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MCPIP1 negatively regulate cellular antiviral innate immune responses through DUB and disruption of TRAF3-TBK1- $\text{IKK}\epsilon$ complex

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ABSTRACT

IFN β innate immune plays an essential role in antiviral immune. Previous reports suggested that many important regulatory proteins in innate immune pathway may be modified by ubiquitin and that many de-ubiquitination (DUB) proteins may affect immunity. Monocyte chemotactic protein-inducing protein 1 (MCPIP1), one of the CCH Zn finger-containing proteins, was reported to have DUB function, but its effect on IFN β innate immune was not fully understood. In this study, we uncovered a novel mechanism that may explain how MCPIP1 efficiently inhibits IFN β innate immune. It was found that MCPIP1 negatively regulates the IFN β expression activated by RIG-I, STING, TBK1, IRF3. Furthermore, MCPIP1 inhibits the nuclear translocation of IRF3 upon stimulation with virus, which plays a key role in type I IFN expression. Additionally, MCPIP1 interacts with important modulators of IFN β expression pathway including IPS1, TRAF3, TBK1 and $\text{IKK}\epsilon$. Meanwhile, the interaction between the components in TRAF3-TBK1- $\text{IKK}\epsilon$ complex was disrupted by MCPIP1. These results collectively suggest MCPIP1 as an innate immune regulator encoded by the host and point to a new mechanism through which MCPIP1 negatively regulates IRF3 activation and type I IFN β expression.

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1. Introduction

The innate immune system stands the first line of the defense that protects the host from viral intrusion, depending on pattern recognition receptors (PRRs) and the corresponding pathways [1–4]. The pathogen-associated molecular patterns (PAMPs) of the invading viruses may be recognized by PRRs. Then, the adaptor proteins (TRIF for TLR3, MyD88 for TLR7/8/9, MAVS/IPS-1 for RIG-I) would be recruited, and the infecting signals would be transmitted to the downstream kinase complexes, followed by the activation of transcription factors, such as interferon regulatory factor-3 (IRF-3), nuclear factor κB (NF- κB) and ATF-2/c-jun [2,5–7]. Upon activated, the transcription factors may regulate the expression of type I Interferons which induce the expression of IFN-stimulated genes (ISGs) and ultimately establish the antiviral function of the host [4,8–10].

Many important regulatory proteins in innate immune pathways may be modified by ubiquitin [11–13]. On the other hand, Many de-ubiquitination (DUB) proteins may affect immunity. We have found that the papain-like proteases (PLPs) encoded by coronavirus (CoV) reduce the ubiquitinated modification of essential regulatory molecules of IFN innate immune pathway, such as RIG-1, MAVS, STING, TRAF3, TBK1 and IRF3. Additionally, CoV PLPs negatively regulate IFN expression of the host, acting as both deubiquitinases and IFN antagonists [14–16].

The C-terminal phosphorylation and activation of IRF3 requires noncanonical $\text{I}\kappa\text{B}$ kinases, TBK1 or IKK, which form signaling complexes with TRAF family members that transmit upstream signals to downstream effectors resulting in the expression of type I IFN. Previous studies suggest that TRAF family members are involved in the regulation of antiviral immune responses [17–20]. We have reported that SARS-CoV PLP blocks the ubiquitination of STING-TRAF3-TBK1 complex and disrupts STING-TRAF3-TBK1 complex [21].

MCPIP1 (monocyte chemotactic protein-induced protein 1), a negative regulator of macrophage activation, was also found to negatively regulate JNK and NF- κB activity by removing ubiquitin moieties from proteins including TRAFs [22]. In this study, we observed that MCPIP1 inhibits the IFN β expression activated by

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RIG-I, STING, TBK1, IRF3. Additionally, MCP1P1 inhibits the nuclear migration of IRF3. Furthermore, MCP1P1 interacts with TRAF3 and disrupts TRAF3-TBK1-IKK ϵ complex which is essential for the activation of IFN β production pathway. This report suggests that MCP1P1 may act as an IFN antagonist antiviral protein encoded by the host and uncovers the mechanism undergoes by MCP1P1 to inhibit IFN β innate immune pathway.

2. Materials and methods

2.1. Cell and plasmids

HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FCS supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).

The plasmid expressing V5-MCPIP1 was cloned according to NCBI Reference Sequence NM_025079.2. The reporter plasmids IFN β -Luc, PRD (III-I)4-Luc, NF- κ B-Luc, and plasmids NL63-PLP2-TM, Myc-IRF3, Flag-IRF3, Flag-IRF7, Flag-IPS-1, Flag-STING, Flag-TBK1, Flag-IKK ϵ , Flag-RIG-IN, A20, HA-TRAF3 were described previously [14,21,23,24].

2.2. SiRNA preparation

MCPIP1-target siRNA sequence (5'-CCAGCGUGUAUA-CUAAGCUTT-3') were designed and chemically synthesized by Genescript Co. A siRNA with the sequence of 5'-

UUCUCCGAACGUGUCACGU-3' was selected as the negative control siRNA (NC-siRNA) as described previously [25,26].

2.3. Luciferase reporter gene assay

HEK293T cells were transfected with the indicated stimulator plasmid DNA (Flag-IPS-1, Flag-STING, Flag-TBK1, Flag-IKK ϵ , Flag-RIG-IN), reporter plasmid DNA (pRL-TK, IFN β -Luc, or PRD (III-I)4-Luc) and either V5-MCPIP1 or NL63PLP2-TM/A20 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and incubated for 24 h. Then, firefly luciferase and renilla luciferase activities were assayed using the Dual Luciferase Reporter Assay Kit (Promega). Data were shown as mean relative luciferase (firefly luciferase activity divided by Renilla luciferase activity) with standard deviation from a representative experiment carried out in triplicate. The luciferase assay was performed as described previously [14,15].

2.4. Immunofluorescence assay

HEK293T or HeLa cells were grown to confluence in a six-well plate. Plasmid DNA expressing V5-MCPIP1 and Flag-IRF3 (1.2 μ g per well) were transfected. Twenty-four hours later, the cells were infected or mock-infected by SeV (100 HAU) and incubated for 18 h. Fluorescence was examined by using a confocal microscope. Immunofluorescence assay was performed as described previously [15].

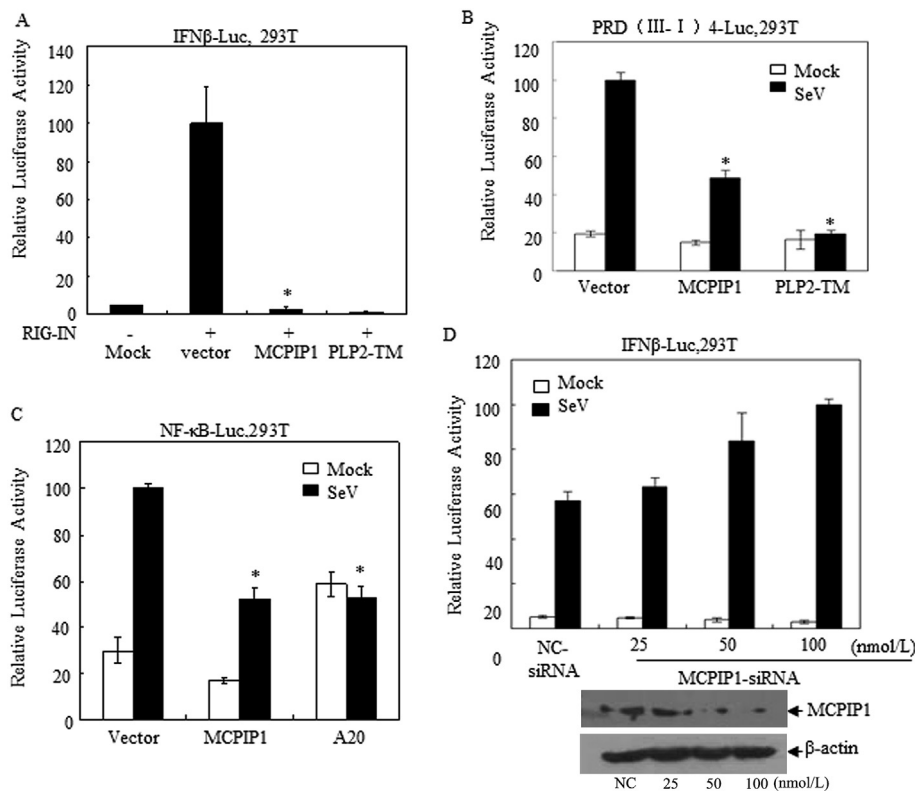


Fig. 1. MCP1P1 inhibits the expression of IFN β in cells. **a** HEK293T cells were co-transfected with the plasmids which expressing IFN β -Luc and either MCPIP1 or NL63 PLP2-TM (positive control). Twenty-four hours later, cells were harvested and subjected to a Dual-luciferase assay. **b** HEK293T cells were transfected with PRD (III-I) 4-Luc and either MCPIP1 or NL63 PLP2-TM expressing plasmids (positive control). Twenty-four hours later, cells were harvested and subjected to a Dual-luciferase assay. **c** HEK293T cells were transfected with the plasmids which expressing NF- κ B-Luc and either MCPIP1 or A20 (positive control). Twenty-four hours later, cells were harvested and subjected to a Dual-luciferase assay. **d** HEK293T cells were respectively co-transfected with the plasmids which expressing IFN β -Luc and MCP1P1-siRNA (25, 50, 100 nmol/L). NC-siRNA (sequence described in the text) was used as control. Twenty-four hours later, cells were harvested and subjected to Dual-luciferase assay. The expression of MCP1P1 was also detected by Western-blotting assay. The results were expressed as mean relative luciferase (firefly luciferase activity divided by Renilla luciferase activity) with standard deviation from a representative experiment carried out in triplicate. Data were presented as mean \pm SEM, $n = 3$. * $p < 0.05$.

2.5. Co-immuno-precipitation (Co-IP) analysis

HEK293T cells were seeded in 100-mm dishes at a density of 1×10^6 cells/dish. Twelve hours later, cells were transiently transfected with a total of 10 μ g of empty vector or indicated expression plasmids using Lipofectamine 2000 (Invitrogen). At 48 h post transfection, cells were lysed in a buffer containing 0.5% TritonX-100, 150 mmol/L NaCl, 12.5 mmol/L β -glycerolphosphate, 1.5 mmol/L $MgCl_2$, 2 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L Na_3VO_4 , 2 mmol/L DTT and protease inhibitor cocktail (Sigma). Cell extracts were clarified by centrifugation at $5000 \times g$ at $4^\circ C$ for 10 min, and protein concentration of lysate determined using BCA Protein Assay kit (Bio-med). The protein concentrations in cell lysates were adjusted to 1 μ g/ μ L, and 500 μ L of each lysate was used for co-IP. Lysates were pre-cleared by adding 20 μ L of protein A + G Agarose (Beyotime) and 1 μ g of normal IgG and incubating for 2 h at $4^\circ C$, followed by spinning down the agarose beads. The pre-cleared supernatant was then incubated with the indicated primary antibody [anti-V5 (MBL) or anti-HA (MBL)/anti-Myc (MBL)/anti-Flag (MBL)] with rocking overnight at $4^\circ C$. Thereafter, the beads-antibody-antigen complex was pelleted and washed 3 times with 1 mL of lysis buffer. The protein complexes were then eluted from the beads in 30 μ L of $2 \times$ SDS-PAGE sample buffer by boiling for 10 min. Samples were separated on SDS-PAGE and transferred to PVDF membranes for Western-blotting. Co-IP assay was performed as described previously [14,27].

3. Results

3.1. MCPIP1 is an IFN β antagonist

According to the previous reports, MCPIP1 showed DUB activity targeting to TRAFs [22]. Our previous work demonstrated that several proteins encoded by viral, such as NL63 PLP2, PEDV PLP2, SARS PLpro, and MERS PLpro, showed DUB activity and negatively regulate IFN immune-response [14–16,21].

In order to further investigate the effect of MCPIP1 on IFN β expression, HEK 293T cells were co-transfected with IFN β -Luc, pRL-TK reporter plasmids and MCPIP1 expression construct. Twenty four hours later, cell lysates were prepared and IFN β promoter-driven luciferase activity was assessed. As shown in Fig. 1, it was observed that MCPIP1 inhibits RIG-I activated IFN β expression (Fig. 1A). Moreover, the SeV stimulated IFN β expression pathway depending on IRF3 and NF- κ B was also negatively-regulated by MCPIP1 (Fig. 1B and C). Meaning while, this negative effect of MCPIP1 could no longer be detected when its expression was silenced by MCPIP1 targeting siRNA in our experiments (Fig. 1D). These results suggest that MCPIP1 may be an IFN β antagonist.

3.2. MCPIP1 antagonizes RIG-I/STING/TBK1/IRF3/IRF7 mediated IFN β expression

We have previously discovered that human coronavirus (NL63 and SARS) encoding anti-viral protein PLPs, which negatively

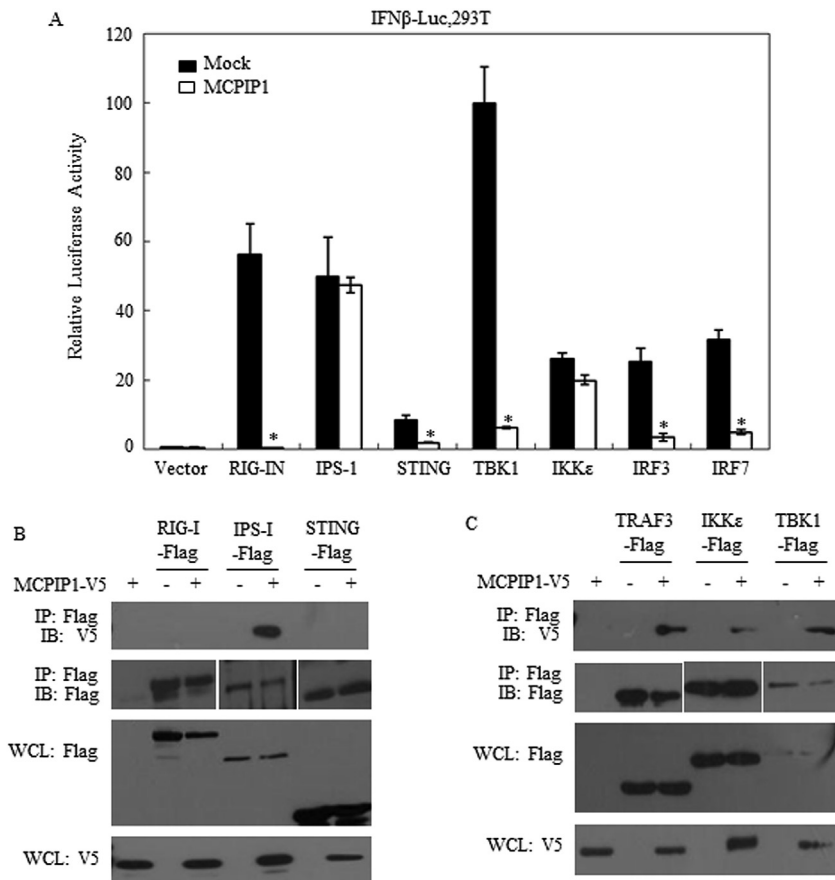


Fig. 2. MCPIP1 negatively regulates the IFN β expression activated by RIG-I, STING, TBK1, IRF3. **a** HEK293T cells were co-transfected with the plasmids which expressing MCPIP1 and IFN β -Luc. RIG-IN, STING, TBK1, IRF3, IRF7, IPS-1 or IKK ϵ was co-transfected to activate IFN expression pathway. Twenty-four hours later, the cells were harvested and subjected to a Dual-luciferase assay. Data were presented as mean \pm SEM, $n = 3$. * $p < 0.05$. **b** HEK293T cells were respectively co-transfected with the plasmids which expressing MCPIP1 and RIG-I, STING, IPS-1, TRAF3, TBK1 or IKK ϵ . Twenty-four hours later, the cells were harvested and Co-IP detection was assayed.

regulate innate antiviral immune response by disrupting STING-mediated IFN induction [14,21]. Based on these findings, we hypothesized that MCP1P1 inhibits the IFN expression pathway through a similar mechanism. To test this hypothesis, we assessed IFN β promoter activity level stimulated by several adaptor proteins, *i.e.* RIG-I, IPS-1, STING, TBK1, IKK ϵ , IRF3 and IRF7, in the presence of MCP1P1. The results in Fig. 2 showed that RIG-I, STING, TBK1, IRF3 and IRF7-mediated IFN β expression were significantly antagonized by MCP1P1 IFN β expression (Fig. 2A). Moreover, Co-IP assay showed that MCP1P1 may interact with IPS-1, TFAF3, TBK1 and IKK ϵ (Fig. 2B). These findings suggested the probable negatively regulating sites of MCP1P1 on IFN β expression pathway.

3.3. MCP1P1 inhibits the nuclear translocation of IRF3

IFN β transcription requires the activation of transcription factors NF- κ B and IRF3 resulting in their subsequent binding to the IFN β promoter [8,28,29]. To investigate the mechanisms used by MCP1P1 to inhibit antiviral IFN β expression, the transcriptional activity of NF- κ B and IRF3 was analyzed using the luciferase reporter gene detection and Co-IP assay. Luciferase reporter gene detection showed that IRF3-dependent IFN β activation was significantly inhibited by MCP1P1 (Fig. 1B). It was uncovered by Co-IP detection that cellular MCP1P1 interacts with IRF3 upon SeV infection (Fig. 3A). Furthermore, to determine whether MCP1P1 prevents IRF3 migration from the cytoplasm to the nucleus, HEK293T cells and HeLa cells infected by SeV were transiently transfected with MCP1P1 and IRF3 expression constructs and the

subcellular localization of the protein was analyzed using confocal microscopy. As shown in Fig. 3B, we observed that IRF3 was activated by SeV infection and migrated from the cytoplasm to the nucleus. Moreover, when MCP1P1 was co-transfected, the nuclear translocation of IRF3 was prevented.

3.4. MCP1P1 disrupts TRAF3-TBK1-IKK ϵ complex

We have previously found that virus-encoded DUB proteins, such as SARS-CoV PLpro-TM, may disrupt STING-TRAF3-TBK1 complex and inhibit IFN β expression [27]. In this research, we detected the effect of MCP1P1 on TRAF3 complex. It was observed that interaction of TRAF3 with TBK1 was disrupted in the presence of MCP1P1 (Fig. 4A, lane 4). In agreement with this, luciferase activity assay showed that the IFN β expression activated by TRAF3 and TBK1 were inhibited by MCP1P1 (Fig. 4B). Meaning while, the interaction of TRAF3 with IKK ϵ , as well as the subsequent IFN β expression, was not disrupted by MCP1P1 (Fig. 4C and D). These results suggest that MCP1P1 may negatively regulating IFN β expression through disruption of TRAF complex.

4. Discussions

MCP1P1 was identified as a regulator of immunity and negatively regulated JNK and NF- κ B activity by removing ubiquitin moieties from TRAFs [22]. However, the mechanisms underwent by MCP1P1 to control immunity have not been deeply understood. In this study, we uncovered a novel mechanism operated by MCP1P1

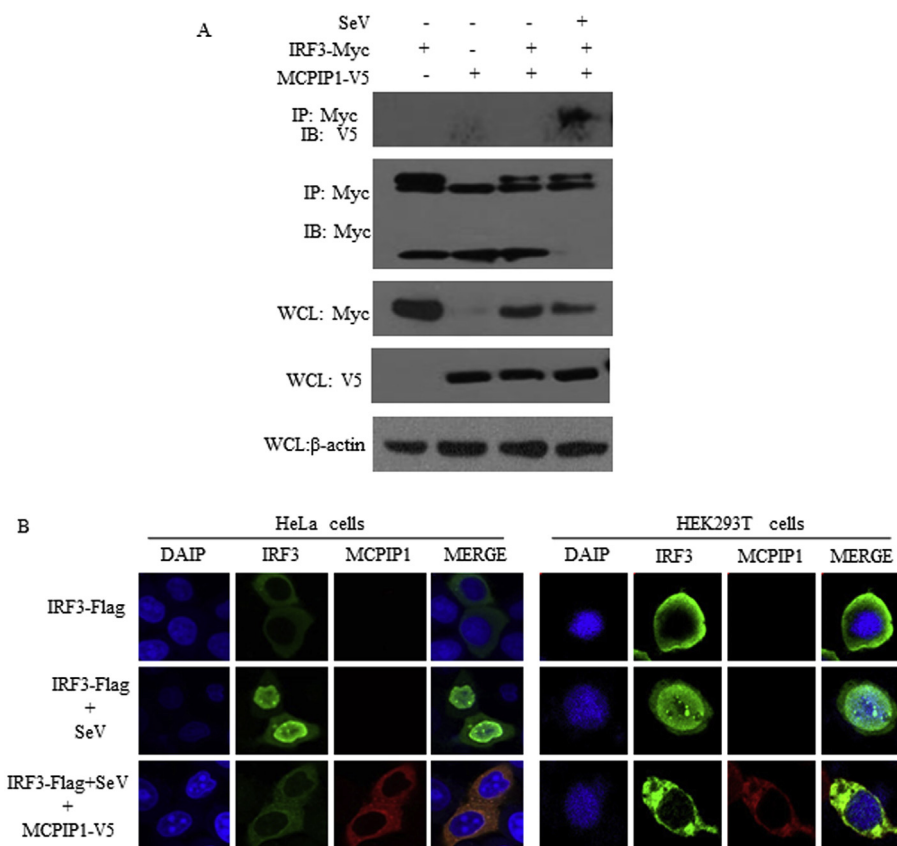


Fig. 3. MCP1P1 inhibits the nuclear translocation of IRF3. **a** HEK293T cells were co-transfected with the plasmids which expressing MCP1P1 and IRF3. Twenty-four hours later, cells were infected with SeV (100 HAU) for another 18 h. Then cells were harvested and detected by Co-IP assay. **b** HEK293T cells and HeLa cells were co-transfected with the plasmids which expressing MCP1P1 and IRF3. Twenty-four hours later, cells were infected with SeV (100 HAU) for another 18 h. Then cells were harvested and detected by immunofluorescence assay.

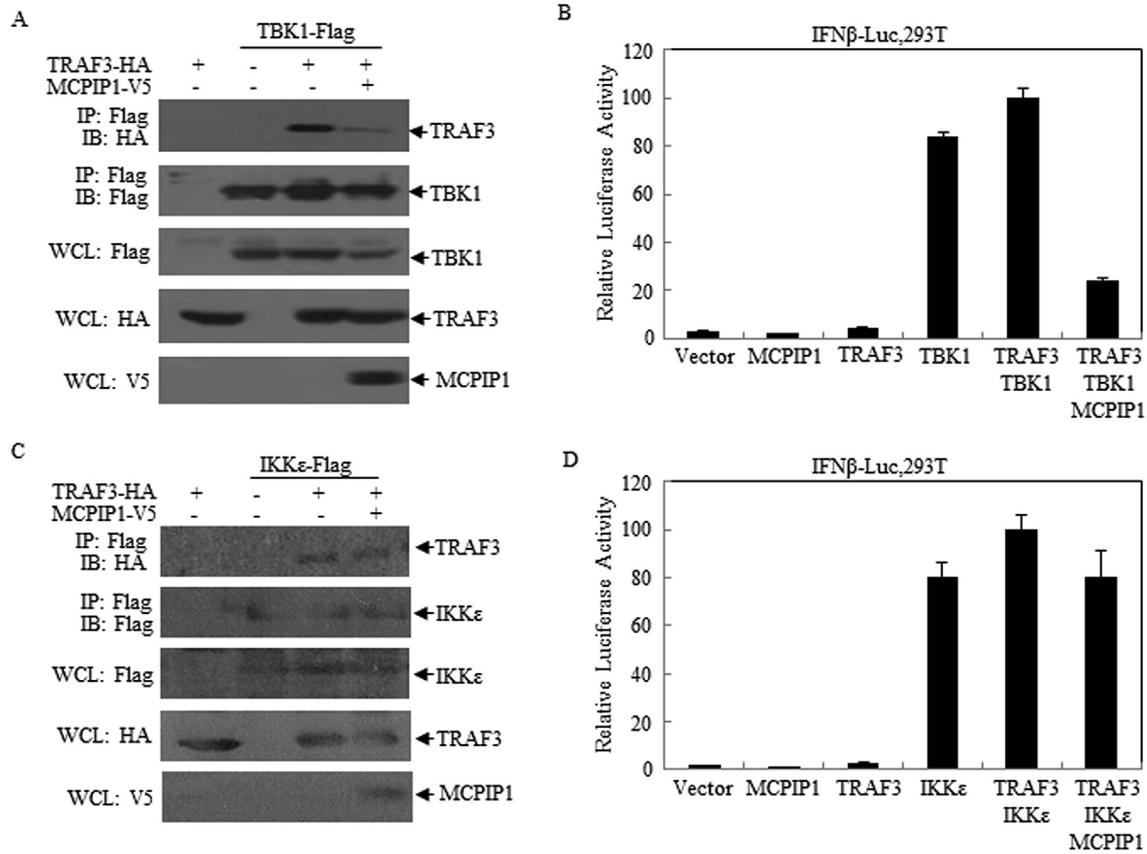


Fig. 4. MCPIP1 disrupts TRAF3-TBK1-IRK ϵ interaction. **a, b** HEK293T cells were respectively co-transfected with the plasmids which expressing MCPIP1, IFN β -Luc, TRAF3 and TBK1. Twenty-four hours later, the cells were harvested and subjected to Co-IP (**a**) or Dual-luciferase (**b**) assay. **c, d** HEK293T cells were respectively co-transfected with the plasmids which expressing MCPIP1, IFN β -Luc, TRAF3 and IKK ϵ . Twenty-four hours later, the cells were harvested and subjected to Co-IP (**c**) or Dual-luciferase (**d**) assay. Data were presented as mean \pm SEM, $n = 3$. * $p < 0.05$.

to negatively regulate IFN β innate immune. We showed that (1) MCPIP1 inhibits NF- κ B activity and IFN β expression pathway activated by RIG-I, STING, TBK1 and IRF3 in a dose-dependent manner. (2) MCPIP1 interacts with IRF3 and inhibits the nuclear translocation of IRF3 upon stimulation with virus. (3) MCPIP1 interacts with the key modulators of IFN β expression pathway including IPS1, TRAF3, TBK1 and IKK ϵ . (4) MCPIP1 disrupts the interaction between the components of TRAF3-TBK1-IRK ϵ complex and inhibits the corresponding IFN β expression. These results collectively suggest a potent mechanism through which MCPIP1 negatively regulates IRF3 activation and IFN β expression.

MCPIP1, also known as ZC3H12A, was identified in human peripheral blood monocytes treated with MCP-1 [30,31]. Several research groups have found that MCPIP1 is an immune response modifier, however, the novel mechanism remain not fully understood [32–36]. MCPIP1 protein contains a CCCH Zn finger domain, which is thought as one of the characteristic structure of antiviral protein [37]. Matsushita et al. reported that MCPIP1 has RNase activity and may control immune response through regulating inflammatory mRNA decay [38].

There is a functional ubiquitin association (UBA) domain at the N terminus of MCPIP1. Liang et al. have discovered that MCPIP1 may be a deubiquitinase and defined a novel DUB domain of MCPIP1 [22]. Ubiquitin and deubiquitin modification emerge as the key mechanisms that regulate the virus-induced type I IFN signaling pathways [39–42]. Several cellular DUB proteins play important roles in negative regulation of host innate immunity. We previously found that several CoV encoded proteins also negatively regulate

IFN β expression of the host through DUB activity [15,16,21]. Based on these, we hypothesize that MCPIP1 may also inhibits IFN β innate immune in cells. The results in this study that MCPIP1 inhibits NF- κ B activity and IFN β expression pathway activated by RIG-I, STING, TBK1 and IRF3 in a dose-dependent manner yielded evidence for our hypothesis.

Innate immune defense mechanism characterized by production of type I interferons was launched by the host upon infection. This innate antiviral response is initiated when viral PAMPs were detected by the host via a number of cellular PRRs, such as the membrane bound Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I), or melanoma differentiation-associated gene 5 (MDA5) [1–3]. These PRRs would recruit different adaptor molecules upon engagement of their respective ligands, submitting signals to downstream kinases that activate IRF3, NF- κ B and other transcription factors that coordinately regulate IFN β transcription [2,7]. IRF3 is a constitutively expressed, latent transcription factor that plays a pivotal role in type I IFN responses. The activation of IRF3 requires specific C-terminal phosphorylation. IRF3 may be activated upon phosphorylation mediated by TBK1 and IKK ϵ , which leads to its homodimerization, nuclear translocation, and collaboration with activated NF- κ B to induce IFN β synthesis [43–45].

IFN β innate antiviral response may be regulated by the host, as well as the viruses. We have previously reported that PLP and 3CLpro encoded by CoVs negatively regulate IFN β expression of the host [14,16]. In this study, we observed that MCPIP1 inhibited NF- κ B activity and IFN β expression pathway activated by RIG-I, STING, TBK1 and IRF3 in the host cells. Furthermore, the formation of

TRAF3-TBK1-IKKe complex is an essential step in the activation of IRF3 [46]. In this study, we found that when MCP1P1 was co-expressed in the cell, the interaction between TRAF3 and TBK1 was significantly disrupted, suggesting the TRAF3-TBK1-IKKe complex and the submitting signals to downstream kinases were blocked. Upon stimulation with virus, IRF3 migrated towards the nuclear. However, the nuclear translocation of IRF3 induced by viral was inhibited when co-expressed with MCP1P1. Taking our previously foundation together with the report by Liang et al. [21,22], we think that the DUB activation of MCP1P1 plays important role in its negatively regulation of IFN expression pathway, which is similar to A20 [12,47–49].

In summary, the results in this study uncovered a novel mechanism used by MCP1P1 to negatively regulated type I IFN antiviral defense. These findings may provide plentiful evidences which may be helpful to deeply understand the working mechanism employed by the antiviral proteins to control innate immune regulation.

Conflicts of interest

The authors have declared that no competing interest exists.

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