Expanded View Figures

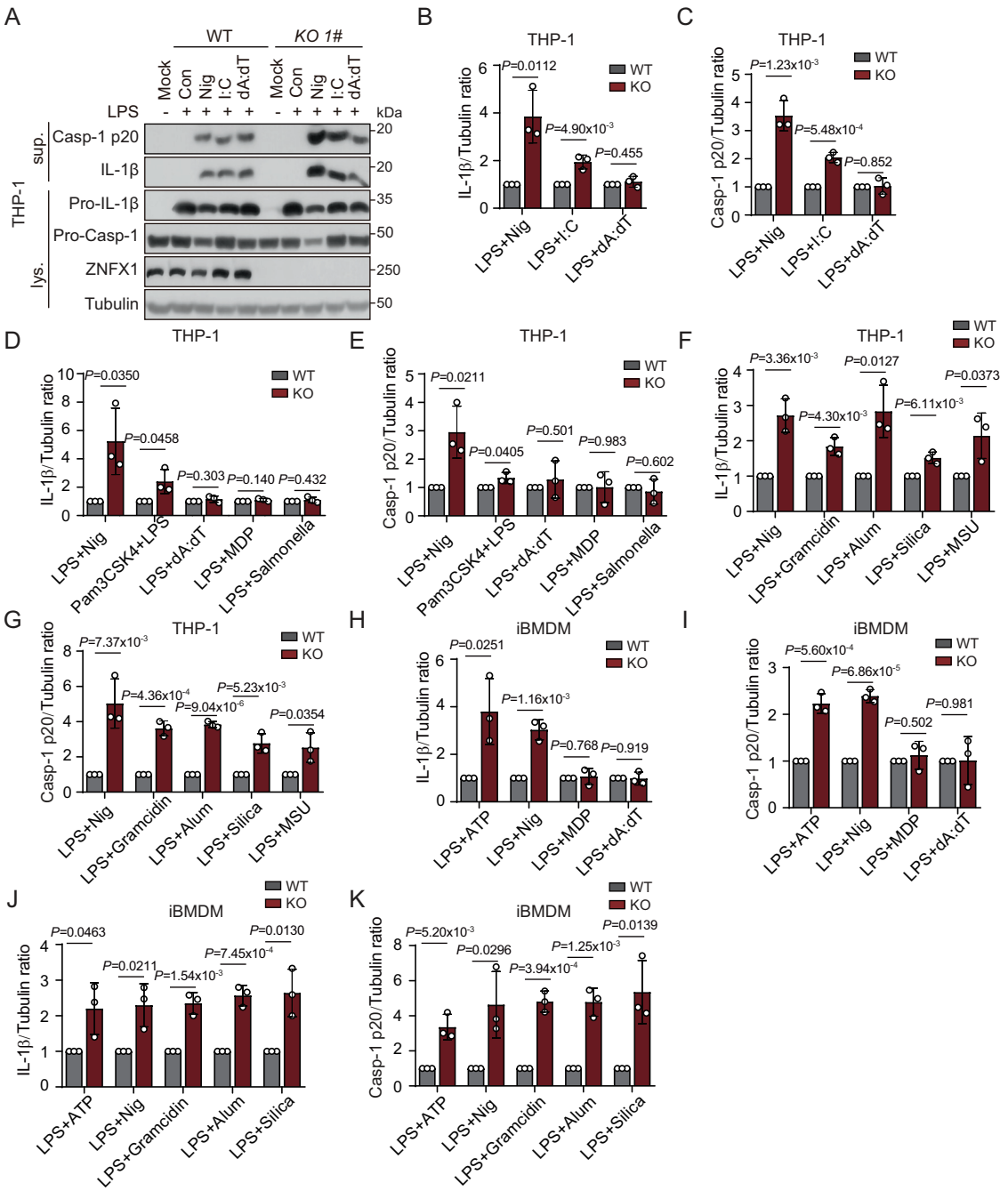


Figure EV1. ZNFX1 specifically suppresses the activation of the NLRP3 inflammasome in vitro (related to Fig. 1).

(**A**) LPS-primed wildtype (WT) or *ZNFX1* knockout (KO) THP-1-derived macrophages were treated with the indicated inflammation agonists, caspase-1 and IL-1β in the supernatant (sup.) and cell lysate (Lys.) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Mock represents macrophages primed with PBS without further stimulation. Con control, Nig nigericin, I:C poly(I:C), dA:dT, poly(dA:dT). (**B**, **C**) Quantification of IL-1β and caspase-1 P20 protein levels in (**A**). $n = 3$ biological replicates, error bar, ±s.d. (**D–G**) Quantification of IL-1β and caspase-1 P20 protein levels in Fig. 1A, B. $n = 3$ biological replicates, error bar ± s.d. (**H–K**) Quantification of IL-1β and caspase-1 P20 protein levels in Fig. 1C, D. $n = 3$ biological replicates, error bar, ±s.d. Student's *t*-test, two-tailed for (**B–K**). For each biological replicate, band intensity was measured using ImageJ. The WT control was set to 1, and the ratio for *ZNFX1* KO cells was calculated by dividing their intensity by the corresponding WT control intensity. Source data are available online for this figure.

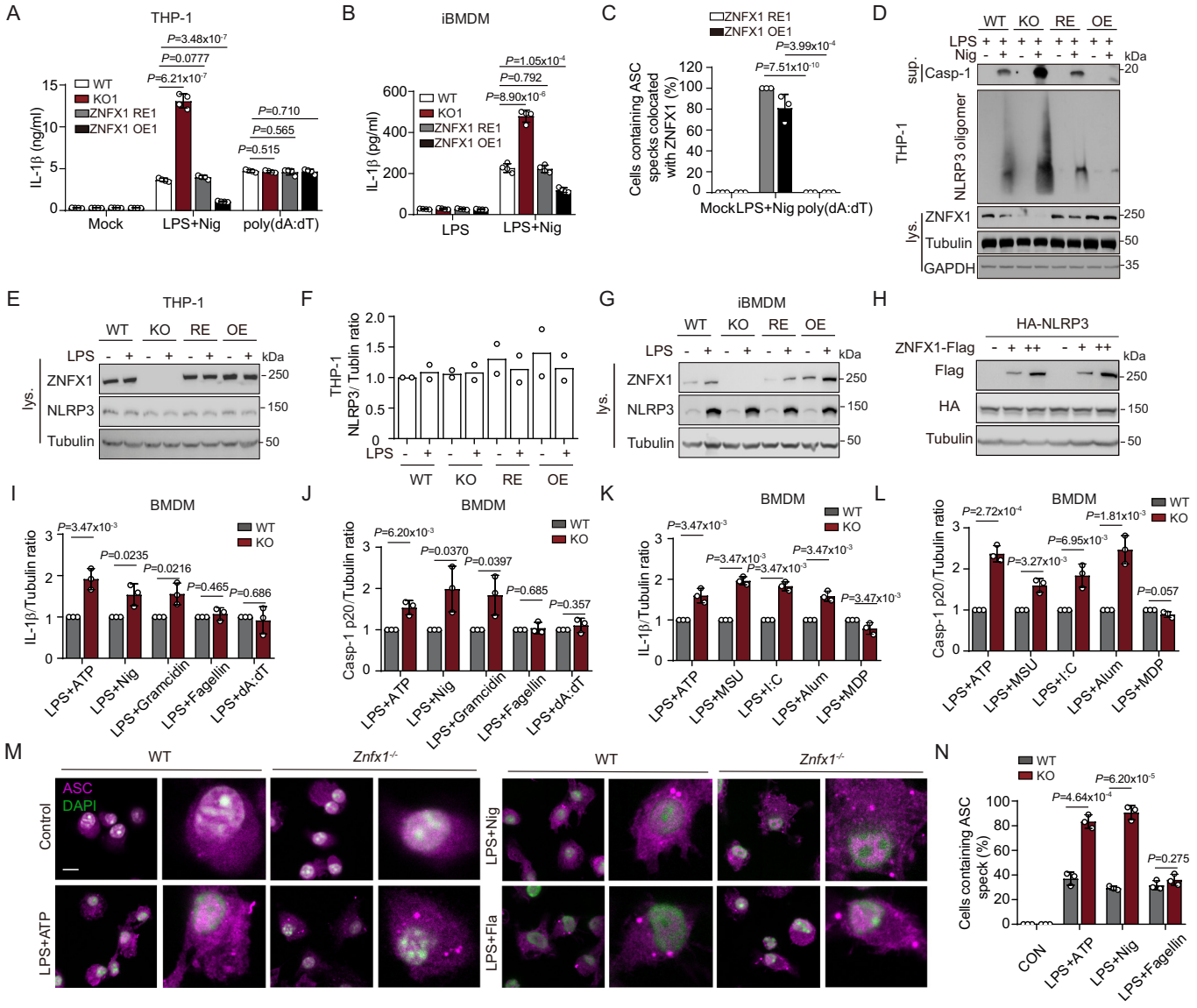


Figure EV2. ZNFX1 inhibits the assembly of mature NLRP3 inflammasome.

(**A**, **B**) ELISA detection of IL-1β in the supernatant as shown in Fig. 1G, H. Student's *t*-test, two-tailed. **C** Quantification of the percentage of cells with ASC specks containing GFP-ZNFX1 as shown in Fig. 1E. Student's *t*-test, two-tailed. (**D**) SDD-AGE analysis of NLRP3 oligomerization in the lysates of nigericin-treated macrophages. (**E**, **F**) Immunoblotting to detect indicated proteins in WT, *ZNFX1* KO, *ZNFX1* rescue (RE), and *ZNFX1* overexpression (OE) THP-1 derived macrophage with or without LPS priming (**E**). Quantification of NLRP3 protein level (**F**). $n = 2$ biological replicates. For each biological replicate, band intensity was measured using ImageJ. The WT control was set to 1, and the ratio for *ZNFX1* KO cells was calculated by dividing their intensity by the corresponding WT control intensity. (**G**) Immunoblotting to detect indicated proteins in WT, *ZNFX1* KO, *ZNFX1* RE, and *ZNFX1* OE IBMDM cells with or without LPS priming. (**H**) HeLa cells were transfected with HA-NLRP3 and increased level 3xFLAG-ZNFX1. Immunoblotting was used to detect indicated proteins. (**I–L**) Quantification of IL-1β and caspase-1 P20 protein level as shown in Fig. 1I, J. $n = 3$ biological replicates. Error bars represent ± s.d. Student's *t*-test, two-tailed. For each biological replicate, band intensity was measured using ImageJ. The WT control was set to 1, and the ratio for *ZNFX1* KO cells was calculated by dividing their intensity by the corresponding WT control intensity. (**M**) Primed WT and *Znfx1* BMDM cells were treated with the indicated inflammasome agonist. ASC speck was detected with anti-ASC antibody followed by Alex fluor-568 conjugated secondary antibody. Scale bar, 10 μm. (**N**) Quantification of the percentage of cells containing ASC specks in (**M**). $n = 3$ biological replicates. Error bars represent ± s.d. For each replicate, 100 cells were quantified. Student's *t*-test, two-tailed. Source data are available online for this figure.

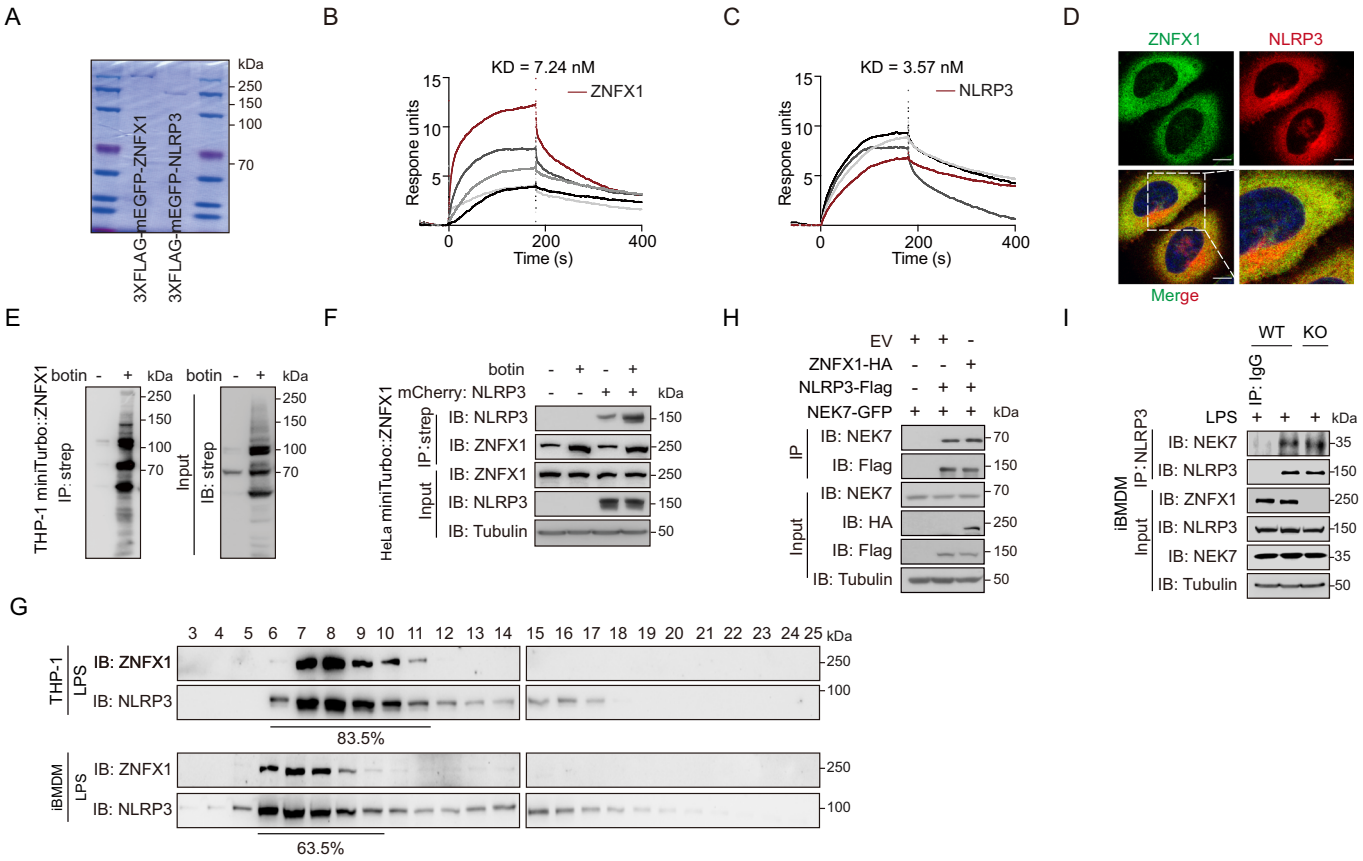
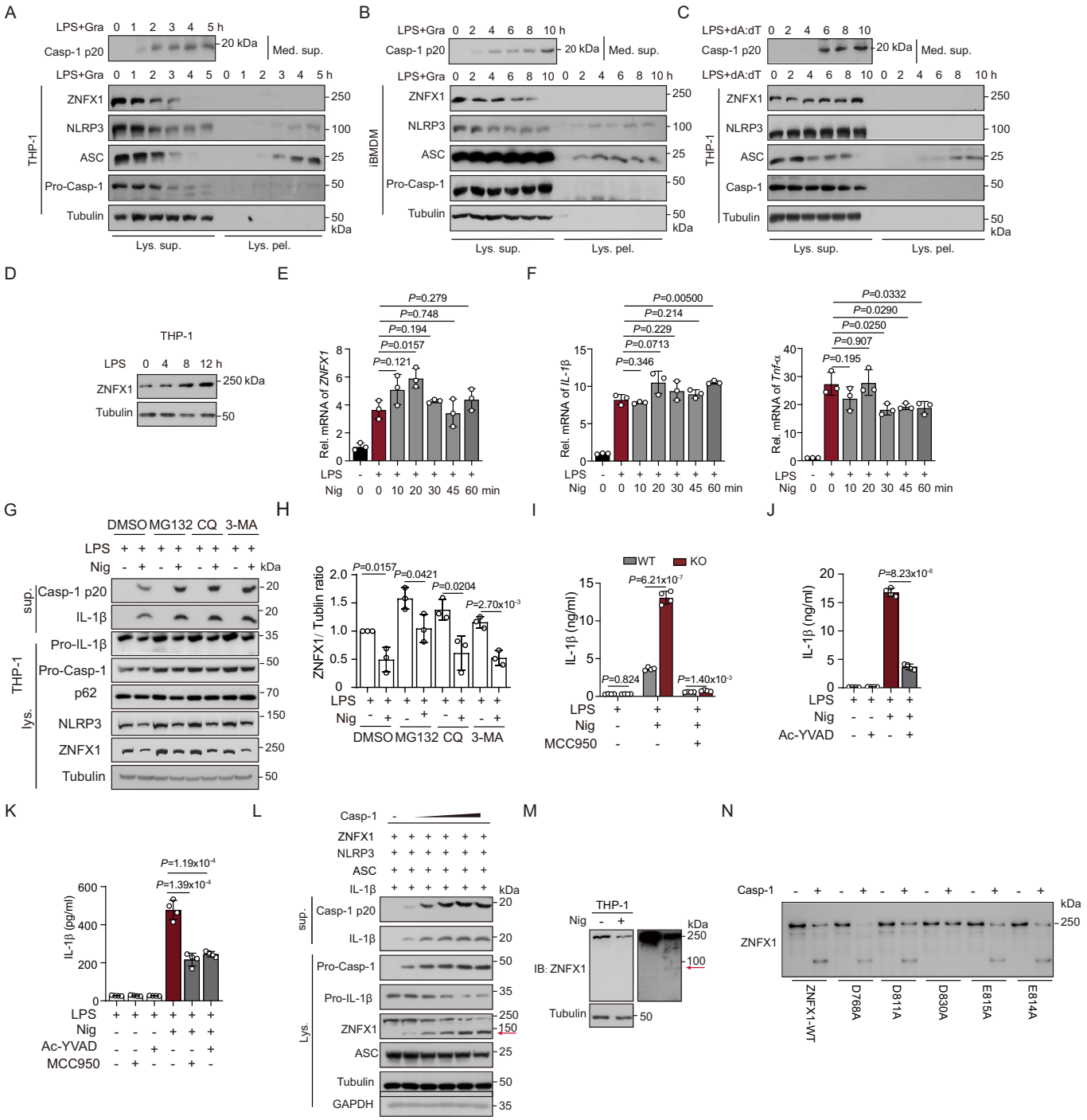


Figure EV3. ZNFX1 interacts with NLRP3 related to Fig. 3.

(**A**) Coomassie brilliant blue staining of purified 3xFLAG-GFP-ZNFX1 and 3xFLAG-GFP-NLRP3 proteins. (**B, C**) Purified 3xFLAG-ZNFX1 or 3xFLAG-NLRP3 proteins were immobilized, and proteins associated with the immobilized proteins were analyzed using surface plasmon resonance (SPR). (**D**) Fluorescence imaging of HeLa cells stably expressed GFP-ZNFX1 and mCherry-NLRP3. Scale bar, 10 μm. (**E**) Immunoblot analysis of proteins in cell lysate and miniTurbo-ZNFX1 catalyzed biotinylated species from THP-1 derived macrophages before and after adding biotin substrate using strep-HRP. (**F**) HeLa cells stably expressing miniTurbo-ZNFX1 or miniTurbo-ZNFX1 and mCherry-NLRP3 were treated with biotin to initiate proximity biotin labeling. Proteins from cell lysate and those enriched by streptavidin were resolved by SDS-PAGE and detected by immunoblot. (**G**) Proteins from resting THP-1-derived macrophages or iBMDM were separated using a size-exclusion Column. Eluted protein fractions were detected by immunoblot. (**H**) NEK7-GFP was co-expressed with empty vector (EV), HA-ZNFX1, or FLAG-NLRP3 in HEK293T cells. Coimmunoprecipitation analysis of indicated proteins was performed using α-GFP agarose beads. (**I**) Cell lysate from WT or *Znfx1* KO iBMDM cells was precipitated with NLRP3. Immunoblot analysis of proteins in a cell lysate or co-precipitated fraction using indicated antibodies. Source data are available online for this figure.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Med. sup.
LPS+Gra 0 1 2 3 4 5 0 1 2 3 4 5 h
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ZNFX1
NLRP3
ASC
Pro-Casp-1
Tubulin
250
100
25
50
50
kDa
Lys. sup.
Lys. pel.
B
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20 kDa
Med. sup.
LPS+Gra 0 2 4 6 8 10 0 2 4 6 8 10 h
iBMDM
ZNFX1
NLRP3
ASC
Pro-Casp-1
Tubulin
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Nig - + - + - + - + kDa
sup.
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THP-1
lys.
Pro-IL-1β
Pro-Casp-1
p62
NLRP3
ZNFX1
Tubulin
20
20
35
50
70
150
250
50
H
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P=0.0421
P=0.0204
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Nig - + - + - + - +
DMSO MG132 CQ 3-MA
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P=1.40x10-3
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Nig - + +
MCC950 - - +
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MCC950 - + - - + -
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Casp-1 -
ZNFX1 + + + + + +
NLRP3 + + + + + +
ASC + + + + + +
IL-1β + + + + + +
kDa
sup.
Casp-1 p20
IL-1β
Lys.
Pro-Casp-1
Pro-IL-1β
ZNFX1
ASC
Tubulin
GAPDH
20
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M
THP-1
Nig - +
kDa
IB: ZNFX1
250
100
Tubulin
50
N
Casp-1 - + - + - + - + - + - +
kDa
ZNFX1
250
ZNFX1-WT
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D811A
D830A
E815A
E814A

Figure EV4. The decreased *ZNFX1* protein level in response to NLRP3 inflammasome activation is largely posttranscriptional and independent of the ubiquitin-proteasome and autophagy pathway.

(**A**, **B**) LPS-primed THP-1-derived macrophages (**A**) or iBMDM (**B**) were treated with gramicidin for the indicated time. Proteins from medium supernatant, cell lysate supernatant, and pellet were detected by immunoblot. (**C**) LPS-primed THP-1 derived macrophages were transfected with poly (dA:dT). Proteins from medium supernatant, cell lysate supernatant, and pellet post-transfection were detected by immunoblot. (**D–F**) THP-1 derived macrophages were primed with LPS and treated with 10 μM nigericin for the indicated time, the protein level of *ZNFX1* (**D**), mRNA level of *ZNFX1* (**E**), and mRNA level of *IL-1β* (**F**) and *TNF-α* (**F**) were measured by immunoblotting or quantitative reverse transcription PCR (qRT-PCR). $n = 3$ biological replicates, mean ± s.d., Student's *t*-test, two-tailed. (**G**, **H**) LPS-primed THP-1 derived macrophages were pretreated with proteasome inhibitor MG132 (10 μM), autophagy inhibitor 3-MA (20 mM), or CQ (100 μM) for 4 h, and then stimulated with nigericin. Proteins from medium supernatant and cell lysate were detected by immunoblot (**G**). Quantification of *ZNFX1* protein was performed using ImageJ (**H**). $n = 3$ biological replicates. Error bars represent ± s.d. Student's *t*-test, two-tailed. For each biological replicate, band intensity was measured using ImageJ. The WT control was set to 1, and the ratio for *ZNFX1* KO cells was calculated by dividing their intensity by the corresponding WT control intensity. (**I–K**) Primed WT THP-1 derived macrophages (**I–J**) or iBMDM (**K**) were subjected to LPS or nigericin treatment with or without MCC950 or caspase-1 inhibitor (Ac-YVAD-cmk) pretreatment. Proteins from culture medium supernatant were detected by ELISA. $n = 3$ biological replicates, mean ± s.d., Student's *t*-test, two-tailed. (**L**) Indicated proteins were co-expressed in HeLa cells. Proteins from medium supernatant and cell lysate were detected by immunoblot. (**M**) Proteins from cell lysate of LPS + nigericin-treated THP-1 cells were detected by immunoblot. The red arrow indicates putative cleaved *ZNFX1* fragments. (**N**) Purified 3xFLAG-mEGFP-ZNFX1 with or without potential caspase-1 cleavage sites mutated were incubated with caspase-1 protein. Products were detected by immunoblot using α-FLAG antibody. This is an independent biological replicate related to Fig. 6D. Source data are available online for this figure.

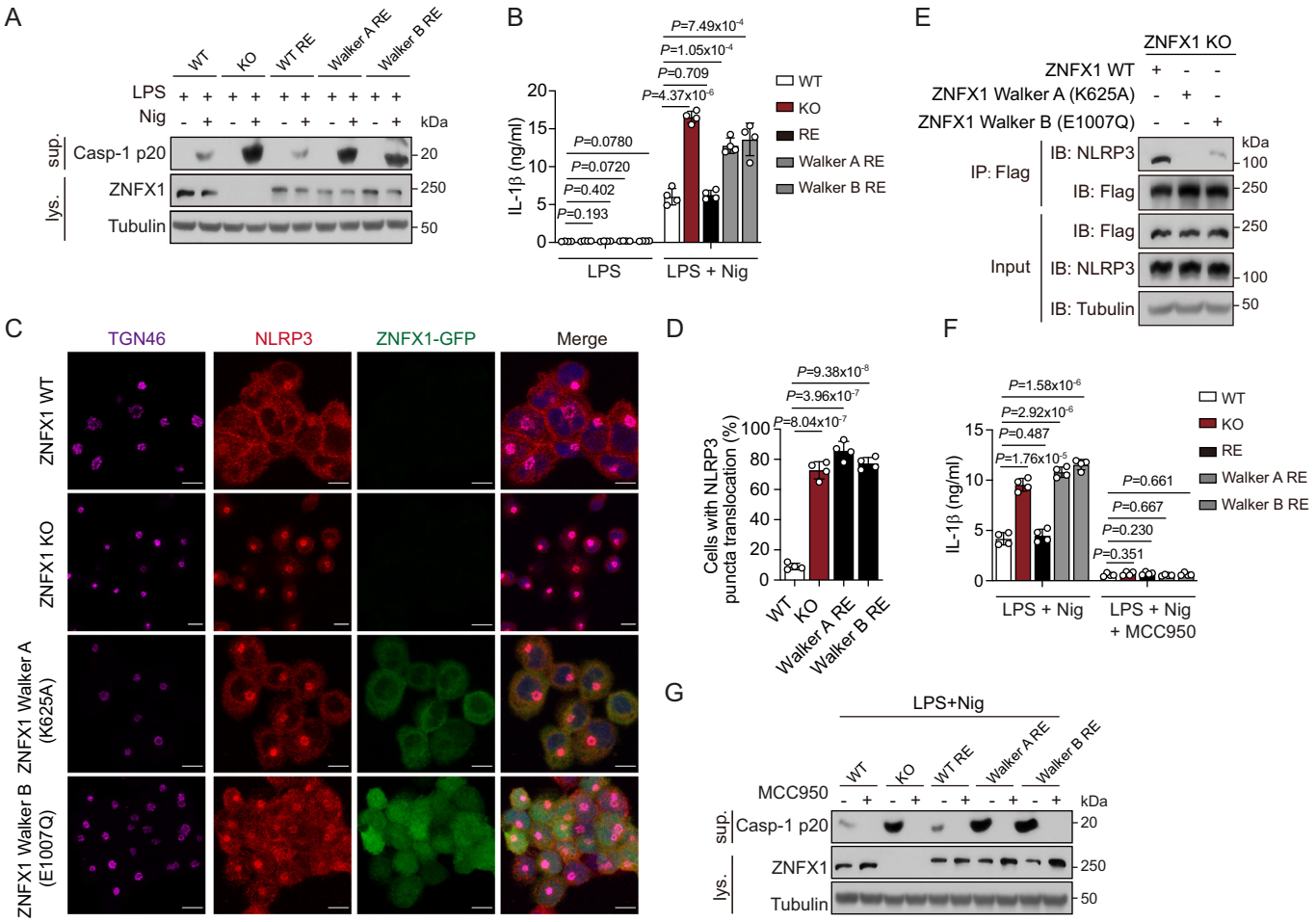

Figure EV5. The helicase activity of *ZNFX1* is critical for NLRP3 inflammasome inhibition.

(**A**, **B**) WT *ZNFX1* or *ZNFX1* containing Walker A or Walker B motif mutations were introduced back to *ZNFX1* KO THP-1 cells by lentivirus-mediated delivery. Cells were primed with LPS and stimulated with nigericin. Proteins from medium supernatant or cell lysate were examined with immunoblot (**A**) and ELISA (**B**). $n = 4$ biological replicates, mean ± s.d., Student's *t*-test, two-tailed. (**C**) WT, *ZNFX1* KO, or *ZNFX1* KO cells complemented with *ZNFX1* harboring helicase mutations were fixed and stained with anti-TGN46 and anti-NLRP3 antibodies, followed by Alex fluor-647 and Alex fluor-568 conjugated secondary antibodies, respectively. Scale bar, 10 μm. (**D**) Quantification of the percentage of cells with NLRP3's TGN translocation from 100 cells in (**C**). $n = 3$, mean ± s.d., two-sided Student's *t*-test. (**E**) Coimmunoprecipitation analysis of NLRP3 with WT or helicase mutants of ZNFX1. Proteins in input cell lysate, as well as precipitation, were detected with indicated antibodies. (**F**, **G**) Cells with indicated genotype were primed with LPS and treated with nigericin, with or without MCC950 pretreatment, NLRP3 inflammation activation was measured by ELISA in (**F**) and immunoblot in (**G**). $n = 4$ biological replicates, mean ± s.d., Student's *t*-test, two-tailed. Source data are available online for this figure.