The E3 ubiquitin ligase MARCH8 negatively regulates IL-1β-induced NF-κB activation by targeting the IL1RAP coreceptor for ubiquitination and degradation

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The proinflammatory cytokine interleukin-1 (IL-1) signals via type I IL-1 receptor (IL-1RI) and IL-1 receptor accessory protein (IL1RAP), which leads to activation of the transcription factor NF-κB and induction of a range of downstream proteins involved in inflammatory and immune responses. Here, we identified the E3 ubiquitin ligase membrane-associated RING-CH (MARCH8) as a suppressor of IL-1β-induced NF-κB- and MAPK-activation pathways. Overexpression of MARCH8 inhibits IL-1β–induced NF-κB and MAPK activation, whereas knockdown of MARCH8 has the opposite effect. Mechanistically, MARCH8 interacts with IL1RAP and targets its Lys512 for K48-linked polyubiquitination and degradation. Our findings suggest that MARCH8-mediated polyubiquitination and degradation of IL1RAP is an important mechanism for negative regulation of IL-1β–induced signaling pathways.

The transcription factor NF-κB plays pivotal roles in many aspects of cellular processes such as inflammation, innate immunity, and cancer. NF-κB is sequestered in the cytoplasm and kept inactive in nonstimulated cells by binding to inhibitor IκB (inhibitor of κB) proteins. Under stimulation with cytokines, infectious pathogens, or genotoxic stresses, the IκB proteins are phosphorylated, ubiquitinated, and ultimately degraded, which frees NF-κB for translocation into the nucleus, where it induces transcription of downstream target genes (1–3).

Interleukin 1 (IL-1) is a critical proinflammatory cytokine that can trigger a cascade of signaling leading to activation of NF-κB. It functions through engagement of two membrane-bound receptors: IL-1 receptor type I (IL-1RI) and IL-1 receptor accessory protein (IL1RAP) (4–6). IL-1RI is the ligand-recognition receptor that binds IL-1β directly (7). Although IL1RAP does not bind IL-1β directly, its recruitment to IL-1RI following IL-1β stimulation is essential for the formation of an activated membrane receptor complex (8–10). The activated complex can then recruit intracellular adaptor proteins and kinases, including MyD88, IRAK4, and IRAK1 (11–14). IRAK4 phosphorylates and activates IRAK1, which in turn recruits TRAF6. IRAK6 and TRAF6 form a complex that is released from the receptor complex (15, 16). TRAF6 possesses an E3 ubiquitin ligase activity that mediates its autoubiquitination. Ubiquitinated TRAF6 further recruits the TGF-β-activated kinase 1 (TAK1)-TAK1-binding protein 2 (TAB2)-TAB3 complex, resulting in the activation of TAK1. Activated TAK1 subsequently activates downstream kinases IKK-α and IKK-β, which phosphorylate IκB proteins, and lead to activation of NF-κB (17).

Several studies have suggested that ubiquitination is a central rhythm of regulation of IL-1β–induced NF-κB activation pathways. It has been shown that the E3 ubiquitin-ligase TRAF6 mediates K63-linked polyubiquitination of IRAK1 for recruiting IKK and activating NF-κB (18). Tripartite motif 8 (TRIM8) catalyzes K63-linked polyubiquitination of TAK1, and this promotes the activation of TAK1 (19). It has also been demonstrated that the E3 ligase Pellino 3b acts as a negative regulator for IL-1 signaling by regulating IRAK degradation (20). RBCK1 ubiquitinates and down-regulates TAB2/3 to negatively regulate IL-1β–induced NF-κB activation (21).

The membrane-associated RING-CH (MARCH) proteins belong to a recently discovered family of RING-finger ubiquitin ligases. Ten mammalian MARCH gene products have been recognized, and the majority shares a similar structure, including an N-terminal C4HC3-type RING finger (RING-CH finger) and two or more C-terminal transmembrane spans (22, 23). Several members of this novel E3 ubiquitin ligase family have been shown to ubiquitinate and down-regulate transmembrane proteins, such as major histocompatibility complex class I and II, B7-2, CD166, and ICAM-1 (24–28).

In this study, we identified MARCH8 (also called c-MIR and RNF178) as a specific inhibitor of IL-1β–induced NF-κB-activation pathways. Overexpression of MARCH8 caused down-regulation of IL1RAP and inhibited IL-1β–induced NF-κB activation; whereas, knockdown of MARCH8 potentiated IL-1β–induced NF-κB activation. MARCH8 interacts with and targeted IL1RAP at K512 for K48-linked polyubiquitination and degradation. Our findings reveal a negative regulatory mechanism on the control of the early events of IL-1β–induced NF-κB-activation pathways.

Results
Identification of MARCH8 as a Negative Regulator of IL-1β–Induced Signaling Pathways. The proinflammatory cytokine IL-1β initiates a cascade of signaling events to activate the transcription factor NF-κB (4, 5). To identify the regulators involved in IL-1β–induced NF-κB-activation signaling pathways, we screened ~10,000 independent human cDNA clones by NF-κB reporter assays in 293 cells. These efforts led to identification of MARCH8, a member of the MARCH family that was recently discovered to possess E3 ubiquitin ligase activity (22, 23). In the screening experiments, overexpression of MARCH8 markedly inhibited IL-1β–induced NF-κB activation (Fig. 1D). Further validation experiments indicated that MARCH8 inhibited IL-1β–induced NF-κB activation in a dose-dependent manner in 293 cells (Fig. 1B). In similar experiments, MARCH8 had no marked inhibitory effect on TNF-α- and Sendai virus (SeV)–induced NF-κB activation (Fig. 1C).

Previously, it had been shown that the tryptophan residue at position 114 of MARCH8 is conserved among the MARCH family members and this residue is required for recruitment of E2s for conjugation of ubiquitin moiety to target proteins (29, 30). To further examine the role of MARCH8 in IL-1β–induced signaling pathways, we examined the effect of MARCH8(W114A) mutant on IL-1β–induced NF-κB activation. As shown in Fig. 1D, MARCH8(W114A) had marked decreased ability to inhibit IL-1β–induced NF-κB activation compared to its wild-type counterpart, suggesting that the recruitment of E2s is required for MARCH8 to suppress IL-1β–induced NF-κB activation.

In quantitative real-time PCR experiments, wild-type MARCH8 inhibited IL-1β–induced transcription of endogenous TNF-α and IκB-α genes; whereas, MARCH8(W114A) had only minimal or no effects (Fig. 1E). These results together suggest that MARCH8 negatively regulates IL-1β–induced NF-κB activation and transcription of downstream genes.

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Knockdown of MARCH8 Potentiates IL-1β–Induced NF-κB Activation.

Because overexpression of MARCH8 inhibits IL-1β–induced NF-κB activation, we next examined the role of endogenous MARCH8 in IL-1β–induced signaling. We made two MARCH8 RNAi constructs that target different sites of MARCH8 mRNA. Transient transfection experiments indicated that both of the RNAi constructs could efficiently down-regulate expression of MARCH8 protein and mRNA levels (Fig. 2A and B). In reporter assays, knockdown of MARCH8 markedly potentiated IL-1β–induced NF-κB activation in 293 cells (Fig. 2C). Similar results were also found in HeLa cells (Fig. S1). In similar experiments, knockdown of MARCH8 had no marked effects on TNF-α– or SeV–induced NF-κB activation (Fig. 2D). Quantitative real-time PCR experiments demonstrated that knockdown of MARCH8 potentiated IL-1β–induced transcription of endogenous TNF-α and IκB-α genes (Fig. 2E). These results suggest that MARCH8 is a physiological suppressor of IL-β–induced NF-κB–activation pathways.

**MARCH8 Negatively Regulates IL-1β–Induced NF-κB Activation by Destabilizing IL1RAP.** The MARCH family is a family of membrane-bound E3 ubiquitin ligases, and several members of this family have been reported to regulate the turnover of cargo...
proteins. To elucidate the mechanism by which MARCH8 functions to negatively regulate the IL-1β-induced NF-κB-activation pathways, we examined whether MARCH8 could regulate the turnover of some key components in IL-1β-induced NF-κB-activation pathways. In transient transfection and immunoblot experiments, we found that the protein level of IL1RAP but not IL-1RI, MyD88, IRAK1, or TRAF6 was dramatically down-regulated when coexpressed with MARCH8 (Fig. 3A). In addition, compared with wild-type MARCH8, MARCH8(W114A) failed to cause obvious down-regulation of IL1RAP (Fig. 3B). This result is consistent with previous finding that MARCH8 (W114A) had only minimal inhibitory effect on IL-1β-induced NF-κB activation. We next examined the effect of knockdown of MARCH8 on expression of endogenous IL1RAP. The results showed that knockdown of MARCH8 up-regulated the level of endogenous IL1RAP (Fig. 3C).

IL1RAP is an essential component of the IL-1 coreceptor complex required for IL-1β-induced signaling (8–10). Coexpression of IL1RAP and IL-1RI can mimic IL-1β stimulation for activation of NF-κB (Fig. S2). As MARCH8 could down-regulate the expression of IL1RAP, we examined the effect of MARCH8 on NF-κB activation mediated by coexpression of IL1RAP and IL-1RI. In reporter experiments, overexpression of MARCH8 inhibited NF-κB activation mediated by coexpression of IL1RAP and IL-1RI in a dose-dependent manner (Fig. 3D and E). In parallel experiments, overexpression of MARCH8 had no obvious effect on NF-κB activation induced by overexpression of downstream components IRAK1 or TRAF6 (Fig. 3D). These results suggest that MARCH8 inhibits IL-1β-induced NF-κB activation by mediating down-regulation of IL1RAP.

**Fig. 3.** MARCH8 down-regulates IL1RAP to inhibit IL-1β-induced NF-κB activation. (A) MARCH8 specifically destabilizes IL1RAP. The 293 cells (4 × 10⁵) were transfected with the indicated plasmids for 24 h and then immunoblots were performed with the indicated antibodies. (B) Effect of MARCH8(W114A) on expression of IL1RAP. The experiments were performed as in A. (C) Knockdown of MARCH8 up-regulates levels of endogenous IL1RAP. The 293 cells (1 × 10⁵) were transfected with control or MARCH-RNAi (no. 1) plasmid (2 μg) for 36 h. The cells were then left untreated or treated with IL-1β (10 ng/mL) for 30 min before immunoblots were performed with the indicated antibodies. (D) MARCH8 inhibits IL-1R1/IL1RAP- but not IRAK1-, TRAF6-, and TNFR1-mediated NF-κB activation. The 293 cells (1 × 10⁵) were transfected with indicated plasmids for 20 h before reporter assays were performed. The expression levels of input proteins were detected by immunoblot analysis. (E) MARCH8 inhibits IL1-R1/IL1RAP-mediated NF-κB activation in a dose-dependent manner. The experiments were performed as in D.

**Fig. 4.** MARCH8 interacts with and ubiquitinates IL1RAP. (A) MARCH8 interacts with IL1RAP in mammalian overexpression system. The 293 cells (2 × 10⁶) were transfected with the indicated plasmids (5 μg each) for 24 h before coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (B) Endogenous interaction between MARCH8 and IL1RAP. HeLa cells (5 × 10⁵) were left untreated or treated with IL-1β for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (C) MARCH8 colocalizes with IL1RAP. HeLa cells (1 × 10⁶) were transfected with CFP-tagged MARCH8 and GFP-tagged IL1RAP. Twenty hours after transfection, cells were fixed with 4% paraformaldehyde and then subjected for confocal microscopy. (D) Overexpression of wild-type MARCH8 but not MARCH8(W114A) promotes ubiquitination of IL1RAP. The 293 cells (2 × 10⁶) were transfected with the indicated expression plasmids. Twenty-four hours after transfection, immunoprecipitation, reimmunoprecipitation, and immunoblot analysis were performed with the indicated antibodies (Top). The expression levels of the transfected proteins were examined by immunoblots with anti-CBP (Bottom). (E) Overexpression of MARCH8 enhances K48-linked polyubiquitination of IL1RAP. The experiments were similarly performed as in D. (F) Wild-type MARCH8 but not MARCH8(W114A) mediates polyubiquitination of IL1RAP in vitro. IL1RAP, MARCH8, and MARCH8(W114A) were translated in vitro, and then biotin-ubiquitin, E1, and the indicated E2s were added for in vitro ubiquitination assays. The ubiquitin–conjugated proteins were detected by immunoblot with HRP-streptavidin (Left and Right). Before ubiquitination analysis, the levels of the translated proteins were detected by immunoblots with the indicated antibodies (Center).

**MARCH8 Interacts with IL1RAP.** Because MARCH8 could cause down-regulation of IL1RAP, we examined whether MARCH8 physically interacts with IL1RAP in mammalian cells. In transient transfection and coimmunoprecipitation experiments, MARCH8 interacted with IL1RAP as well as IL-1RI in 293 cells (Fig. 4A).
Endogenous communoprecipitation experiments showed that MARCH8 constitutively interacted with IL1RAP either in absence or presence of IL-1β treatment (Fig. 4B). In addition to communoprecipitation experiments, we also showed by confocal microscopy experiments that MARCH8 was colocalized with IL1RAP at the plasma membrane and in the cytoplasm in HeLa (Fig. 4C) or 293 (Fig. S3).

IL1RAP is a transmembrane protein that contains an N-terminal extracellular domain, a transmembrane domain and a C-terminal cytosolic Toll/Interleukin-1 receptor (TIR) domain. We constructed a series of IL1RAP deletion mutants and performed domain mapping experiments. The results indicated that the transmembrane domain of IL1RAP was required for its interaction with MARCH8 (Fig. S4A). In similar experiments, we found that the first transmembrane domain and the region between two the two transmembrane domains of MARCH8 were important for its interaction with IL1RAP (Fig. S4B).

**MARCH8 Mediates K48-Linked Polyubiquitination of IL1RAP.** Because MARCH8 is an E3 ubiquitin ligase and its recruitment for E2 is critical for inhibition of IL-1β-induced NF-κB activation, we predicted that MARCH8 could mediate ubiquitination of IL1RAP. To test this, expression plasmids for IL1RAP-GFP and HA-ubiquitin were transfected together with wild-type MARCH8, MARCH8(W114A), or empty control plasmid. Coimmunoprecipitation experiments showed that overexpression of wild-type MARCH8 but not MARCH8(W114A) caused polyubiquitination and downregulation of IL1RAP (Fig. 4D). Using linkage-specific ubiquitin, we showed that overexpression of MARCH8 promoted K48-linked but not K63-linked polyubiquitination of IL1RAP (Fig. 4E). Furthermore, in vitro ubiquitination assays with UBC5C as an E2 also suggested that MARCH8 directly mediates K48-linked ubiquitination of IL1RAP to facilitate its degradation.

**MARCH8 Targets K512 of IL1RAP for Polyubiquitination.** To identify potential ubiquitination residues of IL1RAP, we first mapped the region targeted by MARCH8. We found that aa365-570 but not other truncations of IL1RAP were down-regulated and ubiquitinated by MARCH8 (Fig. 5A and B). These results suggest that MARCH8 targets the C-terminal region of IL1RAP for ubiquitination and degradation. We then mutated all of the 18 lysine residues identified in the C-terminal region to arginine individually and examined whether they can still be ubiquitinated by MARCH8. Cotransfection and immunoblot experiments showed that the level of IL1RAP(K512R) was not affected by MARCH8, whereas the levels of wild-type and the other 17 mutant IL1RAP were down-regulated by MARCH8 (Figs. 5C and Fig. S5). Consistently, in ubiquitination experiments, MARCH8 enhanced the polyubiquitination level of wild-type IL1RAP but not IL1RAP(K512R) (Fig. 5D). These results suggest that MARCH8 targets wild-type IL1RAP and IL1RAP(K512R) were reconstituted into IL1RAP knockdown cells, IL1RAP(K512R) showed an increased ability to mediate IL-1β-induced activation of NF-κB than wild-type IL1RAP (Fig. S6). Taken together, these data suggest that MARCH8 targets K512 of IL1RAP for ubiquitination and degradation.

**MARCH8 Negatively Regulates IL-1β-Induced MAPK Pathways.** In addition to NF-κB activation, IL-1β stimulation also leads to activation of MAPK pathways including Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. We performed experiments to determine whether MARCH8 also regulates these pathways. We found that overexpression of MARCH8 inhibited IL-1β-induced phosphorylation of JNK, p38, and ERK (Fig. 6A), whereas knockdown of MARCH8 enhanced IL-1β-induced phosphorylation of these kinases (Fig. 6B). Consistent with our earlier observations, overexpression of MARCH8 also inhibited IL-1β-induced phosphorylation and degradation of IκBα, whereas, knockdown of MARCH8 had opposite effects (Fig. 6). To further confirm the regulation of MARCH8 on IL-1β-induced MAPK signaling, we examined the effects of MARCH8 on expression of MAPK-responsive genes. The results showed that overexpression of MARCH8 inhibited transcription of IL8, EGR1, and MKP-1 genes (Fig. S7A), and knockdown of MARCH8 enhanced their transcription (Fig. S7B).
MARCH8 Attenuates the Assembly of IL-1R-Proximal Signaling Complex.

Because MARCH8 targets at IL1RAP level to inhibit IL-1β-induced signaling, we next examined the effects of MARCH8 on assembly of IL-1β-initiated IL-1R-proximal signaling complex. We transfected the 293 cells with Flag-tagged IL-1RI, Myc-tagged MyD88, and HA-tagged IRAK1 combined with empty vector or HA-tagged MARCH8, and performed communoprecipitation experiments. The results showed that overexpression of MARCH8 attenuated IL-1β-induced interaction between IL-1RI and MyD88 or IRAK1 (Fig. S8). This data suggest that MARCH8 inhibits the assembly of IL-1R-proximal signaling complex.

Discussion

The transcription factor NF-κB plays pivotal roles in a broad range of physiological and pathological processes, such as development, immune response, inflammation, and cancer (31). IL-1β is a potent proinflammatory cytokine that can strongly elicit activation of NF-κB (4, 5). However, this NF-κB-activation process needs to be tightly regulated to keep inflammatory homeostasis. In a screening for regulators of IL-1β-activated NF-κB, we identified MARCH8, a member of the MARCH family proteins.

Overexpression of MARCH8 inhibited IL-1β-induced NF-κB activation, whereas knockdown of MARCH8 potentiated IL-1β-induced activation of NF-κB. In similar experiments, neither overexpression nor knockdown of MARCH8 had marked effects on TNF-α- or SeV-induced activation of NF-κB. These results suggest that MARCH8 is a specific inhibitor of IL-1β-induced NF-κB-activation pathways.

Overexpression of MARCH8 caused substantial down-regulation of IL1RAP and inhibition of IL1RAP-mediated NF-κB activation. However, MARCH8 had no obvious effects on IRAK1-, TRAF6-, or TNFR1-triggered NF-κB activation. Further communoprecipitation and confocal microscopy experiments showed that MARCH8 can physically interact and colocalize with IL1RAP in the absence and presence of IL-1β, which suggests that MARCH8 constitutively targets IL1RAP for degradation to inhibit IL-1β-induced NF-κB activation.

Ubiquitination plays important roles in different steps of NF-κB activation. It has been shown that MARCH8 is a RING finger E3 ubiquitin ligase, and the W114 is a conserved site among MARCH family and many other RING finger E3 ubiquitin ligases that directly involved in E2 recruitment (30, 32). Correspondingly, overexpression of wild-type MARCH8 but not ligase-deficient mutant MARCH8(W114A) caused the ubiquitination and degradation of IL1RAP. Using link-specific ubiquitin, we identified that MARCH8 catalyzed K48-linked but not K63-linked polyubiquitination of IL1RAP. In the in vitro ubiquitination assays, we found that Ubiquitin-conjugating enzyme 5b/5c (UBCH5b/5c) were specific E2 enzymes for IL1RAP ubiquitination. Further site-directed mutagenesis experiments indicated that MARCH8 ubiquitinated IL1RAP at K512. Although it has been known that ubiquitination is involved in regulation of IL-1β-induced signaling pathways, however, as a key step of IL-1β-initiated signaling, whether the IL-1 receptor complex could also be regulated by ubiquitination is unknown. Our study provides evidence that IL-1β-induced NF-κB-activation pathways are regulated by ubiquitination at the IL1RAP coreceptor level.

Our results indicated that overexpression of MARCH8 inhibited IL-1β-induced activation of ERK, JNK, and p38; whereas, knockdown of MARCH8 had opposite effect. These results suggest that MARCH8 also regulates IL-1β-induced MAPK pathways. This further supports our conclusion that MARCH8 targets IL1RAP, a coreceptor that mediates both IL-1β-induced NF-κB and MAPK pathways.

In confocal microscopy experiments, we found that in addition to plasma membrane, overexpressed MARCH8 and IL1RAP were also colocalized in the cytoplasm. Previously, it has been demonstrated that IL-1β-induced internalization of IL-1RI and IL1RAP requires ubiquitination of these proteins (33). In light of this published study, it would be interesting to elucidate whether MARCH8-mediated polyubiquitination is required for internalization of the IL-1 receptor complex.

In our experiments, we have also routinely noticed that MARCH8 causes faster migration of IL1RAP. The simplest explanation for this observation is that MARCH8, either directly or indirectly, causes decreased posttranslational modifications of IL1RAP. More experiments are needed in the future to elucidate the mechanisms behind this observation.

IL-1β-induced NF-κB and MAPK activation contributes to inflammatory response. However, this process must be tightly regulated by MARCH8-mediated polyubiquitination, which provides a potential therapeutic target for immune disorders.
Materials and Methods

Reagents and Constructs. The reagents and constructs used in this study are described in SI Materials and Methods.

Commmunoprecipitation and Immunoblot Analysis. Transfected 293 cells from each 10-cm dish were lysed in 1 ml Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.4–7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, 0.8 mL cell lysate was incubated with 0.5 μg of the indicated antibody and 25 μL of 50% (vol/vol) slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) at 4 °C for 2 h. The Sepharose beads were washed three times with 1 mL lysis buffer containing 500 mM NaCl. The precipitates were fractionated by SDS/PAGE and subsequent immunoblot analysis was performed with the indicated antibody.

Reimmunoprecipitation Experiments. Reimmunoprecipitation experiments were performed as described (38, 39). Briefly, after first-round immunoprecipitation, the immunoprecipitates were reextracted in lysis buffer containing SDS and denatured by heating for 5 min. The sample was diluted with regular lysis buffer till the concentration of SDS was decreased to 0.1%. The diluted sample was reimmunoprecipitated with the indicated antibody, and the immunoprecipitates were analyzed by immunoblot with anti-HA.

Transfection, Reporter Assays, RNAi Experiments, and Real-Time PCR. These procedures are described in SI Materials and Methods.

Fluorescent Confocal Microscopy. The 293 or HeLa cells were transfected with the indicated plasmids with Fugene HD (Roche). At 24 h after transfection, the cells were fixed with 4% paraformaldehyde for 15 min at 4 °C. The cells were then observed with a Leica confocal microscope under a ×100 oil objective.

In Vitro Ubiquitination Assays. The tested proteins were expressed with a TNT Quick Coupled Transcription/Translation Systems kit (Promega) following instructions of the manufacturer. Ubiquitination was analyzed with an ubiquitination kit (Enzo Life Sciences) following protocols recommended by the manufacture.

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Supporting Information

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SI Materials and Methods

Reagents and Antibodies. Recombinant human TNF-α and IL-1β (R&D Systems); mouse monoclonal antibodies against Flag, HA, and β-actin (Sigma); mouse polyclonal antibody against GFP (Santa Cruz); rabbit polyclonal antibodies against calmodulin-binding peptide (CBP) (Upstate), and MARCH8 (Proteintech) were purchased from the indicated manufacturers. Mouse anti-MARCH8, mouse anti-IL1RAP, and mouse anti-IκB-α antibodies were raised against recombinant human MARCH8, IL1RAP(389-570), and IκB-α respectively.

 Constructs. Mammalian expression plasmids for Flag- or HA-tagged IL-1RI, IRAK1, IRAK4, MyD88, TRAF6, TNFRI, β-actin, IL1RAP, and its mutants, MARCH8 and its mutants, CBP-tagged IL1RAP, CFP-tagged MARCH8, and GFP-tagged IL1RAP, and Myc or HA-tagged ubiquitin were constructed by standard molecular biology techniques. Mammalian expression plasmids for K48- and K63-only ubiquitin mutants (all lysine residues except for Lys-48 or Lys-63 are mutated), Flag-tagged IL1RAP point mutants, RNAi off-target IL1RAP, and IL1RAP(K512R) (two bases in the RNAi target region of IL1RAP were mutated without changing the amino acid sequence to avoid being targeted by IL1RAP RNAi vectors) were made by a site-directed mutagenesis method. NF-κB lucerase reporter plasmid was provided by Gary Johnson, University of North Carolina, Chapel Hill, NC.

Transfection and Reporter Assays. The 293 cells were seeded on 24-well plates and transfected the next day by standard calcium phosphate precipitation method. HeLa cells were transfected by lipofectamine 2000 (Invitrogen). Where necessary, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, pRL-TK (Renilla luciferase) reporter plasmid (0.01 μg) was added to each transfection. Reporter assays were performed by using a dual-specific luciferase assay kit (Promega). Firefly lucerase activities were normalized based on Renilla luciferase activities. All reporter assays were repeated for at least three times.

RNAi Experiments. Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine). The following sequences were targeted for human MARCH8 and IL1RAP mRNA, respectively.

MARCH8-1: 5’- GTGTAAAGTGATGTGCAAA -3’; MARCH8-2: 5’- GAAACAAGCAAAAAGAATA -3’; andIL1RAP: 5’- GATGAAACAAGAACTCAGA -3’.

Real-Time PCR. Total RNA from transfected 293 cells was isolated using TRIZol reagent (Invitrogen). The RNA was reverse-transcribed with oligo(dT) primer using a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas). An aliquot of the product was subjected to real-time PCR analysis to measure mRNA expression levels of the tested genes. Gene-specific primer sequences were as follows:

MARCH8- CTCTCGCACTTTCTATACGCCA (forward) and AAGTGGAGGCTTCCTGTGCAGT (reverse); IκB-α- CGGGCTGAAGAAGGAGCGGC (forward) and ACGAGTCCCGGCTCCTCGGTG (reverse); TNF-α-GCCGCATCGGCGCTTCTCCTAC (forward) and CTCAGCCCCCTCTGGGCTC (reverse); IL8- GAGAGTGATTGAGAGTGGAACAC (forward) and CA-CAACCCTCTCGACCAGTCTT (reverse); EGR1- AGACGCACCTTTCAACCTCAGG (forward) and GAAGTTGTGCTGGGCTGGGTAACT (reverse); and MKP-1- CAAACCCAAAGCAGACACATCAGC (forward) and GAAAGCAGACAGATGGTGCT (reverse).
Fig. S1. Knockdown of MARCH8 potentiates IL-1β-induced NF-κB activation in HeLa cells. HeLa cells (1 x 10⁵) were transfected with NF-κB reporter and MARCH8-RNAi plasmids (0.5 μg each) for 36 h. The cells were then treated with IL-1β (10 ng/mL) or left untreated for 8 h before reporter assays were performed.

Fig. S2. Cotransfection of IL1RAP with IL-1RI activates NF-κB in 293 cells. The 293 cells (1 x 10⁵) were transfected with NF-κB luciferase reporter (20 ng) and indicated plasmid (50 ng) for 20 h before luciferase assays.
**Fig. S3.** MARCH8 colocalizes with IL1RAP in 293 cells. The 293 cells (1 × 10⁵) were transfected with RFP-MARCH8 and GFP-IL1RAP (0.1 μg each). Twenty hours after transfection, cells were fixed with 4% paraformaldehyde before confocal microscopy.

**Fig. S4.** Domain mapping of the interaction between MARCH8 and IL1RAP. (A) The transmembrane domain of IL1RAP is required for its binding to MARCH8. The 293 cells (2 × 10⁶) were transfected with HA-tagged MARCH8 (5 μg) and Flag-tagged IL1RAP and its truncation mutants (5 μg each) for 20 h. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (B) The first transmembrane domain and the region between the two transmembrane domains of MARCH8 are important for its interaction with IL1RAP. The experiments were performed as in A.
**Fig. S5.** Effects of MARCH8 on expression of IL1RAP mutants. The 293 cells (2 × 10^5) were transfected with HA-β-actin, Flag-tagged IL1RAP mutants, and HA-MARCH8. Twenty-four hours after transfection, cell lysates were analyzed by immunoblots with the indicated antibodies.

**IL1RAP-Flag**

**HA-MARCH8:**

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**IL1RAP-Flag**

**HA-MARCH8:**

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**NF-κB**

![Graph showing NF-κB activity](image)

**Fig. S6.** The effects of wild-type IL1RAP and IL1RAP(K512R) on IL-1β-induced NF-κB activation. The 293 cells (1 × 10^5) were transfected with control RNAi or MARCH8-RNAi plasmids (0.5 μg) for 36 h, then the cells were further transfected with NF-κB luciferase reporter and indicated RNAi off-target wild-type IL1RAP or IL1RAP(K512R) plasmids (50 ng). Twenty hours later, the cells were treated with IL-1β (10 ng/mL) for 8 h before luciferase assays. The amounts of input proteins were analyzed by immunoblots with anti-Flag.

![Graph showing NF-κB activity](image)
Fig. S7. Effects of MARCH8 on transcription of IL-1β–induced target genes. (A) Overexpression of MARCH8 inhibited IL-1β–induced transcription of IL8, EGR1, and MKP-1 genes. The 293 cells (2 × 10^5) were transfected with the indicated plasmids (0.2 μg). Twenty hours after transfection, cells were treated with IL-1β (10 ng/mL) or left untreated for 2 h before real-time PCR experiments were performed. (B) Knockdown of MARCH8 promoted IL-1β–induced transcription of IL8, EGR1, and MKP-1 genes. The 293 cells (2 × 10^5) were transfected with the indicated MARCH-RNAi plasmids (0.5 μg) for 36 h. The cells were then treated with IL-1β (10 ng/mL) or left untreated for 2 h before real-time PCR experiments were performed.

Fig. S8. Effects of MARCH8 on assembly of IL-1R–proximal complex. The 293 cells (2 × 10^6) were transfected with the indicated plasmids (3 μg). Twenty hours after transfection, cells were treated with IL-1β (10 ng/mL) for the indicated times. The cells were then lysed and immunoprecipitated with the indicated antibodies. The levels of expressed proteins and the immunoprecipitates were analyzed by immunoblots with the indicated antibodies.