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Supplemental Data

Pyrin Activates the ASC Pyroptosome in Response to Engagement by Autoinflammatory PSTPIP1 Mutants

Je-Wook Yu, Teresa Fernandes-Alnemri, Pinaki Datta, Jianghong Wu, Christine Juliana, Leobaldo Solorzano, Margaret McCormick, ZhiJia Zhang, and Emad S. Alnemri

Supplemental Experimental Procedures

Full-Length cDNAs and Expression Constructs

The mammalian expression plasmids for full-length human pyrin (pcDNA-pyrin-*myc*-His), cryopyrin (pcDNA-cryopyrin-Flag), pro-IL-1β (pcDNA-pro-IL-1β) and ASC (pMSCVpuro-ASC) were described previously (Yu et al., 2006). Full-length cDNAs for WT and the disease-associated mutants of human PSTPIP1, A230T and E250Q were generated by PCR using the IMAGE clone 4180398 (Genbank accession # BC008602) from Invitrogen as a template. For expression studies in 293T cells, each PSTPIP1 cDNA was cloned into the mammalian expression vector pcDNA3 with C-terminal T7 or Flag epitope tags. For expression studies in THP-1 cells, each PSTPIP1 cDNA was cloned without tags into the *Hpa* I site of a modified retrovirus expression vector, pMSCVgfp, in which the puromycin selection marker was replaced with GFP. cDNAs for the C-terminal truncated pyrin 1-580, pyrin 1-410 and pyrin 1-343 were generated by PCR using the pcDNA-pyrin-*myc*-His plasmid (Yu et al., 2006) as a template. The cDNAs were then cloned into the *Nde* I/*Xho* I sites of the bacterial expression vector pET-21a (+) in-frame with the vector's C-terminal His6 tag to generate pET-21a-pyrin1-580, pET-21a-pyrin1-410 and pET-21a-pyrin1-343 plasmids, or the *Nhe* I/*Xho* I sites of the mammalian expression vector pcDNA3.1-myc-His (-) B (Invitrogen) in-frame with the vector's C-

terminal myc-His tag to generate the pcDNA-pyrin1-580-*myc*-His, pcDNA-pyrin1-410-*myc*-His and pcDNA-pyrin1-343-*myc*-His plasmids. The full-length pyrin in pET-21a (pET-21a-pyrin-*myc*-His) was generated by removing the 3' *Sac* II/*Xho* I fragment from pET-21a-pyrin1-580 and replacing it with the *Sac* II/*Afl* II fragment from pcDNA-pyrin-*myc*-His plasmid after blunting the *Xho* I and *Afl* II ends. cDNAs for the truncated pyrin mutants pyrin-LN-BB-CC, pyrin-LN-BB and pyrin-LN, or the pyrin-Trim5α chimeras PT-CC-SPRY and PT-BB-CC-SPRY were generated by PCR using pcDNA-pyrin-*myc*-His plasmid and pcDNA-Trim5α as a template. The cDNAs were then cloned into appropriate sites of the bacterial expression plasmid pET21a or mammalian expression plasmid pcDNA3.1-myc-His (-) B. pMSCVpuro-ASC-EGFP-N1 plasmid was generated by excising the ASC-GFP fusion cDNA from the pEGFP-N1-ASC construct (Yu et al., 2006) with *Bgl* II and *Not* I and inserting it in the *Bgl* II and *Hpa* I sites of pMSCVpuro after blunting the *Not* I end. pMSCVgfp-ASC was generated from pMSCVpuro-ASC by excising the puromycin selectable marker and replacing it with GFP. The nucleotide sequences of all constructs were confirmed by automated sequencing.

Generation of Antibodies

The anti-pyrin polyclonal antibody was raised in rabbits against the N-terminal 343 amino acids of pyrin. Pyrin 1-343 was produced in bacteria with a C-terminal His6 tag to facilitate purification using the pET-21a-pyrin1-343 plasmid. The anti-PSTPIP1 polyclonal antibody was raised in rabbits against the full-length protein. Full-length PSTPIP1 was produced in bacteria with a C-terminal His6 tag using the pET-21a-PSTPIP1 plasmid. The human anti-caspase-1 antibody was a kind gift from Dr. Douglas Miller (Merck). The anti- IL-1β monoclonal antibody (32D) was obtained from the NCI preclinical repository, Biological resource branch.

Generation of Stable THP-1 Cell Lines

The monocytic cell line THP-1 was cultured in RPMI 1640 supplemented with 10 mM N-(2-hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid), 1 mM sodium pyruvate, 55 µM b-mercaptoethanol, 10 % fetal bovine serum and 200 $\mu g \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin sulfate. The stable THP-1 cell lines used in this study were all generated by retrovirus infection with recombinant MSCV retroviruses produced in the amphotropic packaging cell line Phoenix (G.P. Nolan's laboratory, Stanford University Medical Center, Stanford, CA). Phoenix cells were transfected with the empty vector pMSCVgfp or pMSCVgfp-PSTPIP1 (WT, A230T, E250Q) or pMSCVpuro-ASC-EGFP-N1 vectors using the LipofectAMINE transfection method. Forty-eight hours after transfection, the GFP expressing cells were sorted three times over a period of 4 weeks by flow cytometry until more than 95% of the cells were GFP positive. To infect THP-1 cells, the stable Phoenix cells were seeded in THP-1 culture medium for 24 h and culture supernatants containing retroviral particles were collected and filtered through 0.45 m membrane. THP-1 cells (1×10^6 cells/well) were then centrifuged in 6-well plates for 60 min at 2500 rpm at 32°C in the presence of 3 ml of retrovirus-enriched culture supernatant supplemented with 4 µg/ml of polybrene (Sigma). Plates were placed back in a CO_2 incubator at 37°C for 2 h. Fresh THP-1 medium was then added and the cells were allowed to recover for 24 h. The cells were subjected to another cycle of infection and then allowed to recover for 72 h before sorting by flow cytometry. The GFP-expressing cells were sorted three times over a period of 4 weeks until more than 95% of the cells were stable GFP positive. The stable expression of the various transgenes was verified by western blotting.

Generation of Stable HEK293T Cell Lines

HEK293T cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum, 200 $\mu g \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin sulfate. 293-caspase-1, 293-caspase-1-ASC, 293-ASC and 293-ASC-EGFP-N1 cells have been described before (Yu et al., 2006). To generate the 293-caspase-1-ASC-pyrin (293-C1AP) and 293-caspase-1-ASC-cryopyrin (293-C1AC) stable cell lines, the parental 293-caspase-1-ASC cells were transfected with pcDNA-pyrin-*myc*-His or pcDNA-cryopyrin-Flag plasmid together with a construct containing the hygromycin selectable marker (pMSCVhygro) using LipofectAMINE (Invitrogen). After few weeks of selection in hygromycin containing media stable 293-C1AP and 293-C1AC clones were isolated and characterized. The expression of pyrin and cryopyrin in the selected clones was verified by western blot analysis. To generate the 293-caspase-1-pyrin stable cells, the parental 293-caspase-1 were transfected with pcDNA-pyrin-*myc*-His and pMSCVhygro and stable cell clones were selected as described above. The 293-C1P-ASC-EGFP-N1 stable cell line was generated from the parental 293-caspase-1-pyrin cells by transfection with pMSCVpuro-ASC-EGFP-N1 constructs. The stable GFP expressing cells were then sorted three times over a period of 4 weeks by flow cytometry until more than 95% of the cells were GFP positive. The 293-C1-ASC-EGFP-N1 was generated from the 293-caspase-1 cells by transfection with pMSCVpuro-ASC-EGFP-N1 followed by sorting by flow cytometry as described above.

Immunoprecipitation and Pull-Down Assays

293-ASC (Fig. 3E) or 293T (Fig. 5E) cells were transfected with pcDNA-pyrin-*myc*-His plasmid together with empty vector or pcDNA3-PSTPIP-1-Flag expression plasmids encoding WT, A230T or E250Q PSTPIP1 variants in 100 mm dishes and 24 h after transfection the transfected cells were lysed by syringing (20X) in an IP buffer (20 mM

Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml leupeptin, 1 mM Na_3VO_4, 5 mM NaF) and centrifuged at 16,000g for 10 min. The pyrin-ASC or pyrin-PSTPIP1 complexes were immunoprecipitated from the lysates with an anti-pyrin antibody and immobilized on protein G-sepharose beads. The bead-bound proteins were then fractionated by SDS-PAGE and immunoblotted with anti-pyrin antibody, anti-ASC or anti-Flag antibody to detect PSTPIP1. In some experiments (Fig. 5D and F) 293T cells were transfected with full length, or different truncate or chimeric pyrin plasmids together with empty vector or pcDNA3-PSTPIP-1-Flag expression plasmids encoding WT, A230T or E250Q PSTPIP1 variants and then immunoprecipitated and analyzed as above.

In vitro pull-down assays (Fig. 5G) were performed with bacterially expressed GST-tagged PYD of pyrin. GST or GST-PYD was isolated from bacterial lysates by glutathione-affinity purification on glutathione-agarose beads. The bead-bound proteins were then incubated for 2 h at 4°C with in vitro translated ^{35}S-methione-labeled mutant pyrin proteins in the presence or absence of bacterially produced PSTPIP1-A230T protein. After incubation, the complexes were fractionated by SDS-PAGE and detected by autoradiography.

Confocal and Fluorescence Microscopy

The 293-ASC-EGFP-N1 cells were seeded on cover slips or in 6-well plates and then transfected with empty vector or pyrin or cryopyrin expression constructs together with or without PSTPIP1 expression plasmids. The transfections were done using Lipofectamine PLUS-reagent (Invitrogen) according to the manufacturer's instructions. In some experiments, 293-caspase-1-ASC-EGFP-N1 cells or 293-C1P-ASC-EGFP-N1 cells were seeded in 6-well plates and then transfected with empty vector or different PSTPIP1 plasmids as above. After 24-48 h of transfection, cells were stained with DAPI. Cells on

cover slips were observed using a Zeiss LSM 510 Meta confocal microscope, while cells in 6-well plates were observed with a fluorescent microscope.

Chemical Crosslinking

Chemical cross-linking of pyrin, truncated pyrin mutants and PSTPIP1 proteins using ethylene glycol bis (succinimidylsuccinate) (EGS) (Pierce) was performed essentially as described (Javanbakht et al., 2006). For analysis of the oligomerization of wild-type pyrin, cell lysates were prepared from pyrin-transfected 293T cells, THP-1 cells or pyrin-expressing *E. coli* in NP40 lysis buffer [0.5% Nonidet P40 (NP40), 1x protease inhibitor cocktail (complete EDTA-free, Roche Diagnostics) in PBS]. Lysates were centrifuged at 14,000 rpm in an Eppendorf 5417R table-top centrifuge for 15 min at 4°C. Lysates (200 µl) were then cross-linked with varying concentrations of EGS for 20 min at room temperature. The reaction mixes were quenched with 0.1 M Tris–HCl, pH 7.5 and briefly centrifuged. The cleared, cross-linked lysates were precipitated with anti-pyrin antibody and protein G-Sepharose beads for 2 h at 4°C. The bead-bound proteins were washed four times with NP40 wash buffer (10 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.5% NP40) and boiled in Laemmli sample buffer. The samples were analyzed by SDS-PAGE followed by western blotting with anti-pyrin antibody.

The truncated pyrin mutants (Fig. 4B and C) were expressed in *E. coli* with N-terminal T7-tags. Lysates containing the T7-tagged truncated pyrin mutants were prepared and cross-linked with EGS as described above. The cross-linked proteins were immunoprecipitated with T7-Tag® antibody agarose (Novagen) and then fractionated by SDS-PAGE followed by western blotting with anti-T7-HRP antibody (Novagen).

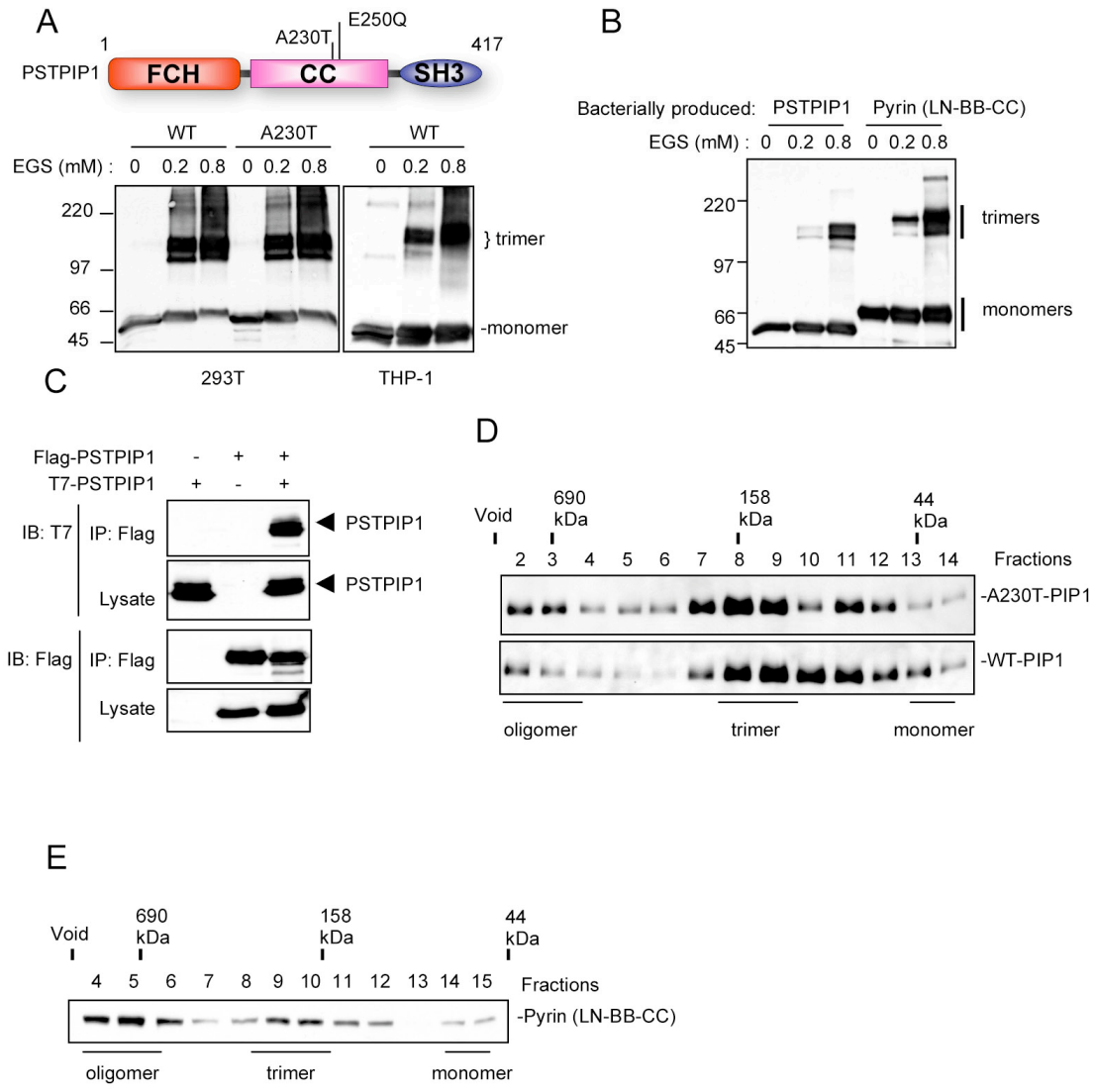

Figure S1. PSTPIP1 Is a Homotrimer

(A) Upper diagram, schematic representation of the domain structure of PSTPIP1. The two PAPA-associated mutations in the coiled-coil (CC) domain are indicated.

Lower panels, lysates containing full-length PSTPIP1 (WT and A230T) expressed in 293T cells (left panel) or endogenous PSTPIP1 from THP-1 cells (right panel) was cross-linked with the indicated EGS concentrations. PSTPIP1 was then immunoprecipitated with a PSTPIP1 specific antibody and fractionated by SDS-PAGE

followed by western blotting with PSTPIP1 antibody. Notice the presence of trimeric PSTPIP1 in the 2nd, 3rd, 5th and 6th lanes of left panel, and 2nd and 3rd lanes of right panel.

(B) The indicated bacterially-produced T7-tagged PSTPIP1 (left panel) or the truncated pyrin mutant LN-BB-CC were cross-linked with EGS, immunoprecipitated with T7-agarose and then fractionated by SDS-PAGE followed by western blotting with anti-T7 antibody.

(C) 293T cells were transfected with the indicated PSTPIP1 constructs. Lysates were then immunoprecipitated (IP) with anti-Flag and then fractionated by SDS-PAGE followed by western blotting with anti-T7 antibody (1st panel from top) or anti-Flag antibody (3rd panel from top). Lysates were also western blotted with anti-T7 antibody (2nd panel from top) or anti-Flag antibody (4th panel from top). Notice the association of the Flag-tagged PSTPIP1 with the T7-tagged PSTPIP1 in the 3rd lane, 1st panel from top, indicating that the PSTPIP1 monomers self-associate with each other to form multimers.

(D and E) Bacterially produced T7-tagged PSTPIP1 (WT and A230T) and pyrin-LN-BB-CC were fractionated on Superdex 200 FPLC column in phosphate buffered saline pH 7.0, containing 0.5% NP40. The indicated fractions (0.5 ml) were then western blotted with anti-T7 antibody. Notice that the basic units of both pyrin and PSTPIP1 are homotrimers, which further oligomerize to form large multimers.

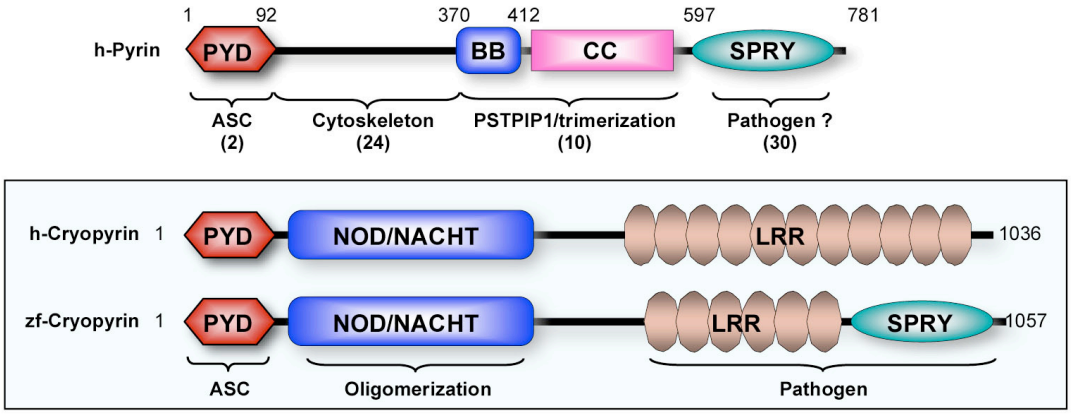

Figure S2. Domain Structures of Pyrin and Related Proteins

The top diagram shows the domain structure of human pyrin and the regions that have been shown to interact with ASC, the cytoskeleton and PSTPIP1. By analogy to Trim5α, the PRY-SPRY domain of pyrin might interact with pathogen-associated molecules. The numbers in parenthesis represent the numbers of FMF-associated mutations identified in these domains (for more updates see http://fmf.igh.cnrs.fr/infevers/).

The bottom panel shows the domain structures of human cryopyrin (h-Cryopyrin) and zebrafish cryopyrin (zf-Cryopyrin). To sense pathogens, human cryopyrin contains a C-terminal LRR domain whereas the zebrafish cryopyrin contains both LRR and PRY-SPRY domains.

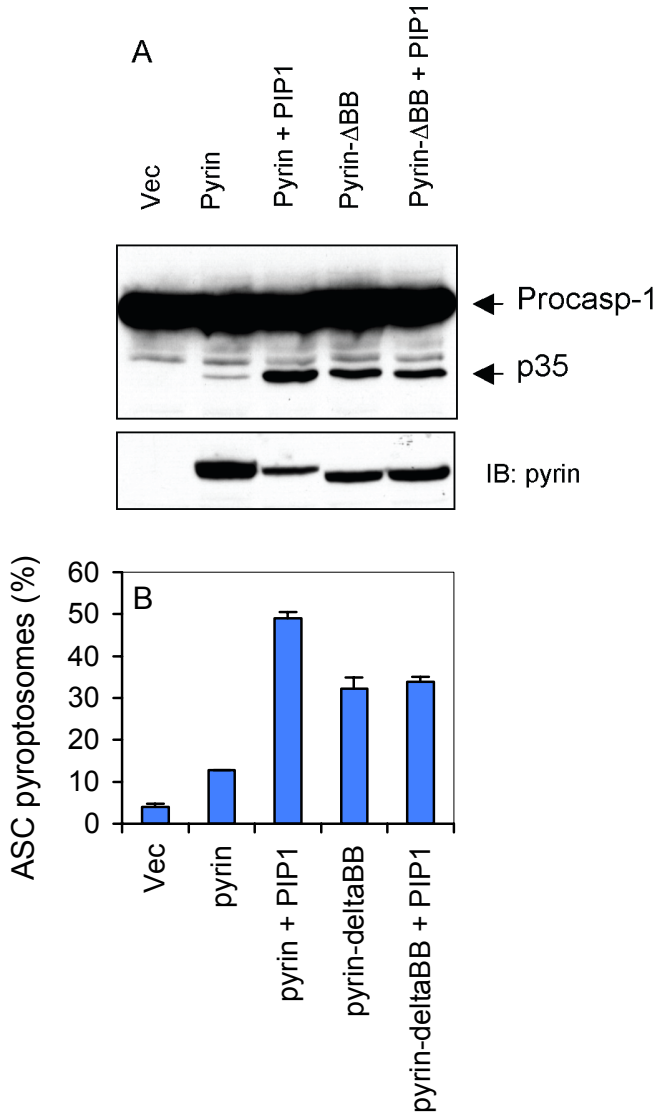

Figure S3. Deletion of the B-Box Activates Pyrin

(A) 293-caspase-1-ASC cells were transfected with an empty vector (1st lane), or expression constructs for pyrin or pyrin-ΔB-box (pyrin-ΔBB) together with an empty vector (2nd and 4th lanes) or A230T PSTPIP1 mutant plasmid (3rd and 5th lanes) as indicated. 28 h after transfection, the cells were lysed in hypotonic CHAPS buffer and the resulting cell lysates were western blotted with the anti-Flag (caspase-1) antibody (upper panel) or anti-pyrin antibody (lower panel).

(B) 293-ASC-EGFP-N1 cells were transfected with an empty vector (1st column), or expression constructs for pyrin or pyrin-ΔB-box (pyrin-deltaBB) together with an empty vector (2nd and 4th columns) or A230T PSTPIP1 mutant plasmid (3rd and 5th columns) as indicated. 24 h after transfection, the cells were observed by fluorescence microscopy and the percentages of cells containing ASC-GFP pyroptosomes were calculated as described under the "Experimental Procedures" (mean ± SD; n = 5).

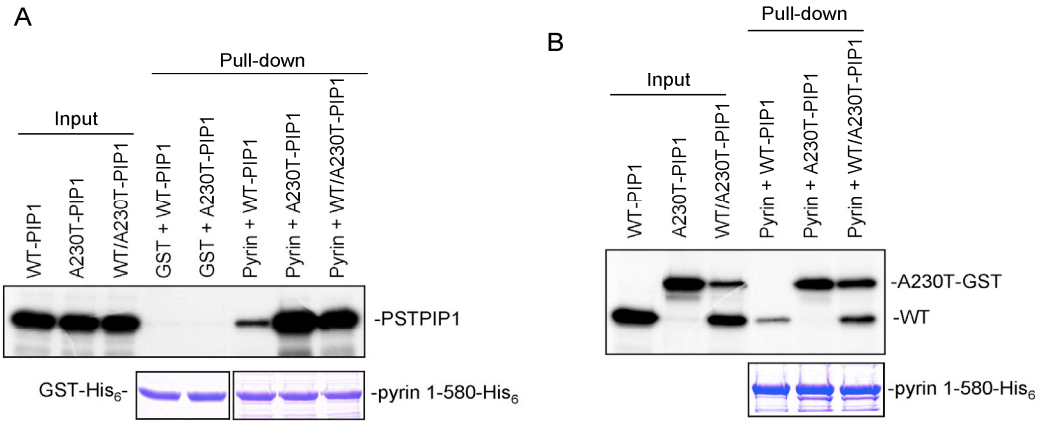

Figure S4. Binding of PSTPIP1 WT/A230T Heterotrimer to Pyrin

(A) Wildtype (WT), A230T, or a 1:1 mixture of WT and A230T expression plasmids were in vitro transcribed/translated using TNT® coupled reticulocyte lysates system (Promega) in the presence of ^{35}S-methionine. 10 µl of each translation mixture was then incubated at 4°C for 1.5 h with TALON™-agarose-bound GST-His$_6$ (4th and 5th lanes) or pyrin 1-580-His$_6$ (6th to 8th lanes) in a 100 µl reaction as indicated. The bead-bound complexes were washed several times and then boiled in 30 µl of sample buffer. Each sample was loaded onto a 12.5% SDS polyacrylamide gel and separated by electrophoresis. The gel was dried and exposed to x-ray film. Lanes 1 to 3 show the input of each translation mixture (1 µl). Notice that unlike WT PSTPIP1 (6th lane), the

PSTPIP1 WT/A230T heterotrimer (8^{th} lane) exhibits strong binding to pyrin, explaining why mutant PSTPIP1 behave dominantly.

(B) Untagged wildtype (WT) PSTPIP1 (lane 1), GST-tagged A230T (A230T-GST) PSTPIP1 (lane 2), or a 1:1 mixture of WT and A230T-GST (lane 3) expression plasmids were in vitro transcribed/translated using TNT® coupled reticulocyte lysates system. The translation mixtures were bound to TALON^{TM}-agarose-bound pyrin 1-580-His_6 (4^{th} to 6^{th} lanes) and analyzed as described in A above. The GST-tagged A230T was used to differentiate between the binding of WT and A230 subunits. Notice the increased binding of the WT subunit(s) (6^{th} lane) in the presence of A230T-GST subunit(s).

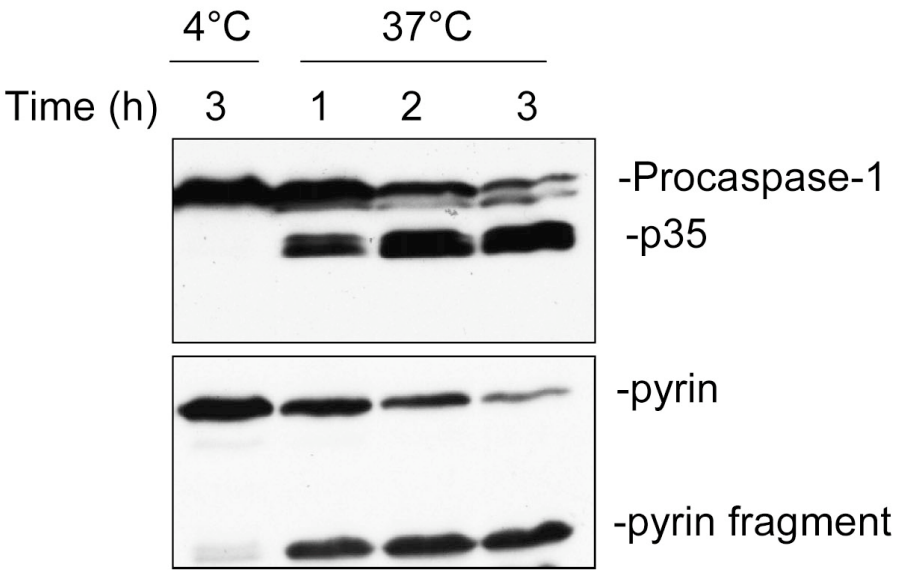

Figure S5. Activated Caspase-1 Cleaves Pyrin

Lysates from stable 293-C1AP cells (10 µg/µl), which express Flag-procaspase-1, ASC and pyrin were activated by incubation at 37°C or left at 4°C for the indicated times. The lysates were then analyzed by SDS-PAGE and western blotted with anti-Flag (upper panel) or anti-pyrin (lower panel) antibodies.

Incubation at 37°C activates the ASC pyroptosome which in turn activates caspase-1 (Fernandes-Alnemri et al., 2007). Notice the cleavage of pyrin by the activated caspase-1. No pyrin cleavage occurs in lysates that do not contain ASC or caspase-1 (not shown).

Supplemental References

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