Meng et al., http://www.jcb.org/cgi/content/full/jcb.201505091/DC1

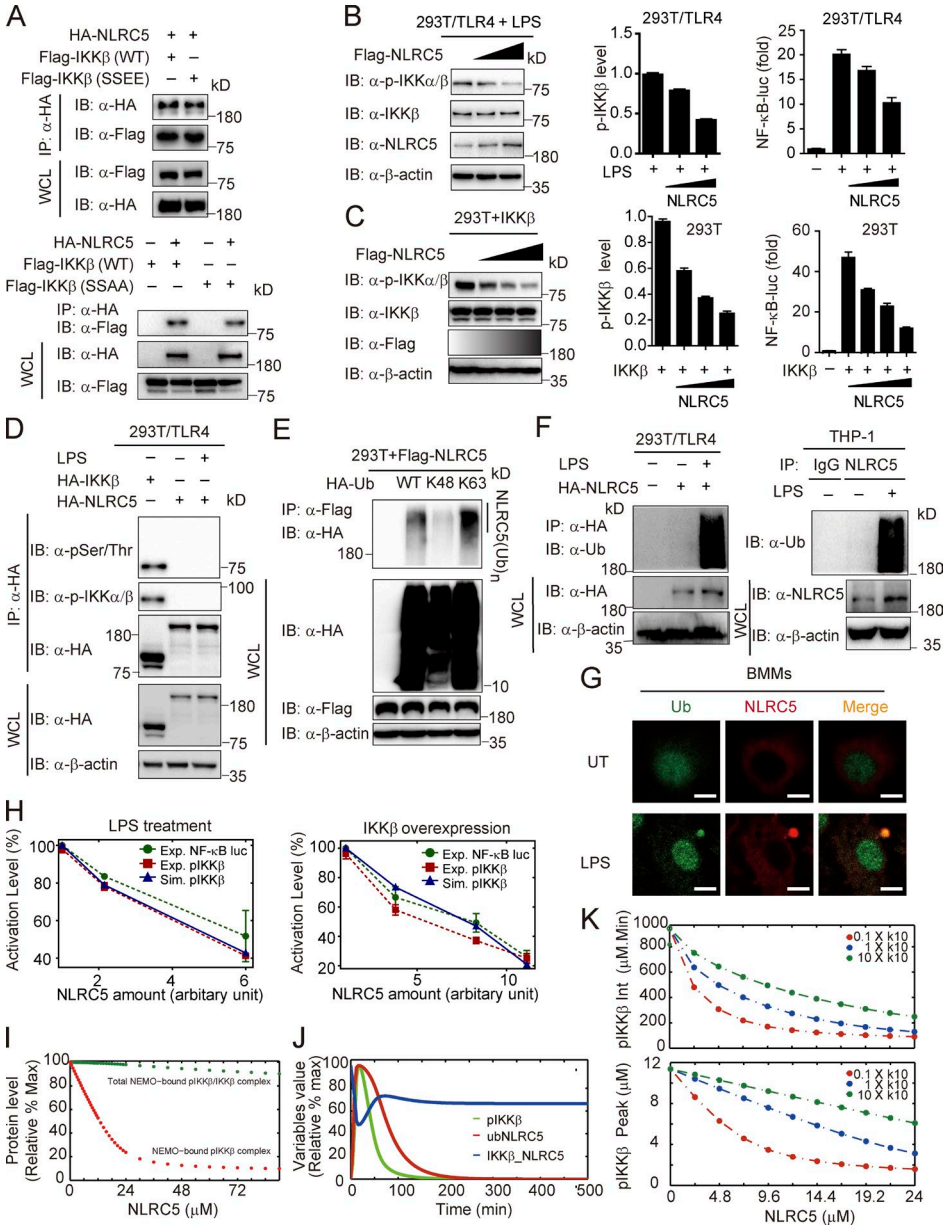

Figure S1. **NLRC5 ubiquitination is critical for its inhibitory function on NF-κB activation.** (A) HEK293T cells were transfected with *HA-NLRC5*, *Flag-IKK-β* (WT), and *Flag-IKK-β* (SSEE; top) or *IKK-β* (SSAA; bottom). Cell lysates were immunoprecipitated with anti-HA beads and detected with the indicated antibodies by immunoblot analysis. (B and C) HEK293T/TLR4 or HEK293T cells were transfected with increasing amounts of HA-NLRC5, followed by LPS treatment (B) or IKK-β overexpression (C), respectively. The lysates were used to analyze the pIKK-β by immunoblot analysis (left). The relative protein levels of pIKK-β were quantified by density scanning (middle). HEK293T/TLR4 or HEK293T cells were transfected with NF-κB–luc reporter plasmid, pRL-TK–luc reporter plasmid, and increasing amounts of NLRC5, followed by LPS treatment (B) or IKK-β overexpression (C), respectively. The cell lysates were analyzed for NF-κB–dependent luciferase activity (right). Data are representative of three independent experiments. $n = 3$. Error bars indicate the SEM. (D) HEK293T/TLR4 cells transfected with *HA-NLRC5* and *HA-IKK-β* were treated with LPS. Cell lysates were immunoprecipitated with anti-HA beads and immunoblotted with the indicated antibodies. (E) HEK293T/TLR4 cells transfected with *Flag-NLRC5* and WT *HA-Ub*, *HA-K48-Ub*, or *HA-K63-Ub* were treated with LPS. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with the indicated antibodies. (F) HEK293T/TLR4 and THP-1 cells were treated with 200 ng/ml LPS for 2 h. Cell lysates were preboiled with 1% SDS before incubation in lysis buffer containing the anti-HA affinity gel (Sigma-Aldrich) or NLRC5 antibody with protein A/G agarose overnight. (G) Fluorescence microscopy of NLRC5 (red) and Ub (green) in BMMs with or without LPS stimulation. Bars, 10 μm. (H) Comparison of experimental data, three independent experiments; $n = 3$. Error bars indicate the SEM (B and C) and simulation results of pIKK-β level by model II. (I) Simulation results of total NEMO–IKK-β complex (green; NEMO–IKK-β + NEMO–pIKK-β) and NEMO–pIKK-β (red) with different levels of NLRC5 by model II. (J) Temporal simulation for pIKK-β (green), ubiquitinated NLRC5 (red), and IKK-β–NLRC5 complex (blue) in model II. (K) The integrated and peaked value of pIKK-β with increasing amounts of NLRC5 under different ubiquitination conditions (i.e., 0.1 × k10, 1 × k10, and 10 × k10; k10 = 5.91×10^{-1} min^{-1}). The parameter k10 quantifies the ubiquitination rate of NLRC5. IB, immunoblotting; IP, immunoprecipitation; UT, untreated; WCL, whole cell lysate.

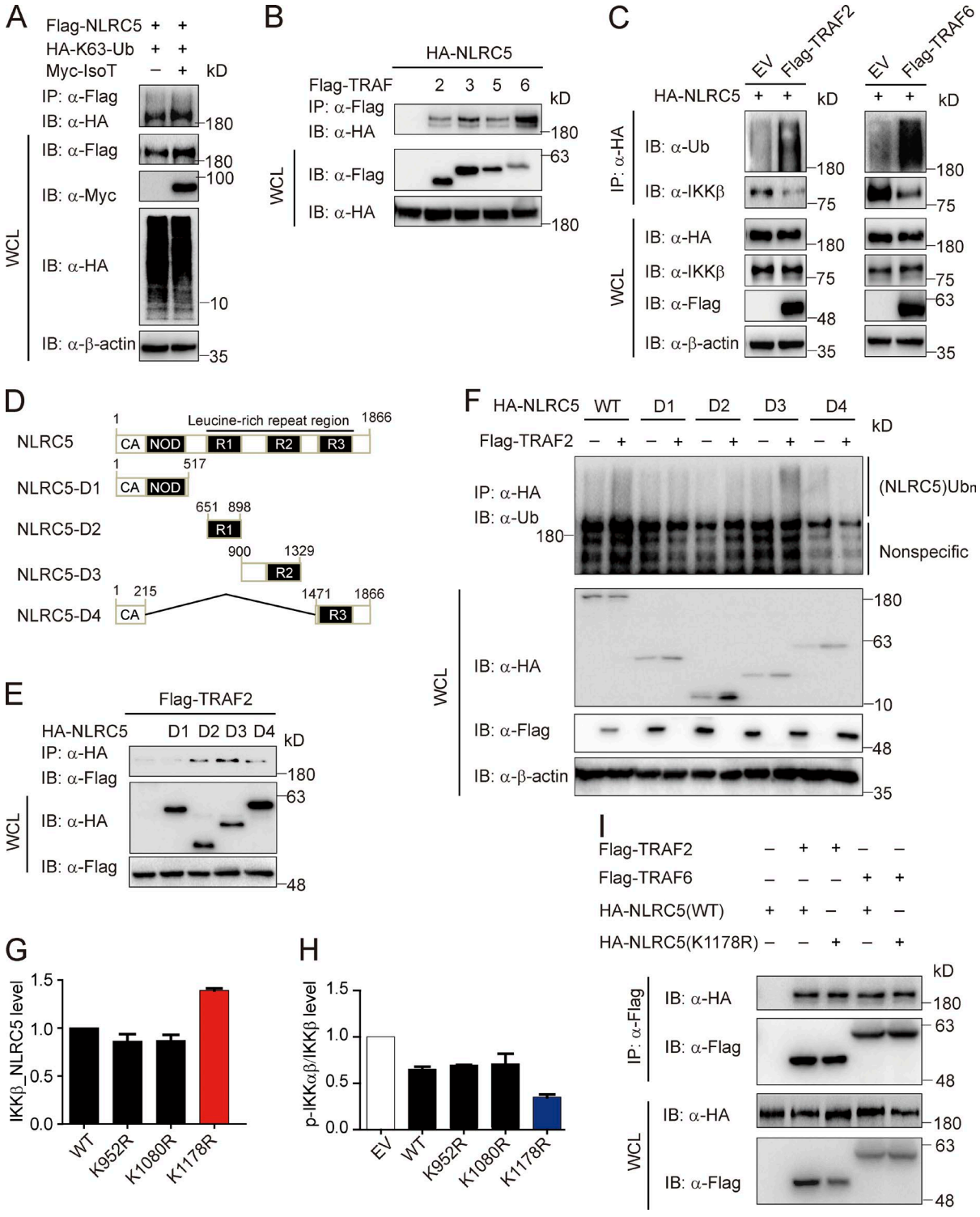

Figure S2. **TRAF2 and TRAF6 mediate the ubiquitination of NLRC5 on its functional domain.** (A) HEK293T cells were transfected with *Flag-NLRC5* and *HA-K63-Ub* with or without *Myc-IsoT*. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with the indicated antibodies. (B) HEK293T cells were transfected with *HA-NLRC5*, *Flag-TRAF2*, *Flag-TRAF3*, *Flag-TRAF5*, or *Flag-TRAF6*. Cell lysates were immunoprecipitated with anti-Flag beads followed by immunoblot analysis with the indicated antibodies. (C) HEK293T cells were transfected with plasmids encoding *HA-NLRC5*, *EV*, *Flag-TRAF2* (left), or *Flag-TRAF6* (right). Cell lysates were immunoprecipitated with anti-HA beads and immunoblotted with the indicated antibodies. (D)Various constructs of NLRC5 domains. (E) HEK293T cells were transfected with *HA-NLRC5-D1*, *HA-NLRC5-D2*, *HA-NLRC5-D3*, *HA-NLRC5-D4*, or *Flag-TRAF2*. Cell lysates were immunoprecipitated with anti-HA beads, and immunoblotting was performed with the indicated antibodies. (F) HEK293T cells were transfected with *HA-NLRC5*, *HA-NLRC5-D1*, *HA-NLRC5-D2*, *HA-NLRC5-D3*, *HA-NLRC5-D4*, and *Flag-TRAF2* or EV (–). Cell lysates were immunoprecipitated with anti-HA beads and immunoblotted with anti-Ub antibody. (G and H) Quantitative comparison of IKK-β–NLRC5 (G) or pIKK (H) level by density scanning of the blots in Fig. 3 F. Data are representative of three independent experiments. $n = 3$. Error bars indicate the SEM. (I) HEK293T cells were transfected with *HA-NLRC5(WT)*, *HA-NLRC5(K1178R)*, *Flag-TRAF2*, or *Flag-TRAF6*. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-HA antibody. IB, immunoblotting; IP, immunoprecipitation; Ubn, Ub chain; WCL, whole cell lysate.

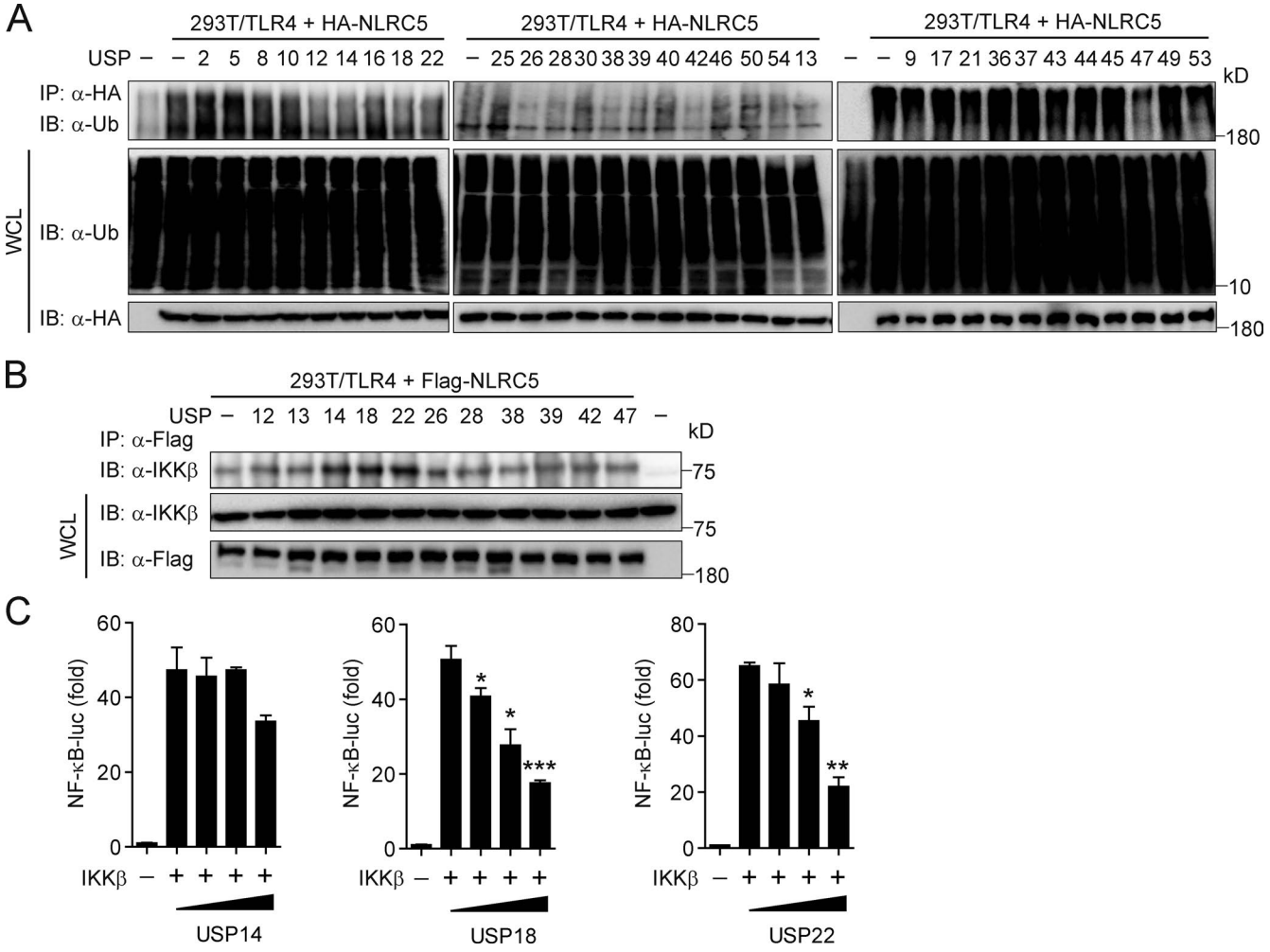

Figure S3. **Identification of NLRC5-specific DUBs.** (A) HEK293T/TLR4 cells were transfected with *HA-NLRC5* and EV (–) or the indicated *USP* plasmids, followed by LPS treatment for 30 min. Cell lysates were immunoprecipitated with anti-HA beads and immunoblotted with anti-Ub antibody. (B) HEK293T/TLR4 cells were transfected with *Flag-NLRC5* and EV (–) or USP family plasmids, followed by LPS treatment for 30 min. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti–IKK-β antibody. (C) HEK293T cells were transfected with *NF-κB–luc* and *pRL-TK–luc* reporters, the plasmids encoding *IKK-β*, increasing amounts of *Myc-USP14* (left), *Myc-USP18* (middle), or *Myc-USP22* (right), and analyzed for NF-κB–dependent luciferase activity. Data are representative of three independent experiments. $n = 3$. Error bars indicate the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the cells with IKK-β overexpression alone (two-tailed Student's *t* test). IB, immunoblotting; IP, immunoprecipitation; WCL, whole cell lysate.

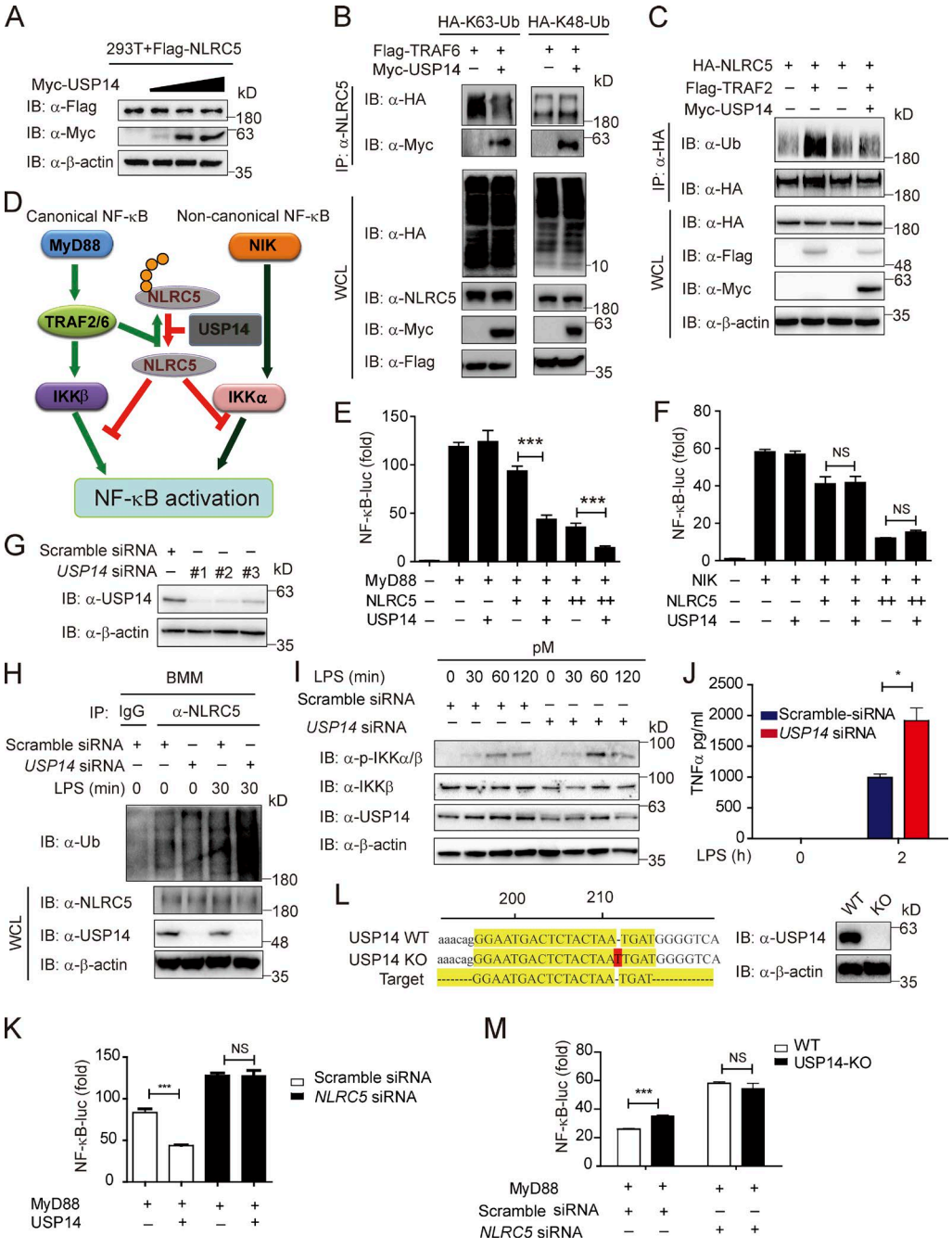

Figure S4. **USP14 deubiquitinates NLRC5 and enhances NLRC5 inhibition of NF-κB signaling.** (A) HEK293T cells were transfected with plasmids encoding *Flag-NLRC5* and increasing amount of *Myc-USP14*, followed by immunoblot analysis with the indicated antibodies. (B) HEK293T cells were transfected with plasmids encoding *Flag-TRAF6*, *Myc-USP14*, and *HA-K63-Ub* (left) or *HA-K48-Ub* (right). Cell lysates were immunoprecipitated with anti-NLRC5 antibody and immunoblotted with the indicated antibodies. (C) HEK293T cells were transfected with plasmids encoding *HA-NLRC5* or *Flag-TRAF2* with or without *Myc-USP14*. Cell lysates were immunoprecipitated with anti-HA beads and immunoblotted with the indicated antibodies. (D) A schematic representation of canonical and noncanonical NF-κB signaling pathway regulated by NLRC5 and USP14. (E and F) HEK293T cells were transfected with plasmids encoding *MyD88* (E) or *NIK* (F) and the other plasmids with the indicated combinations. Cell lysates were analyzed for NF-κB–dependent luciferase activity. ***, P < 0.001 versus the cells with MyD88 and NLRC5 overexpression (two-tailed Student's *t* test). (G) HEK293T cells were transfected with *USP14* siRNAs or control siRNA, and cell lysates were immunoblotted with the anti-USP14 antibody. (H) BMMs were transfected with scrambled siRNA or mouse *USP14* siRNA, followed by LPS treatment at the indicated time points. Cell lysates were immunoprecipitated with anti-NLRC5 antibody and immunoblotted with anti-Ub antibody. (I and J) pMs were transfected with scrambled siRNA or mouse *USP14* siRNA, followed by LPS treatment at the indicated time points. Cell lysates were immunoblotted with the indicated antibodies (I). ELISA was performed to detect the level of TNF in cell supernatant (J). (K) HEK293T cells were transfected with *NLRC5* siRNAs or control siRNA, followed by the transfection of *MyD88*, *USP14*, and NF-κB–luc reporter plasmid and pRL-TK–luc reporter plasmid. The cell lysates were analyzed for NF-κB–dependent luciferase activity. (L) Characterization of *USP14* KO cells. The sequence (left) and immunoblot (right) of USP14 in *USP14* KO cells were determined. Yellow highlighting indicates the target area of USP14 guide RNA, and red highlighting indicates the insertion mutation of the USP14 gene. (M) WT or USP14 KO HEK293T cells were transfected with *NLRC5* siRNAs or control siRNA, followed by the transfection of *MyD88* and NF-κB–luc reporter plasmid or pRL-TK–luc reporter plasmids. The cell lysates were analyzed for NF-κB–dependent luciferase activity. *, P < 0.05; ***, P < 0.001 versus the cells transfected with control siRNAs (two-tailed Student's *t* test). Data in E, F, J, K, and M are representative of at least three independent experiments. *n* = 3. Error bars indicate the SEM. IB, immunoblotting; IP, immunoprecipitation; WCL, whole cell lysate.

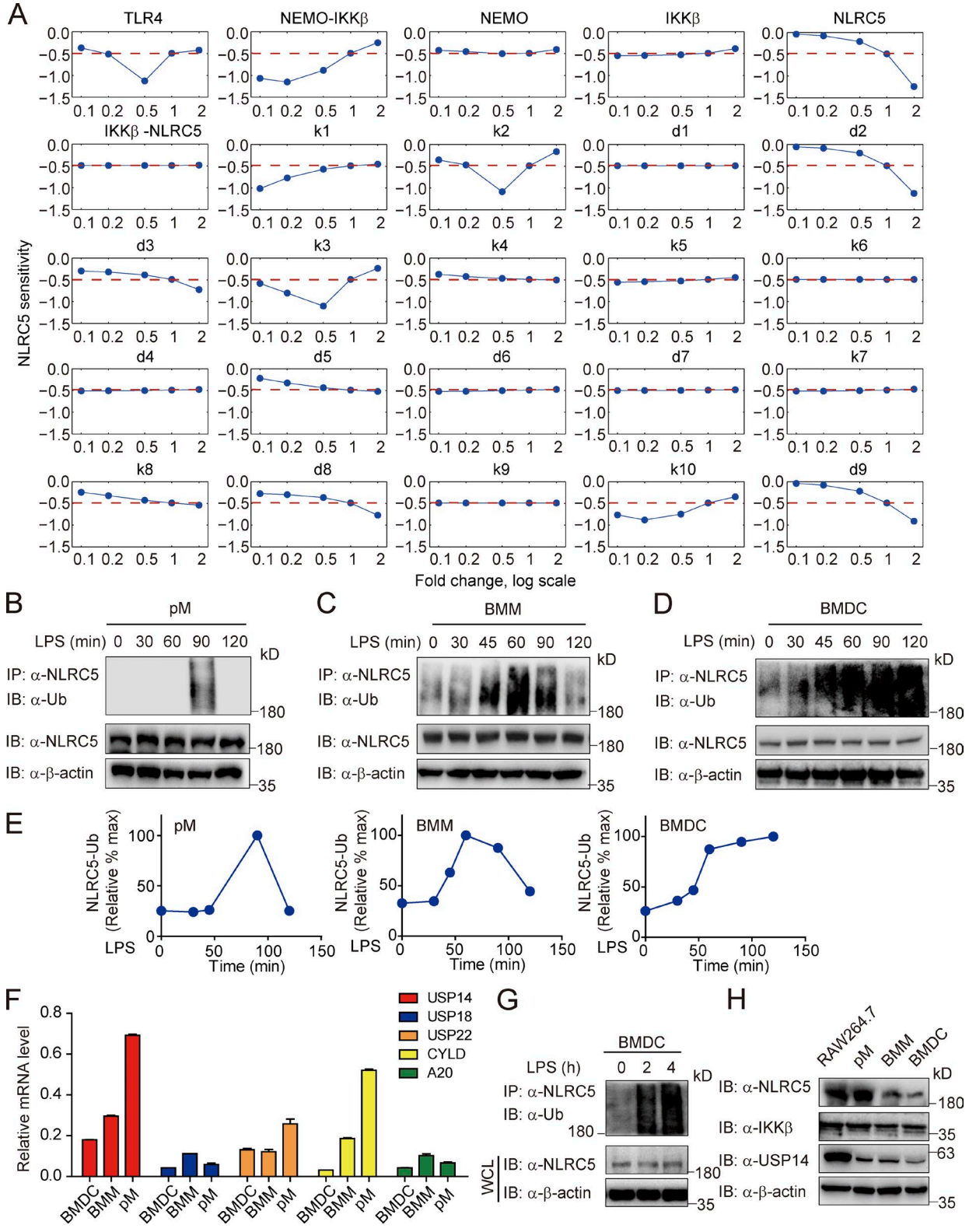

Figure S5. **NLRC5 and USP14 levels determine the NLRC5 sensitivity on NF-κB activation in different cell types.** (A) Local sensitivity coefficients for NLRC5 on IKK activity under varying parameters or initial nonzero conditions. Dotted red lines indicate the NLRC5 sensitivity for pIKK-β with no perturbation of the parameter set. (B–D) The ubiquitination level of NLRC5 with LPS stimulation at the indicated time points from pMs (B), BMMs (C), and BMDCs (D). (E) The quantified ubiquitination level of NLRC5 in pMs, BMMs, or BMDCs. The ubiquitination level of NLRC5 was normalized by the maximum. (F) mRNA levels of indicated DUBs and NLRC5 in pMs, BMMs, and BMDCs. Data are representative of three independent experiments. $n = 3$. Error bars indicate the SEM. (G) The ubiquitination levels of NLRC5 upon LPS stimulation at the indicated time points from BMDCs. (H) The protein levels of NLRC5, IKK-β, and USP14 from pMs, BMDCs, BMMs, or RAW264.7 cells. IB, immunoblotting; IP, immunoprecipitation; WCL, whole cell lysate.

Table S1. **Reactions and rates are shown**

Reactions	Reaction rates	Abbreviation
Ligand binding		
TLR4 + LPS → TLR4-LPS	k1[TLR4][LPS]	V1
TLR4-LPS → S + TLR4-LPS	k2[TLR4-LPS]	V2
TLR4-LPS → TLR4 + LPS	d1[TLR4-LPS]	V3
TLR4-LPS → null	d2[TLR4-LPS]	V4
S → null	d3[S]	V5
IKK-β activation		
NEMO–IKK-β + S → NEMO–pIKK-β + S	k3[NEMO–IKK-β][S]	V6
NEMO–IKK-β → NEMO + IKK-β	d4[NEMO–IKK-β]	V7
NEMO + IKK-β → NEMO–IKK-β	k4[NEMO][IKK-β]	V8
NEMO–pIKK-β → NEMO + pIKK-β^{free}	d5[NEMO–pIKK-β]	V9
NEMO + pIKK-β^{free} → NEMO–pIKKβ	k5[NEMO][pIKK-β^{free}]	V10
pIKK-β^{free} + IKK-β → 2pIKK-β^{free}	k6[pIKK-β^{free}][IKKβ]	V11
NEMO–pIKK-β → NEMO–IKK-β	d6[NEMO–pIKK-β]	V12
pIKK-β^{free} → IKKβ	d7[pIKK-β^{free}]	V13
NLRC5 regulation		
IKK-β + NLRC5 → IKK-β–NLRC5	k7[NLRC5][IKK-β]	V14
pIKKβ^{free} + NLRC5 → IKKβ–NLRC5	k8[pIKKβ^{free}][NLRC5]	V15
IKK-β–NLRC5 → IKK-β + NLRC5	d8[IKK-β–NLRC5]	V16
NLRC5 ubiquitination (adding in model II)		
NLRC5 + S → ubNLRC5 + S	k9[NLRC5][S]	V17
IKK-β–NLRC5 + S → ubNLRC5 + S + IKK-β	k10[IKK-β–NLRC5][S]	V18
ubNLRC5 → NLRC5	d9[ubNLRC5]	V19

Table S2. **Parameters for the WT model I and model II**

Symbols	Description	Values[a]		References
		Model I	Model II	
[TLR4]	Initial concentration of TLR4	2.45	2.15	Fitted
[LPS]	Initial concentration of LPS	2.00	2.00	Rivière et al., 2009
[NEMO–IKK-β]	Initial concentration of NEMO–IKK-β	1.78×10^{1}	1.49×10^{1}	Fitted
[NEMO]	Initial concentration of NEMO	2.31	6.68	Fitted
[IKK-β]	Initial concentration of IKK-β	2.34	4.33	Fitted
[NLRC5]	Initial concentration of NLRC5	8.99	9.21	Fitted
[IKK-β–NLRC5]	Initial concentration of IKK-β–NLRC5	2.40	2.35	Fitted
[USP14]	Initial concentration of USP14	–	24	Estimated
k1	Association rate of TLR4 and LPS	3.3×10^{-1}	3.3×10^{-1}	Shin et al., 2007
d1	Dissociation rate of TLR4-LPS	1.0×10^{-7}	1.0×10^{-7}	Shin et al., 2007
d2	TLR4-LPS degradation rate	2.0×10^{-1}	2.0×10^{-1}	Rivière et al., 2009
k2	S production rate	2.96×10^{-1}	5.26×10^{-1}	Fitted
d3	S degradation rate	2.66×10^{-2}	2.42×10^{-2}	Fitted
k3	S-mediated phosphorylation of NEMO–IKK-β	3.29×10^{-1}	1.66×10^{-1}	Fitted
k4	Association rate of NEMO and IKK-β	4.2×10^{2}	4.2×10^{2}	Lo et al., 2008
d4	Dissociation rate of NEMO–IKK-β	1.5	1.5	Lo et al., 2008
k5	Association rate of NEMO and pIKK-β^{free}	4.40×10^{1}	2.20×10^{1}	Fitted
d5	Dissociation rate of NEMO–pIKK-β	7.53	1.25×10^{1}	Fitted
k6	Autophosphorylation of pIKK-β^{free} rate constant	1.41×10^{-1}	5.40×10^{-1}	Fitted
d6	Dephosphorylation of NEMO–pIKK-β	7.37×10^{-2}	5.83×10^{-1}	Fitted
d7	Dephosphorylation of pIKK-β^{free} rate constant	5.84×10^{-1}	3.48×10^{-1}	Fitted
k7	Association rate of NLRC5 and IKK-β	1.34	6.64	Fitted
k8	Association rate of NLRC5 and pIKK-β^{free}	6.62×10^{-1}	5.33	Fitted
d8	Dissociation rate of IKK-β–NLRC5	7.03	1.89	Fitted
k9	S-mediated ubiquitination of NLRC5 rate	–	8.29×10^{-4}	Fitted
k10	S-mediated ubiquitination of IKK-β–NLRC5 rate	–	5.91×10^{-1}	Fitted
d9	Rate of ubNLRC5 deubiquitination	–	2.43×10^{-1}	Fitted
d9′	Rate of ubNLRC5 deubiquitination for USP14		1.01×10^{-2}	Estimated[b]

[a]The first and second order rate constants are expressed in units of min^{-1} and $\mu M^{-1} \cdot min^{-1}$, respectively. Initial conditions were in units of μM. Other initial conditions were set to zero.
[b]The parameter d9′ was calculated by simply dividing d9 by [USP14]. [USP14] only occurs in Fig. 7.

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