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Supplementary Materials for

Noncanonical STAT1 phosphorylation expands its transcriptional activity into promoting LPS-induced IL-6 and IL-12p40 production

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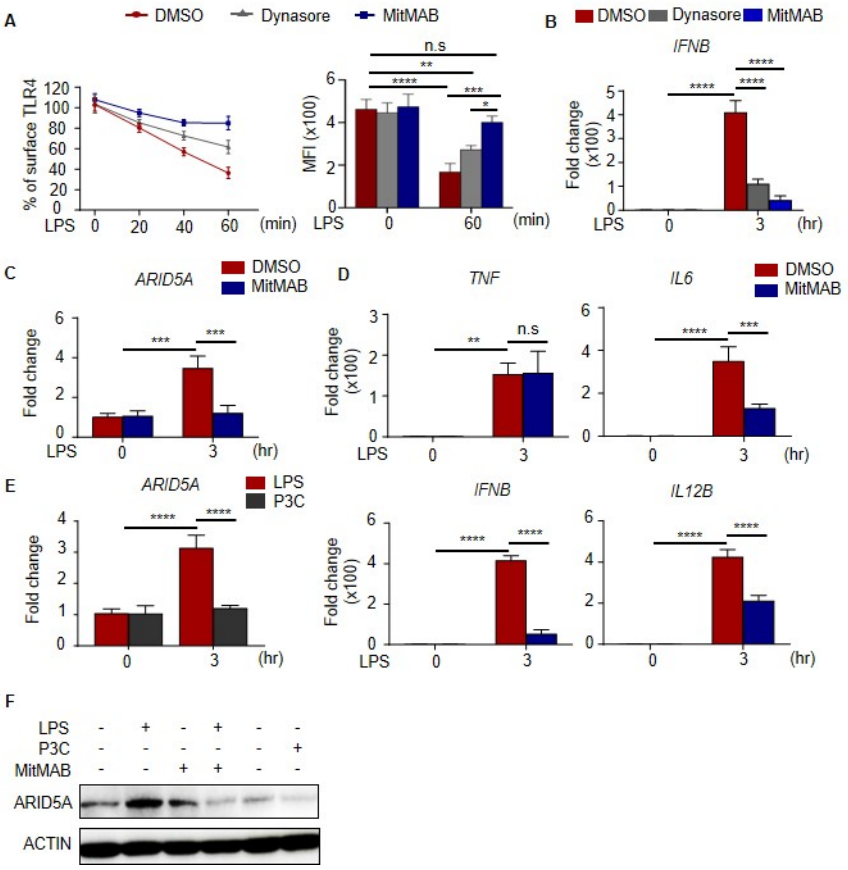

Fig. S1. LPS-induced TLR4 endocytosis promotes ARID5A, IL-6, and IL-12p40 production. (**A** and **B**) WT dTHP-1 cells were stimulated with LPS (100 ng/ml) for the indicated times in the presence or absence of 10 μM MitMAB or 80 μM Dynasore. (A) Left: Flow cytometry analysis of TLR4 endocytosis to measure the remaining percentage of its cell surface abundance as previously described (*18*). Right: Mean fluorescence intensities (MFIs) of the remaining cell surface TLR4 to determine the extent of its endocytosis. (B) Total RNA was isolated and *IFNB* transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**C** and **D**) WT dTHP-1 cells were stimulated with LPS (100 ng/ml) for the indicated times in the presence or absence of 10 μM MitMAB. Total RNA was isolated, and the indicated transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**E**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) or P3C (100 ng/ml) for the indicated times. Total RNA was isolated, and *ARID5A* transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**F**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) or P3C (100 ng/ml) in the presence or absence of 10 μM MitMAB. Whole-cell lysates were harvested 3 hours after stimulation and separated by SDS-PAGE. The indicated endogenous proteins were then detected by Western blotting. Blots are representative of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 as measured by one-way ANOVA with post-hoc Tukey's test (A to E). n.s, not significant.

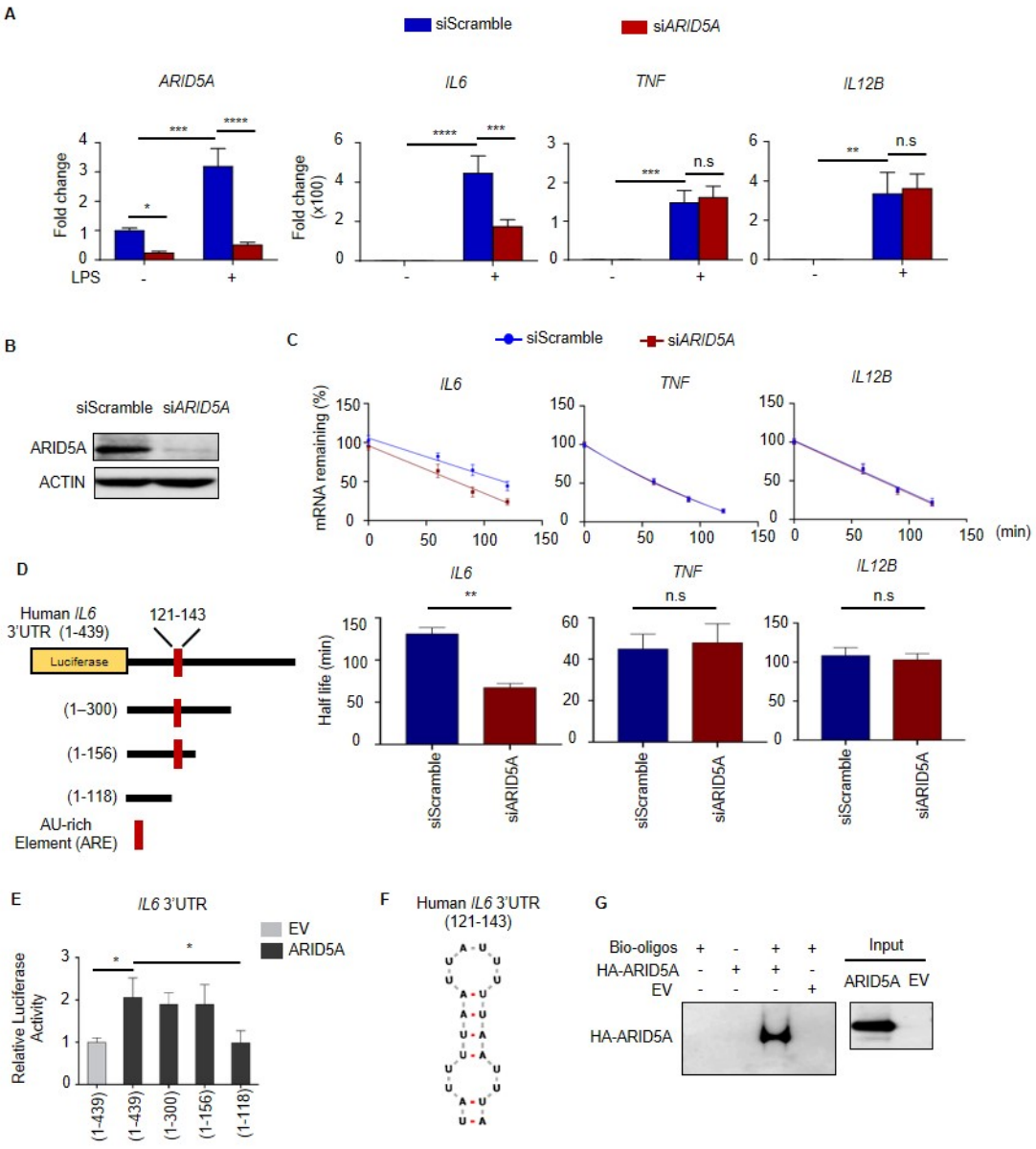

Fig. S2. ARID5A stabilizes *IL6* mRNA, but not *IL12B* or *TNF* mRNA, through binding to its 3′UTR. (**A**) MDMs were transfected with scrambled siRNA or siRNA targeting *ARID5A*. Forty-eight hours later, the cells were then left unstimulated or were stimulated with LPS (100 ng/ml), and total RNA was isolated 3 hours after stimulation. The indicated transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**B** and **C**) WT dTHP-1 cells were transfected with scrambled siRNA or siRNA targeting *ARID5A* and then cultured for 48 hours. (B) Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (C) Cells expressing the indicated siRNAs were stimulated with LPS (100 ng/ml) for 3 hours, which is shown as the zero time point. At that point, the amounts of the indicated transcripts were normalized to 100%. Cells were then treated with actinomycin D (2 μg/ ml), and total RNA was harvested at the indicated times. The indicated transcripts were quantified by qRT-PCR analysis. The graphs show the percentage of mRNA remaining compared to the 0 min time point (Top) and the mRNA decay rate

and half-life of cytokine mRNAs were determined by nonlinear regression curve fitting (one-phase decay) using GraphPad Prism (Bottom). The following parameters were used: Least squares (ordinary fit) Confidence level–95% Asymmetrical (likelihood) CI. Goodness of fit was quantified with R square Convergence criteria–medium. Data are representative of three independent experiments and are presented as means ± SD. (**D**) Scheme of the 3' UTR of human *IL6* mRNA and its deletion constructs. (**E**) Measurement of luciferase activity in 293T cells 48 hours after transfection with the luciferase reporter plasmids described in (D), together with control plasmid (EV) or expression plasmid for ARID5A. Results are presented relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. (**F**) Predicted stem-loop structure of ARID5A–responsive AU-rich Element (ARE) in the 3' UTR of human *IL6* mRNA. (**G**) Binding assay of ARID5A to biotinylated nucleotides containing the sequence shown in (F). Cleared lysates from 293T cells expressing control plasmid (EV) or expression plasmid for ARID5A were incubated with the indicated biotinylated nucleotides. Bound proteins were immunoprecipitated with streptavidin beads and analyzed by Western blotting. Input samples were prepared before incubation with biotinylated nucleotides. Blots are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ as measured by one-way ANOVA with post-hoc Tukey's test (A, C, and E). n.s, not significant.

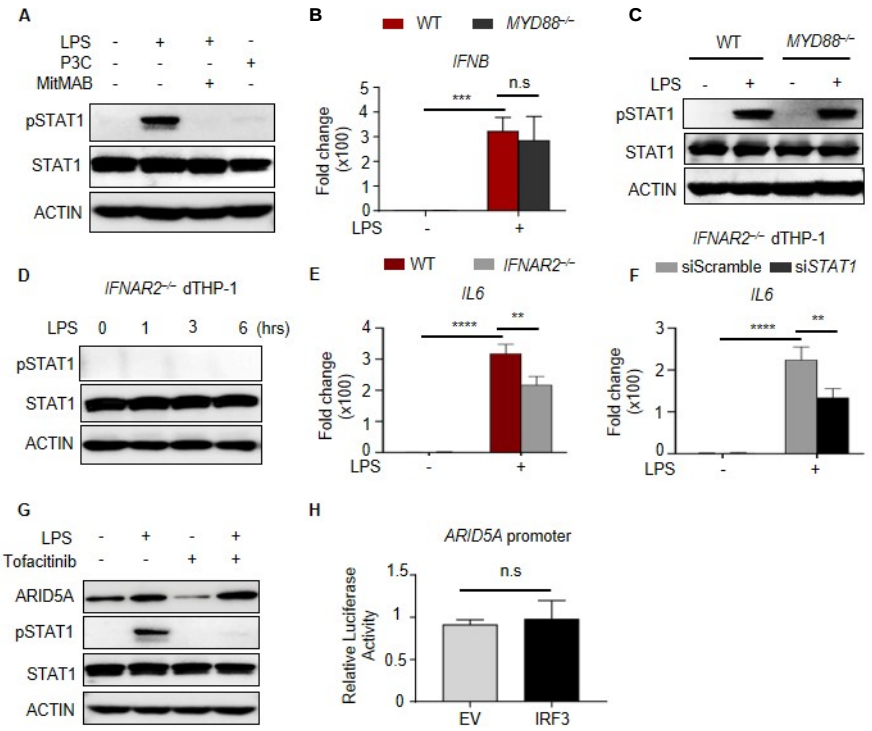

Fig. S3. TLR4 endocytosis–dependent IFN-β–JAK–STAT1-pTyr^{701} signaling is dispensable for *ARID5A* expression. (**A**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) in the presence or absence of 10 μM MitMAB or were stimulated with P3C (100 ng/ml) for 3 hours. Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (**B** and **C**) WT or *MYD88*$^{-/-}$ dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) for 3 hours. (B) Total RNA was isolated, and *ARID5A* transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (C) Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (**D**) *IFNAR2*$^{-/-}$ dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) for the indicated times. Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (**E**) WT or *IFNAR2*$^{-/-}$ dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) for 3 hours. Total RNA was isolated, and *IL6* transcripts were quantified by qRT-PCR analysis. Fold-change data are representative of three independent experiments and are presented as means ± SD. (**F**) *IFNAR2*$^{-/-}$ dTHP-1 cells were transfected with scrambled siRNA or siRNA targeting *STAT1*. Forty-eight hours later, the cells were then left unstimulated or were stimulated with LPS (100 ng/ml). Total RNA was isolated, and *IL6* transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**G**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) in the presence or absence of 1 μM Tofacitinib for 3 hours. Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (**H**) Measurement of luciferase activity in 293T cells 48 hours after transfection with a luciferase reporter plasmid containing the human *ARID5A* promoter, together with control plasmid (EV) or expression plasmid for IRF3. Results are presented relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as measured by one-way ANOVA with post-hoc Tukey's test (B, E, and F) or by paired Student's *t* test (H). n.s, not significant.

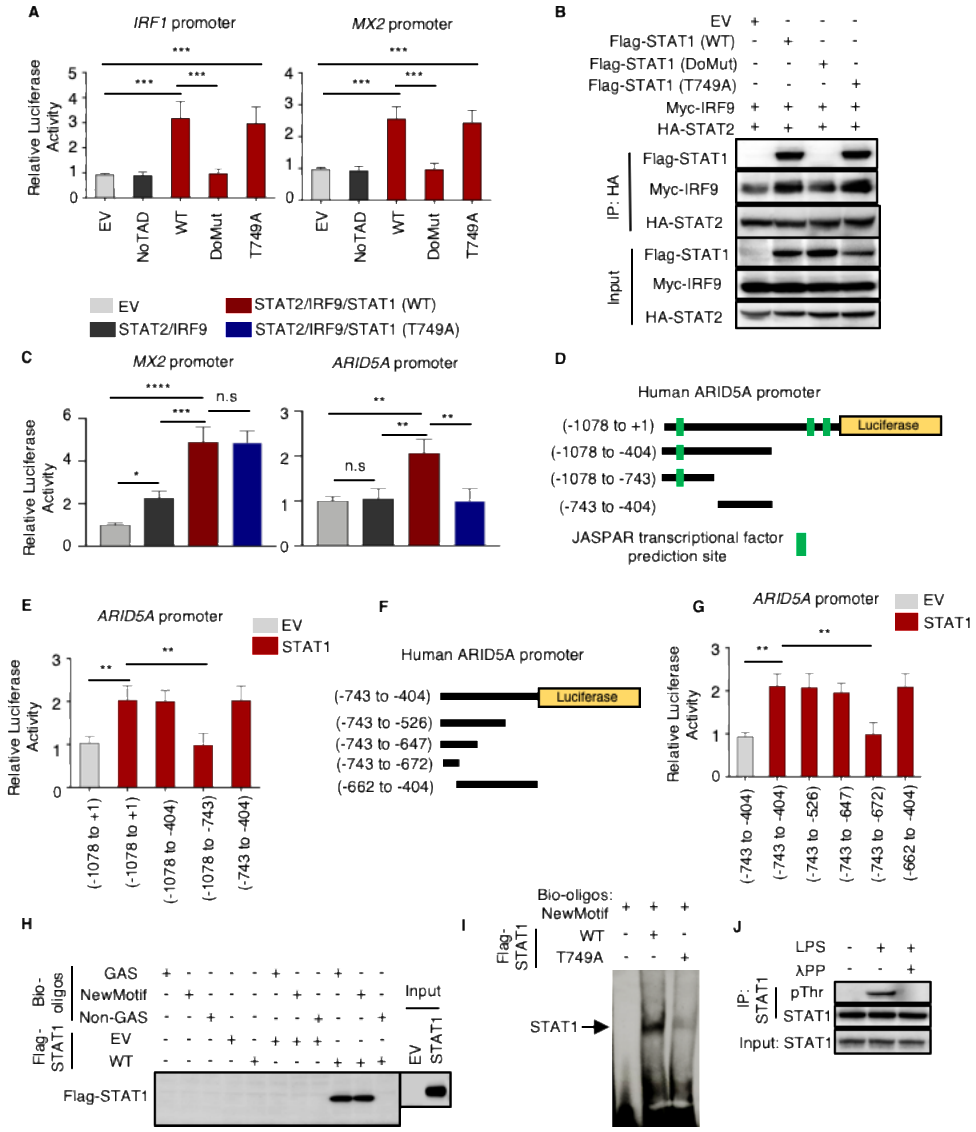

Fig. S4. Phosphorylation of STAT1 at Thr^{749} facilitates its binding to a noncanonical DNA motif in the *ARID5A* promoter region. (**A**) Measurement of luciferase activity in U3A cells 48 hours after transfection with luciferase reporter plasmids containing the human *IRF1* or *MX2* promoter, together with control plasmid (EV) or expression plasmid for STAT1-WT or one of the indicated mutants. Results are presented relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. (**B**) U3A cells were transiently co-transfected with the indicated expression plasmids. Twenty-four hours later, HA-tagged proteins were immunoprecipitated from cleared cell lysates. The immunoprecipitated samples were analyzed by Western blotting to detect the indicated proteins. Input samples were prepared before immunoprecipitation. Blots are representative of three independent experiments. (**C**) Measurement of luciferase activity in U3A cells 48 hours after transfection with luciferase reporter plasmids containing the human *MX2* or *ARID5A* promoter, together with control plasmid (EV) or the indicated expression plasmids. Results are presented relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. (**D**) Scheme of the full-length human *ARID5A* promoter and its deletion constructs. (**E**) Measurement of luciferase activity in U3A cells 48 hours after transfection with the luciferase reporter plasmids described in (D), together with control plasmid (EV) or expression plasmid for WT STAT1. Results are presented

relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. (**F**) Scheme of a region (-743 to -404) of the human *ARID5A* promoter and its deletion constructs. (**G**) Measurement of luciferase activity in U3A cells 48 hours after transfection with the luciferase reporter plasmids described in (F), together with control plasmid (EV) or expression plasmid for WT STAT1. Results are presented relative to *Renilla* luciferase activity. Data are representative of three independent experiments and are presented as means ± SD. (**H**) Binding assay of STAT1 to biotinylated nucleotides containing the noncanonical DNA motif (5'-TTTGAGGC-3') of the human *ARID5A* promoter. Cleared lysates from U3A cells transfected with control plasmid (EV) or a plasmid expressing STAT1-WT were incubated with the indicated biotinylated nucleotides. Bound proteins were immunoprecipitated with streptavidin beads and analyzed by Western blotting. Input samples were prepared before incubation with biotinylated nucleotides. Blots are representative of three independent experiments. (**I**) Electromobility shift assay to assess the binding of STAT1-WT or STAT1-T749A to biotinylated nucleotides containing the noncanonical DNA motif of the human *ARID5A* promoter. Cleared lysates from U3A cells transfected with plasmids expressing the indicated STAT1 proteins were incubated with biotinylated nucleotides containing the 5'-TTTGAGGC-'3 motif. Blots are representative of three independent experiments. (**J**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml) for 4 hours. Whole-cell lysates were harvested, cleared, and subjected to immunoprecipitation of STAT1. The immunoprecipitated samples were left untreated or were treated with λPP and analyzed by Western blotting for the indicated proteins. Input samples were prepared before immunoprecipitation. Blots are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ as measured by one-way ANOVA with post-hoc Tukey's test (A, C, E, and G). n.s, not significant.

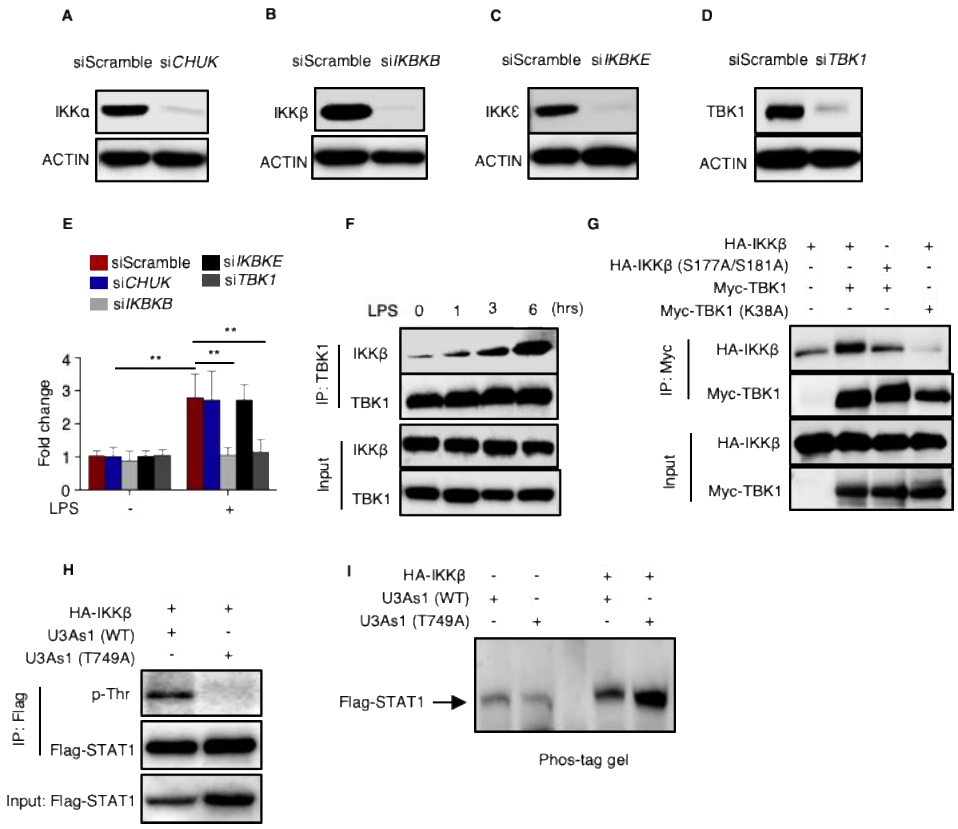

Fig. S5. A noncanonical TBK1-IKKβ heterodimer mediates the phosphorylation of STAT1 at Thr^{749} downstream of TLR4 endocytosis. (**A** to **E**) WT dTHP-1 cells were transfected with scrambled siRNA or siRNAs targeting *CHUK*, *IKBKB*, *IKBKE*, or *TBK1* and then were cultured for 48 hours. (A to D) Whole-cell lysates were harvested and separated by SDS-PAGE. The indicated endogenous proteins were detected by Western blotting. Blots are representative of three independent experiments. (E) The transfected cells were stimulated with LPS (100 ng/ml) for 3 hours. Total RNA was isolated, and *ARID5A* transcripts were quantified by qRT-PCR analysis. Data are representative of three independent experiments and are presented as means ± SD. (**F**) WT dTHP-1 cells were left unstimulated or were stimulated with LPS (100 ng/ml), and whole-cell lysates were harvested at the indicated times. TBK1 was immunoprecipitated from cleared lysates and the immunoprecipitated samples were analyzed by Western blotting to detect the indicated proteins. Input samples were prepared before immunoprecipitation. Blots are representative of three independent experiments. (**G**) U3A cells were transiently co-transfected with the indicated expression plasmids. Twenty-four hours later, Myc was immunoprecipitated from cleared cell lysates and the samples were analyzed by Western blotting for the indicated proteins. Input samples were prepared before immunoprecipitation. Blots are representative of three independent experiments. (**H)** U3As1 cells expressing STAT1-WT or STAT1-T749A were transiently transfected with expression plasmid for IKKβ. Twenty-four hours later, whole-cell lysates were harvested, STAT1 was immunoprecipitated, and the samples were analyzed by Western blotting to detect threonine-phosphorylated proteins. Input samples were prepared before immunoprecipitation. Blots are representative of three independent experiments. (**I**) U3As1 cells expressing STAT1-WT or STAT1-T749A were transiently transfected with an expression plasmid for IKKβ. Twenty-four hours later, whole-cell lysates were separated by Phos-tag acrylamide gel, and STAT1 migration mobility was determined. Blots are representative of three independent experiments. $^{**}P < 0.01$ as measured by one-way ANOVA with post-hoc Tukey's test (E).

Table S1. List of primer sequences for RT-qPCR analysis, plasmid construction, and ChIP-qPCR assays.

RT-qPCR analysis		
Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
ACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
ARID5A	GACACACGCCCATCGAGAG	CCCCAGCTCGTCGTACAC
IL6	ATGCAATAACCACCCCTGAC	AAAGCTGCGCAGAATGAGAT
IL12B	TGTCACCAGCAGTTGGTCATC	CATCCGGATACCAATCCAATTC
IFNB	CATTACCTGAAGGCCAAGGA	CAATTGTCCAGTCCCAGAGG
CCL5	GCCTCCCCATATTCCTCGGAC	TCGGGTGACAAAGACGACTGC
IL1B	AAGTGCTCCTTCCAGGACCTG	GTCCATGGCCACAACAACTG
TNF	GCAAGGACAGCAGAGGACCA	TGTGGCGTCTGAGGGTTGTT
IRF1	GATGCCTGTTTGTTCCGGAG	TCACCTCCTCGATATCTGGCAG
IFIT2	GAGTCCAGAGCTTGACTGTGAGG	GAGGTGAATTCTGGGTTCTTTGG
RSAD2	TTGGTGAGGTTCTGCAAAGTAGAG	TCACAGGAGATAGCGAGAATGTCC
Plasmid construction		
Target	**Forward (5' to 3')**	**Reverse (5' to 3')**
ARID5A	ATGGCAGCCCCTGTCAAAGGGAACAGGAAGC	CTACAGCTTGGTGTTGAGGTGGAAGAAGTG
IRF3	ATGGGAACCCCAAAGCCACG	TTATTGGTTGAGGTGGTGGGGAAC
STAT1	ATGTCTCAGTGGTACGAACTTC	CTATACTGTGTTCATCATACTGTC
IKKB	ATGAGCTGGTCACCTTCCCTGAC	TCATGAGGCCTGCTCCAGGCAGC
TBK1	ATGCAGAGCACTTCTAATCATCTG	CTAAAGACAGTCAACGTTGCGAAG
STAT2	ATGGCGCAGTGGGAAATGC	TTAGAAGTCAGAAGGCATCAAGGGTC
IRF9	ATGGCATCAGGCAGGGCAC	TTACACCAGGGACAGAATGGCTG

IL6 3’ UTR	ATGGGCACCTCAGATTGTTG	TTGCTGAATTTTTTAAAATGCCAT
ARID5A promoter	TGTGTAGCTTCTATGTGCTAGGAAC	CCATGGCCCGGAGAGGTC
IRF1 promoter	AGGCAAGAGTGCTAGGAGGGATC	CTCTGCTGCAGGAGCGATTC
MX2 promoter	ATGACTTAGCCAAGAGGAGCTCAG	ATCATCTCTTCCCTTTCCTCCAC
IL12B promoter	TGAGACGGCGAGGAAAGTTAG	CTTGCTCTGGGCAGGACGGAGAGTC
ChIP-qPCR assays		
Promoter	**Forward (5’ to 3’)**	**Reverse (5’ to 3’)**
IRF1	AGCAGCCGCCCTGTACTTC	TTAGCGGGATTCCCCAGC
ARID5A	TCCTTGTCTAGCTCACACTAACCAC	AAGGTCGAGGCTGCAGTGTG
IL12B	TATGCCTCCCTGAGGGTATTTCAC	TACAGACACAGACCTGGGGAGATG

Table S2. List of antibodies used for Western blotting and immunoprecipitations.

Antibody	Company	Catalog#
Mouse monoclonal IgG anti-ARID5A (clone P18112)	Invitrogen	#MA5-18292
Rabbit monoclonal IgG anti-β-Actin (clone D6A8) (HRP Conjugate)	Cell Signaling Technology	#12620
Rabbit monoclonal IgG anti-MYD88 (clone D80F5)	Cell Signaling Technology	#4283
Rabbit polyclonal IgG anti-TRIF	Cell Signaling Technology	#4596
Rabbit monoclonal IgG anti-phospho-Stat1 (Tyr701) (clone 58D6)	Cell Signaling Technology	#9167
Rabbit polyclonal IgG anti-STAT1	Cell Signaling Technology	#9172
Rabbit monoclonal IgG anti-IRF-3 (clone D83B9)	Cell Signaling Technology	#4302
Rabbit monoclonal IgG anti-Stat2 (clone D9J7L)	Cell Signaling Technology	#72604
Rabbit monoclonal IgG anti-Stat3 (79D7)	Cell Signaling Technology	#4904
Rabbit polyclonal IgG anti-NF-κB (p65 subunit)	Santa Cruz	#sc-109
Rabbit polyclonal IgG anti-IkBα	Santa Cruz	#sc-847
Rabbit monoclonal IgG anti-phospho-NF-κB p65 (Ser536) (clone 93H1)	Cell Signaling Technology	#3033
Rabbit monoclonal IgG anti-phospho-IRF-3 (Ser396) (clone 4D4G)	Cell Signaling Technology	#4947
Rabbit polyclonal IgG anti-Lamin A/C	Cell Signaling Technology	#2032
Rabbit monoclonal IgG anti-β-Tubulin (clone D2N5G) (HRP Conjugate)	Cell Signaling Technology	#56739
Mouse monoclonal IgG anti-phospho-Tyrosine (P-Tyr-102)	Cell Signaling Technology	#9416
Rabbit monoclonal IgG anti-phospho-Threonine Antibody (P-Thr-Polyclonal)	Cell Signaling Technology	#9381
Mouse monoclonal IgG anti-pThr (clone H-2)	Santa Cruz	#sc-5267
Rabbit polyclonal IgG anti-IKKα	Cell Signaling Technology	#2682
Rabbit monoclonal IgG anti-IKKβ (clone D30C6)	Cell Signaling Technology	#8943
Rabbit monoclonal IgG anti-IKKε (clone D20G4)	Cell Signaling Technology	#2905
Rabbit monoclonal IgG anti-TBK1/NAK (clone D1B4)	Cell Signaling Technology	#3504
Mouse monoclonal IgG anti-Flag (clone M2)	Sigma-Aldrich	#F3165
Mouse monoclonal IgG anti-HA (clone TANA2)	MBL	#M180-3
Rabbit polyclonal IgG anti-Myc	MBL	#562