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eEF1Bγ is a positive regulator of NF-κB signaling pathway

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ABSTRACT

Mitochondrial antiviral-signaling protein (MAVS), as a critical adaptor of RIG-I signaling, bridges viral RNA recognition and downstream signal activation. However, the regulating mechanisms of MAVS are not well understood. In this study, we demonstrated that eukaryotic elongation factor 1B gamma (eEF1Bγ) activates NF-κB signaling pathway through targeting MAVS. GST-pull down and mass spectrometric analysis suggested that eEF1Bγ binds to the CARD domain of MAVS. The interaction and mitochondrial colocalization of eEF1Bγ and MAVS were further verified by co-immunoprecipitation (co-IP) and immunofluorescence microscopy assays. The dual-luciferase assays showed that ectopic expression of eEF1Bγ significantly promotes the activities of transcription factor NF-κB and promoters of downstream proinflammatory cytokines *Interleukin-8* (*IL-8*) and *Interleukin-6* (*IL-6*). eEF1Bγ increases the abundance of MAVS by promoting its K63-linked polyubiquitination and attenuating its K48-linked polyubiquitination. Besides, proline-rich (Pro) region and CARD domain of MAVS are indispensable for the process of eEF1Bγ mediated ubiquitination. Collectively, these results demonstrated that eEF1Bγ functions as a positive regulator of NF-κB signal by targeting MAVS for activation, which provides a new regulating mechanism of antiviral responses.

1. Introduction

Innate immunity is the first line to protect host cells from viral infection. Following viral invasion, pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), can recognize viral RNAs, which initiate a series of signaling cascades [1,2]. RIG-I-like receptors (RLRs), such as RIG-I and MDA5, detect viral RNA through their C-terminal regulatory domain (RD) [3], leading to a conformational change that exposes the N-terminal caspase activation and recruitment domains (CARDs). Then the exposed CARD domains of RIG-I interacts with the CARD domain of the mitochondrial adaptor protein MAVS (also termed as VISA) [4,5], which catalyzes the conversion of MAVS on the mitochondrial membrane to prion-like aggregates [6]. Subsequently, MAVS activates downstream kinases TBK1/IKKε (IKK-related kinase) and IKKα/β (canonical IKKs), leading to the activation of transcription factors IRF3/7 and NF-κB. Then these transcription factors translocate into nucleus and induce type I interferons and other proinflammatory cytokines [7].

Eukaryotic elongation factor 1 (eEF1) complex catalyzes the transfer of aminoacyl-tRNAs to ribosomes. In higher eukaryotes, eEF1 proteins consist of two families: a G-protein named eEF1A and a nucleotide exchange factor termed eEF1B [8]. eEF1A binds and transfers aminoacyl-tRNA to the A site of the ribosome in a GTP-dependent manner, leading to GTP hydrolysis and release of eEF1A-GDP [9]. Subsequently, guanine nucleotide exchange factor eEF1B promotes the regeneration of eEF1A-GTP for the next cycle of elongation [10]. eEF1Bγ (formerly named eEF1γ), a member of eEF1B complex, does not have nucleotide exchange activity but harbors a stimulatory function for the exchange activity of the complex [8]. It has been reported that eEF1Bγ mRNA overexpresses in tumour tissue, which may provide useful information for predicting the aggressiveness of tumours [11]. The interaction between eEF1Bγ and the keratin cytoskeleton plays critical roles in protein synthesis [12]. Moreover, eEF1Bγ interacts with the 3′UTR of human vimentin mRNA *in vivo* and *in vitro* [13]. And further study suggested that the eEF1Bγ subunit regulates vimentin gene by binding vimentin promoter region, which is important to cellular shape and mitochondria localization [14]. However, how

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eEF1Bγ regulates innate immunity signaling pathway remains largely unknown.

In this study, we uncovered that eEF1Bγ positively regulates NF-κB signal cascades. eEF1Bγ significantly promotes the activation of transcription factor NF-κB and downstream proinflammatory cytokines *Interleukin-8* (*IL-8*) and *Interleukin-6* (*IL-6*). Further study showed that eEF1Bγ forms complex with NF-κB signaling adaptor MAVS and increases the abundance of MAVS by intensifying its K63-linked polyubiquitination and attenuating its K48-linked polyubiquitination. Collectively, eEF1Bγ activates NF-κB signal by enhancing MAVS activity.

2. Materials and methods

2.1. Plasmids

Myc-eEF1Bγ was kindly provided by Prof. Claudio Passananti (Rome, Italy). Flag-MAVS were gifts from Prof. Xin Ye at Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). MAVS truncations, including HA-ΔTM, HA-ΔProΔTM and HA-ΔCARDΔTM, were kindly provided by Prof. Zhijian Chen at South-western Medical Center (Dallas, TX); and GST-CARD was generated by cloning corresponding cDNAs into pGEX4T-1 vector (Invitrogen). His/Myc-Ub was from Prof. Eli Song at Institute of Biophysics, Chinese Academy of Sciences, and HA-tagged wild-type Ub, K48-Ub and K63-Ub was friendly provided by Prof. Feng Shao (NIBS, China). Expression vectors encoding NF-κB-Luc, IL-8-Luc and IL-6-Luc were gifts from Ying Zhu (Wuhan University, Wuhan, China).

2.2. Reagents

The mouse antibodies against Myc (sc-40), HA (sc-7392) and β-actin (47778) were from Santa Cruz Biotechnology (CA, USA). The mouse anti-Flag (F3165) was ordered from Sigma (USA). The goat HA (A00168) and rabbit Myc (A00172) antibodies were bought from Genscript (Nanjing, China). Protein G beads and GST beads were purchased from Santa Cruz Biotechnology and GE Healthcare (USA), respectively. The dual-luciferase reporter assay system was bought from Promega (Wisconsin, USA).

2.3. Cell culture and transfection

293T and Hela cells were cultured in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 °C in 5% CO_2. Indicated plasmids were transfected into cells with Entranster-H (Engreen, Beijing, China).

2.4. Dual-luciferase reporter assay

293T cells were transfected with plasmids encoding NF-κB, IL-6 or IL-8 luciferase reporter gene together with pRL-TK and other plasmids for 24 h. Then collected and lysed cells. Subsequently, luciferase activity was measured with the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols. Data were normalized by the ratio of firefly luciferase activity to renilla luciferase activity.

2.5. Immunoblot and immunoprecipitation

Cells were harvested and lysed with 1× RIPA buffer (Cell Signaling Technology), and the supernatants were collected after centrifugation and boiled for 5 min together with 2× loading buffer to be used to perform SDS–PAGE. Subsequently, separated proteins were further transferred onto nitrocellulose membranes (Bio-Rad). The membrane was blocked with 5% fat-free milk in TBS-T for 2 h at room temperature, then incubated with appropriate primary antibody at 4 °C overnight. Then the membrane was washed three times with TBS-T and incubated with a HRP-conjugated secondary antibody for 2 h at room temperature. After washing three times with TBS-T, bands were detected with ECL (Applygen, Beijing, China).

For the immunoprecipitation, transfected cells were collected and lysed with 1× lysis buffer (Cell Signaling Technology), and then lysates were centrifugated and incubated with appropriate antibodies and protein G beads (Santa Cruz Biotechnology) at 4 °C overnight. The beads were washed three times with IP buffer (50 mM Tris–HCl (PH 7.4), 150 mM NaCl and 1% Nonidet P40), boiled in 2× SDS loading buffer for 5 min, and then analyzed by immunoblot (IB).

2.6. GST pull-down assay

GST and GST-CARD proteins were expressed and purified from *Escherichia coli* strain (BL21 Star), respectively. The lysates from 293T cells were incubated with prepared GST or GST-fused protein at 4 °C overnight. The beads were washed three times with PBS and further boiled for 5 min with 2× loading buffer. Prepared samples were analyzed by IB and further were used to perform mass spectrometric detection.

2.7. Immunofluorescence microscopy

Hela cells were transfected with plasmids encoding Flag-MAVS and Myc-eEF1Bγ for 24 h, further were eluted, fixed and blocked with 5% BSA at 4 °C overnight. Then the cells were incubated with primary antibodies (mouse anti-Flag and rabbit anti-Myc Abs) at 37 °C for 30 min. After washing three times with PBS, the samples were incubated with secondary antibodies (FITC-conjugated anti-mouse IgG Ab and TRITIC-conjugated anti-rabbit IgG Ab) at 37 °C for 30 min. The final result was observed by using laser confocal fluorescence microscopy (Leica TCS SP2, Germany).

2.8. Statistical analysis

All experiments were performed for more than three replicates. And two-tailed Student *t* test with a *P* value <0.05 was used to identify the significance of these data.

3. Results

3.1. Identification of proteins binding to the CARD domain of MAVS

It has been reported that caspase activation and recruitment domain (CARD) of MAVS plays vital roles in the activation of signaling cascades [6]. Therefore, we performed GST-pulldown and mass spectrometric (MS) screening to identify candidate proteins that interact with caspase activation and recruitment domain of MAVS. GST and GST-CARD proteins were expressed and purified from *E. coli* strain (BL21) (Fig. 1A and B), and were further used to incubate with the lysates from HEK293T human embryonic kidney cells (293T cells). The result suggested there were some proteins involving in the association with MAVS-CARD, and a band was cut for further study (Fig. 1C, indicated with arrow). Then MS analysis identified these candidate proteins, and eEF1Bγ has the top score (Fig. 1D). These data showed that eEF1Bγ participates in the interaction with MAVS and hinted that eEF1Bγ may be involved in the regulation of innate immunity signal pathway.

We next evaluated the physical interaction between eEF1Bγ and wild-type MAVS in 293T cells. Plasmids encoding Myc-eEF1Bγ and Flag-3.0 or Flag-MAVS were transfected into 293T cells, and

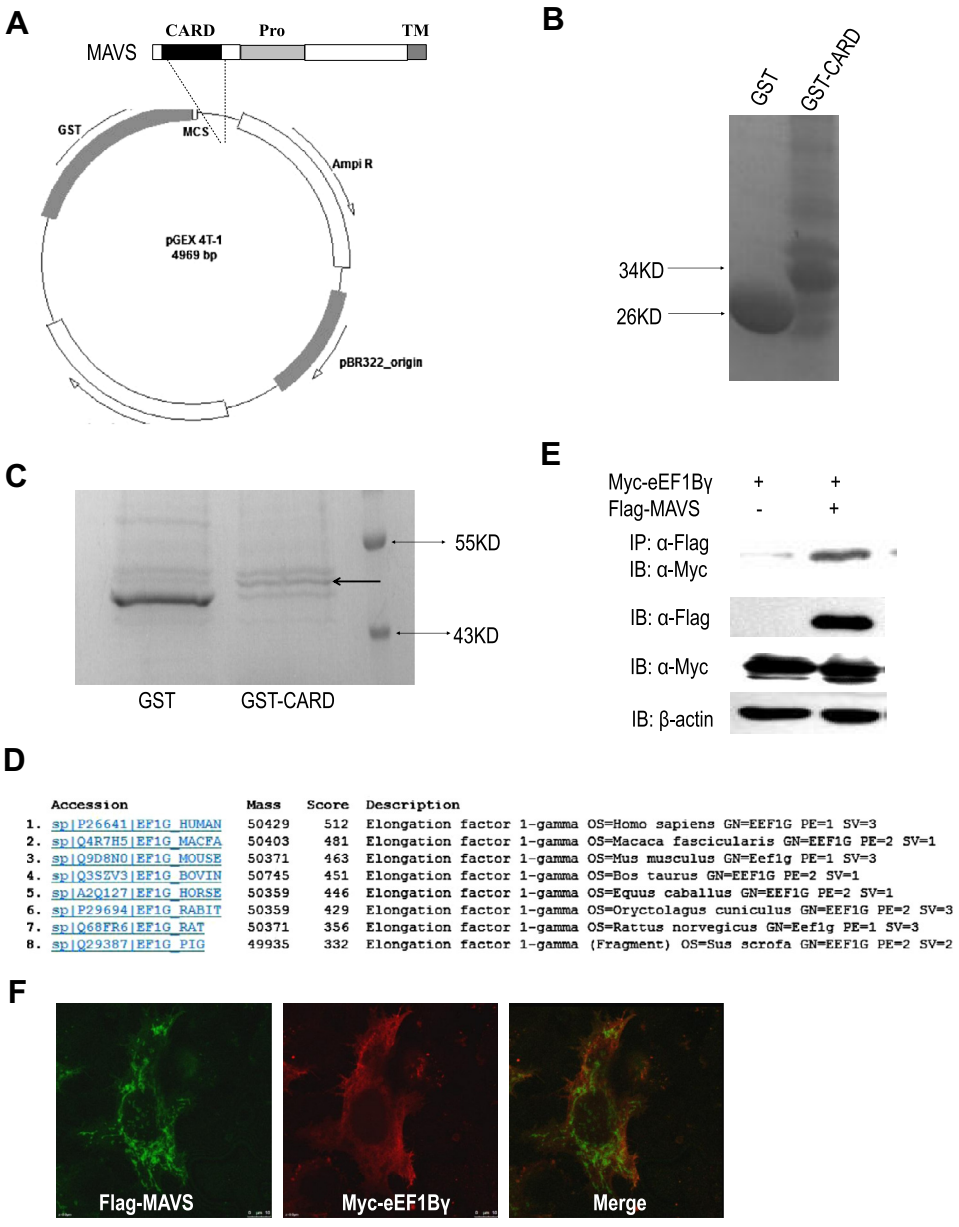

Fig. 1. Identify proteins interact with MAVS-CARD. (A) The construct of GST-tagged CARD plasmid. (B) The expression and purification of GST and GST-CARD proteins from *E. coli* strain (BL21). (C) GST or GST-CARD proteins were incubated with the lysates of 293T cells at 4 °C overnight and then binding proteins were obtained and stained by coomassie blue staining after SDS–PAGE. The band indicated by arrow was cut and send for mass spectrometric analyses. (D) The result of mass spectrometric detection. Proteins with high score are shown in the list. (E) Myc-eEF1Bγ vector was transfected into 293T cells with or without Flag-MAVS co-expression for 24 h, and the lysates were incubated with anti-Flag and protein G beads at 4 °C overnight. Immunoblot was carried out to analyze the association between eEF1Bγ and MAVS. (F) Plasmids encoding Myc-eEF1Bγ and Flag-MAVS were transfected into Hela cells for 24 h, and immunofluorescence microscopy was performed to detect their colocalization.

the lysates were used to perform coimmunoprecipitation (co-IP) assays with anti-Flag. The result confirmed the interaction between these two proteins (Fig. 1E). It has been reported that MAVS is localized to the mitochondria [4]. Here, immunofluorescence microscope assay was performed to identify the colocalization of eEF1Bγ and MAVS in Hela cells. We found eEF1Bγ is mainly localized in the cytoplasm and partially colocalizes with MAVS to the mitochondria (Fig. 1F). Collectively, eEF1Bγ forms complex with MAVS, which indicates that eEF1Bγ may be a potential regulator of MAVS mediated signaling cascades.

3.2. eEF1Bγ positively regulates NF-κB signaling pathway

We performed dual-luciferase reporter assays to explore eEF1Bγ mediated regulation of NF-κB signaling. 293T cells were transfected with expression vectors encoding NF-κB luciferase reporter and internal control renilla luciferase in the absence or presence of MAVS or eEF1Bγ plasmid. The result showed that eEF1Bγ significantly promotes the activation of NF-κB reporter (Fig. 2A). Moreover, NF-κB activation is stimulated in an eEF1Bγ-dose dependent manner (Fig. 2B). Further, the promoter activities of NF-κB downstream proinflammatory cytokines, including *Interleukin-8* (*IL-8*) and *Interleukin-6* (*IL-6*), were evaluated by dual-luciferase reporter experiments. Both *IL-8* and *IL-6* promoters are significantly activated by the ectopic expressed eEF1Bγ (Fig. 2C and D). These results demonstrated that eEF1Bγ is a positive regulator of NF-κB signal pathway.

3.3. eEF1Bγ increases protein stability of MAVS

We next sought to identify how eEF1Bγ activates MAVS-induced NF-κB signaling cascades. 293T cells were transfected with

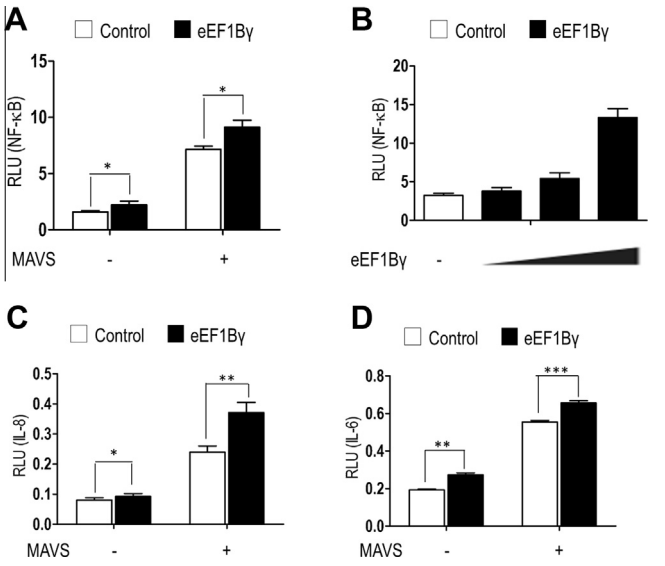

Fig. 2. eEF1Bγ activates NF-κB signal pathway. (A, B) Luciferase assays reveal eEF1Bγ promotes NF-κB activation. 293T cells were transfected with plasmids encoding NF-κB reporter together with indicated plasmids for 24 h, and the cells were lysed and the luciferase activities were monitored. (C, D) Luciferase assays were used to evaluate eEF1Bγ mediated activation of *IL-6* and *IL-8* promoters. Error bars indicate SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

a series of plasmids encoding NF-κB signal adaptors (MAVS, TAK1, IKKα, TRAF6, TAB1 or P65) without or with eEF1Bγ co-expression. IB analysis suggested eEF1Bγ greatly enhances the expression of MAVS, but not others (Fig. 3A). We further found that eEF1Bγ promotes the abundance of MAVS in a dose dependent manner (Fig. 3B). Taken together, eEF1Bγ positively regulates the expression of MAVS at protein level, thus enhancing the activity of NF-κB signaling.

3.4. Proline-rich region (Pro) and caspase activation and recruitment domain (CARD) of MAVS participate in eEF1Bγ mediated polyubiquitination

Subsequently, we detected whether eEF1Bγ increases the abundance of MAVS by ubiquitination. 293T cells were transfected with plasmids encoding Flag-MAVS and wild-type or mutated ubiquitin vectors without or with Myc-eEF1Bγ, and the lysates were used for ubiquitination assays. As shown in Fig. 4A, ubiquitination of MAVS is markedly enhanced by eEF1Bγ co-expression (left panels). Meanwhile, we included two ubiquitin mutants K48 and K63, and their lysine residues were replaced by arginine residues except the site 48 or 63, respectively. And the ubiquitination experiments showed that eEF1Bγ significantly promotes the K63-linked ubiquitination of MAVS whereas attenuates its K48-linked ubiquitination (Fig. 4A). Because studies have shown that K63-linked ubiquitination is related to the activation of MAVS [15,16], whereas K48-linked polyubiquitination mostly involves in proteasomal degradation of target proteins [15,17], both of these results we got support the observation that eEF1Bγ is a positive regulator of MAVS.

To evaluate the domains of MAVS which is responsible for eEF1Bγ induced polyubiquitination, we included some MAVS mutants in the experiments (Fig. 4B), which include ΔTM (lacking the transmembrane domain), ΔProΔTM (lacking the transmembrane domain and Proline-rich region), ΔCARDΔTM (lacking the transmembrane domain and caspase activation and recruitment domain). Then these truncations were separately transfected into 293T cells together with His-Ub in the absence or presence of eEF1Bγ expression vector, and the lysates were used to perform ubiquitination assays. The data suggested that eEF1Bγ overexpression failed to promote the polyubiquitination of ΔProΔTM and ΔCARDΔTM mutants, but not ΔTM truncation (Fig. 4C), which indicated that Pro and CARD domains of MAVS are responsible for eEF1Bγ mediated polyubiquitination. Therefore, eEF1Bγ target

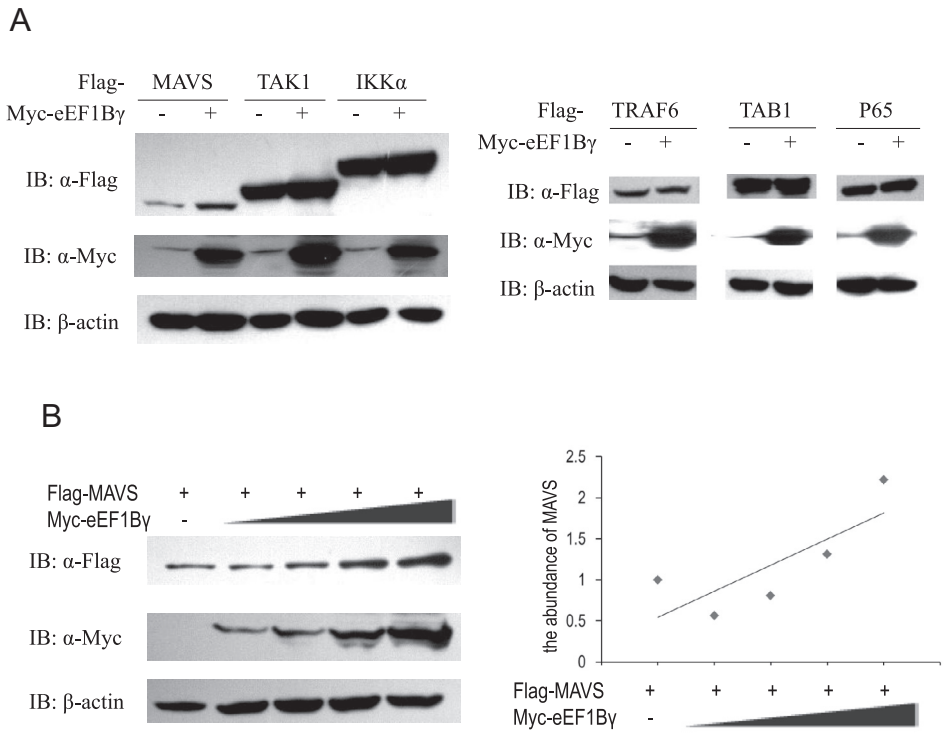

Fig. 3. eEF1Bγ increases MAVS expression. (A) Plasmids encoding adaptors of NF-κB signal pathway (Flag-tagged MAVS, TAK1, IKKα, TRAF6, TAB1 and P65) were separately transfected into 293T cells with or without Myc-eEF1Bγ co-transfection. IB was performed to detect the abundance of these proteins. (B) Flag-MAVS and increasing amounts of Myc-eEF1Bγ were transfected into 293T cells for 24 h, and the lysates were used to perform IB analysis (left). The density of each band normalized with that from β-actin were also shown (right).

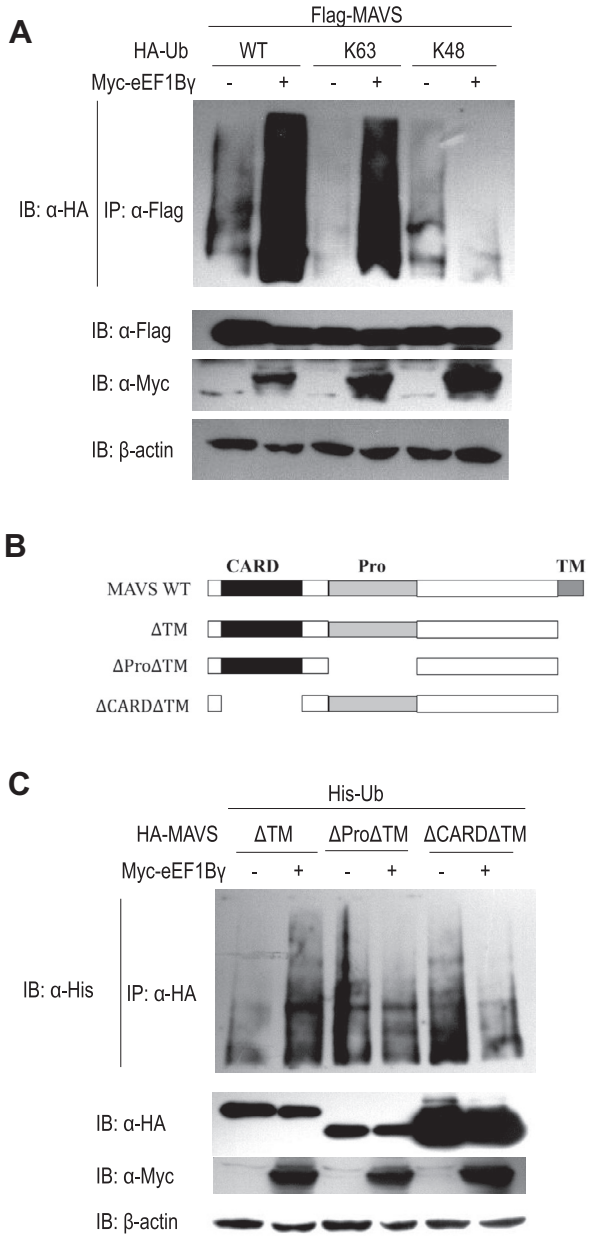

Fig. 4. Pro and CARD of MAVS are critical for eEF1Bγ mediated polyubiquitination. (A) 293T cells were transfected with HA-tagged wildtype Ub, K63Ub or K48Ub and Flag-MAVS in the absence or presence of Myc-eEF1Bγ for 24 h, then the lysates were incubated with anti-Flag Ab and protein G beads at 4 °C overnight. The samples were used to perform IB analysis. (B) Schematic diagram of wildtype MAVS and its truncations. (C) 293T cells were transfected with His/Myc-Ub and MAVS truncations (ΔTM, ΔProΔTM or ΔCARDΔTM) without or with Myc-eEF1Bγ for 24 h. The lysates were incubated with protein G beads plus anti-HA at 4 °C overnight, followed by immunoblot analysis.

the Pro and CARD domains of MAVS for polyubiquitination and the increasement of protein stability.

4. Discussion

MAVS (also termed VISA/IPS-1/Cardif) has been demonstrated plays important roles in connecting RIG-I recognition of viral RNA and downstream signaling cascades [4,5,18,19]. Moreover, caspase activation and recruitment domain (CARD) of MAVS is indispensable for the prion-like behavior of MAVS, which propagates RIG-I signaling to defend viral replication [6,20]. In addition to its essential role in antiviral innate immunity, CARD also acts as a bridge between innate immunity signaling and other research areas such as autophagy and apoptosis [21,22]. Consequently, it is vital to highlight more regulatory mechanism of MAVS-CARD.

We performed GST-pulldown and MS assays to screen the proteins that associate with CARD of MAVS, and the result suggested that eEF1Bγ is a candidate regulator (Fig. 1). eEF1Bγ forms complex with MAVS, and they partially colocalize to mitochondria (Fig. 1E and F), which is consistent with the report of Nicoletta Corbi et al. [14]. It has been reported that eEF1B participate in the regulation of viral replication [8]. eEF1Bδ associates with viral proteins to affect the translational efficiency of cellular or viral mRNAs [23,24]. Besides, eEF1Bγ overexpression involves in the aggressiveness of the tumor [11,25–28]. In this study, we found ectopic expression of eEF1Bγ, another member of eEF1B complex, leads to the activation of transcription factor NF-κB and downstream proinflammatory cytokines (Fig. 2). Further study showed that eEF1Bγ up-regulates the abundance of MAVS by ubiquitination (Figs. 3 and 4), which hinted that eEF1Bγ may recruit some ubiquitin-related enzymes to MAVS. Our report exerts a new role of eEF1Bγ in the regulation of signaling pathway and further broadens its functions.

MAVS, as a critical adaptor of RIG-I, plays vital roles in activating antiviral innate immunity signaling pathway. It has been reported that MAVS activity can be regulated in multiple ways, such as ubiquitination [17,29], MAVS-containing complexes disruption [30]. However, the regulation mechanism of MAVS activity remains largely unknown. In this study, we found that eEF1Bγ increases the abundance of MAVS by inhibiting its K48-linked polyubiquitination (Figs. 3 and 4), which is recognized and degraded by the proteasome system. We speculate eEF1Bγ may recruit certain deubiquitinating enzyme to MAVS to erase its K48-linked ubiquitination or blocks the interaction between MAVS and certain enzyme responsible for K48-linked ubiquitination. Simultaneously, our result showed that eEF1Bγ also intensifies K63-linked polyubiquitination of MAVS (Fig. 4A), which is involved in the activation of this protein. The mechanism of eEF1Bγ mediated regulation of MAVS ubiquitination is to be further studied.

Taken together, we found that eEF1Bγ positively regulates NF-κB signal cascades through promoting K63-linked polyubiquitination of MAVS and erasing its K48-linked polyubiquitination and proteasomal degradation, which provides a new regulating mechanism of antiviral innate immune responses.

Acknowledgments

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