SUPPLEMENTAL INFORMATION

Unified Polymerization Mechanism for the Assembly of ASC-dependent Inflammasomes

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Inventory of Supplemental Information

SUPPLEMENTAL FIGURES

SUPPLEMENTAL TABLES

SUPPLEMENTAL MOVIE

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SUPPLEMENTAL REFERENCES

SUPPLEMENTAL FIGURES

Figure S1. ASC^{PYD} Forms filamentous Structures as Visualized by Electron Microscopy, Related to Figure 1

A. Size-exclusion chromatograph of the ASC^{PYD}/AIM2^{PYD} complex. ASC^{PYD} is fused with an N-terminal His-tag and co-expressed with untagged AIM2^{PYD}. The complex was first purified by Ni-affinity chromatography. It elutes in the void position on a Superdex 200 gel filtration column.
B. A negative stain EM image of Sumo-AIM2^{PYD} filaments.
C. Ni-NTA-gold (5 nm) labeling of Biotin-AIM2^{PYD}/His-ASC^{PYD} complex.
D. Size-exclusion chromatography of His-MBP tagged ASC^{PYD}. His-MBP-ASC^{PYD} fusion protein is monomeric on a Superdex200 column.
E. Proteolysis time course of MBP-tagged ASC^{PYD} labeled with Alexa488 by the TEV protease.
F. AIM2^{PYD} nucleated ASC^{PYD} filaments after the *in vitro* polymerization assay.

Figure S2. NLPR3^{FL} and NLRP3$^{PYD-NBD}$ but not NLRP3^{PYD} Promote ASC^{PYD} Filament Formation, Related to Figure 2

A. NLRP3$^{PYD-NBD}$ nucleates ASC^{PYD} filament formation (1.4 μM) at the physiological intracellular condition of 140 mM KCl and 10 mM NaCl at pH7.4. Data are represented as mean±SD (N=3).
B. SDS-PAGE of size-exclusion chromatography fractions of insect cell expressed MBP-NLRP3^{FL}.
C. A negative stain EM image of recombinant NLRP3$^{PYD-NBD}$.

Figure S3. Cryo-EM Structure of the ASC^{PYD} Filament at Near Atomic Resolution, Related to Figure 3

A. Fourier shell correlation (FSC) plot of the reconstruction, generated by dividing the images into two halves.
B. FSC plot of ASC^{PYD} filament reconstruction generated by comparison of the cryo-EM map with the final model, using DireX with occupancy refinement (solid line) and Phenix with TLS refinement (dashed line). The former gave a nominal resolution of 3.6 Å at FSC=0.143.
C. The actual EM reconstruction, filtered to 3.8 Å, is shown on the left as a transparent grey surface. Three adjacent subunits are shown as a ribbon model, each in a different color. On the right, the grey surface is that generated from the model when filtered to 3.8 Å and with refinement by DireX. The comparison shows that the 3.8 Å resolution, as claimed, is reasonable.
D. Structure-based sequence alignment of 11 PYDs with known structures. These include PYDs of ASC (Liepinsh et al., 2003), NLRP1 (Hiller et al., 2003), ASC2 (Espejo and Patarroyo, 2006; Natarajan et al., 2006), hNLRP10 (Su et al., 2013), mNLRP10 (PDB code 2DO9), NLRP7 (Pinheiro et al., 2010), NLRP12 (Pinheiro et al., 2011), NLRP3 (Bae and Park, 2011), NLRP4 (Eibl et al., 2012), MNDA (PDB code: 2DBG) and AIM2 (Jin et al., 2013). Residues conserved in more than 7 proteins are shown in red. Yellow-highlighted residues indicate conservation with those in the ASC^{PYD} interfaces. The PYD of MEFV (also known as Pyrin), whose mutations are associated with FMF, is included at the bottom of the alignment. Locations of disease mutations in NLRP3 and pyrin are highlighted in green.

Figure S4. Detailed Cryo-EM Model of the ASC^{PYD} Filament, Related to Figure 4

A. Cryo-EM density superimposed with ASC^{PYD} structure in the filament in the α2-α3 region
B. Superposition of all 11 known PYD structures, showing the short α3 and the variation at the α2-α3 loop.
C, D, E. Superposition of the ASC/ASC dimers with RAIDD/PIDD dimers (top) and MyD88/MyD88 dimers (bottom) in the type I (**C**), type II (**D**) and type III (**E**) interactions.
F, G, H. Electrostatic surface representation of the interaction patches.
I. High salt significantly disrupts filament formation.

Figure S5. Structure-based Mutations Disrupts ASC Filament Formation and AIM2/ASC and NLRP3/ASC Interactions *in Vitro* and in Cells, Related to Figure 5

A. Morphology of ASC^{PYD} filaments visualized by fluorescence microscopy. eGFP control, WT (the arrowhead depicts filaments) and mutant (D48R, K21E/K22E, F59E, E13R and R41E) eGFP-tagged ASC^{PYD} (1-106) constructs were expressed in COS-1 cells. n: nucleus; scale bars = 10 μm.
B. Expression levels of depicted constructs shown by Western blotting using anti-ASC specific antibody.
C. Morphology of ASC^{PYD} filaments visualized by EM. eGFP control, WT and mutant (D48R, K21E/K22E, F59E, E13R and R41E) eGFP-tagged ASC^{PYD} constructs were expressed in HEK 293T cells. ASC immunoprecipitated complex was eluted followed by negative stain EM visualization. Scale bar = 100 nm.
D. ASC immunoprecipitated complex was eluted and analyzed by Western blotting using anti-ASC specific antibody. Lower band at ~40 kDa (denoted by arrowhead) represents eluted ASC, and upper band at ~55 kDa represents IgG heavy chain (denoted by asterisk).

Figure S6. Reconstitution of Full AIM2 Inflammasome, Related to Figure 6

A. Kinetics of ASC^{FL} aggregation upon removal of the His-MBP tag.
B. Quantification of relative ASC^{PYD} (Mutant/WT) binding to ASC^{FL} using anti-eGFP antibody, shown as mean ± S.E. *** denotes $p < 0.001$ by Student's *t* test, n=4.
C. Ni-NTA pulldown of $AIM2^{PYD}$ by His-GFP-caspase-1^{CARD} through WT or mutant ASC^{FL} with mutations in the PYD. GFP-caspase-1^{CARD}, MBP-ASC^{FL}, and MBP-$AIM2^{PYD}$ were incubated on ice overnight with TEV protease. MBP and uncut MBP-$AIM2^{PYD}$ and MBP-ASC^{FL} were removed by incubating with amylose resin for 1 hr. Unbound fractions were incubated with Ni-NTA resin for 1 hr, washed 3 times, eluted and subjected to SDS-PAGE.

Figure S7. Morphology, Stoichiometry and ProIL-1β Processing in Inflammasomes, Related to Figure 7

A, B. Morphology of anti-ASC immunoprecipitated NLRP3 inflammasome clusters from uric acid crystal activated THP-1 cells analyzed by negative stain EM (A) in comparison with in vitro reconstituted inflammasomes incubated overnight (B).
C. Control images of immunogold EM on ultrathin cryosections. Left: Lack of labeling in the ASC-eGFP transfected cells in the absence of primary anti-ASC antibody. Arrowheads delineate perinuclear punctum. Right: eGFP alone transfected cells showed neither punctum nor anti-ASC gold labeling. Scale bar: 500 nm
D. Standard curve of quantitative Western blot of recombinant His-MBP-ASC.
E. Standard curve of quantitative Western blot of recombinant His-GFP-caspase-1.

SUPPLEMENTAL TABLES

Table S1. Comparison Between Interactions in the ASC^{PYD} Filament and Those in the PIDDosome and the Myddosome, Related to Figure 4. Shown are rotations needed to match the second subunit when the first subunit in the interaction pairs is aligned, Summary: 15-21° for type I, 21-35° for type II and 17-52° for type III interactions.

	ASC/ASC_Type I	ASC/ASC_Type II	ASC/ASC_Type III
PIDDosome			
RAIDD/RAIDD	16.2°	22.5°	33.2°
RAIDD/PIDD	21.0°	21.1°	22.2°
PIDD/PIDD	15.1°	N/A	17.9°
Myddosome			
MyD88/MyD888	17.2°	34.2°	52.3°
MyD88/IRAK4	25.5°	35.3°	35.4°
IRAK4/IRAK2	20.3°	22.4°	41.6°

Table S2. Summary of Mutagenesis Results on ASC^{PYD} Interfaces, Related to Figure 5. -: disruption of filament formation; +/-: weakening of filament formation.

	Residues	Structure-based mutants *in vitro*	Structure-based mutants in cells	Existing defective mutants (Moriya et al., 2005)
Type I				
Ia	R3	R3E (+/-)		
Ia	R5			
Ia	L9			
Ia	D48	D48R (-), D48N (-)	D48R (-)	D48A, D48N, D48E, D48R
Ia	L50	L50A (+/-)		
Ia	D51	D51R (-)		D51A, D51N, D51K
Ia	D54			
Ib	E19			E19A
Ib	K21	K21Q (-)		K21A, K21Q, K21E
Ib	K22	K21E/K22E (-)	K21E/K22E (-)	
Ib	L25			
Ib	K26	K26E (-)		K26A, K26Q
Ib	L28			
Ib	S29			
Ib	R41	R41E (-)	R41E (-)	R41A, R41Q, R41W
Type II				
IIa	Y36	Y36A (+/-)		
IIa	S58			
IIa	F59	F59E (-)	F59E (-)	
IIa	L61			
IIa	E62			E62A
IIb	L78			
IIb	Q79			
IIb	E80	E80R (+/-)		
Type III				
IIIa	P40			
IIIa	R41	R41E (-)	R41E (-)	R41A, R41Q, R41W
IIIa	G42			
IIIb	E13	E13R (-)	E13R (-)	
IIIb	N14			
IIIb	L15			

Table S3. Relative Rates of ASC^{PYD} Polymerization in the Presence of Sub-stoichiometric NLRP3 or AIM2, Related to Figure 5. Control rate: rate of ASC^{PYD} filament formation in the absence of nucleator. Nucleation rate: initial rate of filament formation in the presence of nucleator. +: indicates normal nucleation; +/-: indicates partially defective nucleation; -: indicates abolishment of nucleation

Activator	Mutation	Interface	Nucleation Ability	Polymerization rate ratio between with and without the activator
NLRP3$^{PYD-NBD}$	WT (@1/100 molar ratio)		+	10.61
	K23E,K24E	Type Ib	-	1.10
	M27E	Type Ib	-	0.88
	E64R	Type IIa	-	0.98
	D82R	Type IIb	-	1.02
	R43W	Type IIIa	-	1.11
	E15R	Type IIIb	-	0.98
AIM2^{PYD}	WT (@1/4 molar ratio)		+	4.91
	L10A,L11A	Type Ia	-	0.87
	R24E	Type Ib	-	0.95
	F27G	Type Ib	-	1.54
	Y74R	Type IIb	-	1.17
	G38E	Type IIIa	+/-	2.39
	K39E	Type IIIa	+/-	2.92
	D15R	Type IIIb	-	1.12

Table S4. Structure-based Sequence Alignment of PYDs Relative to ASC^{PYD} Shows Conservation and Variability at the Three Asymmetric Interfaces, Related to Figure 7. The number of residues conserved with ASC^{PYD} and the total number of residues at each interface are shown in the table and in parentheses, respectively. The total percentages of conservation are shown in the right column for those PYDs with ≥ 50% homology to ASC. See Figure S3C for the alignment.

	Ia (7)	Ib (7)	IIa (5)	IIb (3)	IIIa (3)	IIIb (3)	Total (28)
AIM2	6	3	0	1	1	3	14, 50%
NLRP1	2	3	1	1	1	2	10
NLRP3	4	4	2	1	3	3	17, 61%
hNLRP10	6	1	2	2	1	2	14, 50%
mNLRP10	3	2	2	2	0	3	12
NLRP12	4	4	1	1	2	3	15, 54%
NLRP4	3	3	1	1	1	3	12
NLRP7	2	2	1	2	1	3	11
MNDA	4	0	0	0	0	2	6
ASC2	6	6	4	3	3	3	25, 89%

SUPPLEMENTAL MOVIE

Movie S1. Averaged Power Spectrum Animation, Related to Figure 3

This three-frame animation was generated by computing the averaged power spectrum from filament segments in the central three bins of the histogram in Figure 3C. The large shifts of the near-equatorial layer line (n=-6) as well as the almost fixed meridional intensity (at ~ 1/14 $Å^{-1}$) arise from a variable twist with little variability in the axial rise per subunit. The power spectra, which are unbiased (due to the fact that they are invariant under translations of the images and do not require alignments of the images), demonstrate that the sorting in Figure 3C worked properly.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Purification

Various methods were attempted to obtain high quality filaments of ASC^{PYD}. The best results were obtained with ASC^{PYD} (residues 1-106), which was cloned into pDB-His-MBP vector (Berkeley Structural Genomics Center) using the NdeI and NotI cloning sites and expressed as an MBP-fusion protein with an additional N-terminal 6 x His tag. This construct was transformed and expressed in BL21(DE3) cells by growing the culture at 37 ˚C to OD of 0.8 and inducing with 0.5 mM IPTG overnight at 16 ˚C. The *E. coli* cells were harvested and lysed by sonication in a buffer containing 20 mM Tris at pH 8.0, 200 mM NaCl, 5 mM imidazole, 5 mM β-ME, and 10% glycerol. The cell lysate was centrifuged at 40,000 g for 40 minutes. The supernatant containing soluble MBP-ASC^{PYD} fusion protein was passed through a pre-equilibrated Ni-NTA column by gravity. The column was washed with 20 column-volume sonication buffer containing 20 mM imidazole. The fusion protein was eluted from the Ni-NTA beads with sonication buffer containing 300mM imidazole and passed through a Superdex 200 10/300 GL size-exclusion column with elution buffer containing 20 mM HEPES, 150 mM NaCl, and 1 mM TCEP. To avoid aggregation, all the purification steps were carried out at 4 ˚C.

The ASC^{PYD} filaments were formed by cleavage of the MBP tag with TEV protease at a molar ratio of 1/10 of the fusion protein. This reaction was left overnight at room temperature, which resulted in complete cutting and no precipitation. Cleaved MBP and TEV were removed

by passing this mixture through a small Ni-NTA column. The flow-through, containing ASC^{PYD} filaments formed *in vitro,* was diluted accordingly for EM studies.

For nucleation assay, $NLRP3^{PYD\text{-}NBD}$ construct (residues 2-550) was over-expressed in BL21(DE3) cells with an N-terminal His-MBP tag and purified by Ni-NTA resin followed by gel filtration. The void peak (about 7-8 ml fractions) was concentrated for assay. All mutations in this construct were introduced using the QuikChange mutagenesis protocol. $NLRP3^{FL}$ was cloned into a modified pFastBac HTa vector containing an N-terminal His-MBP tag. This construct was expressed in High Five cells (Invitrogen) with the Bac-to-Bac system using a manufacturer-recommended protocol. Lysis and purification steps were the same as above. $AIM2^{FL}$ and $AIM2^{PYD}$ (residues 1-100) were cloned into pDB-His-MBP vector and over-expressed in BL21(DE3) cells with an N-terminal MBP tag. Mutants for FP assay were generated by QuikChange mutagenesis. $AIM2^{FL}$ were purified by Ni-NTA resin followed by gel filtration. Only fractions corresponding to the monomeric position were used for assay. MBP-tagged $AIM2^{PYD}$ wildtype and mutant constructs were expressed as monomer for assay and pulldown purposes (see below).

ASC^{PYD} Polymerization Assay

Since ASC^{PYD} contains no cysteine residue for convenient labeling, a cysteine residue was introduced to C-terminus of the above MBP-ASC^{PYD} fusion construct (S106C) by QuikChange mutagenesis. This fusion protein was expressed and purified similarly to the wildtype except a buffer at pH 7.0 was used during the gel filtration step. About 30 nmol of the purified protein was incubated with a Cys-reactive fluorophore, Alexa Fluor 488 C_5 maleimide, with 2-fold excess. The reaction was left overnight on ice and passed through a Superdex 200 to remove excess dye. The fraction containing labeled fusion protein was diluted to about 2 to 3 μM for assay in a black round-bottom 384-well Greiner Bio-One plate.

For each well, three parts of labeled fusion protein was added to one part of reaction buffer containing 20 mM HEPES pH 8.0, 150 mM NaCl, 1 mM TCEP, 1 % Triton-X100, and other reaction components (such as TEV, nucleator proteins, DNA, and/or ATP). Data were collected with the SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices) using excitation at 495 nm and emission at 519 nm with an auto-cutoff filter at 515 nm.

Caspase-1^{CARD} Polymerization Assay

Labeling caspase-1^{CARD} by Alexa Fluor 488 C_5 maleimide abolished filament formation due to three exposed cysteine residues on the surface of the molecule. We used the sortase method (Theile et al., 2013) for labeling the monomeric "sandwich"-tagged caspase-1^{CARD} (N-terminal MBP, C-terminal Sumo). Labeling reagents, including an engineered, Ca^{2+}-independent sortase and the peptide-fluorophore conjugate Gly-Gly-Gly-TAMRA (GGG-TAMRA), were kindly provided by Dr. Hidde Ploegh. MBP-caspase-1^{CARD}-Sumo containing a C-terminal sortase motif, "LPETGG", was purified to homogeneity similar to the ASC^{PYD} construct. Two mg of the gel filtration monomer was incubated in a mixture containing protein: sortase: GGG-TAMRA = 50 μM: 30 μM: 500 μM at 4˚C overnight. The labeled MBP-casp1^{CARD}-Sumo was separate from free peptide-fluorophore by gel filtration in 20 mM HEPES pH 8.0, 150 mM NaCl, and 1 mM TCEP. MBP-tagged ASC^{PYD}, ASC^{CARD}, and ASC^{FL} in this assay were purified as monomer in the same buffer by gel filtration. Upon removal of the MBP tag, caspase-1^{CARD} polymerized into filaments and the C-terminal Sumo did not interfere with filament formation (as confirmed by EM). TAMRA labeled caspase-1 was used at ~4 μM in this assay. The same setup as the ASC^{PYD} polymerization assay was used with excitation/emission at 561 nm/585 nm.

Binary PYD/PYD Complexes and Nanogold Labeling

To form the $AIM2^{PYD}$/ASC^{PYD} binary complex, the two gene fragments (AIM2 1-100, ASC 1-106) were inserted into the pDW363 biotinylation vector (Tsao et al., 1996) to co-express $AIM2^{PYD}$

with an N-terminal biotin acceptor peptide (BAP) and ASC^{PYD} with an N-terminal 6 x His tag. The vector contains the BirA enzyme gene for $AIM2^{PYD}$ biotinylation *in vivo.* The complex was expressed in BL21(DE3) cells. Similar expression protocol as above was used except that during overnight induction, 50 µM of biotin was supplemented. Since ASC^{PYD} forms filaments inside the *E. coli* cells resulting in reduced solubility compared to monomeric proteins, a lower speed (30,000 g for 30min) was used for clarifying the cell debris after sonication. The complex was purified by Ni-NTA resin with the batch method followed by gel filtration.

For the $NLRP3^{PYD\text{-}NACT}$/ASC^{PYD} complex, a different approach was used. $NLRP3^{PYD\text{-}NBD}$ (residues 2-550) was cloned into a modified pDB-His-MBP vector containing the biotin acceptor peptide. This construct was expressed with an N-terminal His-MBP tag and a C-terminal BAP and biotinylated *in vivo* by co-expressing with the empty pDW363 vector (harboring the BirA enzyme) using the co-transformation method. The biotinylated $NLRP3^{PYD\text{-}NBD}$ was mixed with 50-fold excess of the monomeric MBP-fused ASC^{PYD}. The polymerization of ASC^{PYD} was achieved by adding TEV to cleave off the MBP tag, forming the $NLRP3^{PYD\text{-}NBD}$/ASC^{PYD} complex.

Nanogold labeling for biotinylated binary complexes was done using streptavidin-gold conjugate (Electron Microscopy Sciences, 6 nm diameter gold). A carbon-coated copper EM grid was covered with 5 µl of sample and let sit for 1 minute. Excess sample was blotted with filter paper, and the grid was washed upside-down on 25 µl of incubation buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 1 mM TCEP, and 0.1 % gelatin) three times of 1 minute each. The grid was floated for 60 minutes on 25 µl of 6 nm streptavidin-gold conjugate diluted in incubation buffer for 60 minutes. The grid was washed three times with incubation buffer and stained for 1 minute in 2 % uranyl acetate. Images were taken on a Tecnai G^2 Spirit BioTWIN Electron Microscope.

Ternary Complexes and Immunogold Labeling

MBP-fused ASC^{FL}, MBP-fused $AIM2^{PYD}$, and GFP-fused caspase-1^{CARD} were expressed separately in BL21(DE3) cells and purified by Ni-NTA resin and gel filtration chromatography. The ternary complex was formed *in vitro* by mixing AIM2/ASC/caspase-1 in a ratio of 1:1:3 and incubating with TEV for 2 hrs in room temperature to cleave off the MBP tag. The mixture was passed through amylose resin to remove cleaved MBP tag and uncut ASC^{FL} and $AIM2^{PYD}$. The complex was purified by Ni-NTA resin using 6 x His-GFP as a handle. Untagged ASC^{FL} and $AIM2^{PYD}$ in the core ternary complex were detected by Western blot.

Visualization of AIM2 and ASC were achieved by immunogold labeling using appropriate commercially available primary antibodies (rabbit polyclonal IgG, against PYD). After applying 5 µL of samples, the grids were washed with incubation buffer (gel filtration buffer + 0.1 % gelatin) for three times of 1 minute each. The grids were floated on respective primary antibody diluted to appropriate concentrations recommended by EMS for immunogold labeling (for anti-AIM2, 0.5 µg/ml; for anti-ASC, 2 µg/ml). After 1 hr incubation, grids were washed three times with incubation buffer, then floated on 25 µL of secondary antibody-gold conjugate (goat anti-rabbit IgG, either 15 nm or 6 nm) diluted 1/20 in incubation buffer for 1 hr. The grids were washed and stained for 1 minute in uranyl acetate for visualization. For caspase-1 labeling, Ni-NTA-nanogold conjugate (Nanoprobes, 5nm) was incubated with the ternary complex on grid for 30 minutes (similar to the streptavidin-gold labeling).

Cryo-Electron Microscopy and Image Processing

Samples (2.5 µl) were applied to glow-discharged lacey carbon grids and vitrified using an FEI Vitrobot Mark IV. Grids were imaged using an FEI Titan Krios electron microscope operating at 300 keV, and recorded using a 4k x 4k Falcon II direct electron detector with a backthinned CMOS chip, with a sampling of 1.08 Å/px. The CTFFIND3 program (Mindell and Grigorieff, 2003) was used for determining the defocus, which ranged from ~ 1.0 µ to ~ 4.0 µ. A total of 370 images were used. The e2helixboxer routine from EMAN2 (Tang et al., 2007) was used for

boxing filaments from the images. These long filaments were then cut into overlapping boxes, each 400 px (432 Å) long, with a shift of 20 px between adjacent boxes (an overlap of 380 px). The SPIDER software package (Frank et al., 1996) was used for most of the image processing, and the IHRSR algorithm (Egelman, 2000) was implemented within SPIDER. The helical indexing was unambiguous (Egelman, 2010) due to the strong meridional intensity at ~ 1/(13.9 Å) in averaged power spectra, showing that the rise per asymmetric unit in the filament was ~ 13.9 Å. The layer line at ~ 1/(31 Å) is n=3, and the near-equatorial layer line is n=-6. There is a C3 point-group symmetry, so every layer line contains a Bessel order that is a multiple of three. An initial reconstruction was generated using this symmetry. The broadness of the n=-6 layer line in the averaged power spectrum, combined with the sharpness of the meridional layer line, suggested that the filaments had a fairly constant axial rise but a variable twist (Egelman and DeRosier, 1982). We therefore generated multiple references, having different values of the twist and axial rise, to use for a reference-based sorting of the images. The results showed that almost all of the variation was in the twist, as suspected, and power spectra generated from bins with different twists confirm the sorting (movie S1). Using references with a spacing of 0.3° in twist, the central bin (with a twist of 52.9°) had 24,665 segments among the ~80,000 total segments, and these were used for the final reconstruction.

Without treating out-of-plane tilt, the reconstruction reached a resolution of ~6-7 Å. The absolute hand of the reconstruction was unambiguous at this resolution given the comparison with the NMR structure (1UCP.PDB). Using references with out-of-plane tilt showed that the mode of the distribution was ~6° of tilt from the normal to the beam. The final cycles of the IHRSR algorithm involved generating references with azimuthal angle and out-of-plane tilt increments of 1.5°. The symmetry converged to a twist of 52.91° and an axial rise of 13.95 Å. The stated resolution of ~3.8 Å has been determined by comparison with the atomic model which provides a reality-check on any claims, absent from the Fourier Shell Correlation (FSC) approach (Yu et al., 2012). It is clear, however, that the resolution is not uniform, and the helices on the inside of the filament that are more tightly packed are better resolved than those on the outside. The right-handed nature of the α-helices is clearly visible. The standard FSC method (dividing the data into two sets, aligned against the same reference) yielded FSC = 0.26 at 1/(3.8 Å), while the resolution at FSC = 0.143 (Rosenthal and Henderson, 2003) gave a resolution of 2.9 Å.

Structural Refinement

The ASC^{PYD} NMR structure (PDB ID 1UCP) (Liepinsh et al., 2003) was chosen as the starting model for refinement. A region of the filament EM density with a length of 108 Å was extracted and 15 protomers were docked as a rigid-body into the EM density. The radial structure factor distribution of the EM density was then scaled to match that of the 15 subunit starting model. The density was then further sharpened with a B-factor of -60 $Å^2$ and a cosine shaped smooth cutoff at a resolution of 3.5 Å was applied.

The model was optimized by iterations over real-space refinement in DireX (Schröder et al., 2007) and manual model building with Coot (Emsley and Cowtan, 2004). For the refinement, a mask was generated by computing a 15 Å density map from the initial atomic model. The edges of this low resolution density were steepened by applying a cosine mapping function with the program apply-cos-mapping, which is part of DireX, using the values 0.4 and 0.3 as upper and lower threshold values, respectively. A simple Babinet bulk solvent model with ksol = 0.65 and Bsol = 45 $Å^2$ was used.

A cross-validation approach was used to identify the optimally fitted model and to prevent over fitting (Falkner and Schröder, 2013). In brief, only Fourier components of the cryo-EM density map lower than 3.7 Å were used for fitting, while Fourier components from the so called 'free' interval 3.5–3.7 Å were used for validation only. For this, the cross-correlation coefficient, C_{free}, is calculated between the model density map and the cryo-EM density map,

both of which were band-pass filtered using the free interval, thus containing information that has not been used for fitting.

To further assist model building and correction a 20 ns MD simulation was performed using Gromacs (Hess et al., 2008) with the Amber99SB-ILDN force field (Lindorff-Larsen et al., 2010). The simulation included 12 protomers solvated in explicit water. From this simulation, 1200 snapshots of structures were extracted and refined with DireX into the EM density. The structure with the lowest C_{free} value was compared with the refined NMR model and the fragment between residues 31 to 53 fitted significantly better into the density was therefore used to replace the corresponding fragment in the model.

For final refinement, 20 iterations over DireX refinement and energy minimization with CNS (Brunger, 2007) (without experimental data) were performed. The DireX refinement used grouped and restrained occupancy refinement. This final refinement procedure yields C_{work} and C_{free} values of 0.826 and 0.108, respectively. The Fourier Shell Correlation (FSC) between the final model and the EM density yields a cross-resolution of 3.6 Å, 4.8 Å, and 5.9 Å for FSC=0.143, 0.5, and 0.71 criteria. Phased reciprocal space refinement with Phenix (Adams et al., 2010) including TLS refinement resulted in a very similar FSC curve (Figure S3A). Resolution measurements have been much more problematic and controversial in the EM field than in X-ray crystallography, and attempts have been made to devise measures when there is no atomic model that can be compared to the reconstruction (Sousa and Grigorieff, 2007). However, when near atomic resolution is achieved, we think that the ultimate measure of resolution is provided by the interpretability of the map and the comparison between the model and the map (Figure S3B, S3C). Therefore, based upon both the FSC (Figure S3A) and the comparisons of the map with the model (Figure S3B, S3C), we estimate the resolution of our reconstruction at ~3.8 Å.

Plasmids and Antibodies

Full length human ASC was cloned in EcoRI/XhoI sites of pCDNA3.1/myc-HisA and XhoI/HindIII sites of pEGFP-N1. ASC PYD (residues 1-106) (WT), encoding the PYD domain of human ASC, was amplified via PCR using full-length human ASC as template. The PCR product was cloned into the XhoI/HindIII sites of pEGFP-N1. Mutants (D48R, KK2122EE, F59E, E13R, R41E, K21E/D48K/D51K and E67R) of ASC^{PYD} (1-106) domain were generated by standard sit-directed mutagenesis kit (Stratagene). These mutants were cloned into the XhoI/HindIII sites of pEGFP-N1. All constructs were sequence verified.

The following antibodies were diluted and used as described below: anti-EGFP, 1:5,000 (polyclonal, Thermo Scientific); anti-ASC (N-15), 1:5,000 (polyclonal, Santa Cruz Biotechnology); anti-caspase-1 p12 subunit, 1:5,000 (monoclonal, Thermo Scientific).

Cell Culture and Transfection

The cells were grown in 100 mm dish and six-well plate containing coverslip for protein extraction and morphological studies respectively. HEK 293T cells were grown in DMEM with L-glutamine supplemented with 10 % fetal bovine serum. COS-1 cells were grown in DMEM supplemented with 10 % fetal bovine serum. THP-1 cells were maintained in RPMI with L-glutamine supplemented with 10 % fetal bovine serum, and 0.1 mM non-essential amino acids solution. All cells were maintained at 37 $^{\circ}$C with 5 % CO_2. COS-1 and HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions.

For NLRP3 inflammasome activation, THP-1 cells were treated overnight with PMA (300 ng/ml final concentration). This was followed by overnight LPS induction (10 µg/ml final concentration). Uric acid crystals (200 µg/ml final concentration) treatment was carried out for 6 hours. During whole procedure cells were maintained at 37 $^{\circ}$C with 5 % CO_2.

Protein Extraction, Immunoprecipitation, Immunoblotting, and Electron Microscopy

Wildtype and mutant (D48R, KK2122EE, F59E, E13R and R41E) human ASC^{PYD} domain (residue 1-106) constructs were expressed in HEK 293T cells, and were processed for recombinant protein extraction as follows. The Petri dishes were placed on ice, and cells were harvested with a rubber scraper and sedimented at ~2,600 g at 4 °C for 20 min. Cells were washed once with 20 ml of ice-cold PBS to remove serum proteins. The cell pellet was resuspended in 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, and protease inhibitor) and pipetted up and down 10 times. The preparation was sedimented at ~500 g at 4 °C for 5 min to remove the nuclear fraction. The supernatant was collected and resedimented at 16,000 g at 4 °C for 30 min. The supernatant was measured for protein concentration with Bradford assay, and normalized to 1 mg/ml.

For immunoprecipitation, the precleared supernatant was immunoprecipitated with anti-ASC antibody overnight at 4 °C. The immobilized immune complex on protein A-sepharose was washed thrice using 1 ml of lysis buffer, and eluted using 0.2 M glycine at pH 2.6. Samples were immediately neutralized with Tris-HCl at pH 8.0. The eluted immunopurified complex was resuspended in 5 µl of 5 x sample buffer and heated at 95 °C for 5 min. Proteins were resolved on 12.5 % SDS-PAGE gels, and immunoblotting was performed with anti-ASC and anti-eGFP antibodies.

Co-immunoprecipitation experiments were performed in transfected HEK 293T cells. Precleared supernatants were immunoprecipitated with monoclonal anti-His antibodies (Qiagen), overnight at 4 °C. The immobilized immune complex on protein A-sepharose was washed thrice using 1 ml of lysis buffer. The bound complex was eluted using 20 µl of 5 x sample buffer and heated at 95 °C for 5 min. Proteins were resolved on 12.5 % SDS-PAGE gels, and immunoblotting was performed with anti-His antibodies, and co-immunoprecipitated component was detected using polyclonal anti-eGFP antibodies (Thermo Scientific). Western blots (ECL detection) were documented using Chemidoc MP system (BioRad). Binding was quantified using densitometric measurement of band intensity using NIH ImageJ software.

For negative staining electron microscopy, glow discharged copper grids containing 2 µl of eluted immunopurified complex were stained with 5 % uranyl acetate for 1 minute and air-dried. Samples were imaged using JEOL 1200EX 80kV Transmission Electron Microscope (TEM) and images were recorded with an AMT 2k CCD camera (Harvard Medical School core facility).

Fluorescence and Confocal Laser Scanning Microscopy

COS-1 cells were seeded at a density of 2 x 10^5 for morphological studies. 12 hours post transfection cells were monitored by Zeiss Axiovert 135 fluorescence microscope (Carl Zeiss) at various time points. Cells were washed once with PBS, fixed with 4 % PFA for 5 minutes at room temperature, and were mounted in *N*-propyl gallate (NPG) antifade. Fluorescence microscopy was performed using a Zeiss Axiovert 200M microscope (Carl Zeiss) equipped for conventional epifluorescence microscopy with the respective filter sets for enhanced green fluorescent protein (eGFP). Images were captured using a 40 x objective (0.75 numerical aperture) with AxioVision Rel. 4.6 software. Confocal sections were obtained with confocal laser scanning microscope FluoView FV1000 (Olympus). Images were captured using a 60 x objective (1.2 numerical aperture) with Olympus FluoView version 3.0 viewer software. The images were identically processed with Adobe Photoshop.

Immunogold EM on Ultrathin Cryosections

Transfected COS-1 cells (human ASC full length-eGFP and eGFP alone) were used for immunogold electron microscopy as previously described (Griffiths, 1993) and as detailed below. For preparation of cryosections the cells were rinsed once with PBS and were removed from the dish using 0.5mM EDTA (PBS). 800 µl of the cell suspension was layered on top of a 200 µl cushion of 8 % paraformaldehyde (in 0.1 M Sodium Phosphate buffer, pH 7.4) in an

eppendorf tube and was sedimented at ~510 g for 3 minutes at room temperature (RT). The supernatant was carefully removed and fresh 4 % paraformaldehyde was added. Cells were fixed for 2 hours at room temperature and later fixative was replaced with PBS. Prior to freezing in liquid nitrogen the cell pellets were infiltrated with 2.3 M sucrose (PBS) (containing 0.2 M glycine to quench free aldehyde groups) for 15 minutes at RT. Frozen samples were sectioned at -120 °C, the sections were transferred to formvar-carbon coated copper grids. Grids were floated on 2 % gelatin dish at 4 °C until the immunogold labeling was carried out. The gold labeling was carried out at RT on a piece of parafilm. Grids were floated on drops of 1 % BSA for 10 minutes to block for unspecific labeling, transferred to 5 µl drops of primary antibody (anti-ASC, 1:50) and was incubated for overnight at 4 °C. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5 µl drops of Protein-A gold (10 nm) for 20 minutes, washed in 4 drops of PBS for 15 minutes and 6 drops of double distilled water at RT. Protein-A gold (10 nm) labeling alone served as a control. The labeled sections were contrasted by floating the grids on drops of 0.3% uranyl acetete in 2% methyl cellulose for 10 minutes, the excess liquid was removed with a filterpaper (Whatman #1), leaving a thin coat of methyl cellulose (bluish interference color when dry). The grids were examined in a JEOL 1200EX-80kV Transmission electron microscope (TEM) and images were recorded with an AMT 2k CCD camera (Harvard Medical School core facility).

AIM2 Inflammasome Reconstitution

Transient reconstitution of the human AIM2 inflammasome was performed as previously described (Burckstummer et al., 2009; Hornung et al., 2009; Jin et al., 2012). In brief, HEK293T cells were transfected with pEFBOS-C-term-Guassia luciferase/Flag pro-IL-1β (~54 kDa), pro-caspase-1, HA-ASC, and the full-length wildtype or mutant Flag-AIM2 expression constructs using GeneJuice (Novagen). Cell lysates were probed with mouse anti-IL1β monoclonal antibody (clone 3zD, National Cancer Institute, NIH). Expression of ASC and AIM2 was detected using anti-Flag (Sigma) and anti-HA antibodies (Roche Applied Biosystems), respectively.

SUPPLEMENTAL REFERENCES

Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W.*, et al.* (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr *66*, 213-221.

Bae, J.Y., and Park, H.H. (2011). Crystal structure of NALP3 protein pyrin domain (PYD) and its implications in inflammasome assembly. J Biol Chem *286*, 39528-39536.

Brunger, A.T. (2007). Version 1.2 of the Crystallography and NMR system. Nat Protoc *2*, 2728-2733.

Burckstummer, T., Baumann, C., Bluml, S., Dixit, E., Durnberger, G., Jahn, H., Planyavsky, M., Bilban, M., Colinge, J., Bennett, K.L.*, et al.* (2009). An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol *10*, 266-272.

Egelman, E.H. (2000). A robust algorithm for the reconstruction of helical filaments using single-particle methods. Ultramicroscopy *85*, 225-234.

Egelman, E.H. (2010). Reconstruction of helical filaments and tubes. Methods Enzymol *482*, 167-183.

Egelman, E.H., and DeRosier, D.J. (1982). The Fourier transform of actin and other helical systems with cumulative random angular disorder. Acta Cryst *A38*, 796-799.

Eibl, C., Grigoriu, S., Hessenberger, M., Wenger, J., Puehringer, S., Pinheiro, A.S., Wagner, R.N., Proell, M., Reed, J.C., Page, R.*, et al.* (2012). Structural and Functional Analysis of the NLRP4 Pyrin Domain. Biochemistry *51*, 7330-7341.
Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr *60*, 2126-2132.
Espejo, F., and Patarroyo, M.E. (2006). Determining the 3D structure of human ASC2 protein involved in apoptosis and inflammation. Biochem Biophys Res Commun *340*, 860-864.
Falkner, B., and Schröder, G.F. (2013). Cross-validation in cryo-EM–based structural modeling. Proc Natl Acad Sci U S A *in press*.
Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, M., and Leith, A. (1996). SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. J Struct Biol *116*, 190-199.
Griffiths, G. (1993). Fine Structure Immunocytochemistry (Heidelberg, Germany, Spinger Verlag).
Hess, B., Kutzner, C., van der Spoel, D., and Lindahl, E. (2008). GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. J Chem Theory Comput *4*, 435-447.
Hiller, S., Kohl, A., Fiorito, F., Herrmann, T., Wider, G., Tschopp, J., Grutter, M.G., and Wuthrich, K. (2003). NMR structure of the apoptosis- and inflammation-related NALP1 pyrin domain. Structure (Camb) *11*, 1199-1205.
Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514-518.
Jin, T., Perry, A., Jiang, J., Smith, P., Curry, J.A., Unterholzner, L., Jiang, Z., Horvath, G., Rathinam, V.A., Johnstone, R.W.*, et al.* (2012). Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. Immunity *36*, 561-571.
Jin, T., Perry, A., Smith, P.T., Jiang, J., and Xiao, T.S. (2013). Structure of the AIM2 pyrin domain provides insights into the mechanisms of AIM2 autoinhibition and inflammasome assembly. J Biol Chem.
Liepinsh, E., Barbals, R., Dahl, E., Sharipo, A., Staub, E., and Otting, G. (2003). The death-domain fold of the ASC PYRIN domain, presenting a basis for PYRIN/PYRIN recognition. J Mol Biol *332*, 1155-1163.
Lindorff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J.L., Dror, R.O., and Shaw, D.E. (2010). Improved side-chain torsion potentials for the Amber ff99SB protein force field. Proteins *78*, 1950-1958.
Mindell, J.A., and Grigorieff, N. (2003). Accurate determination of local defocus and specimen tilt in electron microscopy. J Struct Biol *142*, 334-347.
Moriya, M., Taniguchi, S., Wu, P., Liepinsh, E., Otting, G., and Sagara, J. (2005). Role of charged and hydrophobic residues in the oligomerization of the PYRIN domain of ASC. Biochemistry *44*, 575-583.
Natarajan, A., Ghose, R., and Hill, J.M. (2006). Structure and dynamics of ASC2, a pyrin domain-only protein that regulates inflammatory signaling. J Biol Chem *281*, 31863-31875.
Pinheiro, A.S., Eibl, C., Ekman-Vural, Z., Schwarzenbacher, R., and Peti, W. (2011). The NLRP12 pyrin domain: structure, dynamics, and functional insights. J Mol Biol *413*, 790-803.
Pinheiro, A.S., Proell, M., Eibl, C., Page, R., Schwarzenbacher, R., and Peti, W. (2010). Three-dimensional structure of the NLRP7 pyrin domain: insight into pyrin-pyrin-mediated effector domain signaling in innate immunity. J Biol Chem *285*, 27402-27410.
Rosenthal, P.B., and Henderson, R. (2003). Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J Mol Biol *333*, 721-745.

Schröder, G.F., Brunger, A.T., and Levitt, M. (2007). Combining efficient conformational sampling with a deformable elastic network model facilitates structure refinement at low resolution. Structure *15*, 1630-1641.
Sousa, D., and Grigorieff, N. (2007). Ab initio resolution measurement for single particle structures. J Struct Biol *157*, 201-210.
Su, M.Y., Chang, C.I., and Chang, C.F. (2013). (1)H, (13)C and (15)N resonance assignments of the pyrin domain from human PYNOD. Biomol NMR Assign.
Tang, G., Peng, L., Baldwin, P.R., Mann, D.S., Jiang, W., Rees, I., and Ludtke, S.J. (2007). EMAN2: an extensible image processing suite for electron microscopy. J Struct Biol *157*, 38-46.
Theile, C.S., Witte, M.D., Blom, A.E., Kundrat, L., Ploegh, H.L., and Guimaraes, C.P. (2013). Site-specific N-terminal labeling of proteins using sortase-mediated reactions. Nat Protoc *8*, 1800-1807.
Tsao, K.L., DeBarbieri, B., Michel, H., and Waugh, D.S. (1996). A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in Escherichia coli. Gene *169*, 59-64.
Yu, X., Goforth, C., Meyer, C., Rachel, R., Wirth, R., Schroder, G.F., and Egelman, E.H. (2012). Filaments from Ignicoccus hospitalis show diversity of packing in proteins containing N-terminal type IV pilin helices. J Mol Biol *422*, 274-281.