responses during infection and it has Differential effect on bacterial replication

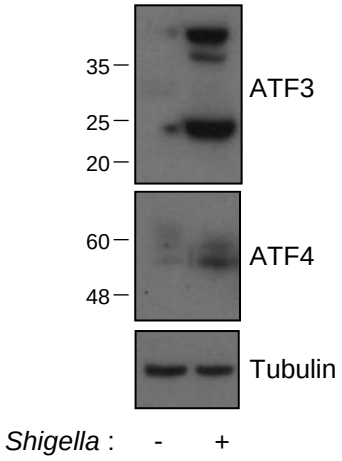


A-C. Percentage of infected cells with Tia-1 positive stress granules during infection with *Shigella* (**A**) *Salmonella* (**B**) and *Listeria* (**C**) in wild-type (WT) and eIF2α S51A knock-in (KI) MEFs.

D-E. Cxcl1 secretion from the supernatants of WT and KI MEFs infected with *Salmonella* (**D**) and *Listeria* (**E**) as measured by ELISA.

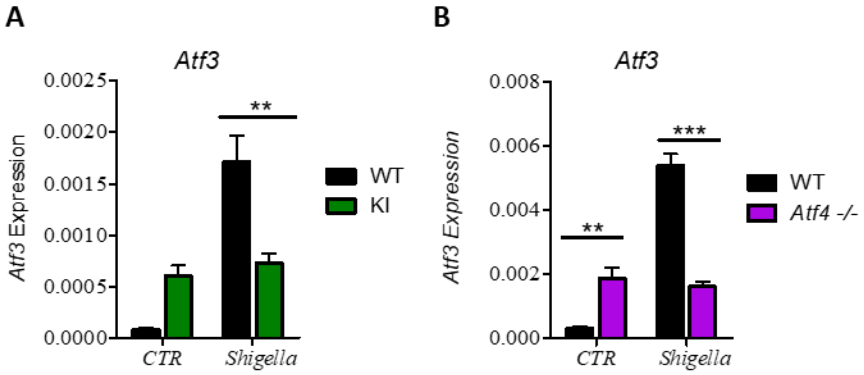
F-H. IL-6 secretion from the supernatants of WT and KI MEFs infected with *Shigella*, (**F**) *Salmonella* (**G**) and *Listeria* (**H**) as measured by ELISA.
I-K. Colony forming unit (CFU) analysis of intracellular bacterial replication in WT and KI MEFs infected with *Shigella* (**I**) *Salmonella* (**J**) or *Listeria* (**K**).
Line graphs represent the means +/- S.D. from 3 or more independent experiments (*, ** and ***, $p < 0.05$, 0.01 and 0.001, respectively).

Fig. S2. Shigella infection induces ATF3 and ATF4 expression.



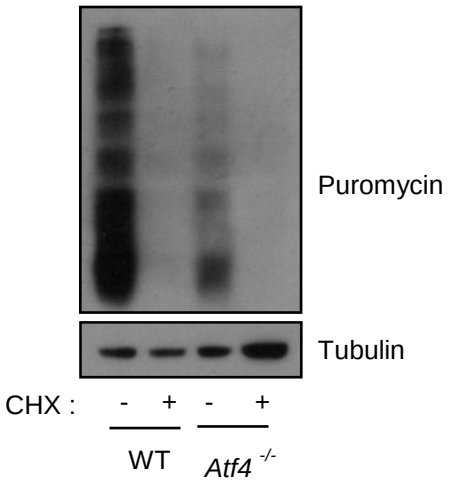
HeLa cells left unstimulated or infected with *Shigella* for 4 hours and analyzed by Western blotting using anti-ATF3, anti-ATF4 and anti-Tubulin antibodies.

Fig S3. Phosphorylation of eIF2α and ATF4 are required for Atf3 expression during bacterial infection.



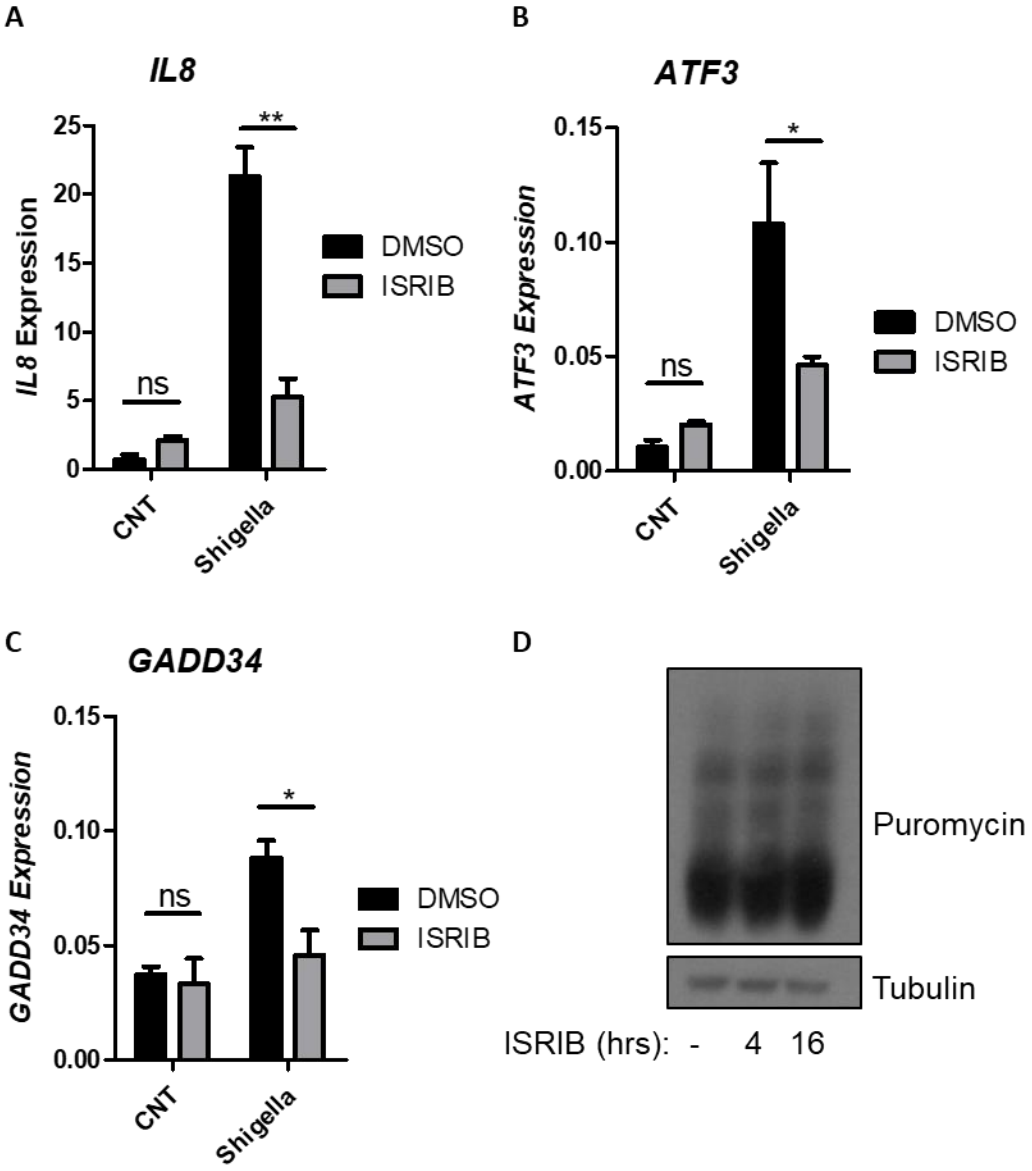
A. Expression of *Cxcl1* in WT (Wild type) and eIF2α S51A knock-in (KI) MEFs following infection with *Shigella* for 4 hours measured by qPCR.
B. Expression of *Cxcl1* in WT and Atf4 knockout (*Atf4* $^{-/-}$) MEFs left unstimulated or infected with *Shigella* for 4 hours measured by qPCR.
Bar graphs display the means +/- S.D. from 3 independent experiments (** and ***, $p < 0.01$ and 0.001, respectively).

Fig S4. Atf4 knockout cells perform less translation at baseline.



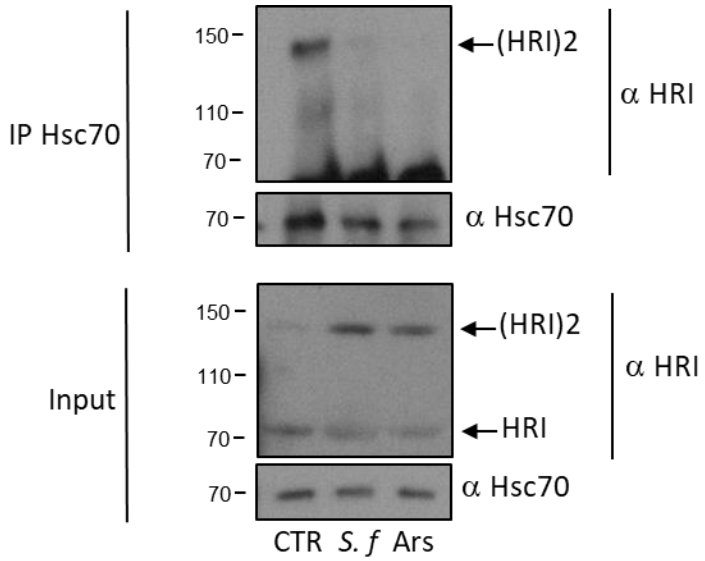
SUNSet assay to measure translation levels in wild-type (WT) and Atf4 knockout (Atf4 $^{-/-}$) MEFs left unstimulated or treated with 10 μg /mL cycloheximide (CHX) for 4 hours, treated with 10 μg /mL puromycin for 10 minutes and analyzed by Western blotting using anti-puromycin antibodies. Cycloheximide was used as a positive control for a treatment which induces translation block and therefore no puromycin staining.

Fig S5. Acute inhibition of eIF2α phosphorylation is sufficient to inhibit inflammatory responses during infection.



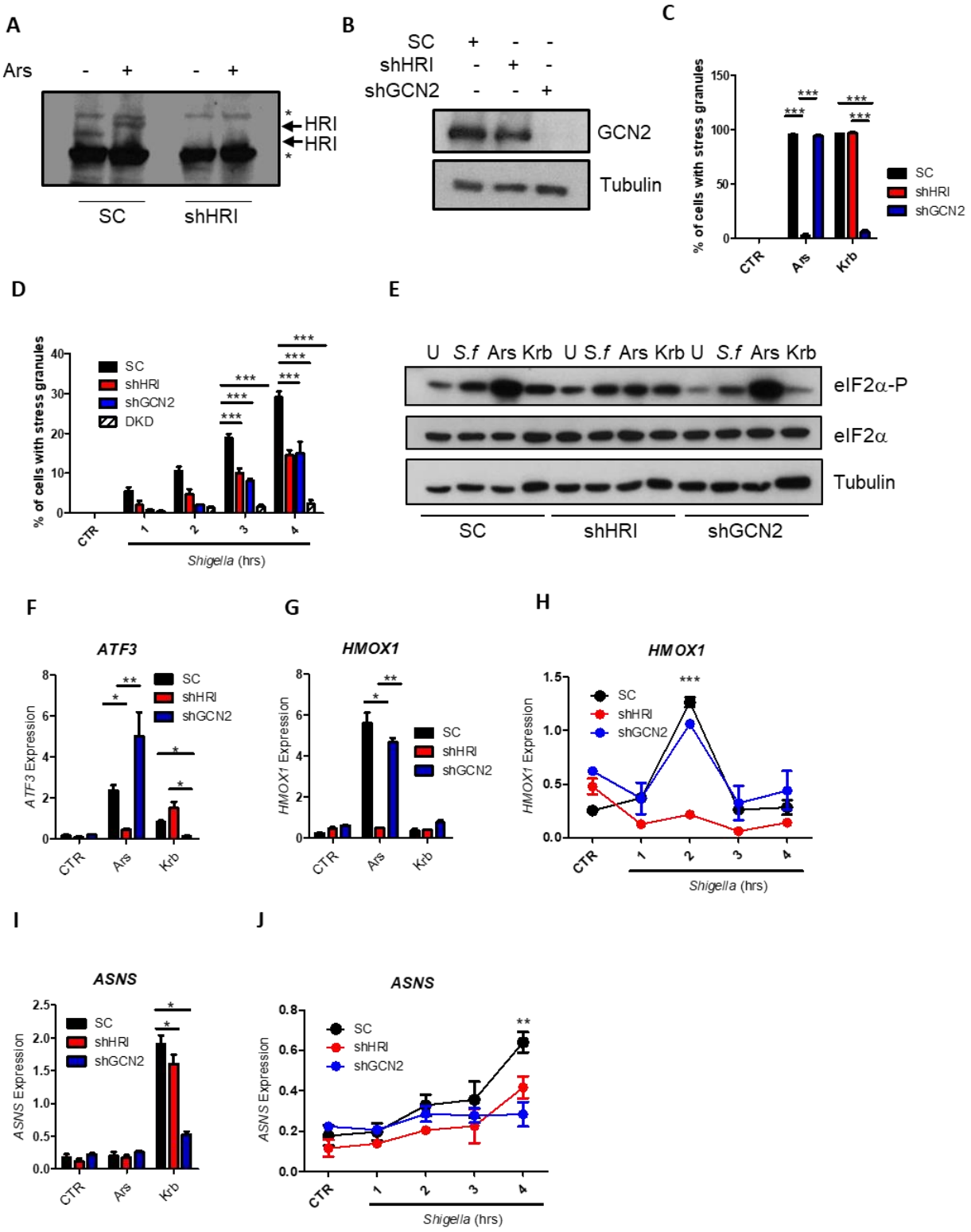
A-C. Expression of *IL8* (**A**), *ATF3* (**B**) and *GADD34* (**C**) in HeLa cells treated with DMSO or
1 μg/mL ISRIB and infected with *Shigella* for 4 hours measured by RT-qPCR.
D. SUNSet assay to measure translation levels in HeLa cells treated with 1 μg /mL ISRIB for the indicated time points. Cells were treated with 10 μg /mL puromycin for 10 minutes before collection and analyzed by Western blotting using anti-puromycin antibodies.
Bar graphs represent the means +/- S.D. from 3 independent experiments (*,** and ***, p <0.05, 0.01 and 0.001, respectively).

Fig S6. HRI dissociates from Hsc70 during *Shigella* infection and Arsenite treatment.



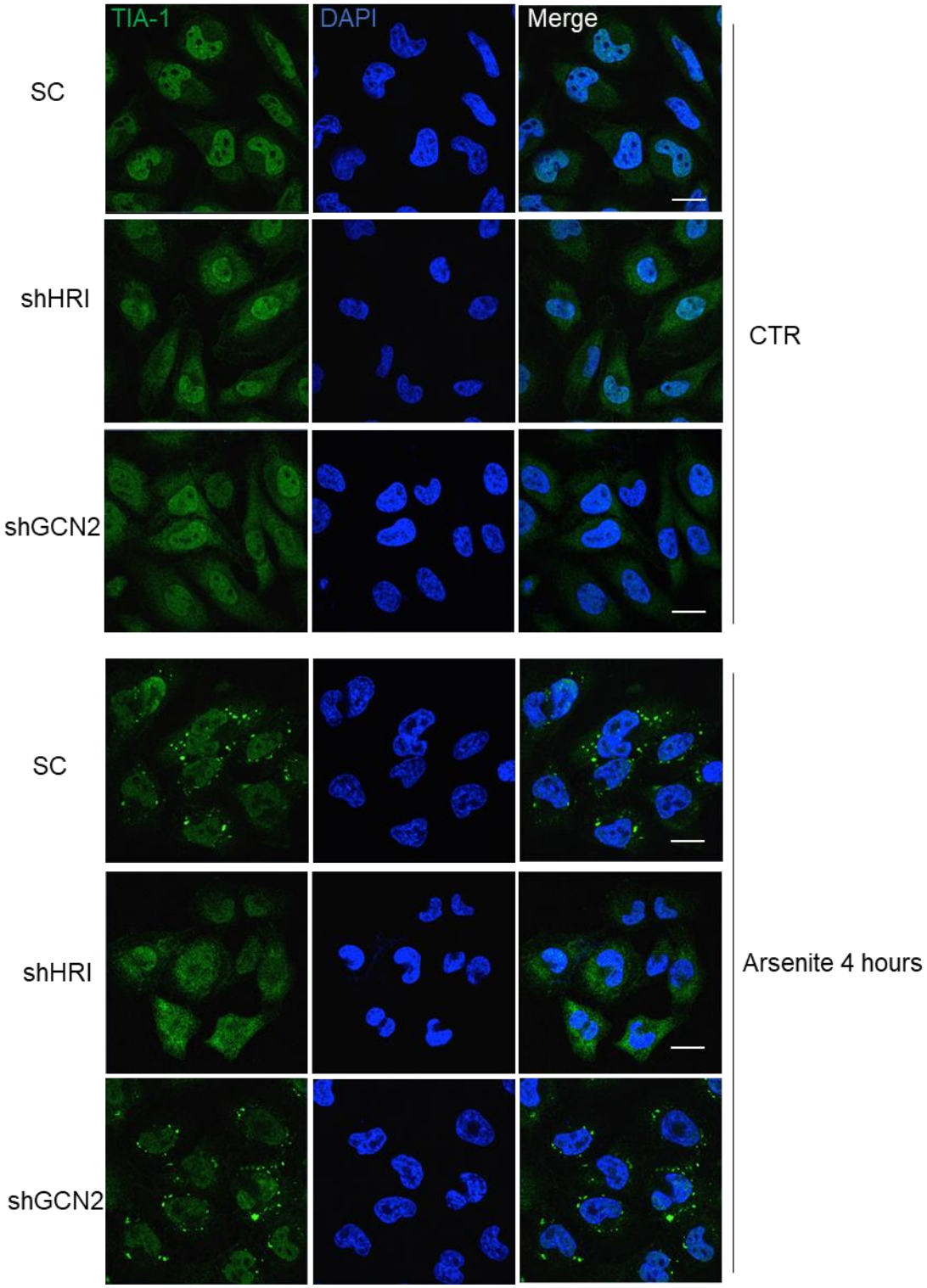
Co-immunoprecipitation experiments with the lysates of HeLa cells that were left unstimulated (CTR), infected with *Shigella (S.f)* and treated with 300 µM sodium arsenite (Ars) for 30 minutes.

Fig S7. Validation of HRI and GCN2 silencing and effect on stress granule formation and gene expression.



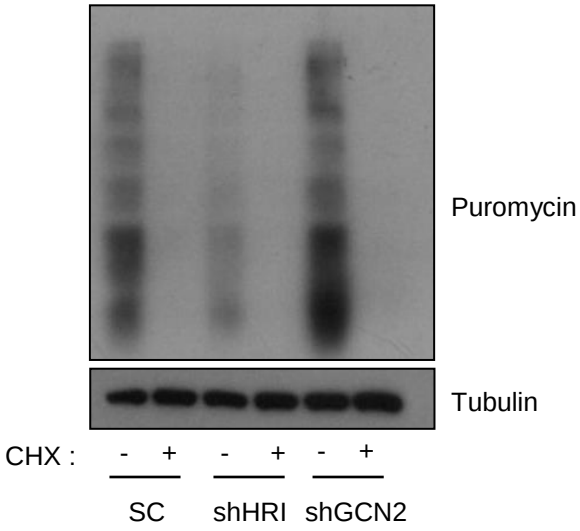
A-B. HeLa cells transduced with lentiviral particles targeting a scrambled (SC) sequence, HRI (shHRI) or GCN2 (shGCN2) with or without stimulation with 300 µM sodium arsenite for 30 minutes were analyzed by western blotting using anti-HRI antibodies (**A**) or left untreated and analyzed by western blotting using anti-GCN2 and anti-Tubulin antibodies (**B**).
C. HeLa cells transduced with lentiviral particles targeting a scrambled sequence, HRI or GCN2, were stimulated with 300 µM sodium arsenite for 4 hours or overnight stimulation with KRB and visualized by immunofluorescence using anti-Tia1 antibodies and the percentage of cells with Tia-1 positive stress granules was quantified.
D. HeLa cells transduced with HRI or GCN2 targeting lentiviral particles or both (DKD) and infected with *Shigella* for the indicated time points and visualized by immunofluorescence using anti-Tia1 antibodies and the percentage of cells with Tia-1 positive stress granules was quantified.
E. HeLa cells transduced with HRI or GCN2 targeting lentiviral particles left untreated (U), infected for 1 hour with *Shigella (S.f)*, treated with 300 µM sodium arsenite for 1hr (Ars) or treated with KRB starvation buffer for 45 minutes (Krb) and analyzed by Western blotting with anti-Phospho eIF2α (S51), anti-eIF2α antibodies or anti-tubulin antibodies.
F-G. Expression of *ATF3* (F) and *HMOX1* (G) in HeLa cells transduced with HRI or GCN2 targeting lentiviral particles and treated for 4 hours with 300 µM sodium arsenite or KRB measured by qPCR.
H. Expression of *HMOX1* in HeLa cells transduced with HRI or GCN2 targeting lentiviral particles infected with *Shigella* for the indicated time points measured by qPCR.
I. Expression of *ASNS* in HeLa cells transduced with HRI or GCN2 targeting lentiviral particles and treated for 4 hours with 300 µM sodium arsenite or KRB.
J. Expression of *ASNS* in HeLa cells transduced with HRI or GCN2 targeting lentiviral particles infected with *Shigella* for the indicated time points measured by qPCR.
* in panel **A** denotes non-specific bands. Bar graphs represent the means +/- S.D. from 3 or more independent experiments (** and ***, $p < 0.01$ and 0.001, respectively).

Fig S8. Validation of HRI and GCN2 silencing and effect on stress granule formation.



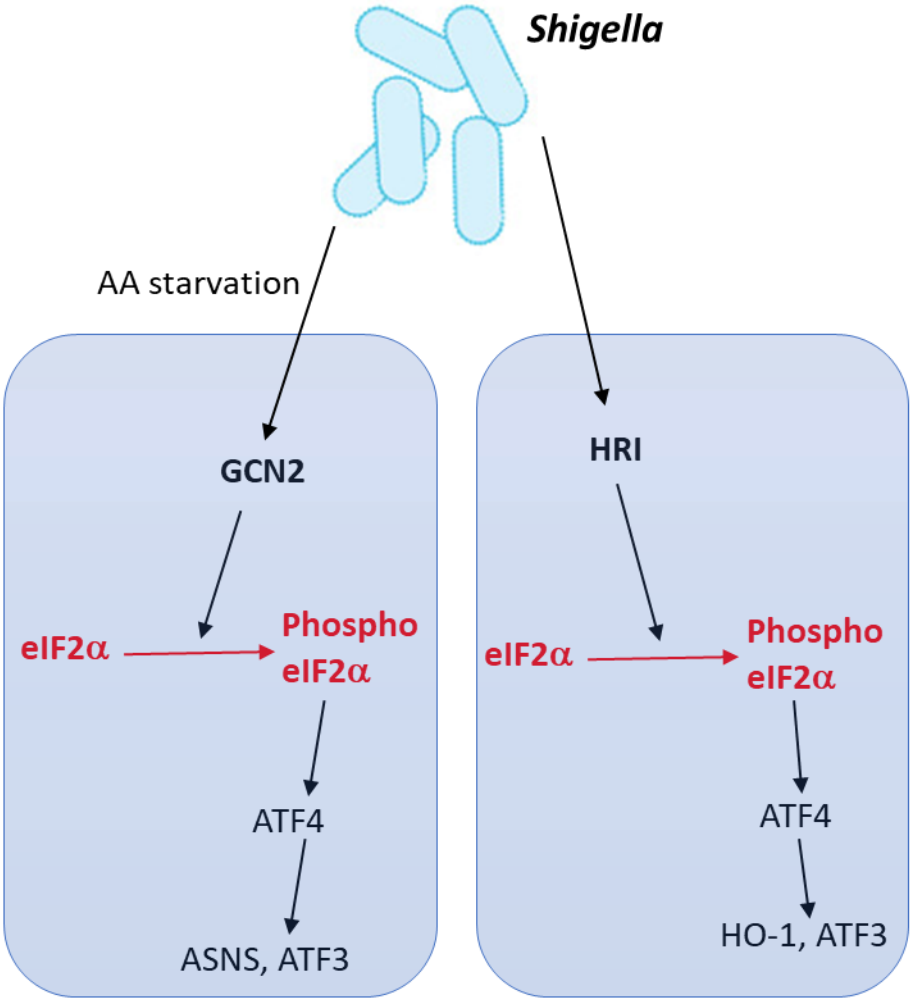
Representative immunofluorescence images of HeLa cells transduced with shRNAs targeting a scrambled (SC) sequence, HRI(shHRI) and GCN2(shGCN2) after being left unstimulated or treated with 300 μM sodium arsenite using anti-Tia1 antibodies. Scale bars denote 20 μm.

Fig S9. HRI knockdown cells have lower baseline levels of translation.



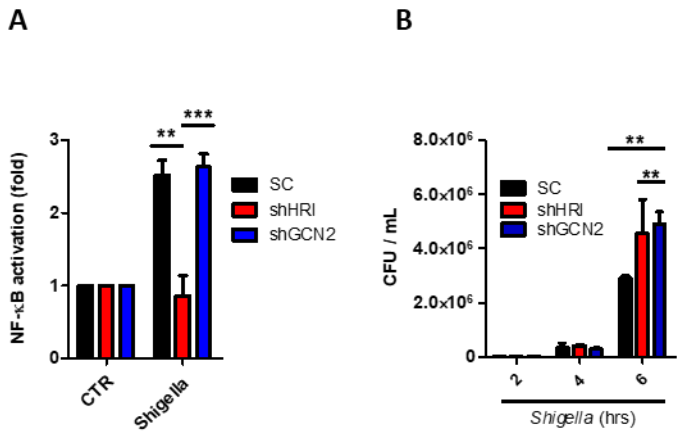
SUNSet assay to measure translation levels in HeLa cells transduced with lentiviral particles targeting a scrambled (SC) sequence, HRI (shHRI) or GCN2 (shGCN2) and left unstimulated or treated with 10 μg /mL cycloheximide (CHX) for 4 hours, treated with μg /mL puromycin for 10 minutes and analyzed by Western blotting using anti-puromycin antibodies. Cycloheximide was used as a positive control for a treatment which induces translation block and therefore no puromycin staining.

Fig S10. Model depicting the roles of HRI and GCN2.



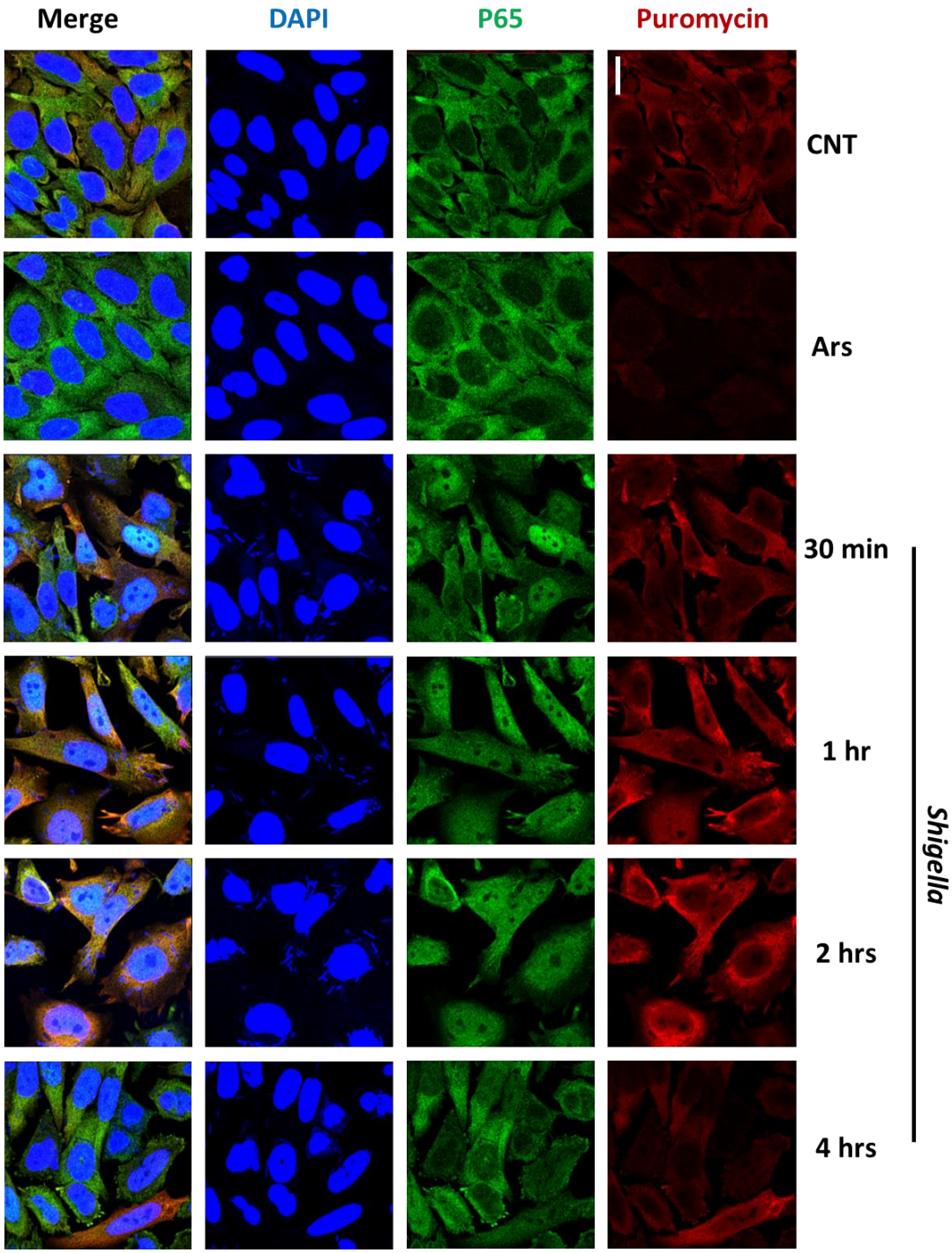
Model summarizing the findings of Figure 1, where both HRI and GCN2 are responsible for inducing eIF2 alpha phosphorylation during infection, although this leads to different albeit partially overlapping transcriptional responses including the induction of ASNS and HO-1 which are GCN2 and HRI dependent respectively, and the induction of ATF3 which is reliant on both arms of the pathway.

Fig S11. HRI is required for NF-κB activation during infection and restricts *Shigella* intracellular replication.



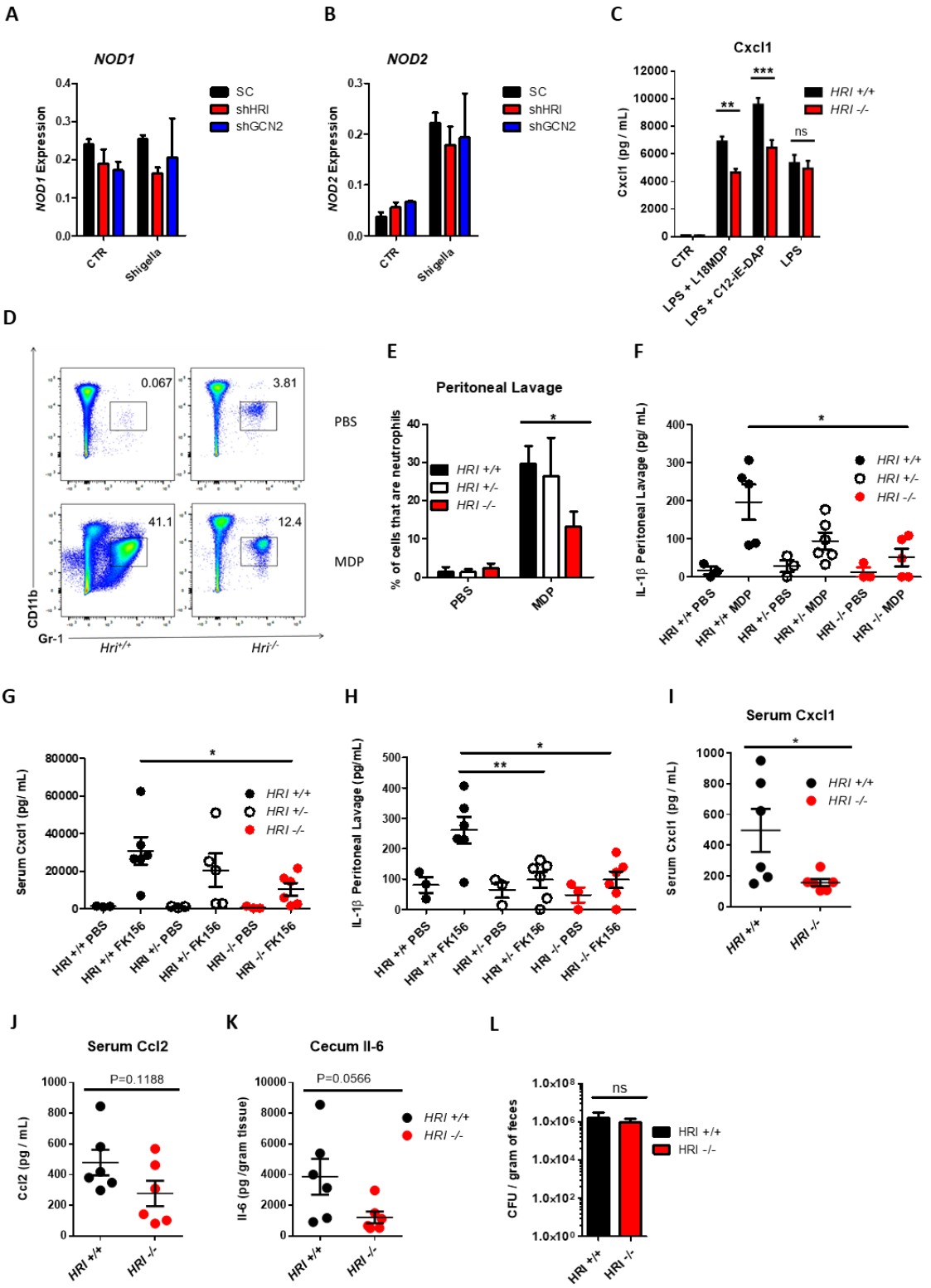
A. HeLa cells transduced with shRNAs targeting a scrambled (SC) sequence, HRI (shHRI) and GCN2 (shGCN2) were transfected with a NF-κB-Luc reporter plasmid and luciferase assays were performed following 4-hour infection with *Shigella*.
B. Scrambled and knock-down HeLa cells were infected with *Shigella* for the indicated time points and intracellular bacterial replication was determined by colony forming unit (CFU) assays.
Bar graphs represent the means +/- S.D. from 3-5 independent experiments (** and ***, $p < 0.01$ and 0.001, respectively).

Fig S12. Nuclear translocation of NF-κB occurs prior to infection-induced translation arrest.



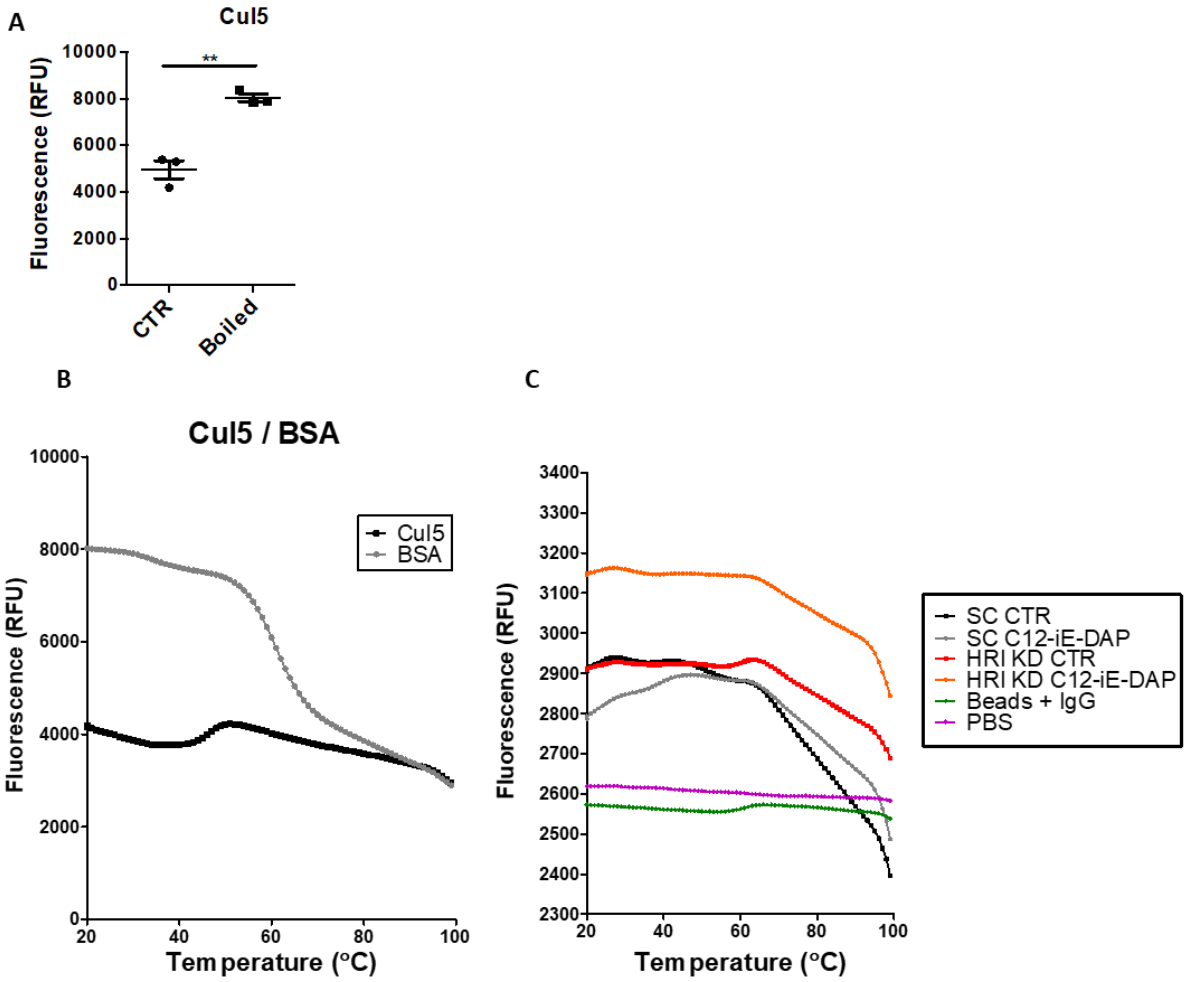
Representative immunofluorescence images of HeLa cells after infection with Shigella for the indicated time points or stimulation with 300 µM sodium arsenite for 2 hours and treated with 10 µg /mL puromycin for 10 minutes . Staining was performed with anti-p65 and anti-puromycin antibodies. Scale bars denote 20 µm.

Fig S13. HRI is essential for NOD1- and NOD2-driven inflammatory responses.



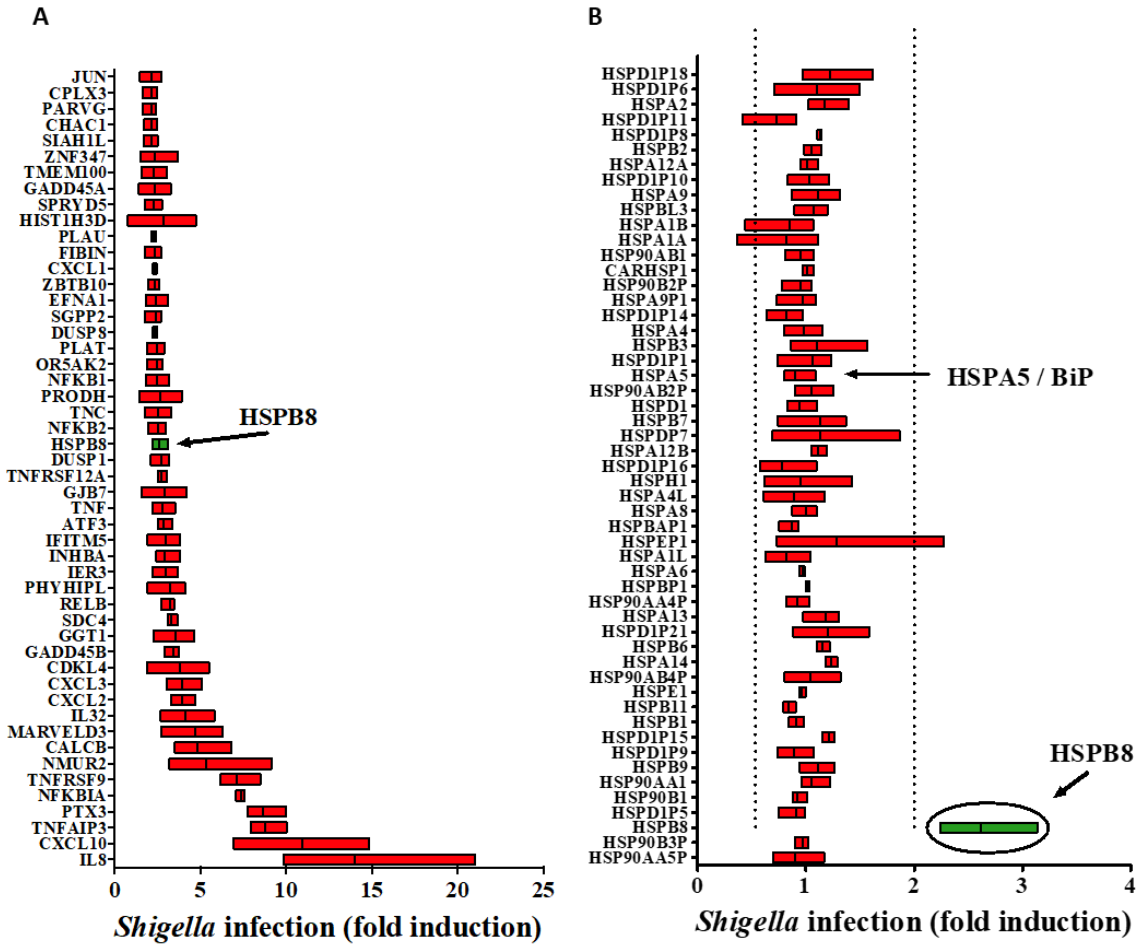
A-B. Expression of *NOD1* (**A**) and *NOD2* (**B**) in HCT116 cells transduced with lentiviral particles targeting a scrambled sequence (SC), HRI (shHRI) or GCN2 (shGCN2) and infected with *Shigella* for 4 hours determined by qPCR.
C. Cxcl1 secretion from the supernatants of wild-type ($HRI^{+/+}$) and HRI knockout ($HRI^{-/-}$) bone marrow derived macrophages (BMDMs) stimulated with 10 ng / mL L18-MDP or 10 µg / mL C12-ie-DAP for 6 hours with 16-hour pretreatment with 100 ng / mL LPS measured by ELISA.
D-E. Percentage of neutrophils in the peritoneal lavage of $HRI^{+/+}$ and $HRI^{-/-}$ and/or HRI heterozygous mice ($HRI^{+/-}$) mice 2 hours following intraperitoneal injection of PBS or 50 µg of MDP measured by fluorescence activated cell sorting (FACS) analysis for Gr1 and CD11b displayed as representative FACS plots (**D**) and as a bar graph (**E**) of the means with 3-6 mice per group.
F. IL-1β in the peritoneal lavage of $HRI^{+/+}$, $HRI^{+/-}$ and $HRI^{-/-}$ mice 2 hours following intraperitoneal injection with 50 µg of MDP (**E**) or 50 µg of FK-156 (**F**) measured by ELISA.
G. Serum Cxcl1 in $HRI^{+/+}$, $HRI^{+/-}$ and $HRI^{-/-}$ mice 2 hours following intraperitoneal injection with 50 µg of FK-156 measured by ELISA.
H. IL-1β in the peritoneal lavage of $HRI^{+/+}$, $HRI^{+/-}$ and $HRI^{-/-}$ mice 2 hours following intraperitoneal injection with 50 µg of FK-156 measured by ELISA.
I-K. Serum CXCL1 (**I**), CCL2 (**J**) or IL-6 from the cecum homogenate (**K**) of $HRI^{+/+}$ and $HRI^{-/-}$ mice 4 days post infection with *Citrobacter rodentium* measured by ELISA.
L. Bacterial load in the feces of mice 4 days post infection with *Citrobacter rodentium* measured by colony forming unit assays.
In panels **A-C**, bar graphs display the means +/- S.D. from 3 or more independent experiments. For panels **E** and **L** bar graphs display the means +/- S.D with 3-6 mice per group. For panels **F-K,** values from individual mice are plotted in scatter plots. Littermate mice from $HRI^{+/-}$ crosses were used (n=2-5 litters).

Fig S14. Validation of SYPRO Orange hydrophobicity measurements.



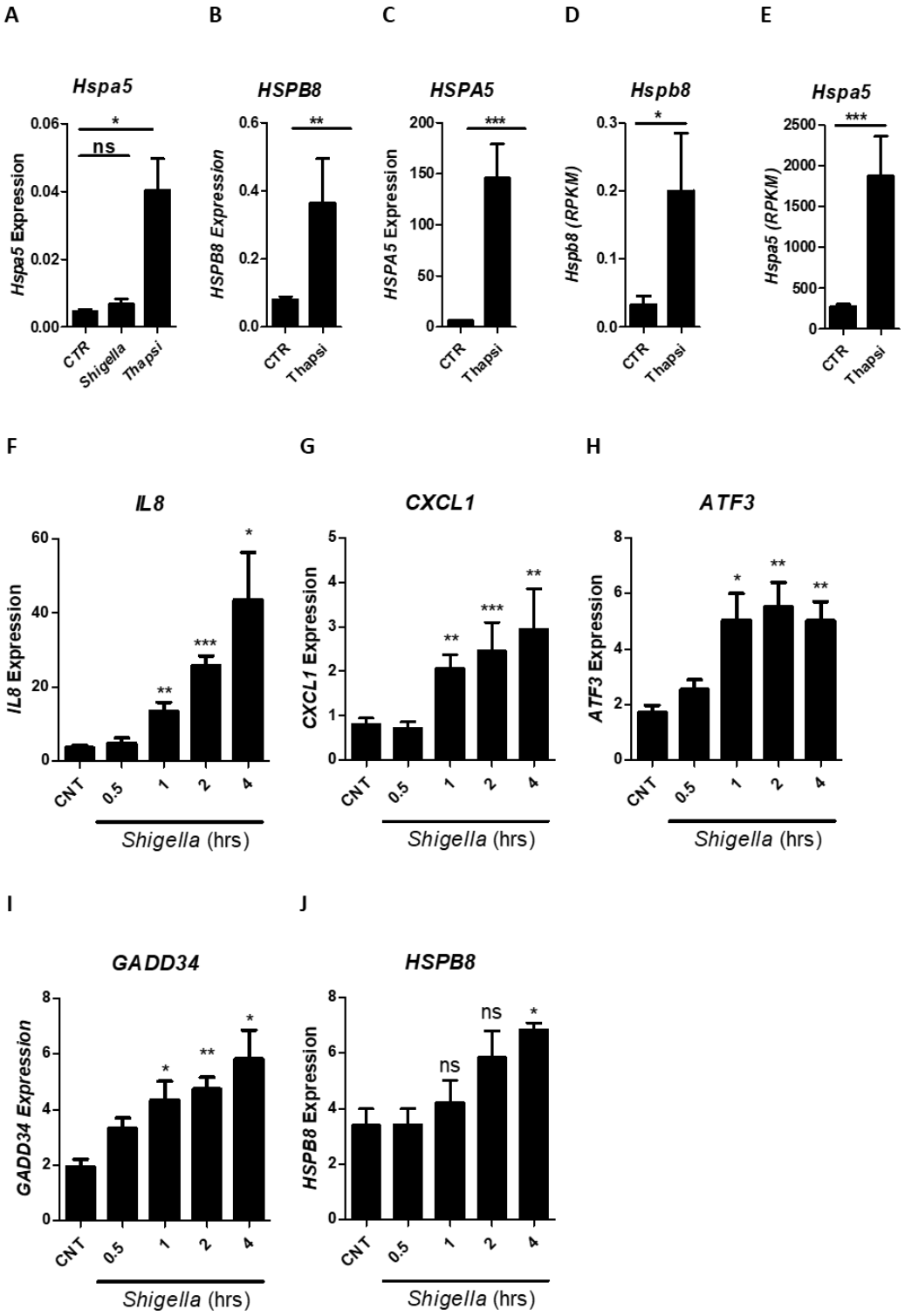
A. Purified Cul5 protein at a concentration of 1 mg /mL was left on ice or boiled for 5 minutes and incubated with SYPRO orange dye and fluorescence was measured.
B. Purified Cul5 protein and Bovine serum albumin (BSA) at a concentration of 0.5 mg / were incubated with SYPRO orange dye and fluorescence was measured as the temperature was raised 1 degree Celsius per minute.
C. Scrambled or HRI knockdown HEK293T cells were transfected with NOD1-HA and stimulated for 30 minutes with 10 ng/mL C12-iE-DAP and NOD1-HA complexes after RIPA cell lysis were immunoprecipitated, resuspended in PBS and incubated with the dye SYPRO orange and fluorescence was measured as the temperature was raised 1 degree Celsius per minute.
In **B** and **C** the general decrease in fluorescence with increasing temperatures is indicative of the progressive aggregation of the protein or protein complexes.
The dot plot from **A** represents the means +/- S.D. from 3 independent experiments (**, $p < 0.01$). Line graphs denote a representative experiment, with n=3.

Fig S15. HSPB8 is the only HSP transcriptionally induced by *Shigella* infection



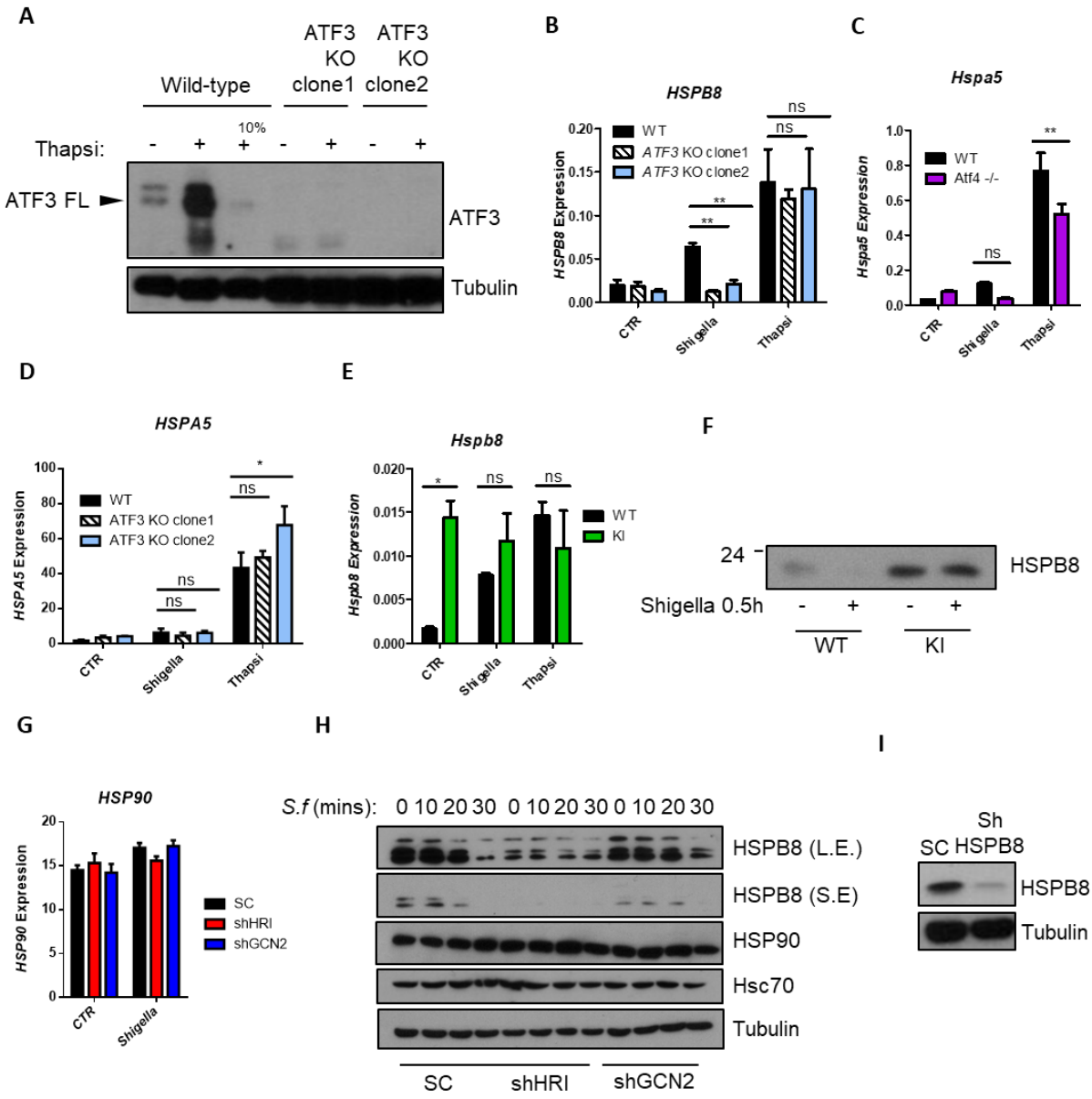
A-B. Microarray analysis of the 50 most transcriptionally upregulated genes (**A**) and of heat shock protein gene expression (**B**) in HeLa cells infected with S*higella* for 4 hours, repurposed from a previous report (Tattoli et al., 2012).

Fig S16. HSPB8 is induced during ER stress and is transcriptionally induced following the induction of inflammatory and primary stress response genes during *Shigella* infection.



A. Expression of *Hspa5* in MEFs infected with *Shigella* or treated with 5µM Thapsigargin for 4 hours measured by qPCR.
B-C. Expression of *HSPB8* (**B**) and *HSPA5* (**C**) in HCT116 cells treated with 5µM Thapsigargin for 4 hours measured by qPCR.
D-E. Expression of *Hspb8* (**D**) and *Hspa5* (**E**) in murine intestinal organoids treated with 5µM Thapsigargin for 4 hours measured by RNA seq.
F-J. Expression of *IL8* (**F**), *CXCL1* (**G**), *ATF3* (**H**), *GADD34* (**I**) and *HSPB8* (**J**) in HeLa cells infected with *Shigella* for indicated time points measured by -qPCR.
Bar graphs represent the means +/- S.D. from 3-5 independent experiments (*, ** and ***, $p<0.05$, 0.01 and 0.001, respectively).

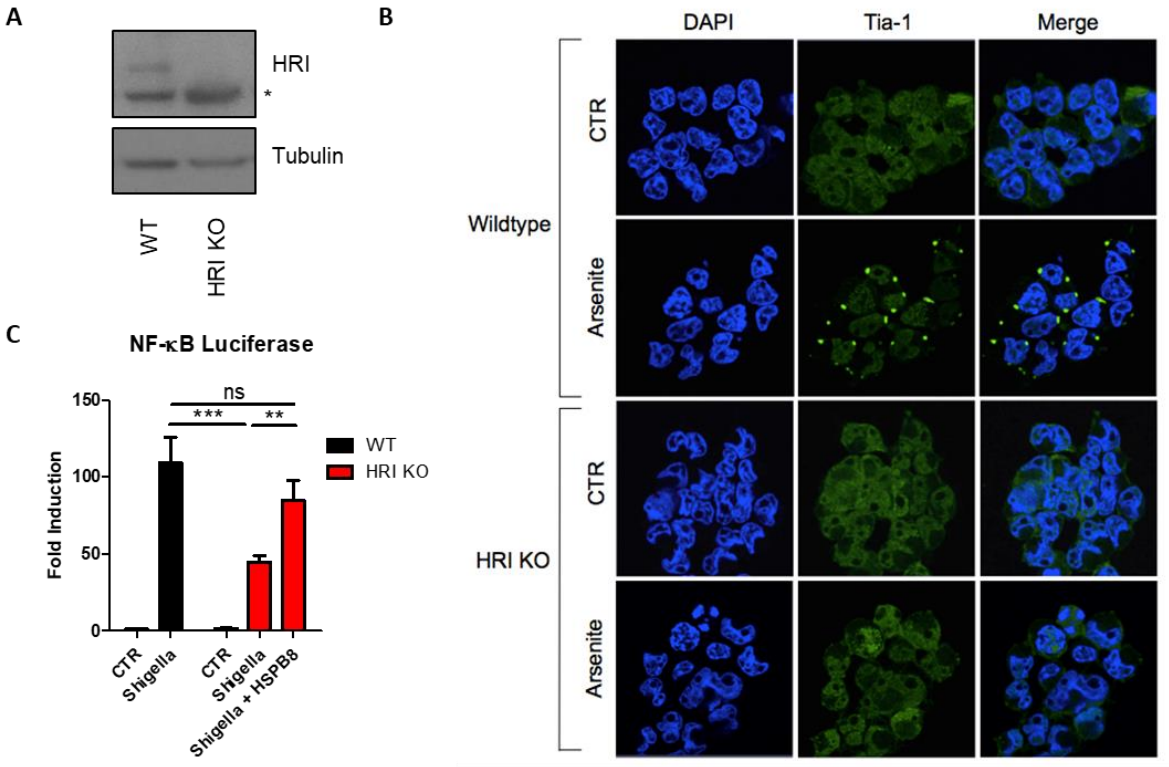
Fig S17. HSPB8 expression is induced by *Shigella* in an eIF2a-dependent manner and validation of the ATF3 CRISPR knockout HCT116 cells and HSPB8 knockdown HeLa cells.



A. Wild-type and two clones of ATF3 knockout HCT116 cells created by the CRISPR/CAS9 technique were analyzed by Western blotting using anti-ATF3 and anti-Tubulin antibodies when left untreated or treated with 5µM Thapsigargin. Lane 3 shows loading of 10% of the lane 2.
B. Expression of *HSPB8* in ATF3 knockout HCT116 cells infected with *Shigella* for 4 hours or treated with 5µM Thapsigargin for 4 hours measured by qPCR.

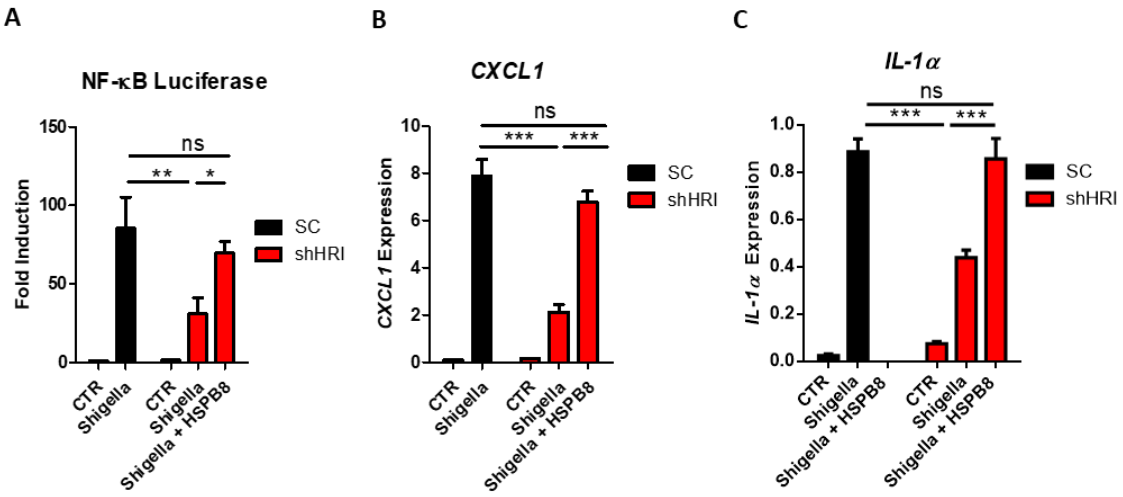
C-D. Expression of *Hspa5/HSPA5* in Atf4 -/- MEFs (**C**) and ATF3 knockout HCT116 cells (**D**) infected with *Shigella* for 4 hours or treated with 5µM Thapsigargin for 4 hours measured by qPCR.
E. Expression of *Hspb8* in wild-type (WT) and eIF2α knock-in (KI) MEFs infected with *Shigella* for 4 hours or treated with 5µM Thapsigargin for 4 hours measured by qPCR.
F. WT and KI MEFs infected with *Shigella* for 0.5 hours and analyzed by Western blotting using anti-HSPB8 antibodies.
G. Expression of *HSP90* in HeLa cells transduced with lentiviral particles against a scrambled sequence (SC), HRI or GCN2 and infected with *Shigella* for 4 hours measured by qPCR.
H. Scrambled and knockdown HeLa cells infected with *Shigella* for the indicated time points and analyzed by Western blotting for indicated proteins. L.E., long exposure. S.E., short exposure.
I. HeLa cells transduced with lentiviral particles targeting either a scrambled sequence or human HSPB8 (shHSPB8) were analyzed by western blotting using antibodies against HSPB8 or Tubulin as a loading control.
Bar graphs represent the means +/- S.D. from 3 independent experiments, (* and**, $p < 0.05$ and 0.01 respectively).

Fig S18. Validation of the HRI CRISPR knockout HEK293T cells.



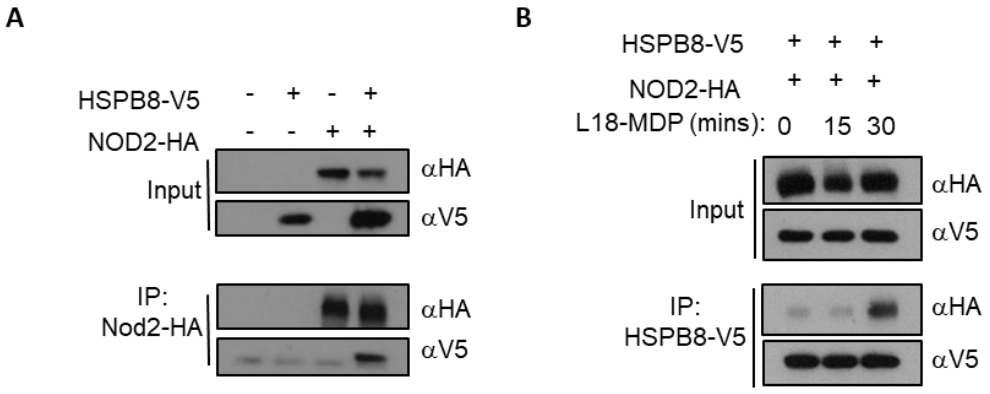
A. Wild-type(WT) and HRI knockout (HRI KO) HEK293T cells created by the CRISPR/CAS9 technique were analyzed by western blotting using anti-HRI and anti-Tubulin antibodies.
B. Representative immunofluorescence images of WT and HRI KO HEK293T cells stimulated with or without 300 µM sodium arsenite for 4 hours and stained using anti-Tia1 antibodies.
C. WT and HRI KO HEK293T cells were transfected with NF-κB reporter and HSPB8 overexpression plasmids and luciferase assays were performed following infection with *Shigella* for 4 hours.
Bar graphs represent the means +/- S.D. from 3 independent experiments, (** and***, $p < 0.01$ and 0.001 respectively). In panel a, * denotes a nonspecific band.

Figure S19. HSPB8 overexpression rescues inflammatory in HRI knockdown cells.



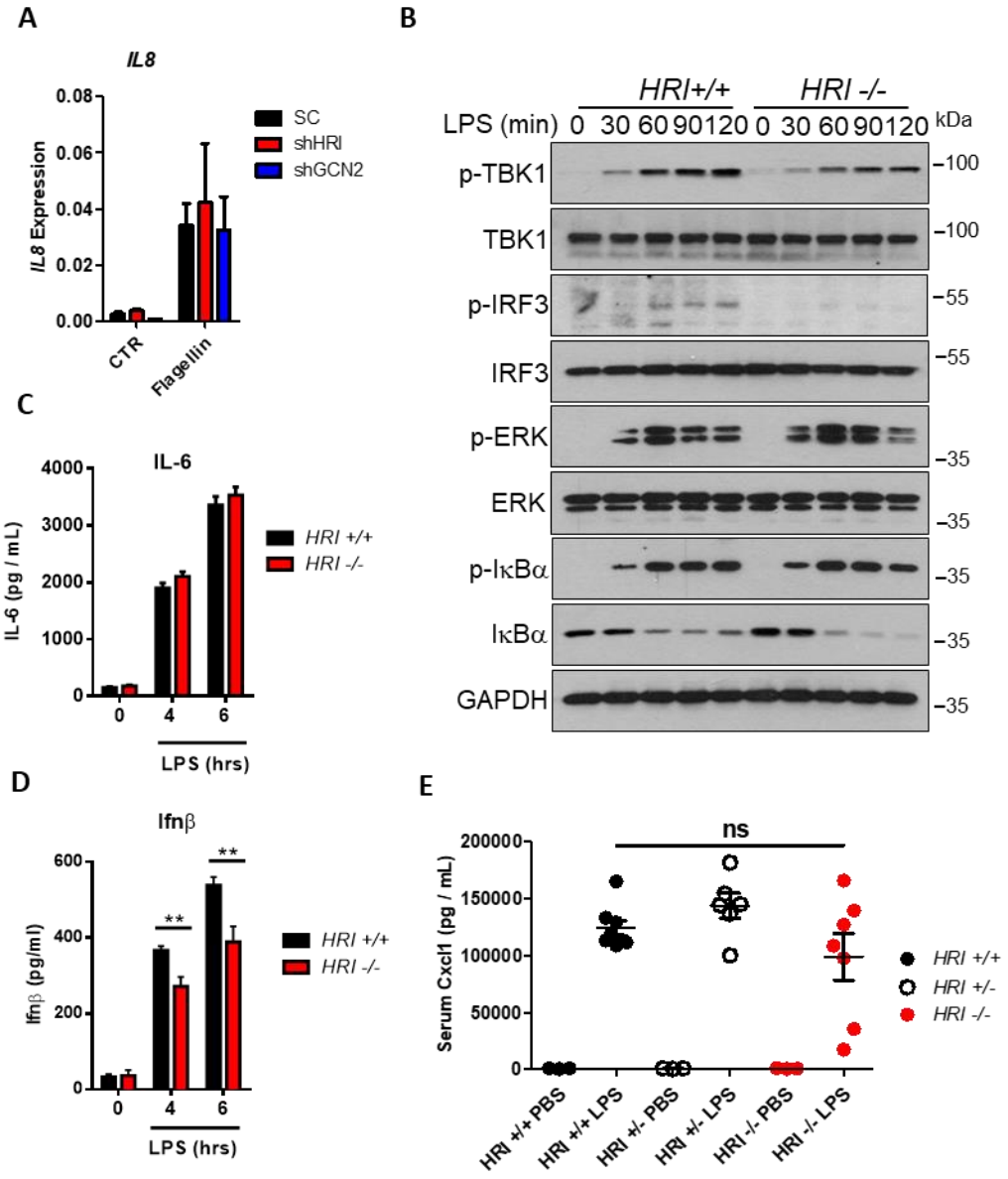
A. Scrambled and and HRI Knockdown HEK293T cells were transfected with NF-κB reporter and HSPB8 overexpression plasmids and luciferase assays were performed following infection with *Shigella* for 4 hours.
B-C. Expression of *CXCL1* (**A**) and *IL-1α* (**B**) in scrambled or HRI knockdown HeLa cells infected with *Shigella* for 4 hours measured by qPCR with or without transient transfection of HSPB8-V5 measured by qPCR.
Bar graphs represent the means +/- S.D. from 3 independent experiments, (***, $p < 0.001$).

Fig S20. **HSPB8 interacts with oligomerizing pattern recognition molecules.**



A-B. Co-Immunoprecipitation assays with the lysates of HEK293T cells transfected with NOD2-HA and HSPB8-V5 (**A**) and NOD2-HA and HSPB8 following stimulation with 10 ng/mL L18-MDP(**B**) for the indicated time points.

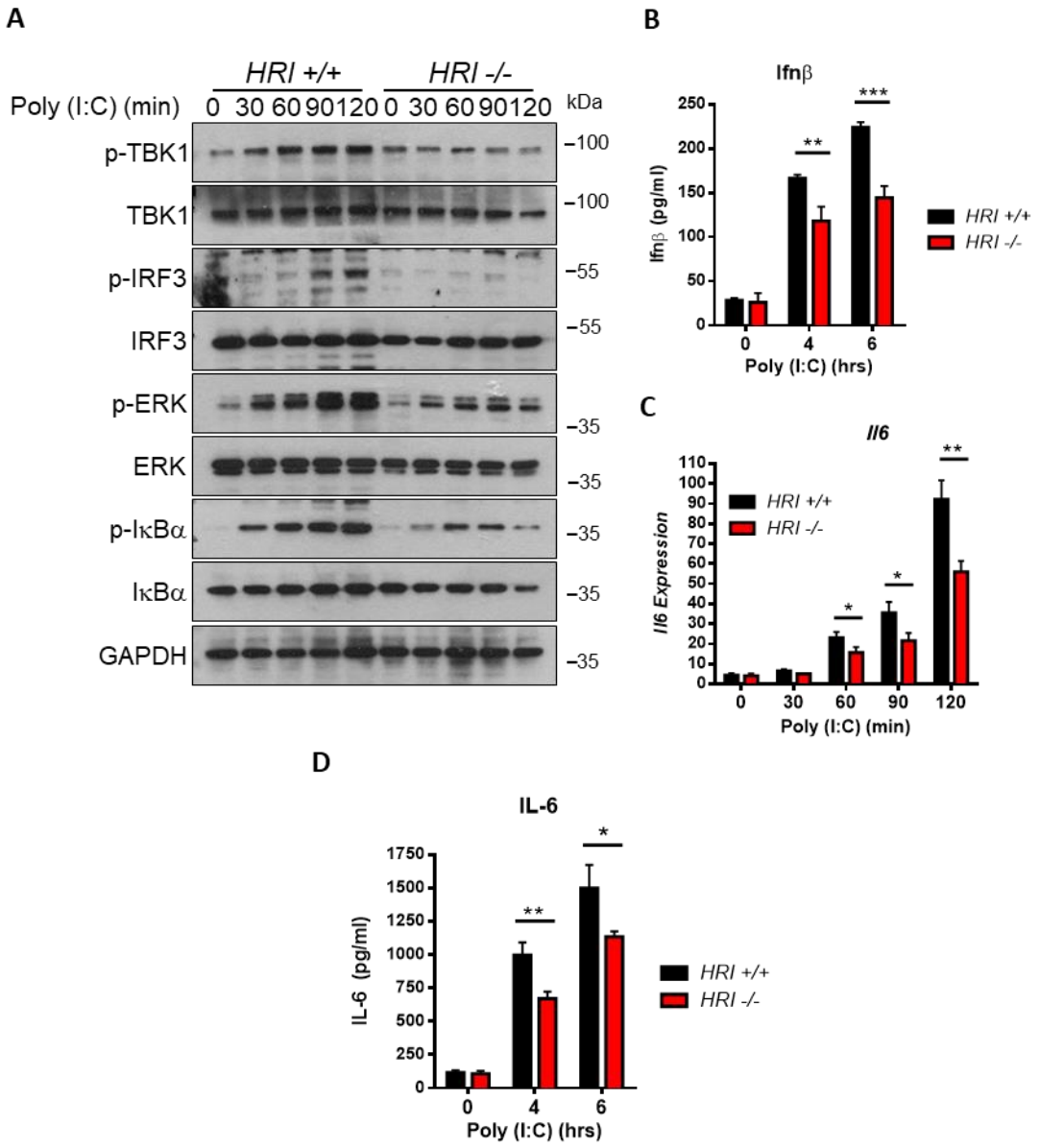
Fig S21. HRI is dispensable for TLR5 and TLR4-MyD88 signaling but required for TLR4-TRIF signaling.



A. Expression of *IL8* in HCT116 cells stimulated for 4 hours with 100 ng /mL of Flagellin measured by qPCR.
B. Wild-type ($HRI^{+/+}$) or HRI knockout ($HRI^{-/-}$) BMDMs treated with 100 ng/mL of LPS for the indicated times and analyzed by Western blotting for the indicated proteins.
C-D. Secretion of *Il6* (**C**) *and Ifnβ* (**D**) in $HRI^{+/+}$ or $HRI^{-/-}$ BMDMs treated with 100 ng/mL LPS for the indicated time points measured by qPCR and ELISA, respectively.
E. Serum Cxcl1 levels measured by ELISA in $HRI^{+/+}$, HRI heterozygous ($HRI^{+/-}$) and $HRI^{-/-}$ mice 2 hours following intraperitoneal injection with 1 µg of LPS.

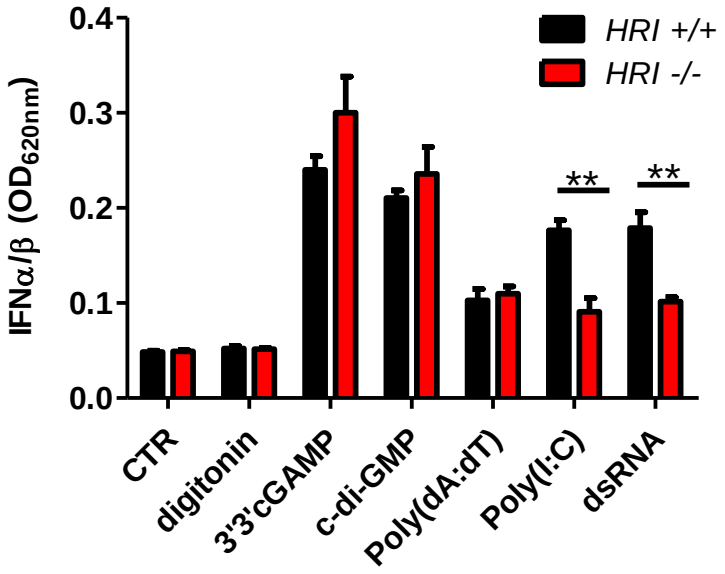
Bar graphs represent the means +/- S.D. from 3-5 independent experiments. For panel **E**, values from individual mice are plotted in scatter plots. Littermate mice from $HRI^{+/-}$ crosses were used (n=4 litters). * and **, $p < 0.05$ and 0.01, respectively.

Fig S22. HRI is required for TLR3 signaling.



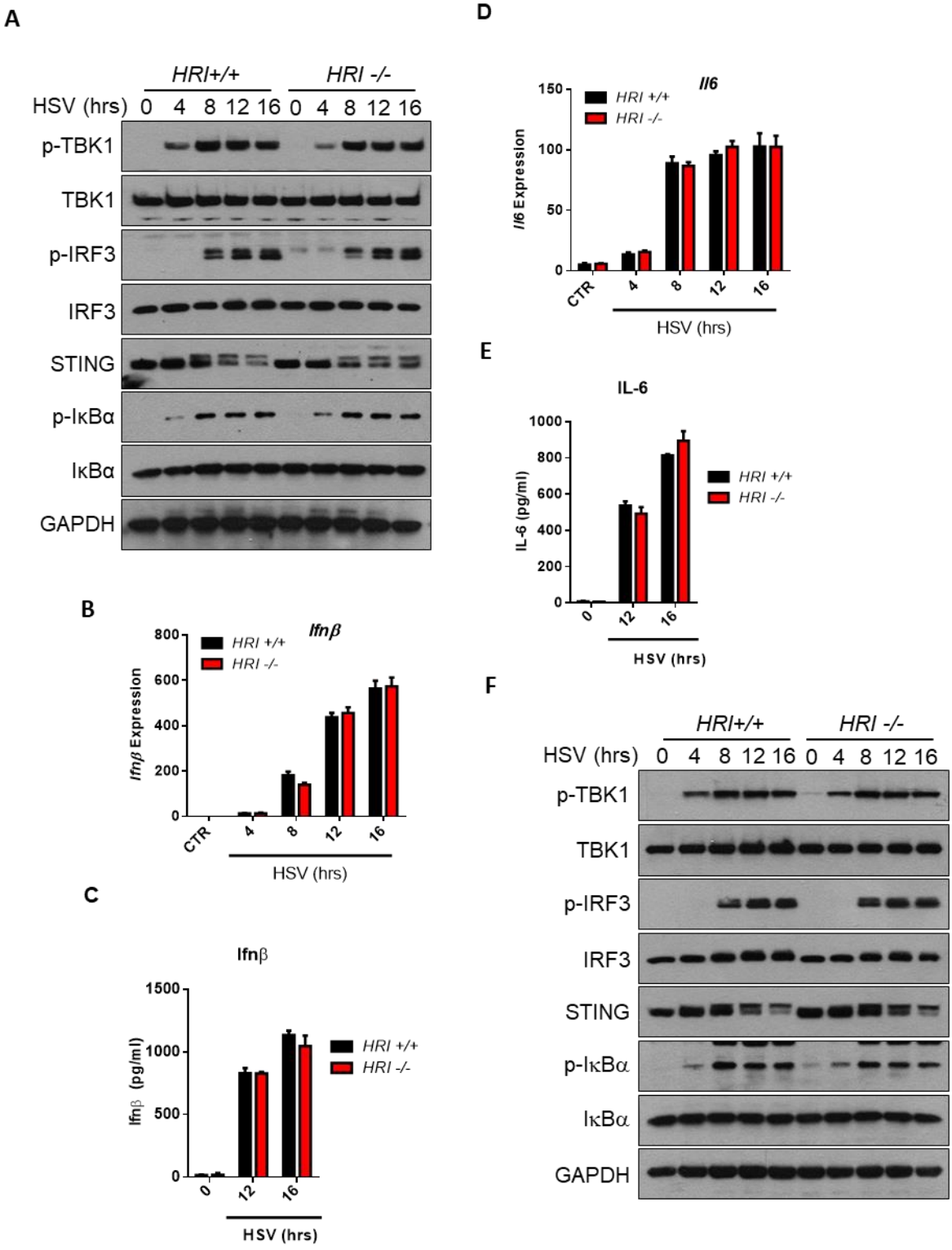
A. HRI$^{+/+}$ or HRI$^{-/-}$ BMDMs treated with 1mg/ml of Poly I/C for the indicated times and analyzed by Western blotting for the indicated proteins.
B. Secretion of *IFNβ* in HRI$^{+/+}$ or HRI$^{-/-}$ BMDMs treated with 1mg/ml Poly I/C for the indicated time points measured by ELISA.
C-D. Expression (**C**) and secretion (**D**) of *Il6* in HRI$^{+/+}$ or HRI$^{-/-}$ BMDMs treated with 1mg/ml Poly I/C for the indicated time points measured by qPCR and ELISA, respectively.
Bar and line graphs represent the means +/- S.D. from 3-5 independent experiments. *,** and ***, $p < 0.05$, <0.01 and <0.001 respectively.

Fig S23. HRI is crucial for antiviral RIG-I like receptor (RLR) but not STING dependent responses.



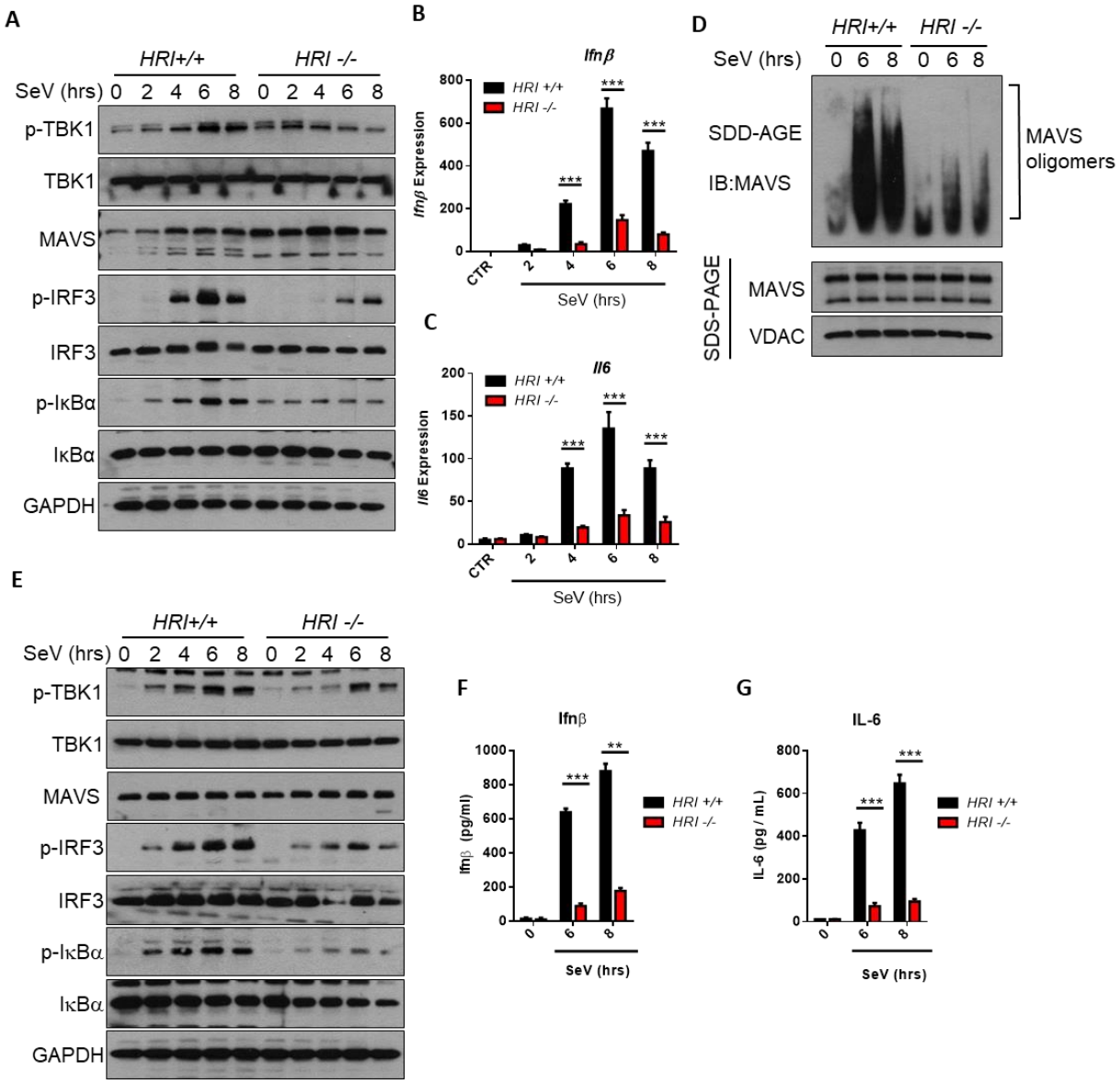
Secretion of Ifnα and Ifnβ (summed) in the supernatant of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs following the indicated stimulations using B16-Blue IFN reporter cells. For stimulations with 3'3' cGAMP and c-di-GMP, ligands were diluted in digitonin permeabilization buffer at a final concentration of 5 µg/mL and incubated for 30 minutes at 37 degrees and replaced with fresh media. Poly(dA:dT), Poly(I:C) and dsRNA was transfected into cells using Lipofectamine 2000. Poly(dA:dT) and Poly(I:C) were used at a final concentration of 5 µg/mL and dsRNA was used at a final concentration of 2 µg/mL. The Bar graph represent the means +/- S.D. from 5 independent experiments (** , $p < 0.01$).
Bar graph represent the means +/- S.D. from 5 independent experiments**.**

Fig S24. HRI is dispensable for STING dependent responses.



A. Wild-type ($HRI^{+/+}$) and HRI knockout ($HRI^{-/-}$) MEFs were infected with HSV for the indicated time points and analyzed by Western blotting for the indicated proteins.
B-C. Expression of *Ifnβ* (**B**) or *Il-6* (**C**) in $HRI^{+/+}$ and $HRI^{-/-}$ MEFs infected with HSV for the indicated time points measured by qPCR.
D-E. IFNβ (**D**) and IL-6 (**E**) secretion from the supernatant of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs infected with HSV measured by ELISA.
F. $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs were infected with HSV for the indicated time points and analyzed by Western blotting for the indicated proteins.
Bar graphs represent the means +/- S.D. from 3-5 independent experiments.

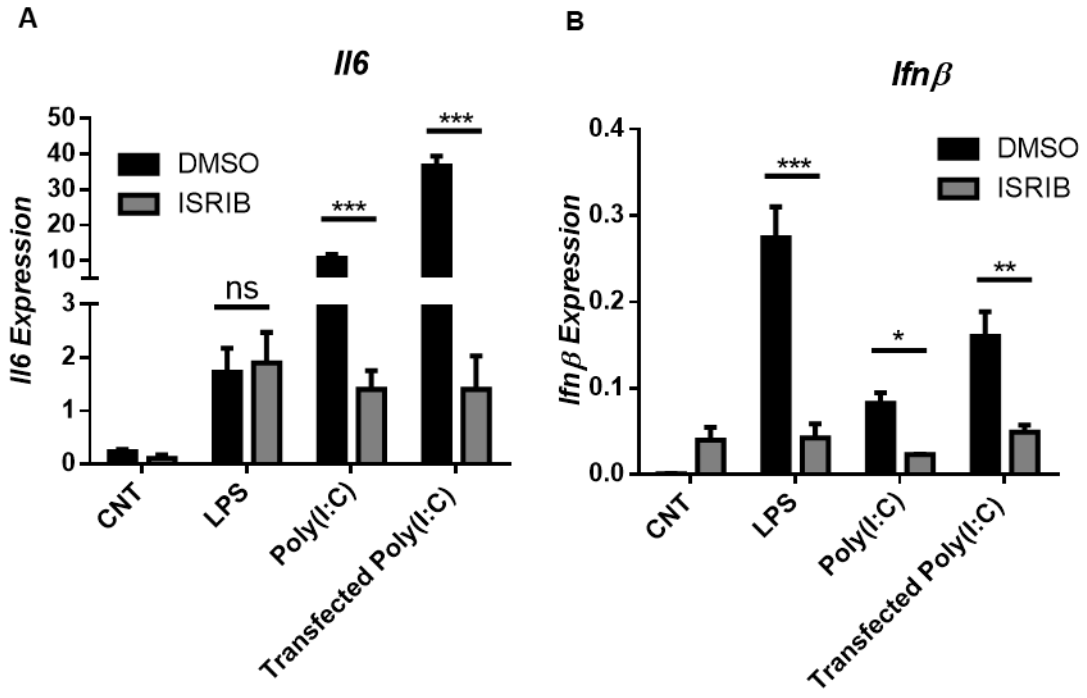
Fig S25. HRI is crucial for antiviral RIG-I like receptor (RLR) signaling.



A. Wild-type ($HRI^{+/+}$) and HRI knockout ($HRI^{-/-}$) MEFs were infected with SeV for the indicated time points and analyzed by Western blotting for the indicated proteins.
B-C. Expression of *Ifnβ* (**B**) or *Il-6* (**C**) in $HRI^{+/+}$ and $HRI^{-/-}$ MEFs infected with SeV for the indicated time points measured by qPCR.
D. $HRI^{+/+}$ and $HRI^{-/-}$ MEFs were infected with SeV for the indicated time points and analyzed by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) and Western blotting for the indicated proteins.
E. $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs were infected with SeV for the indicated time points and analyzed by Western blotting for the indicated proteins.
F-G. IFNβ (**F**) and IL-6 (**G**) secretion from the supernatant of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs infected with SeV measured by ELISA.

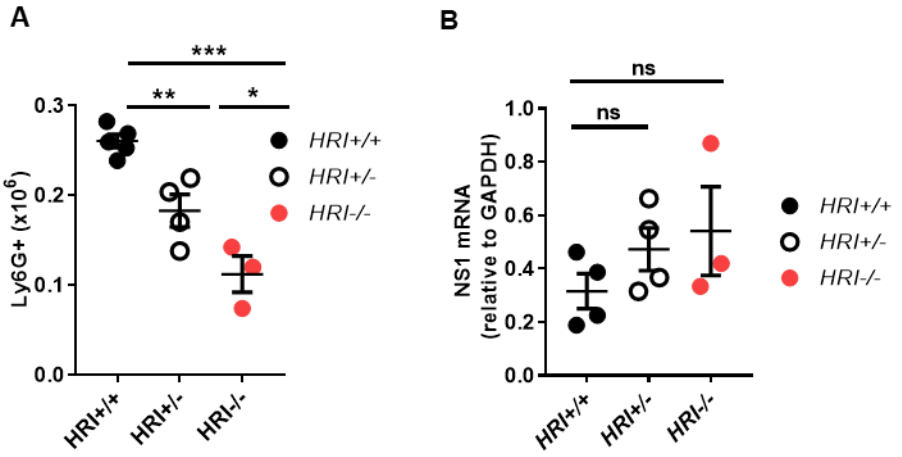
Bar graphs represent the means +/- S.D. from 3-5 independent experiments (** and ***, $p < 0.01$ and 0.001, respectively).

Fig S26. Acute inhibition of eIF2α phosphorylation prevents TRIF and MAVS dependent inflammatory responses but not MyD88 signaling.



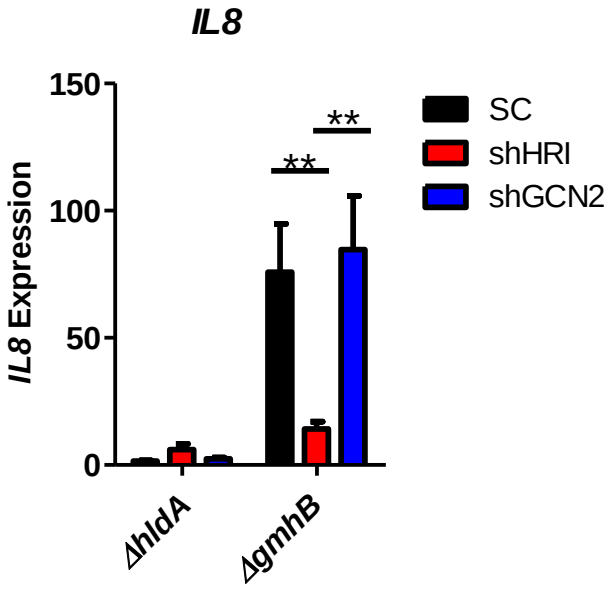
A-**B**. Expression of *Ifnβ* (**A**) and *Il-6* (**B**) in wildtype BMDMs treated with or without 1 μg/mL ISRIB and stimulated with 100 ng /mL LPS, 1 μg/mL (PolyI:C) for 2 hours and 2 μg/mL of transfected (PolyI:C) for 4 hours.
Bar graphs represent the means +/- S.D. from 2 independent experiments with a total of three technical replicates(*,** and ***, p <0.05, 0.01 and 0.001, respectively).

Fig S27. HRI does not influence viral replication *in vivo*.



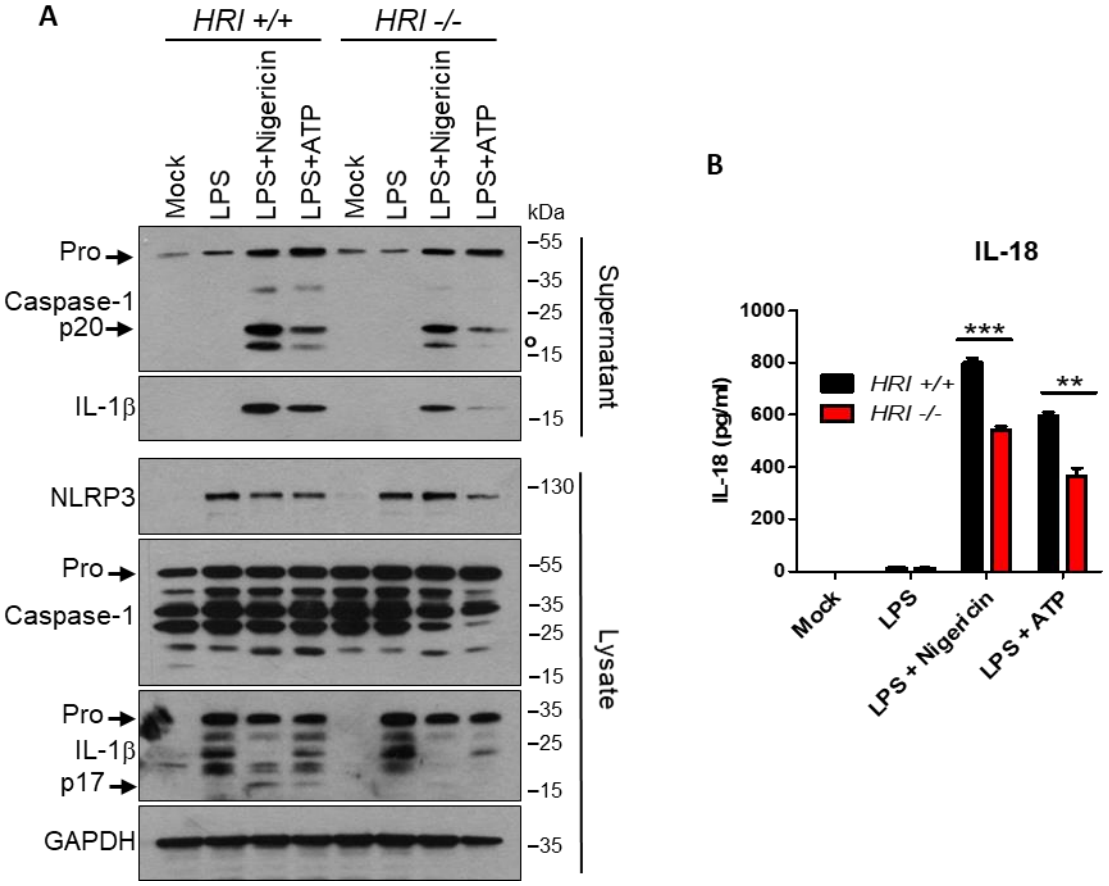
A. Total lung neutrophils (Ly6G+ cells) from HRI$^{+/+}$, HRI$^{+/-}$ and HRI$^{-/-}$ mice infected with a sublethal dose (50 plaque forming units) of Puerto Rico/8/34 (PR8) (H1N1) influenza A virus (IAV) at 6 days post infection were measured by flow cytometry. **B.** HRI$^{+/+}$, HRI$^{+/-}$ and HRI$^{-/-}$ littermate controls were infected with a sublethal dose (50 plaque forming units) of Puerto Rico/8/34 (PR8) (H1N1) influenza A virus (IAV) for 6 days and relative levels of viral NS1 mRNA in the lungs were quantified by qPCR. Iindividual mice are plotted in scatter plots. Littermate mice from HRI$^{+/-}$ crosses were used (n=2-6 litters). *, ** and ***, $p < 0.05$, 0.01 and 0.001, respectively via one-way ANOVA followed by Tukey's multiple comparison test.

Fig S28. HRI is required for TIFA-dependent responses.



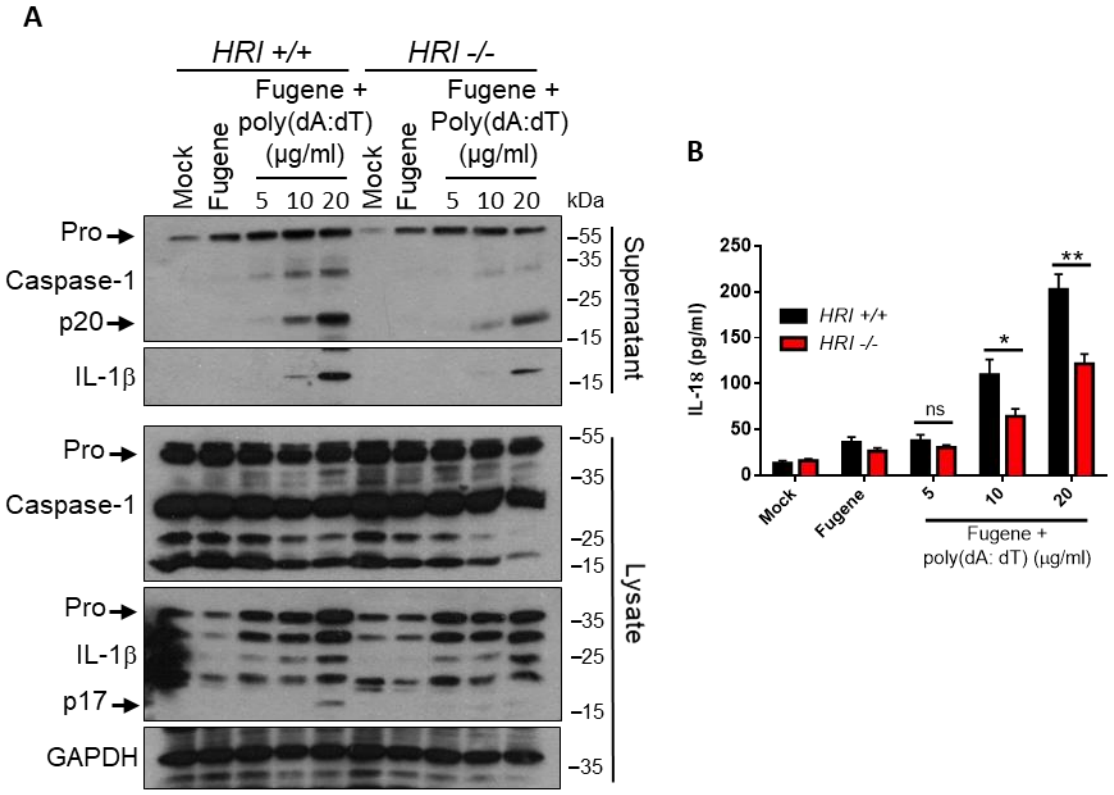
Expression of *IL8* in HeLa cells stimulated with supernatant from the HBP-defective *hldA*$^{-}$ *(ΔhldA)* and *gmhB*- (*ΔgmhB*) *Neisseria meningitidis (ΔgmhB)* (1 in 10 dilutions of overnight culture of *N. meningitidis*) for 4 hours measured by qPCR.
Bar and line graphs represent the means +/- S.D. from 3-5 independent experiments. **, $p < 0.01$.

Fig S29. HRI is required for NLRP3 inflammasome signaling and assembly.



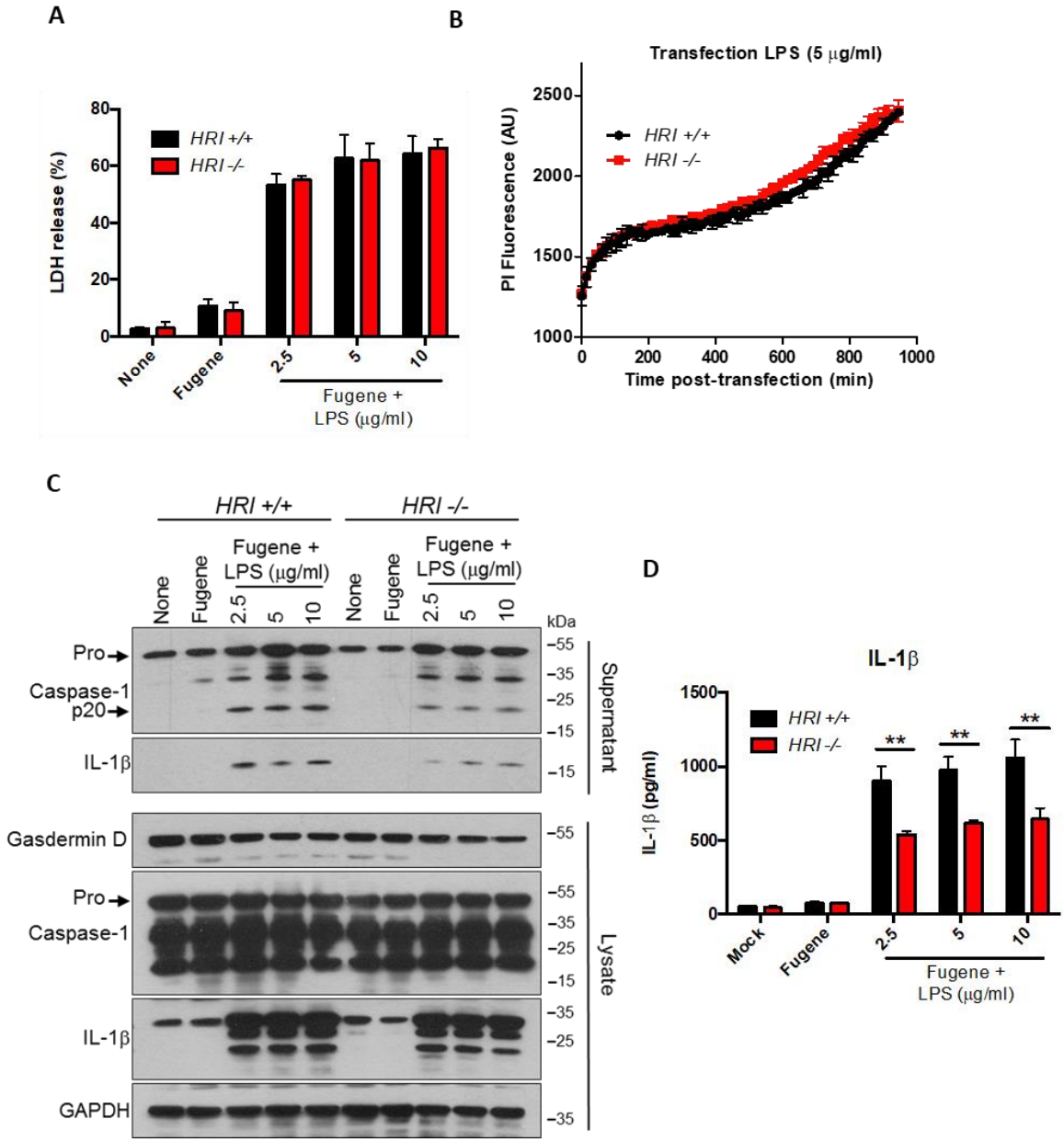
A. HRI$^{+/+}$ and HRI$^{-/-}$ BMDMs were stimulated with either 100 ng/mL of LPS for 3 hours and then subsequently stimulated with 10 μM Nigericin or 5mM ATP for 45 minutes and analyzed by Western blotting for the indicated proteins.
B. IL-18 secretion from the supernatant of HRI$^{+/+}$ and HRI$^{-/-}$ BMDMs stimulated with either 100 ng/mL of LPS for 3 hours and then subsequently stimulated with 10 μM Nigericin or 5mM ATP for 45 minutes measured by ELISA.
The bar graphs represents the means +/- S.D. from 3-5 independent experiments(** and ***, $p < 0.01$ and 0.001, respectively).

Fig S30. HRI is required for AIM2 inflammasome activation.



A. HRI$^{+/+}$ and HRI$^{-/-}$ BMDMs were primed for 4 h with 100 ng/mL LPS in Opti-MEM and stimulated with either Fugene alone or transfected with the indicated concentrations of poly(dA: dT) for 16 hours and analyzed by Western blotting for the indicated proteins. **B.** IL-18 secretion from the supernatant of HRI$^{+/+}$ and HRI$^{-/-}$ BMDMs primed for 4 h with 100 ng/mL LPS in Opti-MEM and stimulated with either Fugene alone or transfected with the indicated concentrations of poly(dA: dT) for 16 hours measured by ELISA. Bar graphs represent the means +/- S.D. from 3 independent experiments. * and **, $p < 0.05$ and 0.01, respectively.

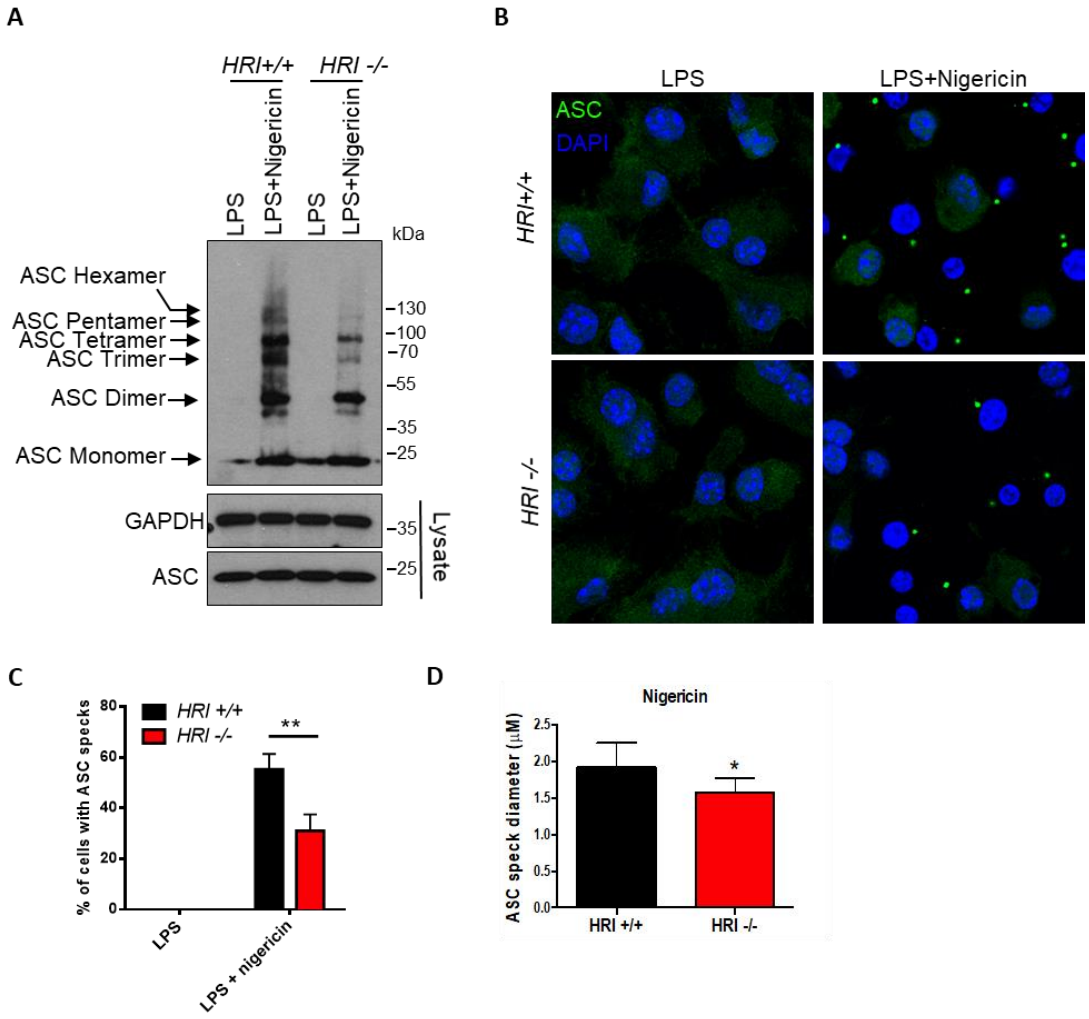
Fig S31. HRI is dispensable for Caspase-11 signaling but is required for NLRP3 activation during sensing of cytosolic LPS



A. Cell death was assessed using lactate dehydrogenase (LDH) release measured in the supernatant of $HRI^{+/+}$ or $HRI^{-/-}$ BMDMs primed for 4 h with 1 μg/mL Pam3CSK4 in Opti-MEM and treated with Fugene alone and transfected with the indicated concentrations of LPS using Fugene for 16 hours.
B. Propidium Iodide staining of $HRI^{+/+}$ or $HRI^{-/-}$ BMDMs primed for 4 h with 1 μg/mL Pam3CSK4 in Opti-MEM transfected with 5 μg /mL LPS using Fugene over 24 hours. Fluorescence was measured using a microplate fluorimeter.

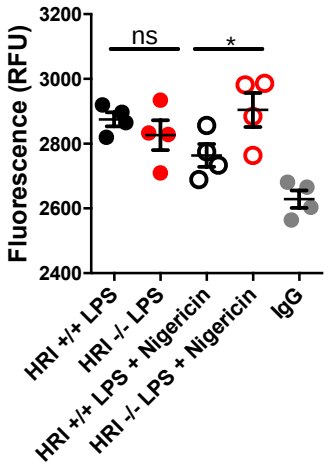
C. $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs primed for 4 h with 1 μg/mL Pam3CSK4 in Opti-MEM and treated with Fugene alone and transfected with the indicated concentrations of LPS using Fugene for 16 hours analyzed by Western blotting for the indicated proteins.
D. IL-1β secretion from the supernatants of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs primed for 4 h with 1 μg/mL Pam3CSK4 in Opti-MEM and treated with Fugene alone and transfected with the indicated concentrations of LPS using Fugene for 16 hours measured by ELISA. The bar graphs represents the means +/- S.D. from 3 independent experiments(**, $p < 0.01$).

Fig S32. HRI is required for NLRP3 inflammasome signaling and assembly.



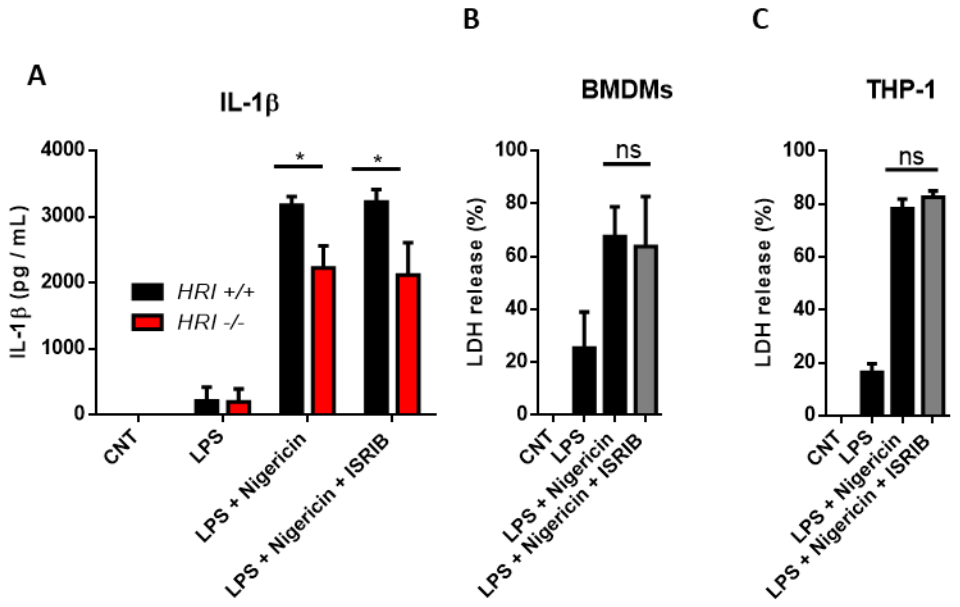
A. Wild-type ($HRI^{+/+}$) and HRI knockout ($HRI^{-/-}$) BMDMs were stimulated with 100 ng/mL of LPS for 3 hours and then subsequently stimulated with 10 μM Nigericin for 45 minutes and analyzed by Western blotting for the indicated proteins following ASC crosslinking with disuccinimidyl suberate.
B-D. Representative immunofluorescence images (**B**), quantification of the percentage of cells with ASC specks (**C**) and quantification of ASC speck diameter (**D**) of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs stimulated with 100 ng/mL of LPS for 3 hours and then subsequently stimulated with 10 μM Nigericin for 45 minutes using anti-ASC antibodies.
The bar graphs represents the means +/- S.D. from 3-5 independent experiments(* and**, $p < 0.05$ and 0.01 respectively).

Fig S33. HRI regulates NLRP3 inflammasome complex formation



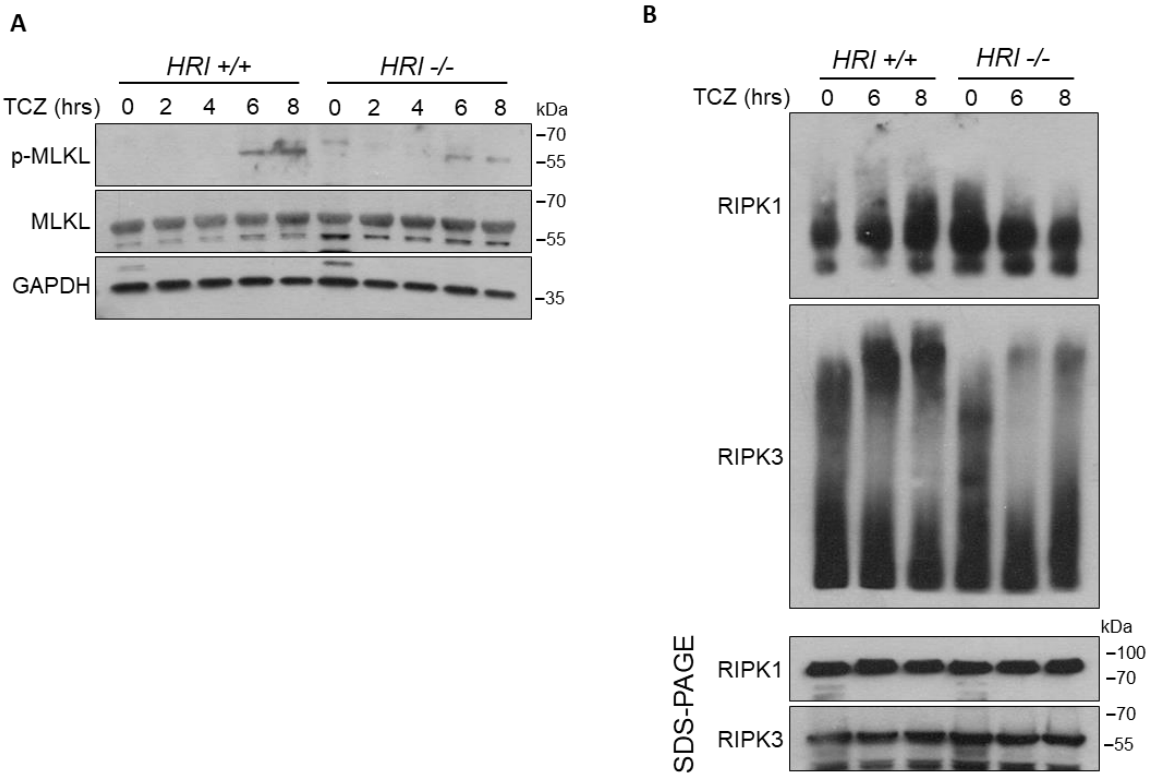
Wildtype ($HRI^{+/+}$) and HRI knockout ($HRI^{-/-}$) BMDMs were stimulated with 100 ng/mL of LPS for 4 hours and then subsequently stimulated with 10 µM Nigericin for 30 minutes, lysed and NLRP3 complexes were immunoprecipitated, resuspended in PBS and incubated with the dye SYPRO orange and fluorescence was measured. Scatter plots display the means from 4 independent experiments (*, $p < 0.05$).

Fig S34. Inhibition of eIF2α phosphorylation does not effect NLRP3 activation.



A. IL-1β secretion from the supernatant of $HRI^{+/+}$ and $HRI^{-/-}$ BMDMs were stimulated with either 100 ng/mL of LPS for 4 hours and then subsequently stimulated with 10 µM Nigericin in the absence or presence of 1 µg/mL ISRIB measured by ELISA.
B-C. Cell death measured by lactate dehydrogenase (LDH) release in the supernatant of wildtype BMDMs (**B**) and THP-1 cells (**C**) treated with or without 1 µg/mL ISRIB and stimulated with 100 ng /mL LPS for 4 hours then subsequently stimulated with 10 µM Nigericin in the absence or presence of 1 µg/mL ISRIB. ISRIB treatment was given 2 hours following priming with LPS.
Bar graphs in panels A and B display the means +/- S.D. from 2 independent experiments with a total of three technical replicates. The bar graph in panel C represents the means +/- S.D. from 3 independent experiments (*,** and ***, p <0.05, 0.01 and 0.001, respectively).

Fig S35. HRI is required for RIPK1 and RIPK3 programmed necrosis



A. Wildtype ($HRI^{+/+}$) or HRI knockout ($HRI^{-/-}$) MEFs were treated with TNF (50 ng/ml), cycloheximide (1 μg/ml) and Z-VAD-FMK (20 μM) (TCZ) for the indicated times and analyzed by Western blotting for the indicated proteins.
B. $HRI^{+/+}$ or $HRI^{-/-}$ MEFs were treated with TCZ for the indicated times and analyzed by Blue Native PAGE (top two panels) or SDS PAGE (bottom two panels) for the indicated proteins.

Table S1. Sequences targeted for Lentiviral knockdown and CRISPR/Cas9 knockout

Target	Sequence (5’ to 3’)
HRI (knockdown)	CAAGAGGCTGTCAAGTCGTC
GCN2	CCCTAAAGAACTGTCGTTAAC
HSPB8	CCTGGAAAGTGTGTGTGAATG
ATF3 (knockout clone1)	CCACCGGATGTCCTCTGCGC
ATF3 (knockout clone1)	TTTGTGATGGACACCCCGAG
HRI (knockout)	GCCATCGACTTTCCCGCCGA

Table S2. Primers for Genotyping

Primer name	Sequence (5’ to 3’)
WT HRI	ATGTGCAGGGCTGAAGAGAT
KO HRI	CATGCTGGGGGTCAAATAGT
Common HRI	AGCTCCACCCTGACGATCTA

Table S3. Murine Primers used in this study for qPCR

Target	Sequence (5’ to 3’)
ActB Forward	GTCCACACCCGCCACCAGTTGG
ActB Reverse	GCTTTGCACATGCCGGAGCCGTT
RPL19 Forward	GCATCCTCATGGAGCACAAT
RPL19 Reverse	CTGGTCAGCCAGGAGCTT
ATF3 Forward	GCAGGCACTCTGTCTTCTCC
ATF3 Reverse	GCGGCGAGAAAGAATAAAA
Cxcl1 Forward	AGACCATGGCTGGGATTCAC
Cxcl1 Reverse	AGTGTGGCTATGACTTCGGT
IFN Beta Forward	TCCAAGAAAGGACGAACATCG
IFN Beta Reverse	TGAGGACATCTCCCACGT
IFN alpha Forward	GGATCACTGTGTACCTGA
IFN alpha Reverse	GGCTGTGTTTCTTCTCTCT
IL-6 Forward	TCCAATGCTCTCCTAACAGATAAG
IL-6 Reverse	CAAGATGAATTGGATGGTCTTG
Hspa5 Forward	GCCTCATCGGACGACTT
Hspa5 Reverse	GGGGCAAATGTCTTGGTT
Hspb8 Forward	CCCTCTAAGTTCGACCAACATC
Hspb8 Reverse	CCAAAGCCATCGTCCAGAAG
GAPDH Forward (Influenza infections)	GGTCCTCAGTGTAGCCCAAG
GAPDH Reverse (Influenza infections)	AATGTGTCCGTCGTGGATCT

Table S4. Human Primers used in this study for qPCR

Target	Sequence (5’ to 3’)
TBP Forward	GGGCATTATTTGTGCACTGAGA
TBP Reverse	TAGCAGCACGGTATGAGCAACT
ATF3 Forward	CTGGGTCACTGGTGTTTGAGGATT
ATF3 Reverse	AGGTGCTTGTTCTGGATGGCAAAC
HO-1 Forward	ATGACACCAAGGACCAGAGC
HO-1 Reverse	GTGTAAGGACCCATCGGAGA
ASNS Forward	GCAGCTGAAAGAAGCCCAAGT
ASNS Reverse	TGTCTTCCATGCCAATTGCA
IL-8 Forward	CCACCGGAAGGAACCATCTC
IL-8 Reverse	TTCCTTGGGGTCCAGACAGA
CXCL1 Forward	CACACTCAAGAATGGGCGGA
CXCL1 Reverse	ACTATGGGGGATGCAGGATTG
IL-1 alpha Forward	CTTCTGGGAAATCACGGCA
IL- 1 alpha Reverse	GTGAGACTCCAGACCTACGC
NOD1 Forward	CAGCACTTTCCCATGTATTGAT
NOD1 Reverse	TCAAATCCCACACTGCACA
NOD2 Forward	GGTTGATGCCTGTGAACTGAA
NOD2 Reverse	AAATGAAATGGAACTGCCTCTT
HSP90 Forward	AGAAATGCCACCCCTTGAAG
HSP90 Reverse	GTAAGTCATCCCTCAGCCAG
HSPA5 Forward	GAACGTCTGATTGGCGATGC
HSPA5 Reverse	TCAACCACCTTGAACGGCAA
HSPB8 Forward	AAAGATGGATACGTGGAGGTG

HSPB8 Reverse	GGGAAAGTGAGGCAAATACTG
GADD34 Forward	ATGTATGGTGAGCGAGAGGC
GADD34 Reverse	GCAGTGTCCTTATCAGAAGGC
CHOP Forward	GCACCTCCCAGAGCCCTCACTCTCC
CHOP Reverse	GTCTACTCCAAGCCTTCCCCCTGCG

Table S5. Viral Primers used in this study for qPCR

Target	Sequence (5’ to 3’)
NS1 Forward	AGAAAGTGGVAGGCCCTCTTTGTA
NS1 Reverse	GGGCACGGTGAGCGTGAACA