

MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF- κ B signaling

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The intensity and duration of macrophage-mediated inflammatory responses are controlled by proteins that modulate inflammatory signaling pathways. MCP1 (monocyte chemoattractant protein-induced protein 1), a recently identified CCH Zn finger-containing protein, plays an essential role in controlling macrophage-mediated inflammatory responses. However, its mechanism of action is poorly understood. In this study, we show that MCP1 negatively regulates c-Jun N-terminal kinase (JNK) and NF- κ B activity by removing ubiquitin moieties from proteins, including TRAF2, TRAF3, and TRAF6. MCP1-deficient mice spontaneously developed fatal inflammatory syndrome. Macrophages and splenocytes from *MCP1*^{-/-} mice showed elevated expression of inflammatory gene expression, increased JNK and I κ B kinase activation, and increased polyubiquitination of TNF receptor-associated factors. In vitro assays directly demonstrated the deubiquitinating activity of purified MCP1. Sequence analysis together with serial mutagenesis defined a deubiquitinating enzyme domain and a ubiquitin association domain in MCP1. Our results indicate that MCP1 is a critical modulator of inflammatory signaling.

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Abbreviations used: AFC, 7-amino-4-trifluoromethylcoumarin; BAP, bacterial alkaline phosphatase; BMDM, BM-derived macrophage; CCR, C-terminal conserved region; DUB, deubiquitinating enzyme; HA, hemagglutinin; IKK, I κ B kinase; IsoT, isopeptidase T; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; mRNA, messenger RNA; NCR, N-terminal conserved region; NEM, N-ethylmaleimide; NEMO, NF- κ B essential modulator; QPCR, quantitative PCR; RIP, receptor-interacting protein; RIPA, radioimmunoprecipitation assay; TRAF, TNF receptor-associated factor; TTP, tristetraprolin; UBA, ubiquitin association domain; UCH, ubiquitin C-terminal hydrolase; UTR, untranslated region; WCE, whole cell extract; ZF, Zn finger domain.

Inflammation is an important component of innate immunity and the host response to pathogens (Henneke and Golenbock, 2004). In response to infection with virus or bacteria, macrophages produce cytokines such as TNF and IL-1 β , which initiate the inflammatory response (Dinarello, 2005). The inflammatory response must be precisely controlled at multiple levels, as uncontrolled inflammation does not benefit organisms but instead causes tissue impairment and drives autoimmunity, septic shock, and inflammation-associated malignancy (Karin and Greten, 2005).

TNF receptor-associated factors (TRAFs) play a central role in the TNF-, IL-1-, and LPS-induced signaling pathways (Lee et al., 1997). Binding of LPS to TLR4 (Toll-like receptor 4) triggers the recruitment of MyD88 and IRAK1/4, which then recruits TRAF6 and triggers downstream signaling (Dong et al., 2006). Downstream of TRAF6, TAK1 (TGF- β -activated kinase 1) and the adaptor proteins TAB2 and

TAB3 mediate the activation of the I κ B kinase (IKK) complex (Sato et al., 2005). TAK1 has also been reported to be important for TNF-mediated NF- κ B activation (Takaesu et al., 2003). Binding of IL-1 to the IL-1R also triggers the recruitment of the adaptor protein MyD88 to the receptor. MyD88 then recruits the kinases IRAK1 and IRAK4, which play an essential role in the recruitment of TRAF6, triggering its oligomerization and autoubiquitination via Lys 63 (K63)-linked ubiquitin chains (Deng et al., 2000). Binding of TNF to TNF-R1 results in the recruitment of the adaptor protein TRADD (TNF receptor type 1-associated death domain protein), which subsequently recruits a signaling complex consisting of TRAF2, TRAF5, and RIP1 (Tada et al., 2001). TRAF2 or RIP1 then plays a role

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in the recruitment of the IKK complex to TNF-R1, leading to oligomerization and activation. In addition to NF- κ B activation, TNF, IL-1, and LPS are potent activators of c-Jun N-terminal kinase (JNK), which regulates the activation of AP-1 transcription factors, including c-Jun and ATF-2 (Song et al., 1997).

JNK and NF- κ B signaling mediate a wide spectrum of cellular responses, including infections, inflammation, and apoptosis (Muzio et al., 1998). Inappropriate regulation of JNK and NF- κ B signaling is involved in a wide range of human diseases, including cancer, neurodegenerative disorders, arthritis, asthma, and chronic inflammation (Karin and Greten, 2005). Thus, JNK and NF- κ B activation must be tightly regulated to maintain transient activation to prevent inflammation-induced tissue damage or malignancy associated with persistent JNK and NF- κ B activation. Ubiquitination plays important regulatory roles in several steps of JNK and NF- κ B signaling events and thus is an important target for several negative regulators of JNK and NF- κ B. The cylindromatosis tumor suppressor CYLD has been shown to inhibit both JNK and NF- κ B signaling mediated by TNF, LPS, CD40, and IL-1 by cleaving K63-linked ubiquitin chains on TRAF2, TRAF6, and IKK- γ (Kovalenko et al., 2003; Regamey et al., 2003; Trompouki et al., 2003; Reiley et al., 2004). Another deubiquitinating enzyme (DUB) that is an important regulator of NF- κ B is A20, which is a transcriptional target of NF- κ B (Wertz et al., 2004). A20-deficient mice develop severe inflammation and cachexia and die prematurely (Lee et al., 2000).

MCPIP1 (monocyte chemotactic protein [MCP]-induced protein 1; also known as *ZC3H12A*) is a recently identified gene in human peripheral blood monocytes treated with MCP-1 (Zhou et al., 2006; Liang et al., 2008b). The gene undergoes rapid and potent transcription induction upon stimulation with proinflammatory molecules such as TNF, MCP-1, IL-1 β , and LPS (Liang et al., 2008a,b; Matsushita et al., 2009; Skalniak et al., 2009; Kasza et al., 2010). Further studies showed that MCPIP1 plays an important role in both physiological and pathological processes related to inflammation. In the experiments on cultured cells, MCPIP1 was proved to be a negative regulator of macrophage activation (Liang et al., 2008b). In a recent study on mice, MCPIP1 deficiency led to a complex phenotype including severe anemia, autoimmune response, and severe inflammatory response, and most mice died within 12 wk of birth (Matsushita et al., 2009). The underlying mechanism of action of MCPIP1 is poorly understood, but it was suggested that it may control messenger RNA (mRNA) stability of a set of inflammatory genes by functioning as an RNase (Matsushita et al., 2009). In this study, we report that MCPIP1 acts as a deubiquitinase to negatively regulate JNK and NF- κ B signaling by targeting TRAFs. These results suggest that MCPIP1 may critically control inflammation and immune response by multiple mechanisms and would be a potential therapeutic target for treatment of human inflammatory diseases.

RESULTS

Disruption of MCPIP1 leads to severe inflammatory response and premature death in mice

Previously, we have identified MCPIP1 as a negative regulator of LPS- and cytokine-induced macrophage activation in vitro probably by antagonizing the NF- κ B signaling pathway (Liang et al., 2008b). To further investigate the physiological function of MCPIP1 in vivo, we generated *Mcip1*^{-/-} mice by homologous recombination in embryonic stem cells from C57BL/6 background mice. We targeted mouse *Mcip1* exons 4 and 5 and most of 6 with a LacZ-neomycin cassette in embryonic stem cells established from C57BL/6 mice and established *Mcip1*^{-/-} mice in pure C57BL/6 background (Fig. 1 a). We confirmed homologous recombination of the *Mcip1* locus by Southern blot analysis (unpublished data). The lack of MCPIP1 protein in *Mcip1*^{-/-} mice was confirmed by immunoblotting (Fig. 1 b). *Mcip1*^{-/-} mice were born from interbred *Mcip1*^{+/-} mice at the expected Mendelian ratios and looked normal at birth. Similar to the findings in a recent study (Matsushita et al., 2009), *Mcip1*^{-/-} mice showed growth retardation after weaning (Fig. 1 c), severe splenomegaly (Fig. 1 d, top), and lymphadenopathy (Fig. 1 d, bottom) and died prematurely (Fig. 1 e). Meanwhile, serum TNF and MCP-1 levels in *Mcip1*^{-/-} mice were >2.5 times of those in *Mcip1*^{+/+} mice (Fig. 1 f). To examine the function of MCPIP1 in macrophages, we isolated BM-derived macrophages (BMDMs) from *Mcip1*^{+/+} and *Mcip1*^{-/-} mice. MCPIP1-deficient BMDMs secreted a greater amount of proinflammatory cytokines TNF, IL-1 β , IL-6, or MCP-1 both in normal culture conditions and in response to LPS stimulation as compared with WT BMDMs (Fig. 1 g). Real-time RT-PCR showed that the mRNA levels of inflammatory cytokines, including TNF, IL-6, and IL-1 β , were also increased in BMDMs from *Mcip1*^{-/-} mice in response to LPS stimulation (Fig. 1 h). As RNA blotting detected abundant mRNA of *Mcip1* in spleen, thymus, lung, and intestine (Fig. S1), we also isolated the total RNA from these tissues of both *Mcip1*^{+/+} and *Mcip1*^{-/-} mice. As expected, the mRNA expression of inflammatory cytokines, including TNF, IL-1 β , IL-6, and iNOS (inducible nitric oxide synthase), was significantly up-regulated in the intestine, lung, and thymus from *Mcip1*^{-/-} mice compared with *Mcip1*^{+/+} mice (Fig. S2). Collectively, these results further indicate that MCPIP1 is a critical modulator of macrophage inflammation in vivo and may play a key role in controlling inflammatory response and immune homeostasis.

Effect of MCPIP1 on LPS or IL-1 β -induced JNK and NF- κ B signaling activation

To understand the mechanisms underlying the inflammatory phenotypes of *Mcip1*-null mice, we first isolated the protein extracts of lungs from both *Mcip1*^{+/+} and *Mcip1*^{-/-} mice. As shown in Fig. 2 a, the JNK phosphorylation was markedly increased in lung tissue from *Mcip1*^{-/-} mice compared with that from *Mcip1*^{+/+} mice. Phospho-IKK- β and phospho-IkBa were moderately increased, whereas total IkBa protein

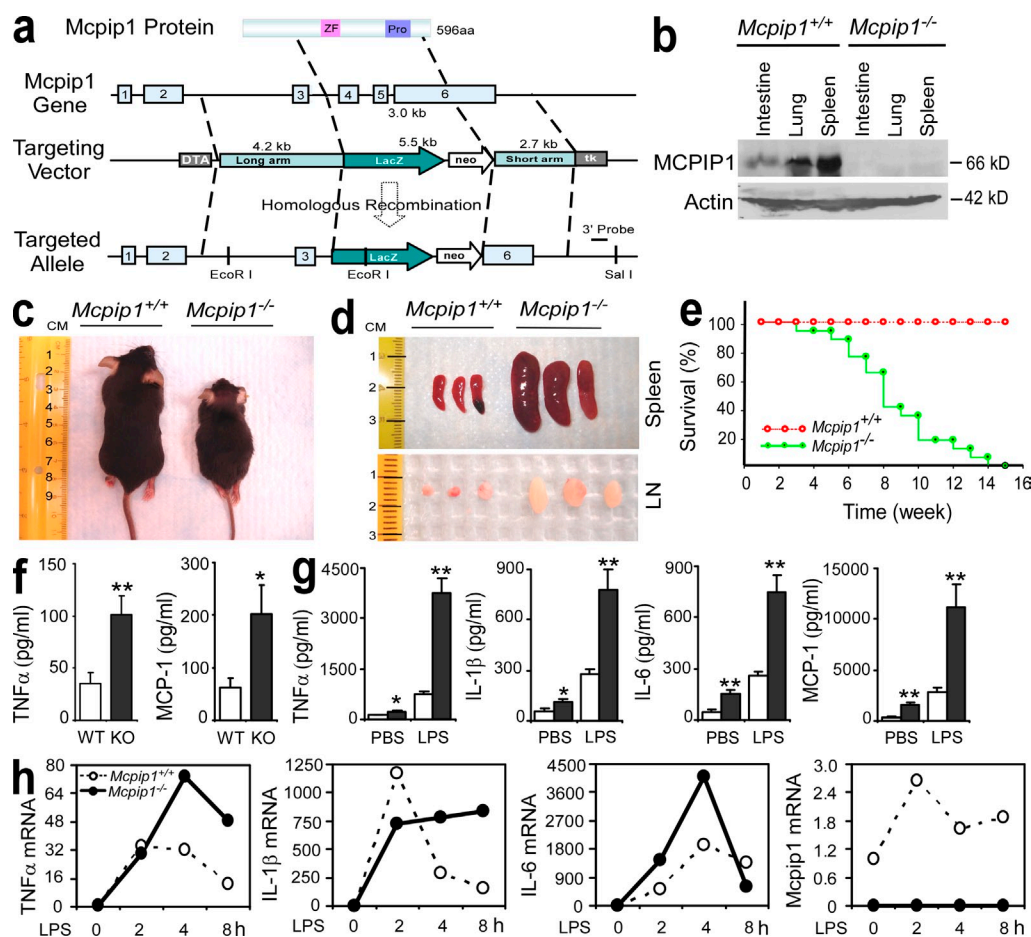


Figure 1. Disruption of MCPIP1 leads to severe inflammatory response and premature death in mice. (a) The structure of the mouse *Mcpip1* gene is shown. The targeting strategy deleted exons 4 and 5 and most of 6, which encode the most part of the MCPIP1 protein, including the ZF and Pro domains. (b) Proteins extracted from the intestine, lung, and spleen of mice were analyzed by immunoblotting with MCPIP1-specific antibody. The same membrane was probed with antiactin to show equal loading. The experiment was performed on three independent sets of animals. (c and d) Gross appearance of *Mcpip1*^{+/+} and *Mcpip1*^{-/-} mice (6–8-wk-old male; c), as well as spleens (d, top) and LNs (d, bottom) from these mice. The ruler (left margin) indicates size in centimeters. Three independent sets of animals showed similar results. (e) Survival rate of *Mcpip1*^{+/+} and *Mcpip1*^{-/-} mice (n = 19). (f) ELISA was used to measure serum cytokine levels in WT and KO mice. Data are presented as mean ± SD (n = 6; * P < 0.05; ** P < 0.01 vs. WT group). (g) BMDMs from WT (open bars) and KO (closed bars) mice were stimulated with PBS or 100 ng/ml LPS for 5 h. ELISA was used to measure cytokine levels in cultured medium. Data are presented as mean ± SD (n = 3; * P < 0.05; ** P < 0.01 vs. WT group). Results are representative of two independent experiments. (h) Total RNA isolated from BMDMs stimulated with LPS for the indicated time periods was reverse transcribed and analyzed by QPCR with gene-specific primers as indicated. Data are presented as mean ± SD (n = 3). Student's t test was performed. The experiment was performed on two independent sets of animals with triplicate measurements.

was decreased in the lung from *Mcpip1*^{-/-} mice compared with those from *Mcpip1*^{+/+} mice. These results suggested that both JNK and NF-κB signaling were constitutively activated in the lung of *Mcpip1*^{-/-} mice. Second, we examined LPS-induced JNK and NF-κB signaling activation in BMDMs from *Mcpip1*^{+/+} or *Mcpip1*^{-/-} mice. As shown in Fig. 2 b, LPS-induced phosphorylation of JNK, ERK1/2, and IKK-β was significantly enhanced in *Mcpip1*^{-/-} BMDMs compared with *Mcpip1*^{+/+} BMDMs. These results suggest that Mcpip1 is essential for the down-regulation of JNK and NF-κB activation upon LPS stimulation. To further confirm the effect of MCPIP1 on LPS-stimulated JNK and NF-κB signaling activation, we transfected MCPIP1 and control vectors into

Raw264.7 cells. Transfected cells were stimulated by 100 ng/ml LPS for 0, 5, 15, and 30 min after 24 h of quiescence. The activation of signaling proteins in the JNK and NF-κB signaling pathways was detected by immunoblotting with phospho-specific antibodies. As shown in Fig. 2 c, MCPIP1 expression completely blocked LPS-induced JNK phosphorylation and partially blocked ERK1/2, p38, and IKK-β phosphorylation. These changes were not caused by protein degradation because JNK, ERK1/2, p38, and IKK-β protein levels were not affected by MCPIP1 overexpression. These results suggest that MCPIP1 expression significantly inhibits LPS-induced JNK activation but moderately affects ERK1/2, p38, and IKK activation.

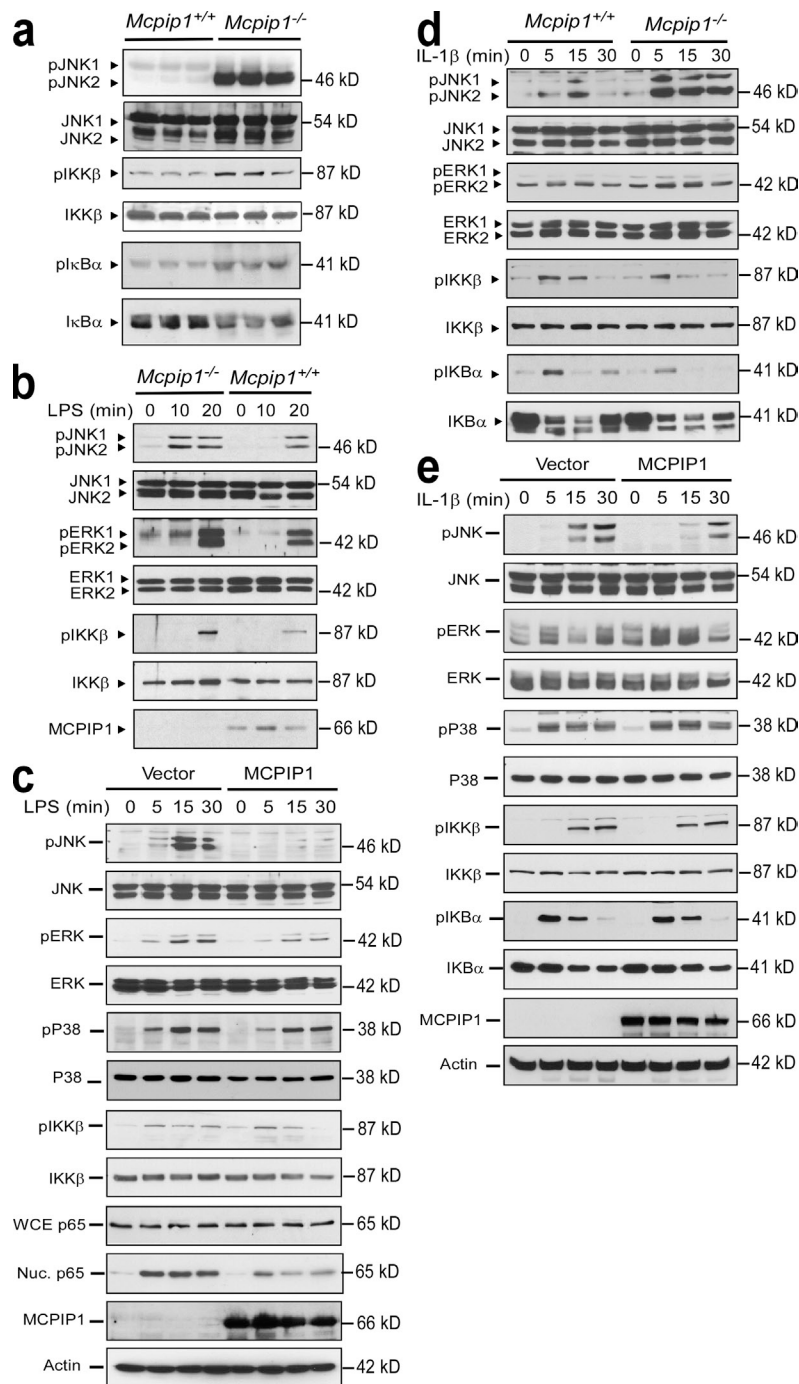


Figure 2. Effect of MCPIP1 on LPS- and IL-1β-induced JNK and NF-κB activation. (a) Proteins extracted from the lung of three pairs of *Mcpip1*^{+/+} and *Mcpip1*^{-/-} mice were analyzed by immunoblot with the indicated antibodies. Results are representative of three independent experiments. (b) BMDMs from *Mcpip1*^{-/-} or *Mcpip1*^{+/+} mice were quiescent for 16 h and then stimulated with 100 ng/ml LPS for the indicated time periods. WCEs were subjected to immunoblot analysis with antibodies as indicated. Similar results were seen in three independent experiments. (c) Raw264.7 cells were transfected with pcDNA3 (vector) or pHA-MCPIP1. After 24 h, the transfected cells were quiescent for 16 h and then stimulated with 100 ng/ml LPS for the indicated time periods. WCEs were subjected to immunoblot analysis with antibodies as indicated. Nuclear fractions (Nuc.) were prepared and subjected to immunoblot analysis using p65-specific antibody. Results are representative of at least three independent experiments. (d) MEFs from *Mcpip1*^{-/-} or *Mcpip1*^{+/+} mice were quiescent for 16 h and then stimulated with 10 ng/ml IL-1β for the indicated time periods. WCEs were subjected to immunoblot analysis with antibodies as indicated. (e) HepG2 cells were transfected with pcDNA3 (vector) or pHA-MCPIP1. After 24 h, the transfected cells were quiescent for 16 h and then stimulated with 10 ng/ml IL-1β for the indicated times. WCEs were subjected to immunoblot analysis with antibodies as indicated.

However, *Mcpip1* deficiency did not significantly affect the activation of ERK1/2 and IKK-β. To further confirm the effect of MCPIP1 on IL-1β-stimulated JNK and NF-κB signaling activation, we transfected MCPIP1 and control vectors into HepG2 cells, a cell type which is easier for transfection and responds efficiently to IL-1β stimulation. Consistently, MCPIP1 expression significantly suppressed IL-1β-induced JNK phosphorylation but did not affect ERK1/2, p38, and IKK-β phosphorylation. These results suggest that MCPIP1 is essential to down-regulate IL-1β-induced JNK signaling but not critical in IL-1β-induced ERK1/2, p38, and NF-κB signaling.

MCPIP1 inhibits NF-κB- and AP-1- but not STAT-dependent gene activation

Next, we examined the effect of MCPIP1 on NF-κB-, AP-1-, or STAT-dependent gene activation. Raw264.7 cells were transiently transfected with a luciferase reporter construct, NF-κB-TK-Luc, AP-1-TK-Luc, or GAS-TK-Luc, together with increasing doses of hemagglutinin (HA)-tagged MCPIP1. After overnight of quiescence, cells were either left untreated or treated with LPS or TNF or IFN-γ plus LPS for 24 h, followed by luciferase assays. As shown in Fig. 3 (a and b), MCPIP1 overexpression did not significantly affect the basal activity of NF-κB reporter and AP-1 reporter. However, overexpression of MCPIP1 significantly inhibited LPS-induced NF-κB reporter activation and TNF-induced AP-1 reporter activation

Consistently, MCPIP1 expression also reduced LPS-induced p65 nuclear translocation in Raw264.7 cells.

To examine the function of MCPIP1 in IL-1R signaling, we analyzed the effect of *Mcpip1* deficiency on IL-1β-stimulated activation of JNK and NF-κB signaling in primary mouse embryonic fibroblasts (MEFs), a cell type which responds efficiently to IL-1β stimulation. As shown in Fig. 2 d, *Mcpip1*-deficient MEFs showed increased activation of JNK in response to IL-1β stimulation, suggesting that MCPIP1 is essential to down-regulate IL-1β-induced JNK activation.

in a concentration-dependent manner. MCPIP1 expression did not affect LPS- and IFN- γ -induced STAT-dependent gene activation (Fig. 3 c). The overexpression of MCPIP1 in the transfected cells was confirmed by immunoblotting with HA antibody (Fig. 3 c, bottom). These results further support the idea that MCPIP1 selectively represses LPS- and cytokine-initiated

NF- κ B and JNK-AP-1 signaling pathways but does not affect the JAK-STAT signaling pathway.

NF- κ B and AP-1 individually or coordinately regulate a large set of inflammatory gene expressions. To examine the whole gene expression changes caused by MCPIP1 expression in an unbiased way, we performed microarray analysis. A macrophage cell line Raw264.7 was transfected with GFP or MCPIP1-GFP constructs. These cells were treated with or without LPS for 8 h before mRNA harvest. Overall, LPS treatment induced 1,783 genes by more than threefold compared with the untreated cells. Among the 1,783 of LPS-induced genes, 30% of them were affected by overexpression of MCPIP1 (Fig. 3 d). Among the MCPIP1-affected genes, 85% of the genes were repressed by MCPIP1, and 15% of the genes were up-regulated by MCPIP1. The 26 genes up-regulated by >10-fold in LPS-treated cells were selected and shown in Fig. 3 e as a heat map. Consistent with our previous results (Liang et al., 2008b), the inflammatory genes *TNF*, *IL-1 β* , *IL-6*, and *MCP-1*, whose transcription is controlled by NF- κ B and JNK signaling, were significantly repressed by MCPIP1. Overexpression of MCPIP1 was confirmed by immunoblotting with MCPIP1 antibody (Fig. 3 e, bottom). These results further support the idea that MCPIP1 may control a set of the inflammatory gene expressions and prevent inflammatory responses in vivo.

MCPIP1 acts as a deubiquitinase

Recent studies have identified a critical role for ubiquitination in JNK and NF- κ B signaling (for review see Skaug et al., 2009). To understand how MCPIP1 regulates JNK and NF- κ B signaling, we first tested the effect of MCPIP1 expression on global protein ubiquitination. To facilitate the detection of ubiquitin conjugates, HEK293 cells were transfected with HA-tagged ubiquitin and MCPIP1 or tristetraprolin (TTP), another well-studied CCCH Zn finger protein. Immunoblotting of whole cell lysates with HA antibody revealed that cells expressing MCPIP1 contained far fewer ubiquitinated proteins than cells not expressing MCPIP1 (Fig. 4 a). Interestingly, TTP also reduced protein ubiquitination, although less potently than MCPIP1. These results indicate that MCPIP1 promotes deubiquitination, although it remains uncertain whether MCPIP1 has intrinsic deubiquitinating activity or regulates ubiquitination indirectly via other cellular proteins. To determine whether MCPIP1 has deubiquitinating activity, we purified Flag-tagged MCPIP1 from HEK293 cells. The purity of the purified protein was confirmed by Coomassie brilliant blue staining and immunoblotting analysis (Fig. 4 b). To test whether MCPIP1 cleaves K48- or K63-linked polyubiquitin, we incubated MCPIP1 with K48- or K63-linked polyubiquitin. Isopeptidase T (IsoT), a well-known deubiquitinase (Hadari et al., 1992), was also included as a positive control. As shown in Fig. 4 c, MCPIP1 cleaved K48-linked polyubiquitin chain into monomers, as did IsoT. MCPIP1 cleaved K63-linked polyubiquitin chain into different oligomers, which suggests incomplete cleavage. To exclude the possibility that this deubiquitinating activity is caused by trace

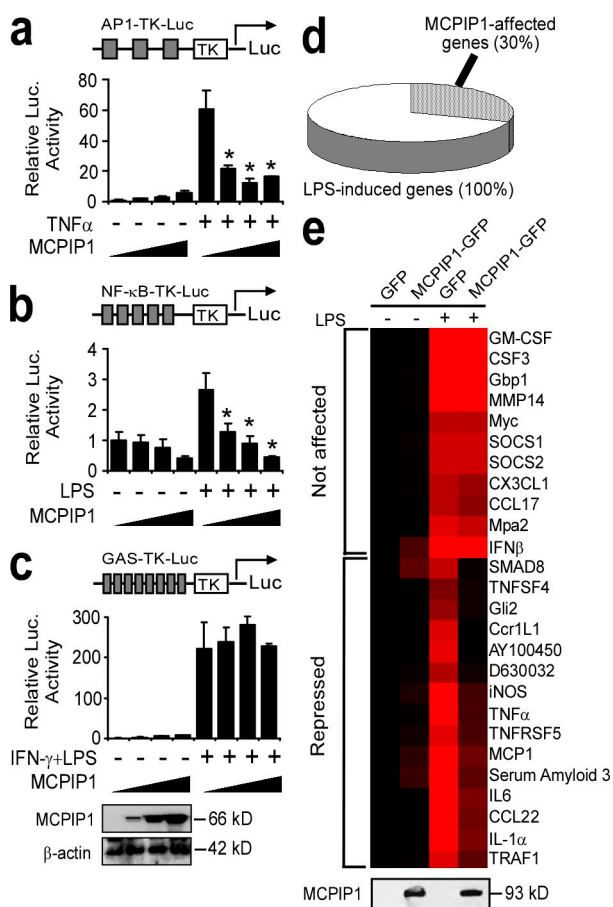


Figure 3. Overexpression of MCPIP1 inhibits NF- κ B and AP-1 but not STAT activation. (a–c) Raw264.7 cells were cotransfected AP-1-TK-Luc (a), NF- κ B-TK-Luc (b), or GAS-TK-Luc (c) with increasing amounts of pHA-MCPIP1 (0, 25, 50, and 100 ng/well, as indicated by wedges) using Eugene6 (Roche). After quiescence for 16 h, cells were treated with or without 10 ng/ml TNF (a), 100 ng/ml LPS (b), or IFN- γ plus LPS (c). Reporter gene activity was analyzed 24 h later. The data are presented as mean \pm SD ($n = 4$; *, $P < 0.01$ vs. stimulated without MCPIP1 transfection group). Analysis of variance with Bonferroni correction was performed. Results are representative of at least three independent experiments. Immunoblotting shows expression of HA-MCPIP1. β -Actin served as a loading control. (d) Raw264.7 cells were transfected with pGFP-N1 or pMCPIP1-GFP plasmids. 16 h later, the transfected cells were treated with or without 1 mg/ml LPS for 8 h. mRNA was labeled and hybridized to the 44K Agilent Mouse Oligo Microarray. 30% of LPS-induced genes were affected by the expression of MCPIP1. (e) 26 selected genes that are induced by LPS >10-fold (some unaffected by MCPIP1 and others affected by MCPIP1) were clustered and presented as a heat map. Red is represented as high expression, whereas black means low expression. The expression of MCPIP1-GFP (93 kD) was detected by immunoblotting with anti-MCPIP1 and shown at the bottom.

DUB contamination from 3xFlag elution buffer or immunoprecipitation procedures, we incubated hexa-ubiquitin chain with 3xFlag elution buffer, immunoprecipitants from empty vector-transfected cells, or Flag-p53. No cleavage was detected in these reactions (Fig. 4 d). Next, we incubated Flag-MCPIP1 protein with K63-linked polyubiquitin chains of different lengths, followed by immunoblotting detection. As shown in Fig. 4 e, MCPIP1 cleaved polyubiquitin with a preference for high molecular polyubiquitin chains like hexa-ubiquitin or octa-ubiquitin. It showed little cleavage toward the

linear diubiquitins. Further experiments confirmed that MCPIP1 cleaved K63-linked octa-ubiquitin chains in dose- and time-dependent manners (Fig. 4 f). Furthermore, we observed that the activity of MCPIP1 to cleave polyubiquitin was completely blocked by *N*-ethylmaleimide (NEM), a Cys protease inhibitor (Fig. 4 g). Interestingly, the deubiquitinating activity of MCPIP1 was also completely abolished by Zn^{2+} and Mn^{2+} but was not affected by Mg^{2+} , Ca^{2+} , and EDTA (Fig. 4 h). To further quantitatively assess the deubiquitinating activity for MCPIP1, we incubated purified

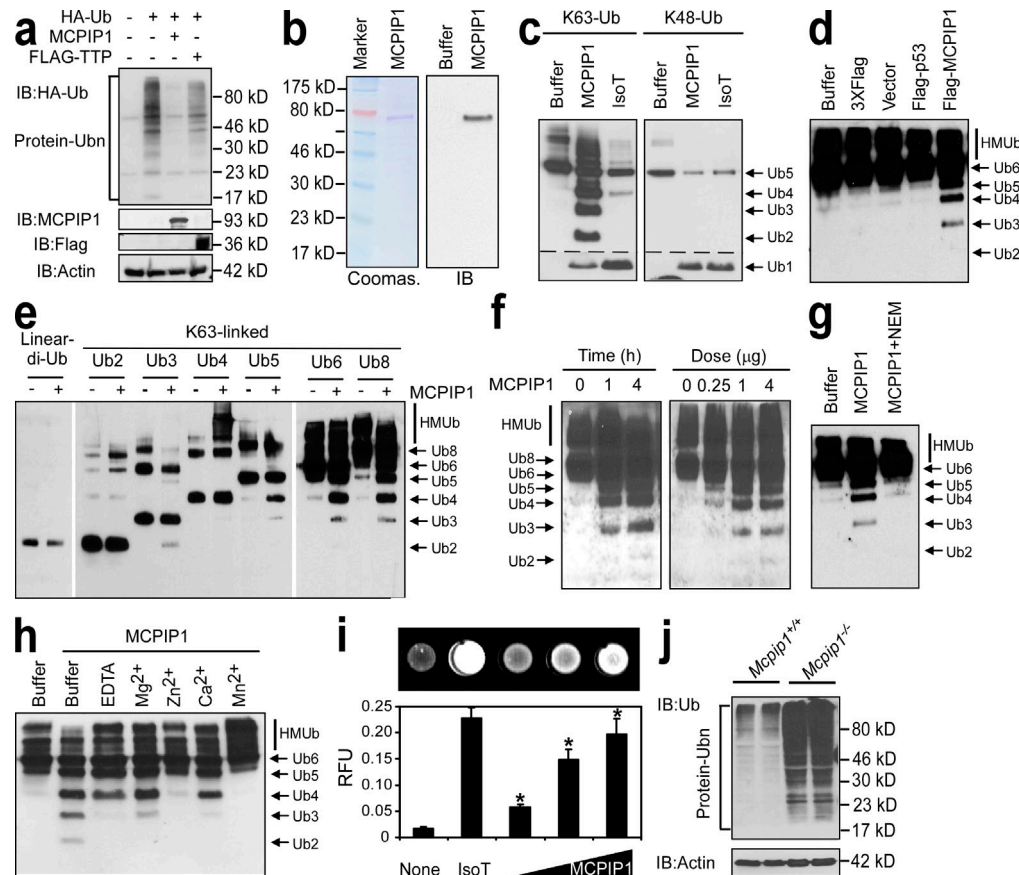


Figure 4. MCPIP1 acts as a deubiquitinase. (a) HEK293 cells were cotransfected with HA-ubiquitin (Ub) and/or MCPIP1-GFP and/or Flag-TTP. 36 h later, the ubiquitinated proteins in denatured cell lysates were detected by immunoblotting (IB) with HA-specific antibody. The expression of MCPIP1-GFP, Flag-TTP, and actin was also detected by immunoblotting with MCPIP1, Flag, and actin-specific antibodies. Results are representative of at least three independent experiments. (b) Flag-tagged MCPIP1 was immunoprecipitated from transfected HEK293 cells. The purified protein was examined by Coomassie blue staining (Coomas.) and immunoblotting using Flag-specific antibody. (c) 1 μg of purified Flag-MCPIP1 was incubated with K63- or K48-linked pentaubiquitin chains (Ub_5) at 37°C for 16 h. Reactions were analyzed by immunoblotting with ubiquitin-specific antibody. Recombinant IsoT was used as a positive control. The blot below the dashed lines had a longer exposure time than the blot above the dashed lines. Long exposure means 2–5 min exposure of the same film. (d) Flag-MCPIP1, 3xFlag elution buffer, immunoprecipitants from empty vector-transfected cells, or Flag-p53 was incubated with K63-linked Ub_6 at 37°C for 16 h. Reactions were analyzed by immunoblotting with ubiquitin-specific antibody. HMUb, high molecular weight ubiquitin. (e) In vitro deubiquitination assays using purified Flag-MCPIP1 showing cleavage of high molecular K63-linked polyubiquitin chains (Ub_{5-8}) but not low molecular polyubiquitin chains (Ub_{2-4}) or linear Ub_2 . (f) In vitro deubiquitination showing that MCPIP1 cleaved Ub_8 in time- and dose-dependent manners. (g and h) MCPIP1 deubiquitinase activity was assessed in the presence of the Cys protease inhibitor NEM, 10 mmol/l Zn^{2+} , 10 mmol/l Mn^{2+} , 50 mmol/l EDTA, 10 mmol/l Mg^{2+} , or 10 mmol/l Ca^{2+} . Data in b–h are representative of at least three independent experiments. (i) 0.5, 1, and 2 μg (indicated by wedge) of purified Flag-MCPIP1 was incubated with 80 μM ubiquitin-AFC at 23°C for 5 min. The protease activity was determined by the release of fluorescent AFC (ex: 400 nm, em: 505 nm). 1 μg IsoT was used as a positive control. Values were represented as relative fluorescent units (RFU; mean ± SD; $n = 3$; *, $P < 0.001$ vs. none group). Two-way analysis of variance with Bonferroni correction was performed. The top shows a fluorescent image of the reactions in a 96-well plate. Results are representative of two independent experiments. (j) The global cellular ubiquitinated proteins in the splenocytes from *Mcpip1*^{+/+} and *Mcpip1*^{−/−} mice were detected by immunoblotting with ubiquitin-specific antibody. Actin was detected and served as a loading control. Three independent experiments showed similar results.

MCPIP1 with ubiquitin-7-amino-4-trifluoromethylcoumarin (AFC) in an in vitro deubiquitination assay. Purified MCPIP1 cleaved ubiquitin-AFC in a dose-dependent manner with less potency compared with IsoT (Fig. 4 i). Moreover, the global ubiquitinated protein level in the splenocytes from *Mcpip1*^{-/-} mice was much higher than that from *Mcpip1*^{+/+} mice (Fig. 4 j). Collectively, these results indicate that MCPIP1 has intrinsic deubiquitinating activity and suggest that MCPIP1 may regulate JNK and NF- κ B signaling through deubiquitination in vivo.

MCPIP1 deubiquitinase targets to TRAFs

TRAFs have been shown to function as ubiquitin ligases that catalyze the synthesis of K63-linked polyubiquitin chains. However, this type of ubiquitination, which occurs early during a cellular response and does not target protein degradation, is important for signal transduction (Lomaga et al., 1999; Lamothe et al., 2007). Stimulus-dependent autoubiquitination of TRAF2 and TRAF6 activates the TAK1, which is critical in the activation of NF- κ B and MAPK (mitogen-activated protein kinase). To examine whether MCPIP1 targets the key components in the JNK and NF- κ B signaling pathways, we transfected HA-ubiquitin with MCPIP1-GFP or Flag-TTP into HEK293 cells. After 2 h of TNF treatment, both TRAF2 and TRAF6 in the denatured cell lysates were immunoprecipitated by TRAF2 or TRAF6 antibodies. The ubiquitinated TRAF2 or TRAF6 in the immunoprecipitates was further detected by immunoblotting with HA antibody. As shown in Fig. 5 a, both TRAF2 and TRAF6 were ubiquitinated upon HA-ubiquitin overexpression. Intriguingly, MCPIP1 expression markedly decreased TRAF2 and TRAF6 ubiquitination. TTP, another CCCH Zn finger protein, did not affect TRAF2 ubiquitination but seemed to also reduce TRAF6 ubiquitination. We also observed the effect of MCPIP1 on the ubiquitination of receptor-interacting protein (RIP) and NF- κ B essential modulator (NEMO). Interestingly, MCPIP1 similarly decreased the RIP ubiquitination but showed less effect on NEMO ubiquitination. Because RIP ubiquitination is dependent on TRAF2 signaling, the effect of MCPIP1 on RIP ubiquitination may be indirect. To further confirm the effect of MCPIP1 on TRAF ubiquitination, we cotransfected Flag-TRAF2 or Flag-TRAF3 with HA-ubiquitin into HEK293 cells. The ubiquitinated TRAF2 or TRAF3 in the denatured cell lysates was immunoprecipitated by Flag antibody and further detected by immunoblotting with HA antibody. Polyubiquitination of both proteins was indeed observed. Transfection of MCPIP1 together with TRAF2 or TRAF3 resulted in a drastic decrease in the ubiquitinated forms of the two proteins (Fig. 5 b). Next, we tested whether MCPIP1 also affects the K48-linked ubiquitination of I κ B α . We transfected HA-ubiquitin with Flag-MCPIP1 into HEK293 cells. After 24 h, the transfected cells were treated with TNF and MG132 for 2 h to prevent the degradation of cellular proteins mediated by K48-linked polyubiquitin chain. I κ B α in the denatured cell lysates was immunoprecipitated by I κ B α antibody. The ubiquitinated

I κ B α in the immunoprecipitates was further detected by immunoblotting with HA antibody. As shown in Fig. 5 c, MCPIP1 expression also dramatically decreased I κ B α ubiquitination. To further examine whether MCPIP1 physiologically regulates TRAF ubiquitination in vivo, TRAF2 in the spleen of *Mcpip1*^{-/-} and *Mcpip1*^{+/+} mice was immunoprecipitated by TRAF2 antibody. The ubiquitinated TRAF2 in the immunoprecipitates was detected by immunoblotting with ubiquitin antibody. As shown in Fig. 5 d, TRAF2 was constitutively ubiquitinated in *Mcpip1*^{-/-} mice but not in *Mcpip1*^{+/+} mice (Fig. 5 d). To further examine the effect of *Mcpip1* deficiency on TRAF ubiquitination in response to LPS stimulation, we isolated splenocytes from *Mcpip1*^{+/+} mice and *Mcpip1*^{-/-} mice. The in vitro cultured splenocytes were stimulated with 100 ng/ml LPS for 30 min. TRAF2, TRAF3, TRAF6, and RIP were immunoprecipitated with the respective individual antibody. The polyubiquitinated TRAF2, TRAF3, TRAF6, and RIP in the immunoprecipitates were detected by immunoblotting with ubiquitin antibody. As shown in Fig. 5 e, *Mcpip1* deficiency led to a drastic increase in both basal and LPS-induced ubiquitination of TRAF2, TRAF3, and TRAF6 but not RIP in splenocytes. To further examine whether MCPIP1 directly cleaves polyubiquitin chain from ubiquitinated TRAF2 and TRAF3, we transfected Flag-TRAF2 or Flag-TRAF3 with HA-ubiquitin into HEK293 cells. The transfected cells were stimulated with TNF for 15 min before harvest. The cellular ubiquitinated TRAF2 was purified by anti-Flag gel and washed with 3xFlag buffer and incubated with 0, 0.5, and 2 μ g Flag-MCPIP1, followed by immunoblotting with HA antibody. As shown in Fig. 5 f, the ubiquitin moieties on TRAF2 can be removed by MCPIP1 in a dose-dependent manner. Similarly, the ubiquitin moieties on TRAF3 can also be removed directly by MCPIP1 (Fig. 5 g). Flag-bacterial alkaline phosphatase (BAP) and IsoT were also included in this assay to serve as negative and positive control, respectively (Fig. 5 h). Collectively, these results suggest that MCPIP1 deubiquitinase may directly target the TRAF family, by which it regulates JNK and NF- κ B signaling.

MCPIP1 contains a novel DUB domain

The aforementioned results strongly suggest that MCPIP1 functions as a deubiquitinase to control the inflammatory signal pathways by removing the polyubiquitin moieties from ubiquitinated TRAFs. DUBs are proteases that cleave ubiquitin chains from protein substrates or their degradation remnants. The human genome encodes 100 DUBs. On the basis of their domain structures, DUBs can be divided into five families (Reyes-Turcu et al., 2009). Four are Cys proteases, including ubiquitin-specific proteases, ubiquitin C-terminal hydrolases (UCHs), otubain proteases, and Machado-Joseph disease proteases. The other is a family of metalloproteases. To determine whether MCPIP1 belongs to any of the five families, we aligned the MCPIP1 protein sequences with the five DUB domains. There is no essential sequence similarity

between MCPIP1 and the five DUB domains except remote homology with UCH domain (27%), which suggests that MCPIP1 may contain a novel DUB domain. To find the conserved domains on MCPIP1 protein, we analyzed MCPIP1

sequence by several programs such as Pfam and SMART. At the N terminus, MCPIP1 contains a ubiquitin association domain (UBA). In the middle, there is a conserved CCCH-type Zn finger domain (ZF). At the C terminus, MCPIP1

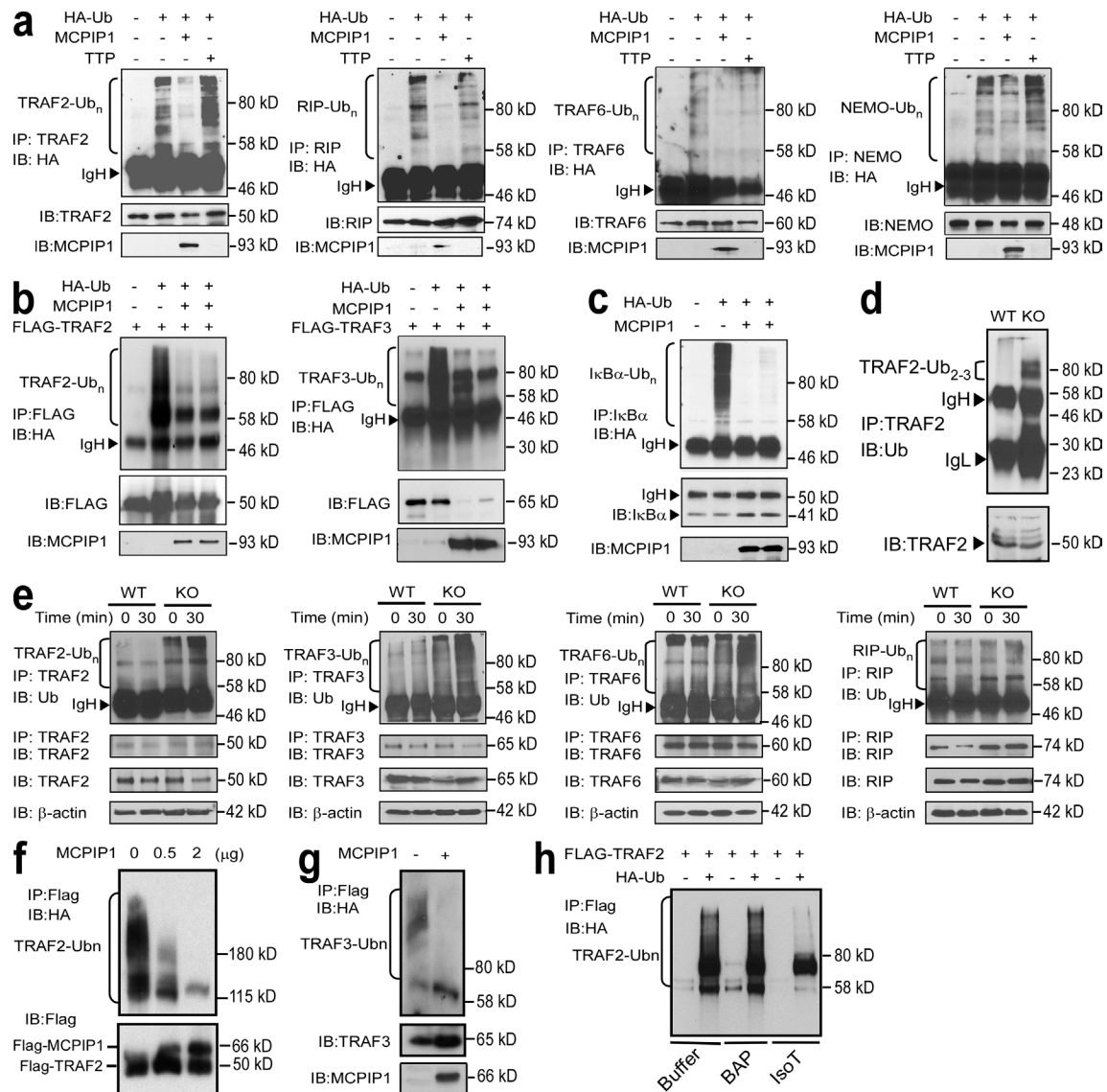


Figure 5. MCPIP1 deubiquitinase targets TRAFs. (a) HEK293 cells were cotransfected with HA-tagged human WT ubiquitin (Ub) with or without MCPIP1-GFP or Flag-TTP. 24 h later, the cells were treated with TNF for 2 h. TRAF2, TRAF6, RIP, and NEMO were immunoprecipitated (IP) and immunoblotted (IB) using anti-HA, anti-TRAF2, anti-TRAF6, anti-RIP, or anti-NEMO as indicated. MCPIP1 expression in WCEs was detected using anti-MCPIP1. IgH, Ig heavy chain. (b) HEK293 cells were cotransfected with HA-ubiquitin, Flag-TRAF2, or Flag-TRAF3 with or without MCPIP1-GFP. 24 h later, the cells were treated with TNF for 2 h. Lysates were subjected to immunoprecipitation and immunoblot with the indicated antibodies. MCPIP1 expression in WCEs was detected using anti-MCPIP1. (c) HEK293 cells were cotransfected with HA-ubiquitin with or without MCPIP1-GFP. After 2 h of TNF and MG132 treatment, the cells were harvested and subjected to immunoprecipitation using IκBα-specific antibody. The immunoprecipitates were examined by immunoblot with anti-HA and anti-IκBα. Data in a–c are representative of at least two independent experiments. (d) Endogenous TRAF2 in the spleens from *Mcpip1*^{+/+} and *Mcpip1*^{−/−} mice was immunoprecipitated with anti-TRAF2, followed by immunoblotting with ubiquitin and TRAF2-specific antibodies. Results are representative of two independent experiments with similar results. (e) Splenocytes from *Mcpip1*^{+/+} and *Mcpip1*^{−/−} mice were stimulated with 100 ng/ml LPS for 30 min. The endogenous TRAF2, TRAF3, TRAF6, and RIP were immunoprecipitated, followed by immunoblotting with antiubiquitin. Results are representative of two independent experiments with similar results. (f and g) Ubiquitinated purified TRAF2 and TRAF3 were incubated with Flag-MCPIP1 at 37°C for 4 h and analyzed by immunoblotting with anti-HA and anti-Flag. The ubiquitin was tagged with HA. Two independent experiments showed similar results. (h) HEK293 cells were cotransfected with Flag-TRAF2 with or without HA-ubiquitin. Flag-TRAF2 protein in cell lysates was purified by anti-Flag matrix and incubated with 3xFlag buffer only, 0.5 μg Flag-BAP, or 0.5 μg IsoT at 37°C for 4 h and analyzed by immunoblotting with anti-HA.

contains a Pro-rich domain. By aligning the MCPIP1 sequences from different species, we further identified a conserved region at N terminus (N-terminal conserved region [NCR]) just preceding CCCH Zn finger and another conserved region at the C terminus (C-terminal conserved region [CCR]; Fig. 6 a).

To examine which domains are functionally important in deubiquitination, we generated serial truncated constructs of Flag-tagged MCPIP1 by PCR amplification of the indicated regions (Fig. 6 a). We cotransfected the truncated plasmids with HA-ubiquitin into HEK293 cells. Immunoblotting with HA antibody showed that WT MCPIP1 markedly decreased overall ubiquitination of cellular proteins. MCPIP1(1–555) and MCPIP1(81–599), with deletion of the UBA or CCR without affecting the NCR and Pro-rich domain, maintained similar deubiquitinating activity as WT MCPIP1. MCPIP1(1–453), with deletion of the Pro-rich domain, had lower deubiquitinating activity. MCPIP1(150–599), MCPIP1(150–453), and MCPIP1(Δ ZF) completely lost deubiquitinating activity (Fig. 6 b). These results suggest that NCR and CCCH Zn finger are critical for MCPIP1 deubiquitinating activity.

To further assess whether the repressor activity of MCPIP1 on NF- κ B reporter is dependent on its deubiquitinating activity, we cotransfected each truncated vector with reporter construct NF- κ B-TK-Luc into HEK293 cells. The transfected cells were left untreated or treated with TNF for 24 h, followed by luciferase assays. As shown in Fig. 6 c, TNF induced NF- κ B reporter activity by 12-fold. Expression of full-length MCPIP1 repressed TNF-induced reporter activity by 80%. MCPIP1(1–555) and MCPIP1(81–599) maintained similar repressing activity as the full-length MCPIP1. MCPIP1(1–453) had lower repressor activity. MCPIP1(150–599), MCPIP1(150–453), and MCPIP1(Δ ZF) lost almost all of the repressor activity. These results suggest that the repressor effect of MCPIP1 on NF- κ B signaling is dependent on its deubiquitinating activity.

The aforementioned results suggest that NCR and CCCH Zn finger may contain a novel DUB domain. As shown in Fig. 6 d, there are several putative Cys boxes and Asp boxes, which usually exist in Cys proteases, on the NCR of MCPIP1. To assess whether these putative boxes constitute the catalytic domain of MCPIP1 deubiquitinase, we generated serial point mutants of MCPIP1, as marked by asterisks in Fig. 6 d. The putative Cys box of MCPIP1 showed 39% sequence homology with that of UCH-L1 and is conserved from fly to human (Fig. 6 e). Luciferase assay showed that the mutants with D141N, C157A, D278A/D279A, and C306R totally lost the repressor activity on NF- κ B reporter (Fig. 6 f). Consistently, the purified protein with D141N, C157A, and C306R but not H88A also lost the deubiquitinating activity (Fig. 6 g). It was recently reported that MCPIP1 also has RNase activity, which targets to the IL-6 3' untranslated region (UTR). We also measured the RNase activity using these mutant proteins. As shown in Fig. 6 h, MCPIP1 digested the IL-6 3' UTR. Similar to a previous study (Matsushita et al., 2009), D141N mutant lost the RNase activity. However, H88A,

C157A, and C306R still efficiently digested the transcript. To further confirm that the repressor effect of MCPIP1 on inflammatory signaling is dependent on its DUB activity but not its RNase activity, HEK293 cells were transfected with control vector, MCPIP1, or its C157A mutant. The transfected cells were quiescent overnight and stimulated with TNF for 0, 15, and 30 min. As shown in Fig. 6 i, MCPIP1 significantly suppressed TNF-induced JNK phosphorylation as it did in other cell lines. C157A mutant, which lost DUB activity but maintained RNase activity, did not suppress TNF-induced JNK phosphorylation. Furthermore, ectopic expression of MCPIP1 in *Mcpip1*^{-/-} MEFs reduced LPS-induced TNF and MCP-1 release; however, C157A mutant lost the repressor effect (Fig. 6 j). These results suggest that inhibition of MCPIP1 on JNK and NF- κ B signaling activation may be dependent on its DUB activity but not its RNase activity.

MCPIP1 interacts with ubiquitin through its UBA

Ubiquitin-binding domains such as the UBA and ubiquitin-interacting motif can influence various cellular events through binding to ubiquitinated proteins, but the function of ubiquitin-binding domains in the deubiquitination reaction remains to be elucidated. As shown in Fig. 6 a, MCPIP1 has a putative UBA at the N terminus. The consensus sequence of UBA in MCPIP1 is conserved from fly to human (Fig. 7 a). To assess whether MCPIP1 directly binds to ubiquitin through these putative UBAs, HEK293 cells were transfected with MCPIP1, the N-terminal truncated form of MCPIP1 without UBA (MCPIP1 Δ N), or the C-terminal truncated form of MCPIP1 (MCPIP1 Δ C; Fig. 7 b). The cell lysates from the transfected cells were incubated with ubiquitin-agarose, and pull-down fractions were detected by immunoblotting with Flag antibody. As shown in Fig. 7 c, WT MCPIP1 was strongly associated with ubiquitin. MCPIP1 Δ C with deletion of the CCR and Pro region reduced the binding. MCPIP1 Δ N with deletion of the UBA abolished the binding with ubiquitin. These results suggest that MCPIP1 contains a functional UBA and may mediate the association of MCPIP1 with ubiquitinated proteins.

DISCUSSION

MCPIP1 (also known as ZC3H12A) is a prototype member of a novel CCCH Zn finger-containing protein family, which was originally identified as an MCP-1-induced gene in human peripheral monocytes through a DNA microarray (Zhou et al., 2006). We previously found that MCPIP1 is a negative regulator of proinflammatory activation of macrophages in vitro (Liang et al., 2008b). Recently, Matsushita et al. (2009) has identified ZC3H12A/MCPIP1 as an immune response modifier that has an essential role in preventing immune disorder through generating Zc3h12a KO mice. In this study, we independently developed an *Mcpip1*/Zc3h12a-null mouse line. Disruption of *Mcpip1* leads to severe inflammatory syndromes, including growth retardation, splenomegaly, lymphadenopathy, and premature death. In the *Mcpip1*^{-/-} mice, inflammatory cytokines, including TNF and MCP-1, were increased by >2.5-fold. BMDMs were more sensitive to LPS stimulation.

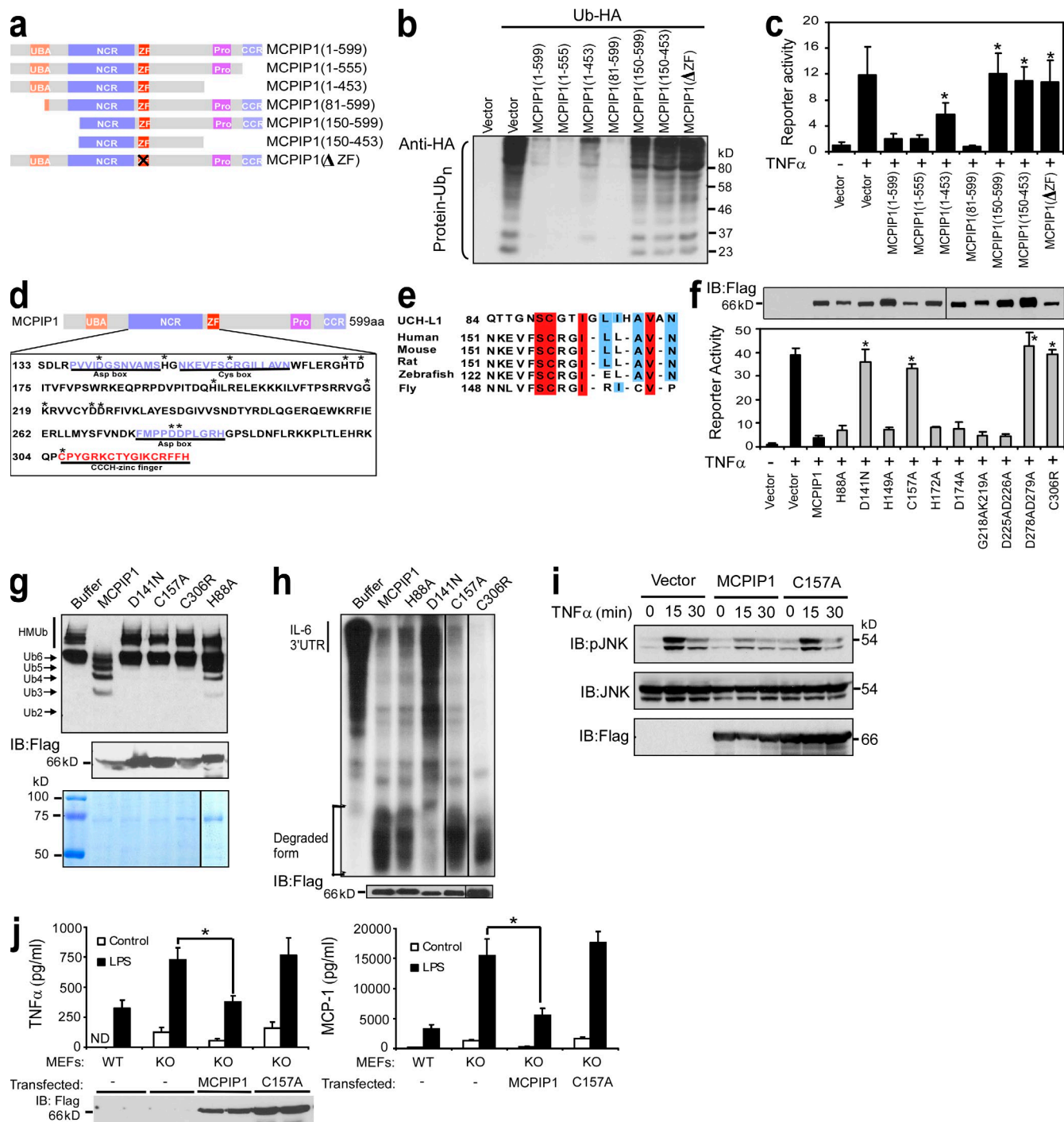


Figure 6. MCPIP1 contains a novel DUB domain. (a) The putative functional domains and conserved regions of MCPIP1 and the deletion strategy are shown. (b) HEK293 cells were cotransfected with HA-ubiquitin (Ub) and with the indicated MCPIP1 constructs. The overall ubiquitination of cellular proteins was examined by immunoblot with HA-specific antibody. Results are representative of at least three independent experiments. (c) The influence of each MCPIP1 construct on NF- κ B reporter activity in TNF-stimulated HEK293 cells. Values are expressed as the fold increase in luciferase expression (\pm SD) compared with the vector alone ($n = 4$; *, $P < 0.01$ vs. MCPIP1(1-599) group). Results are representative of three independent experiments. (d) The putative Asp box and Cys box in the NCR are shown. Point mutants of MCPIP1 were generated as marked with asterisks. (e) Alignment of the Cys region of MCPIP1 with that from the UCH-L1 DUB domain. The NCBI Protein database accession numbers for human UCH-L1 and MCPIP1 of human, mouse, rat, zebrafish, and fly are P09936, NP_079355, NP_766373, NP_001071139, XP_001338688, and NP_650863, respectively. Red indicates completely conserved. Blue indicates mostly conserved. (f) The influence of each MCPIP1 construct on NF- κ B reporter activity in TNF-stimulated HEK293 cells. Values are expressed as the fold increase in luciferase expression (\pm SD) compared with the vector alone ($n = 4$; *, $P < 0.01$ vs. MCPIP1 WT group). Results are representative of three independent experiments. The expression of each construct was detected by immunoblot (IB) with anti-Flag antibody as shown.

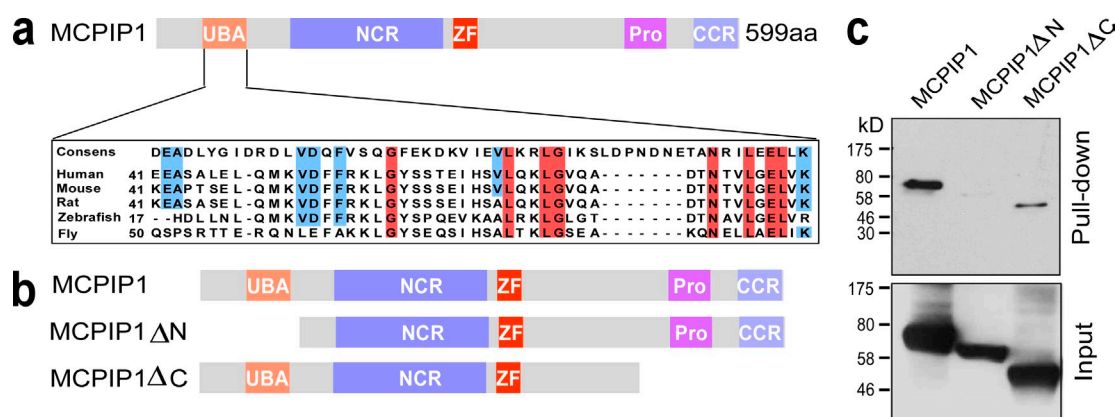


Figure 7. MCPIP1 interacts with ubiquitin through the UBA at the N terminus. (a) The alignment of the putative UBA of MCPIP1 proteins from human, mouse, rat, zebrafish, and fly with UBA consensus sequence. Red indicates completely conserved amino acids. Blue indicates partially conserved amino acids. (b) HEK293 cells were transfected with Flag-tagged MCPIP1 and its C-terminal or N-terminal truncated mutants as indicated. (c) The cell lysates from transfected cells were incubated with ubiquitin-agarose and washed and analyzed by immunoblot with anti-Flag (pull-down). Input levels of each construct in the cell lysates were examined by immunoblot with anti-Flag. Results are representative of two independent experiments.

These results further support the idea that *Mcpip1/Zc3h12a* is a critical modulator of inflammatory response and immune homeostasis. How does MCPIP1 regulate inflammatory response? Two groups recently reported that MCPIP1 might be functioning as an RNase to promote the degradation of some inflammatory mRNA such as IL-6 and IL-1 β (Matsushita et al., 2009; Mizgalska et al., 2009). In this study, we have found that MCPIP1 also acts as a deubiquitinase to negatively regulate JNK and NF- κ B signaling by targeting TRAFs, which suggests that MCPIP1 may control inflammatory response and immune homeostasis by multiple mechanisms. Interestingly, TTP, another well-studied CCCH Zn finger-containing protein and a well-known RNA-destabilizing factor, was also reported to regulate NF- κ B signaling (Liang et al., 2009; Schichl et al., 2009).

Ubiquitination is a fundamental mechanism of signal transduction that regulates immune responses and many other biological processes (Sun, 2008). Similar to phosphorylation, ubiquitination is a reversible process that is counter-regulated by DUBs. Several DUBs such as CYLD, A20, DUBA, and Cezanne are involved in the regulation of inflammatory signaling in macrophages and immune cells (Evans et al., 2003; Reiley et al., 2004; Wertz et al., 2004; Kayagaki et al., 2007). In contrast to CYLD and DUBA, which attenuate NF- κ B

and JNK signaling by selectively removing K63-linked poly-ubiquitin chains from TRAFs, RIP, and NEMO, MCPIP1 also cleaves K48-linked polyubiquitin chains in vitro. Another difference is that CYLD does not affect the global cellular ubiquitination. However, MCPIP1 markedly affects the overall ubiquitination of cellular proteins, which implies that MCPIP1 may target more substrates and regulate multiple biological processes that need to be further identified. As evidence, *MCPIP1*^{-/-} mice showed much more complicated and severe phenotypes than CYLD mutated mice (Jin et al., 2008).

The direct targets of MCPIP1 deubiquitinase in vivo are still uncertain. We observed that the ubiquitination of TRAF2, TRAF3, TRAF6, RIP, and I κ B α was significantly decreased by MCPIP1 expression. Moreover, loss of *Mcpip1* led to a drastic increase in basal and LPS-induced ubiquitination of TRAF2, TRAF3, and TRAF6 but not RIP. Because TRAF2 and TRAF6 are upstream effectors of RIP and I κ B α , the ubiquitination changes of RIP and I κ B α caused by MCPIP1 expression may be indirect. MCPIP1 deubiquitinase may directly target to TRAF family members and regulate JNK and NF- κ B signaling. At least we showed that the purified cellular ubiquitinated TRAF2 and TRAF3 can also be cleaved by MCPIP1 purified protein in vitro. Nevertheless, the direct targets of MCPIP1 deubiquitinase in vivo need to be further characterized.

(g) Flag-tagged MCPIP1 proteins were purified from HEK293 cells and incubated with K63-linked Ub₆ at 37°C for 16 h. The reactions were examined by immunoblotting with ubiquitin-specific antibody. The images of Coomassie blue-stained protein gel and immunoblot with anti-Flag are shown at the bottom. Results are representative of three independent experiments. HMUb, high molecular weight ubiquitin. (h) Flag-tagged MCPIP1 proteins were incubated with ³²P-labeled transcripts from the IL-6 3' UTR at 30°C for 7 h. Samples were run on a 6% polyacrylamide-6M urea gel. The gels were dried and autoradiographed. The proteins loaded were detected by immunoblotting with anti-Flag as shown on the bottom. Two independent experiments showed similar results. (f-h) Vertical black lines indicate that intervening lanes have been spliced out. (i) HEK293 cells were transfected with control vector, Flag-MCPIP1, or Flag-C157A. After 24 h, the transfected cells were quiescent for 16 h and then stimulated with 100 ng/ml LPS for the indicated times. WCEs were subjected to immunoblot analysis with the antibodies as indicated. Two independent experiments showed similar results. (j) *Mcpip1*^{-/-} MEFs were transfected with Flag-MCPIP1 or Flag-C157A. After 24 h, the transfected cells and untransfected MEFs were stimulated with LPS for 5 h. TNF and MCP-1 in medium were measured by ELISA. Data are represented as mean \pm SD ($n = 3$; *, $P < 0.01$). The expression of MCPIP1 constructs was detected by immunoblotting with anti-Flag as shown on the bottom.

MCPIP1 may selectively regulate different signal pathways. As shown in our results, MCPIP1 is a potent suppressor of both LPS- and IL-1 β -induced JNK activation. However, MCPIP1 just moderately affects LPS-induced IKK activation but does not affect IL-1 β -induced IKK activation. This is consistent with the other observation that ubc13, a unique E2 for K63-linked ubiquitination, is essential for JNK activation but not critical for NF- κ B activation (Yamamoto et al., 2006). Interestingly, overexpression of MCPIP1 potently suppresses LPS- and cytokine-induced NF- κ B-dependent reporter activity. It suggests that MCPIP1 may also target the downstream effectors of NF- κ B signaling. Matsushita et al. (2009) did not find any difference between *Mcpip1*^{-/-} and *Mcpip1*^{+/-} mice with respect to LPS-induced NF- κ B and AP-1 activation in peritoneal macrophages. The reason for this discrepancy is not yet known but may be caused by the different cell types used. In the lung from *Mcpip1*^{-/-} mice, both JNK and IKK were constitutively activated. However, JNK and IKK in BMDMs were not constitutively activated; instead, their activation was enhanced by LPS stimulation. The reason for this difference may be because there are many mature macrophages in the lung and these macrophages are exposed to the air.

The human genome encodes almost 100 DUBs (Nijman et al., 2005). Based on their domain structures, these DUBs can be divided into five families: UCHs, ubiquitin-specific proteases, otubain proteases, Machado-Joseph disease proteases, and JAB1/PAB1/MPN domain-containing metalloenzymes. Each contains specific but conserved DUB domains. MCPIP1 does not belong to any of the five known families as it does not contain any of the five specific domains based on sequence alignment. However, we characterized the NCR containing intrinsic deubiquitinating activity, which has remote sequence homology to the UCH domain (27%). In addition, MCPIP1 contains two conserved sequence motifs, the Cys and Asp boxes, in its catalytic domain, which is common in Cys proteases. Mutation of several histidines did not impair the deubiquitinating activity, suggesting that the His box may be located outside of the NCR or that MCPIP1 lacks the His box as does CYLD. Some of the biochemical characteristics of DUB of MCPIP1 are similar to other DUBs. Its deubiquitinating activity can be completely abolished by NEM, a Cys protease inhibitor, which is consistent with MCPIP1 being a Cys protease. In addition, the deubiquitinating activity of MCPIP1 is not dependent on Mg²⁺ and ATP but is inhibited by Zn²⁺ and Mn²⁺, as reported for another DUB (Matsui et al., 1982). Thus, MCPIP1 may represent a member of a novel DUB family.

It was recently reported that MCPIP1 has RNase activity and may control immune response through regulating inflammatory mRNA decay (Matsushita et al., 2009). In this study, we provide evidence that MCPIP1 also acts as a DUB to negatively regulate inflammatory signaling. By deletion experiments, MCPIP1 deubiquitinase domain has also been mapped on NCR regions as its RNase domain. However, serial mutagenesis showed that Asp141, Cys157, and Cys306 in the Asp box, Cys box, and CCCH Zn finger, respectively, are

critically important for its DUB activity. Although Asp141 is also important for its RNase activity, mutation of two other amino acids, C157A and C306R, that abolished DUB activity did not affect its RNase activity. In an NF- κ B reporter assay, the D141, C157, C306, and D278/D279 mutants resulted in the loss of MCPIP1 repressor activity on NF- κ B-dependent reporter activation. Furthermore, C157A mutant, which lost DUB activity but maintained RNase activity, also lost repressor effect on JNK activation and LPS-induced inflammatory cytokine production. These results suggest that the inhibition of MCPIP1 on inflammatory signaling is dependent on its DUB activity. The relationship between the two enzymatic activities is under investigation.

There are many interesting questions that remain to be answered. How does MCPIP1 protein target to specific substrates in response to environmental cues? We postulate that the specific adaptor proteins may recruit MCPIP1 to different substrates and exert different functions: degrading mRNA or cleaving polyubiquitin. In fact, MCPIP1 contains a single conserved CCCH ZF and a Pro-rich domain, which may mediate the interaction with adaptor proteins. There is also a functional UBA at the N terminus of MCPIP1. MCPIP1 may interact with ubiquitinated protein through this domain. However, N-terminal truncation of MCPIP1 without this UBA also decreased protein ubiquitination in cells as WT MCPIP1, suggesting that this UBA may not be required for its DUB activity in vivo. MCPIP1 may also bind to its substrates through other means. Nevertheless, identification of the binding partners of MCPIP1 will greatly enhance our understanding of MCPIP1 functions. In addition, it would be interesting to find out the function of the other three members of the MCPIP1 family. In summary, we here identified MCPIP1 as a deubiquitinase that negatively regulates JNK and NF- κ B signaling by targeting TRAFs and defined a novel DUB domain, which suggests that MCPIP1 may control inflammation and immune response by multiple mechanisms and would be a potential therapeutic target for treatment of human inflammatory diseases.

MATERIALS AND METHODS

Generation of *Mcpip1*^{-/-} mice. The *Mcpip1* targeting construct was built using the pN-Z-TK2 vector, which contains a nuclear LacZ cassette and a neomycin-resistance gene (provided by R. Palmiter, University of Washington, Seattle, WA). The 4.2-kb 5' arm and 2.7-kb 3' arm were amplified by PCR using a BacPac vector (RP23-262L9) as template, which was from C57BL/6 mice and contained the *Mcpip1* gene. The amplified DNA fragments were confirmed by sequencing and inserted upstream and downstream of the nuclear LacZ-Neo cassette by KpnI-Sall and XhoI-NotI, respectively. The targeting construct was linearized and electroporated into C57BL/6-derived embryonic stem cells (inGenious Targeting Laboratory, Inc.). After selection with G418, drug-resistant clones were picked up and screened by PCR and Southern blot analysis. Two *Mcpip1*-targeted embryonic stem clones were individually microinjected into blastocysts derived from C57BL/6 mice and transferred to pseudopregnant females. The resulting chimeric mice were bred to C57BL/6 to obtain germline transmission of the mutant allele. Resulting *Mcpip1*^{+/-} mice were intercrossed to generate *Mcpip1*^{-/-} mice. All animal experiments were performed with the approval of the institutional animal care and use committee at the University of Central Florida.

Cells. The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM Gln, 100 U/ml penicillin and streptomycin, and 10% FBS (endotoxin <1 ng/ml; Sigma-Aldrich). HEK293 cells were cultured in DME supplemented with 2 mM Gln, 100 U/ml penicillin and streptomycin, and 10% FBS. Mouse BMDMs were generated from BM stem cells obtained from femurs of the male *Mcpip1^{+/+}* and *Mcpip1^{-/-}* mice (6 wk old). After lysis of the red blood cells, 4×10^6 of BM stem cells were inoculated in a 6-well plate with complete DME culture medium containing 10% FBS, 30% L-medium, and standard supplements. After 5-d culture, the fully differentiated and matured BMDMs were quiescent for 24 h in macrophage serum-free medium and then treated with 100 ng/ml LPS for different time points.

Plasmids. NF- κ B-TK-Luc, AP-1-TK-Luc, and GAS-TK-Luc were purchased from Agilent Technologies. Flag-TRAF2 plasmid was provided by Y. Sun (University of Michigan, Ann Arbor, MI). Flag-TRAF3 plasmid was provided by G. Chen (University of California, Los Angeles, CA). Flag-TTP was provided by J. Lykke-Andersen (University of California, San Diego, La Jolla, CA). HA-ubiquitin was obtained through Addgene from T. Dawson (Johns Hopkins University, Baltimore, MD). MCPIP1-GFP plasmid was described previously (Zhou et al., 2006; Liang et al., 2008b). HA-tagged MCPIP1 expression plasmid was generated by inserting human MCPIP1 coding fragment into pcDNA3-HA vector at EcoRI and XbaI sites. Flag-tagged MCPIP1 expression plasmid was generated by inserting human MCPIP1 coding fragment into pCMV-MAT-Tag-Flag-1 vector (Sigma-Aldrich) at HindIII and BamHI sites. MCPIP1 serial deletion plasmids were generated by inserting the PCR-amplified fragments into pCMV-MAT-Tag-Flag-1 vector at EcoRI and XbaI sites. MCPIP1 Δ ZF plasmid was generated by mutating K311C312 into GG using Flag-MCPIP1 as template. MCPIP1 serial point mutants were generated using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) using Flag-MCPIP1 as template. IL-6 3' UTR (1–403) was amplified by PCR and inserted into pCR-Blunt II-TOPO (Invitrogen).

Reagents. The MCPIP1 rabbit polyclonal antibody was prepared against the human recombinant MCPIP1 protein as described previously (Zhou et al., 2006; Liang et al., 2008b). Except antiubiquitin antibody from Boston Biochem, all other antibodies used in this paper were purchased from Cell Signaling Technology. Anti-Flag M2 affinity gel, anti-HA immunoprecipitation kit, protein G immunoprecipitation kit, LPS (*Escherichia coli* 026:B6-derived), human recombinant IFN- γ , IL-1 β , and TNF, NEM, and other chemical reagents were purchased from Sigma-Aldrich. Ubiquitin chains, ubiquitin-AFC, ubiquitin-agarose, IsoT, ubiquitin aldehyde, and MG132 were purchased from Boston Biochem. Purified Flag-BAP, an N-terminal Flag fusion protein of *E. coli* BAP, was purchased from Sigma-Aldrich.

Transient transfection and luciferase assay. Transfection and luciferase assay were performed as described previously (Liang et al., 2008b). All transfections were performed in triplicate and repeated at least two times.

Protein isolation and immunoblotting. Protein isolation and immunoblotting were essentially performed as described previously (Liang et al., 2008b). To facilitate the detection of the phosphorylated or ubiquitinated proteins in cell lysates, radioimmunoprecipitation assay (RIPA) buffer was added with protease inhibitor cocktails (Sigma-Aldrich), phosphatase cocktail I and II (Sigma-Aldrich), 20 μ M NEM, and 2 μ M ubiquitin aldehyde.

Coimmunoprecipitation. For endogenous immunoprecipitations, the transfected HEK293 cells were lysed in CellLytic M Cell lysis buffer with protease inhibitors, phosphatase inhibitors, NEM, and ubiquitin aldehyde. Lysates were incubated with specific antibodies. The immune complexes were collected by incubation (2 h at 4°C) with protein G-agarose (Sigma-Aldrich). Coimmunoprecipitation assays were performed by using anti-Flag matrix (Sigma-Aldrich) according to the manufacturer's instruction. After extensive washing, immunoprecipitated proteins were resolved by 6–10%

SDS-PAGE and analyzed by immunoblotting with Flag, HA, or MCPIP1 antibodies. Membranes were developed with enhanced chemiluminescence (GE Healthcare).

RNA isolation, RNA blot, and quantitative PCR (QPCR). Total RNA was isolated from cells or tissues using RNA STAT-60 reagent (Tel-Test, Inc.) according to the manufacturer's instruction. RNA blot and QPCR were performed as described previously (Liang et al., 2008b).

In vivo deubiquitination assay. HEK293 cells were transfected with HA-ubiquitin as well as other plasmids as indicated. 24 h later, cells were stimulated with TNF for 2 h, and the cells were harvested using RIPA buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail, 20 μ M NEM, and 2 μ M ubiquitin aldehyde. The whole cell extracts (WCEs) were directly subjected to immunoblot analysis with HA antibody or further immunoprecipitated using specific antibodies as indicated and then analyzed by immunoblot with HA antibody.

Purification of Flag-MCPIP1 and its mutant proteins. Flag-MCPIP1 or its mutants were transfected into HEK293 cells individually. 48 h later, the transfected cells were harvested using RIPA buffer containing protease inhibitor cocktail. The cell lysates were incubated with Flag M2 beads (Sigma-Aldrich) overnight with rotation at 4°C. After washing three times, the Flag-MCPIP1 was eluted by 3xFlag elution buffer.

Cell-free deubiquitination assay. 0.5 μ g K48- or K63-linked ubiquitin chains (Boston Biochem) were mixed with purified Flag-MCPIP1 or its mutant proteins in DUB assay buffer (50 mM Hepes-NaOH, pH 8.0, 10% glycerol, and 3 mM DTT) at 37°C for 16 h or the indicated times. Samples were prepared for immunoblot analysis with ubiquitin antibody (Boston Biochem).

In vitro cleavage assay of cellular ubiquitinated TRAFs. Flag-TRAF2 or Flag-TRAF3 was cotransfected with HA-ubiquitin into HEK293 cells. 24 h later, the transfected cells were stimulated with TNF for 15 min. The cells were harvested using RIPA buffer containing protease inhibitor cocktail and NEM. The cell lysates were incubated with Flag M2 beads overnight with rotation at 4°C. After washing three times with TBS buffer, Flag-TRAF2 or Flag-TRAF3 was eluted by 3xFlag elution buffer. 15 μ l of eluted Flag-TRAF2 or Flag-TRAF3 was incubated with purified Flag-MCPIP1 in DUB assay buffer at 37°C for 4 h. Samples were prepared for immunoblot analysis with HA and Flag antibodies.

In vitro RNA cleavage assay. The pTOPO-IL-6 3' UTR (1–430) plasmid was used as a template for the synthesis of RNA having an IL-6 3' UTR sequence. In vitro RNA synthesis and 32 P labeling were performed using the Riboprobe in vitro transcription system (Promega) according to the manufacturer's instructions. Equal amounts of in vitro transcribed 32 P-labeled RNAs (5,000 cpm) were incubated with 2 μ g MCPIP1 or its mutants at 30°C for 7 h in 25 mM Hepes, pH 8.0, 50 mM potassium acetate, 5 mM DTT, and 5 mM Mg acetate. The cleaved RNA was purified with TRIZOL (Invitrogen). Samples were run on a 6% polyacrylamide–6M urea gel in 1 \times Tris/Borate/EDTA buffer after RNA sample buffer and loading buffer were added to the samples, and samples were heated at 80°C for 10 min according to the manufacturer's recommendations. Gels were dried and autoradiographed.

Ubiquitin chain binding assay. Flag-MCPIP1 and its mutant proteins purified from HEK293 cells were incubated with ubiquitin-agarose (Boston Biochem) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton buffer overnight at 4°C. Immunoprecipitants were washed with the same buffer for three times and subjected to immunoblot analysis with Flag antibody.

Statistics. Data were expressed as mean \pm SD. For comparison between two groups, the unpaired Student's *t* test was used. For multiple comparisons, analysis of variance followed by unpaired Student's *t* test was used. A value of *P* < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows the tissue distribution of Mcpip1 mRNA in mice. Fig. S2 shows the relative mRNA levels of inflammatory genes, including *TNF*, *IL-6*, *IL-1 β* , and *iNOS*, in intestine, lung, and thymus from *Mcpip1*^{+/+} and *Mcpip1*^{-/-} mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092641/DC1>.

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Author contributions: M. Fu designed the research and analyzed data. J. Liang, M. Fu, T. Lei, J. Wang, and D. Qi performed the experiments. M. Fu generated *Mcpip1*^{-/-} mice. Y. Saad constructed the serial MCP1 point mutants and performed RNase assay. P.E. Kolattukudy and Q. Yang provided advice and critically read the manuscript. M. Fu wrote the manuscript.

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