SUPPLEMENTAL MATERIALS

Extended Experimental Procedures

Protein Expression and Purification

Bacteria were grown at 37°C and induced by 0.5 mM IPTG when OD600 reached 0.5-0.6. Temperature was lowered to 20°C for overnight growth. Bacteria cells were spun down and lysed in buffer containing 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, 20 mM imidazole and 5 mM β-mercaptoethanol. After centrifugation, supernatant was incubated with Ni-NTA resin and protein was eluted in 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, 300 mM imidazole and 5 mM β-mercaptoethanol. N-terminal SUMO tag was removed by incubation with 1/1000 (w/w) Ulp1 protease for two hours. Tag-free HIN1 protein was then loaded onto a Heparin SP column and eluted with a gradient of 0-1.5 M NaCl. The peak corresponding to p202 HIN1 was further purified by size-exclusion chromatography using Superdex 200 (10/30) in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl and 5 mM DTT. Fractions containing HIN1 were pooled, concentrated and flash frozen in liquid nitrogen for future use. Selenomethionyl HIN1, HIN1 mutants, and mouse AIM2 HIN were purified using the same procedure. Wild type p202 HIN2 and HIN2 mutants were expressed and purified similarly but without the heparin step.

Crystallization Conditions

Selenomethionyl HIN1 was mixed with an equal volume of the reservoir solution containing 23-25% PEG 3350, 0.1 M Bis-Tris pH 5.6-5.8 and 0.2 M ammonium sulfate. Crystals were cryo-protected by the reservoir solution supplemented with 30% glycerol and flash frozen with liquid nitrogen. The 20-mer and 12-mer DNA sequences for crystallization are 5'-GCGATGGTTAACCATCGCTG-3' and 5'-GCGATATCGCTG-3'. HIN1/20-mer crystals were grown under the condition of 18% PEG 3350, 0.1 M sodium citrate pH 5.4-6.0. HIN1/12-mer crystals were grown under the condition of 2.2 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.2-5.4. For both complexes, the cryo-protectant was reservoir solution supplemented with 30% ethylene glycol. HIN2 was mixed with an equal volume of the reservoir solution containing 22-25% PEG 3350, 0.1 M Tris pH 8.5 and 0.2 M ammonium sulfate.

Fluorescence Polarization Binding Assay

5'-fluorescein labeled single strand (ss) DNA (5'-CCGATGGTTAGTAGCTATCG-3', 5'-CCGATGTGTATG-3' and 5'-ISD-F) were annealed with equimolar respective complementary non-labeled DNA. 25 nM of dsDNA thus formed was incubated alone or with 1, 2, 4, 8, 16, 32 and 64 μM of wild-type or mutant HIN1 proteins. Final volume is 20 μL. Measurement was performed in 384-well plate using SpectraMax M5 at 25°C. For each concentration, eleven readings in a five-minute time window were averaged for plotting. Data were fitted to a hyperbolic curve, $P = P_0 + P_{max}[protein]/(K_D + [protein])$, where P_0 is the polarization reading without any protein and [protein] is the protein concentration.

Bio-layer Interferometry Binding Measurement

Binding was measured by the BLItz system (ForteBio, Pall Life Sciences). Human AIM2 HIN, IFI16 HIN1 or HIN2 were biotinylated by NHS-PEG4-Biotin following the manufacturer's instructions (Thermo Scientific). Biotinylated HIN proteins were immobilized onto streptavidin sensors, and the unbound proteins were washed off by PBS-based sample dilution buffer

(ForteBio, Pall Life Sciences). The sensor was then immersed into p202 HIN2 of various concentrations for association. Dissociation was carried out by immersion of sensors into sample dilution buffer. Binding affinity was calculated using BLItz Pro 1.0 (ForteBio, Pall Life Sciences).

Electron Microscopy and Image Processing

Purified p202 and HIN2 were prepared by conventional negative staining with 0.75% (w/v) uranyl formate (Ohi et al., 2004). Images were collected with a Tecnai T12 electron microscope (FEI, Hillsboro, OR) equipped with an LaB_6 filament and operated at an acceleration voltage of 120 kV. Images were recorded at a nominal magnification of 67,000x and a defocus value of –1.5 mm on a 2K x 2K or a 4K x 4K charge-coupled device (CCD) camera (Gatan) using low-dose procedures. The pixel size was 1.68 Å at the specimen level.

BOXER, the display program associated with the EMAN2 software package (Tang et al., 2007) was used to interactively select particles. For p202, 34,258 particles were selected from 414 2K x 2K CCD images and 113 4K x 4K CCD images, and for HIN2, 6,093 particles were selected from 97 4K x 4K CCD images. Using the SPIDER software package (Frank et al., 1996), the p202 particles were windowed into 130 x 130-pixel images, and the HIN2 particles into 110 x 110-pixel images. The particles were rotationally and translationally aligned, and subjected to 10 cycles of multi-reference alignment. Each round of multi-reference alignment was followed by K-means classification, specifying 400 output classes for p202 and 50 classes for HIN2. The references used for the first multi-reference alignment were randomly chosen from the particle images.

Isothermal Titration Calorimetry (ITC)

Mouse AIM2 HIN domain and p202 HIN2 domain were dialyzed extensively against running buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0). Protein concentrations were measured using absorbance at 280 nm. Prior to titration, both proteins were centrifuged at 18,000 × g at 25 °C for more than 10 min to remove any debris and air bubbles. The calorimetric titrations were carried out at 25 °C on MicroCal ITC200 instrument with 16 successive injections of 2.4 µL (1.36 mM) mAIM2 HIN, spaced 180 s apart, into the sample cell containing a solution of 200 µL (120 µM) p202 HIN2. The data was analyzed using the ORIGIN software. The association constant (K_A), enthalpy change (ΔH), and the stoichiometry (N) were calculated by fitting the thermograms to one set of binding sites. The dissociation constant (K_D), free energy change (ΔG), and the entropy change ($T\Delta S$) were calculated using the equations: $K_D = 1/K_A$ and $-RT\ln K_A = \Delta G = \Delta H - T\Delta S$

Cell Culture and Electroporation

Mouse work was approved by the University of Queensland animal ethics committee. Mouse bone marrow-derived macrophages (BMM) were cultivated as described (Sester et al., 2006). Calf thymus (CT) DNA (Sigma, St. Louis, MN) and further purified as described (Roberts et al., 2009) was electroporated into cells as described (Roberts et al., 2009) using a Biorad Gene Pulser MXcell at 240V, 1000µF.

Western Blotting

Western blotting was performed as described (Roberts et al., 2009) using antibodies against the following proteins: p202 (S19, Santa Cruz), caspase-1 (M20, Santa Cruz Biotechnology, Santa

Cruz, CA), cleaved caspase-3 (9661, Cell Signaling), ASC (AL177, Enzo), tubulin (T5168, Sigma), S6 ribosomal protein (2317, Cell Signaling Technologies, Boston MA), V5 (MCA1360P, AbD Serotec, Raleigh NC), FLAG (M2, Sigma). Where proteins were analysed in cell medium, the cells were incubated serum-free, and released proteins precipitated with 80% acetone. In some experiments these were proportionally combined with the cell pellet lysate to indicate total caspase cleavage.

Gene Knockdown

p202 was knocked-down with Stealth siRNAs (Life Technologies, Grand Island, NY) as described (Roberts et al., 2009). A control siRNA targeting TLR9 (Roberts et al., 2009) was used in Figure 5A, and a universal control, low GC (Life Technologies) was used in Figure 5B.

Assessment of Cell Death (MTT and PI Staining)

Cell viability was assessed by staining with propidium iodide followed by flow cytometry. Alternatively, cells were plated for assay of MTT cleavage as described (Stacey et al., 1993), except that non-adherent cells were included in analysis by addition of MTT to medium at 1mg/ml. After incubation the colored product was solubilized with addition of an equal volume of isopropanol, 10% Triton X-100, 0.1N HCl. MTT is cleaved by mitochondrial enzyme succinate dehydrogenase and results indicate metabolic activity.

ELISA

ELISA for mouse IFN-β was performed with a kit from PBL Interferon Source (Piscataway NJ).

Protein-Protein Docking

Docking was performed using ClusPro2.0 webserver (Kozakov et al., 2010). p202 HIN2 tetramer was input as receptor, and human AIM2 HIN was used as the ligand. Top 120 models were examined manually for consistency with preliminary experimental data.

Figure S1. Overview of p202 HIN1/dsDNA Structures in Different Crystal Forms (Related to Figure 1)

(A) Alignment of human and mouse HIN domain sequences. Seventeen human or mouse HIN domain sequences are aligned by ClustalW2 with minor manual adjustment. Sequences are grouped in three classes, HIN-A, -C and -B from top to bottom. Residues conserved in all sequences are highlighted in red; while residues conserved in each class are highlighted in green (HIN-A), yellow (HIN-C), or magenta (HIN-B). Residues in p202 HIN1 that interact with dsDNA are colored in blue and shaded in light grey, or colored in white if conserved; while residues in p202 HIN2 that are at tetrameric interface are colored in orange in shaded in dark grey, or colored in white if conserved. Residues in hAIM2 HIN and hIFI16 HIN2 that interact with dsDNA are colored in red. Secondary structure motifs of p202 HIN1 or HIN2 are drawn in proportion above or below its respective sequence, with cylinders, arrows and lines representing α helices, β strands and loops. The region without defined density in p202 HIN2 is represented by dashed lines. | marks every tenth residue.

(B) 2Fo-Fc map of the HIN1/20-mer structure. The 2Fo-Fc map is contoured at the level of 1 σ. DNA and protein are shown as stick models. In the DNA, carbon, oxygen, nitrogen and phosphorus atoms are colored in yellow, red, blue and green, respectively. In the protein, carbon,

oxygen, and nitrogen atoms are colored in grey, red and blue, respectively. The map is clipped for clarity.
(C, D) Cartoon representation of molecules A (C) and B (D) in HIN1/12-mer structure. In the two independent structures of the HIN1/12-mer complexes in the crystallographic asymmetric unit, two neighboring 12-mers stack onto each other to form continuous double helices to interact with the HIN1 molecules. Note that in both cases the dsDNA is formed by a crystallographic two-fold axis (looking into the paper). HIN1 and DNA are colored in cyan (C) or pink (D) while symmetry related molecules are in grey.
(E) Superposition of three independent HIN1/dsDNA structures in two crystal forms. HIN1/20mer, HIN1/12mer copy A and copy B are colored in blue, cyan and pink, respectively. Despite the non-specific nature of the dsDNA recognition, superposition of the three independent complex structures showed that the binding modes are essentially the same.
(F) HIN2 does not bind DNA. dsDNA binding affinities of full-length p202, p202 HIN1 or HIN2 were measured by fluorescence polarization (FP). FP signals were plotted using SigmaPlot.

Figure S2. Quantitative Measurements of HIN1 Interactions with dsDNA (Related to Figure 2)
(A, B) Fluorescence polarization (FP) experiments using wild-type p202 HIN1 and structure-directed mutants in the DNA-binding surface, with fluorescein labeled 20-mer (A) or 12-mer (B) dsDNA.
(C) FP experiment of wild-type p202 HIN1 and p202 mutants in residues that are conserved between p202 and the DNA binding surface of AIM2, with fluorescein labeled 20-mer dsDNA.
(D) Summary of K_D values from (A-C).

Figure S3. HIN2 is Tetrameric in Solution as Measured by MALS and EM (Related to Figure 3)
(A) Molecular masses (MW) of wild-type HIN2 were measured by multi-angle light scattering (MALS) coupled with size-exclusion chromatography.
(B) Averages of HIN2 in negative stain obtained by classifying 6093 particles into 50 classes. The side length of the individual panels is 18.5 nm

Figure S4. Tetrameric but Flexible Nature of Full-length p202 as Demonstrated by EM (Related to Figure 4)
(A) Representative raw image of negatively stained p202. The scale bar is 50 nm.
(B) Averages of p202 in negative stain obtained by classifying 34,258 particles into 400 classes. The side length of the individual panels is 21.8 nm.

Figure S5. p202 Exerts its Inhibitory Role on AIM by a Direct HIN-HIN Interaction (Related to Figure 5)
(A) Knockdown of p202 increases DNA-dependent death of NZB BMM. BMM from C57BL/6 and NZB mice were either untreated, or transfected with siRNAs against p202 and TLR9 (control) for 72hr and then electroporated with 20 µg CT DNA as per Figure 5A. Cell viability was assessed after 1 hour by cleavage of MTT reagent, mediated by the mitochondrial enzyme succinate dehydrogenase. Results shown are MTT cleavage in DNA-treated cells, relative to cells electroporated in the absence of DNA. The mean and SEM for 4 independent experiments are shown. Significance levels assessed by paired one-tailed t test are *$p<0.05$, **$p<0.01$.

(B) A separate experiment for knockdown of p202, carried out as in Figure 5A, showing results for cleaved and uncleaved procaspase-1. With siRNA#2 there was more death and release of caspase-1 into the medium than siRNA#1. However, film exposures are not the same for medium and cell pellet blots, and at this early time point, the majority of cleaved caspase-1 was still within the cell. Thus the cell pellet result represents the bulk of the caspase-1, and demonstrates enhancement of caspase-1 cleavage by both p202 siRNAs.
(C) Cell death in response to electroporated DNA is dependent on AIM2 and ASC at short time points. BMM from C57BL/6 (WT), *Aim2*$^{-/-}$ and *Asc*$^{-/-}$ mice were electroporated with or without 20μg CT DNA as per panel A, and viability assessed by MTT cleavage after 1 hour (upper panel) and 3 hours (lower panel). Results shown are mean and range of duplicate electroporations. For each strain results have been normalized to the result for cells electroporated without DNA ("control"). *Asc*$^{-/-}$ mice were made using 129 strain embryonic stem (ES) cells (Mariathasan et al., 2004) and have been backcrossed onto the C57BL/6 background. *Aim2*$^{-/-}$ mice made on the C57BL/6 background were obtained from Veit Hornung (unpublished).
(D-E) Knockdown of p202 has no effect on IFN-β induction. BALB/c BMM were electroporated with either no siRNA, control siRNA targeting TLR9, or siRNAs targeting p202 mRNA. After 24 hours, cells were harvested and electroporated with or without 5 μg CT DNA and RNA prepared after a further 70 minutes. The levels of mRNA were determined by real time PCR. Results were obtained from 3 independent experiments, which used 1 or 2 p202 siRNAs in each experiment. (D) Induction of IFN-β in response to transfected DNA was unaffected by p202 knockdown. Results for each experiment were determined as IFN-β mRNA relative to hprt mRNA, and were then normalized to the "no siRNA +DNA" sample, to facilitate comparison between different experiments. (E) The degree of knockdown of p202 mRNA was determined after 24 hours. Results are shown for p202 mRNA relative to the control hprt mRNA. The different symbols denote results of different experiments. The degree of knockdown of p202 mRNA was 80-91% for p202#1 and 63-78% for p202#2.
(F) p202 HIN2 specifically binds AIM2 HIN. Real time bio-layer interferometry measurement of 5, 10, 20 and 40 μM of p202 HIN2 to immobilized human AIM2 HIN (solid lines). IFI16 HIN1 (dotted lines) or IFI16 HIN2 (dashed lines) showed minimal binding.
(G) The MFHATVAT motif is buried in HIN domain structures. The MFHATVAT motif in p202 HIN1 is colored in red. This conserved motif constitutes β2 strand, which is sandwiched between β1 and β3 strands and is an integral part of OB fold. This motif is buried in other determined HIN structures as well.

Figure S6. Potential mechanism of the AIM2 inflammasome inhibition by p202 (Related to Figure 6)

(A) Binding of p202 HIN2 has no effect on AIM2 HIN DNA binding affinity. FP measurement of fluorescein labeled 20-mer dsDNA with mouse AIM2 HIN, either alone or with two-fold molar excess p202 HIN2.
(B) p202 HIN2 dimeric mutant binds to mAIM2 HIN with similar affinity and stoichiometry. Binding between R376E mutant and mAIM2 HIN was carried out with ITC as in **Figure 5E**.
(C) An docking model showing end-binding of AIM2 HIN to p202 HIN2 tetramer. HIN2 tetramer is colored as in **Figure 3B**. Two AIM2 HIN molecules are colored in blue.
(D-E) Relatively higher interferon production by NZB cells in response to cytosolic DNA (**Figure 6D**) is not due to differences in DNA detection pathways affecting mRNA induction.

Time course of induction of IFN-β mRNA, following electroporation of C57BL/6 and NZB BMM with either 3 µg CT DNA (D) or 7 µg CT DNA (E). RNA was extracted at the indicated times and assessed for IFN-β mRNA, relative to hprt mRNA control by real time PCR. Error bars show the range calculated from duplicate PCR assays on the one cDNA sample. NZB BMM show minimal cell death over this timecourse, but C57BL/6 BMM have compromised viability which is much more pronounced at the 7 µg dose. It is evident at the 3 µg dose that there is no deficiency in signaling to induce IFN-β mRNA in the C57BL/6 cells compared with NZB cells. Consequently the difference in production of IFN-β protein by cells of the two strains is likely to be due to differential loss of viability, which occurs in a p202-dependent manner.

Table S1. Structure-based Alignments (Related to Figure 1). p202 HIN1 or HIN2 structure is superimposed with known HIN domain structures identified by their names and PDB IDs. The number of Cα atoms and root-mean-square deviation are listed for each pair.

		PDB ID (chain ID)	Aligned Cα No	RMSD (Å)
HIN1	hAIM2 (20-mer)	3RN2 (A)	168	1.298
	hAIM2 (19-mer)	3RN5 (A)	169	1.352
	hIFI16-HIN2 (16-mer)	3RNU (A)	152	1.085
	hIFI16-HIN1	2OQ0 (A)	164	1.043
	hIFI16-HIN2	3B6Y (A)	153	0.898
	hIFI16-HIN2	3RLN	147	0.834
	hIFI16-HIN2 (MT)	3RLO	148	0.886
	p202-HIN1 (20-mer)	-	173	0.527
	p202-HIN1 (12-mer)	(A)	175	0.768
HIN2	hAIM2 (20-mer)	3RN2 (A)	157	1.790
	hAIM2 (19-mer)	3RN5 (A)	154	1.571
	hIFI16-HIN2 (16-mer)	3RNU (A)	150	0.972
	hIFI16-HIN1	2OQ0 (A)	159	1.78
	hIFI16-HIN2	3B6Y (A)	157	1.348
	hIFI16-HIN2	3RLN	151	1.199
	hIFI16-HIN2 (MT)	3RLO	150	1.276
	p202-HIN1	-	157	1.982
	p202-HIN1 (20-mer)	-	155	2.303
	p202-HIN1 (12-mer)	(A)	157	2.560

Supplemental Reference

Kozakov, D., Hall, D. R., Beglov, D., Brenke, R., Comeau, S. R., Shen, Y., Li, K., Zheng, J., Vakili, P., Paschalidis, I., and Vajda, S. (2010). Achieving reliability and high accuracy in automated protein docking: ClusPro, PIPER, SDU, and stability analysis in CAPRI rounds 13-19. Proteins *78*, 3124-3130.