

# UBE2O negatively regulates TRAF6-mediated NF- $\kappa$ B activation by inhibiting TRAF6 polyubiquitination

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**Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is a key regulator of the activation of transcription factor NF- $\kappa$ B by the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily. Recruitment of TRAF6 to the receptor-associated IRAK1-IRAK4-MyD88 adaptor protein complex induces lysine 63 (K63) autopolyubiquitination of TRAF6, which leads to further recruitment of downstream regulators, such as TAB2/3 and TAK1, and subsequently triggers NF- $\kappa$ B activation. Here, we identified the putative E2 ubiquitin-conjugating (UBC) enzyme UBE2O as a novel negative regulator of TRAF6-dependent NF- $\kappa$ B signaling. We found that UBE2O binds to TRAF6 to inhibit its K63-polyubiquitination, and to prevent the activation of NF- $\kappa$ B by IL-1 $\beta$  and lipopolysaccharides (LPS). We further show that the inhibitory effect of UBE2O is independent of its carboxy-terminal UBC domain. In contrast, we found that UBE2O acts to disrupt the IL-1 $\beta$ -induced association of TRAF6 with MyD88. These results provide novel insight into the regulation of signaling by IL-1R/TLR and TRAF6.**

**Keywords:** UBE2O; TRAF6; NF- $\kappa$ B signaling; polyubiquitination

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## Introduction

The interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) family plays a pivotal role in innate immunity and inflammation. These receptors receive and transduce signals from cytokines such as IL-1 $\beta$  and pathogen-associated molecules such as lipopolysaccharides (LPS), and thereby activate the transcription factors nuclear factor  $\kappa$ -light-chain-enhancer of activated B-cells (NF- $\kappa$ B) and activator protein-1 (AP-1) [1, 2]. Upon ligand stimulation, IL-1R or TLR recruits the adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and IL-1R-associated kinase 1/4 (IRAK1/4), which subsequently form a complex tumor necrosis factor (TNF)-associated factor 6 (TRAF6) to trigger lysine 63 (K63) auto-polyubiquitination of TRAF6 with the help of ubiquitin-conjugating (UBC) enzyme 13 (UBC13)/Uev1a [3-

8]. K63 auto-polyubiquitinated TRAF6 forms a recognition signal for the recruitment of TGF- $\beta$ -activated kinase 1 (TAK1)-binding protein 2/3 (TAB2/3) and activation of TAK1 [9]. TAK1 then activates the I $\kappa$ B kinase (IKK) complex, leading to the phosphorylation and subsequent degradation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  [10]. This cascade of events enables the NF- $\kappa$ B complex to translocate to the nucleus and initiate transcriptional responses.

TRAF6 is a member of the TNF receptor (TNFR) associated factors (TRAFs) that mediate TNFR intracellular signaling, but unlike other TRAFs, TRAF6 also mediates IL-1R/TLR signaling [11]. TRAF6 contains an amino (N)-terminal RING-finger domain, followed by several zinc-finger domains and a conserved carboxy (C)-terminal TRAF domain [12]. The N-terminal RING finger domain is responsible for the binding of the E2 enzyme UBC13, which mediates TRAF6 auto-polyubiquitination. The conserved C-terminal TRAF domain enables the interaction with receptors and upstream signaling proteins. A crucial role of TRAF6 in NF- $\kappa$ B signaling has been demonstrated by the fact that TRAF6 knockout cells failed to respond to IL-1 $\beta$  or LPS stimulation [13]. Control of TRAF6 activity depends to a large extent on K63 auto-polyubiquitination, and several deubiquitinases

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have been described to regulate NF- $\kappa$ B signaling by removing ubiquitin chains from TRAF6 [14–18]. Hence, modulation of TRAF6 polyubiquitination is an important way to control NF- $\kappa$ B signaling.

The putative UBC enzyme UBE2O, together with BRUCE [19, 20], constitute the two large E2 enzymes. UBE2O was first purified from rabbit reticulocytes and named as E2-230K [19]. It is ubiquitously expressed, but preferentially in brain, skeletal muscle and heart tissues [21]. In erythroid cells, UBE2O is upregulated during the reticulocyte stage of erythroid differentiation [22, 23]. The function of UBE2O remains mostly unknown. By using yeast two-hybrid screens, Markson *et al.* [24] identified more than 100 E3 ubiquitin ligases interacting with UBE2O, including TRAF6. However, it is as yet unknown whether UBE2O plays a role in TRAF6-mediated signaling.

In this report, we show that UBE2O functions as a negative regulator of TRAF6-mediated IL-1R/TLR4 signaling by inhibiting TRAF6 polyubiquitination. We demonstrate that the inhibitory effect of UBE2O does not require its UBC domain and that UBE2O disrupts the formation of the MyD88-TRAF6 protein complex. As far as we know, this is the first report to show a function for UBE2O.

## Results

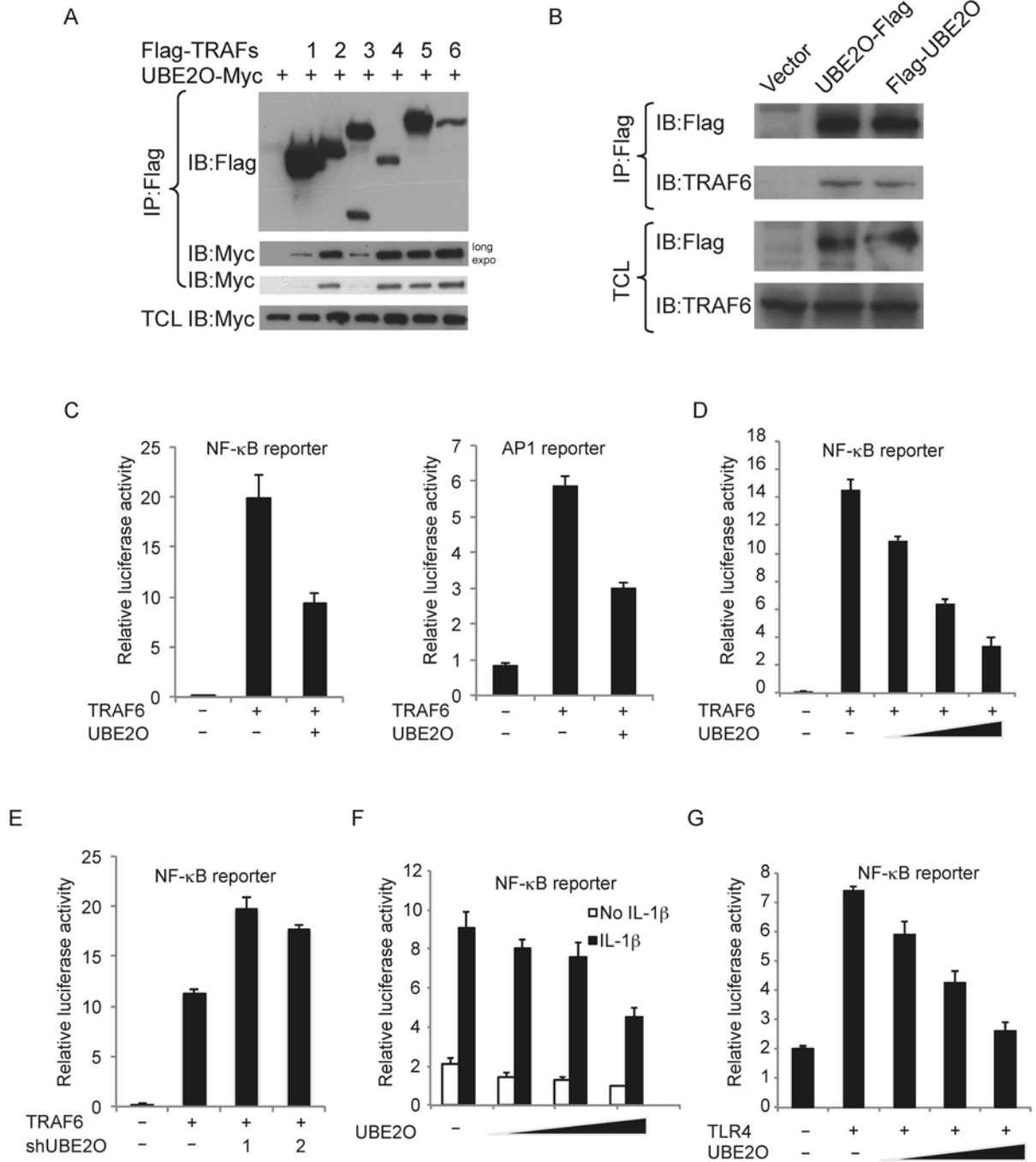
### *UBE2O interacts with TRAF6 and inhibits its transcription stimulatory activity*

The putative E2 enzyme UBE2O has previously been reported to interact with the signaling mediators TRAF4, TRAF5 and TRAF6 in yeast two-hybrid assays [24]. We first verified this interaction by expressing UBE2O-Myc together with Flag-tagged TRAF1, 2, 3, 4, 5, or 6. As shown in Figure 1A and Supplementary information, Figure S1A, UBE2O was found to interact with several TRAFs, including TRAF6. We next analyzed the effect of UBE2O on TRAF-regulated NF- $\kappa$ B reporter gene activity. UBE2O had the strongest effects on TRAF6-induced NF- $\kappa$ B activation (Supplementary information, Figure S1C and S1D), and we therefore further focused on the effects of UBE2O on TRAF6-mediated signaling. Endogenous TRAF6 was also found to interact with ectopic UBE2O, both in primary mouse fibroblasts (MEFs) and HEK293T cells (Figure 1B and Supplementary information, Figure S1B). Moreover, UBE2O inhibited the transcription stimulatory effect of TRAF6 on both NF- $\kappa$ B and AP-1 reporter genes (Figure 1C) in a UBE2O dose-dependent manner (Figure 1D and Supplementary information, Figure S1E). A control reporter lacking the three copies of NF- $\kappa$ B sites of the immunoglobulin  $\kappa$

light chain was not induced by TRAF6 nor inhibited by UBE2O (data not shown). We next verified the effects of UBE2O on TRAF6 function by UBE2O knockdown. Importantly, the ability of TRAF6 to activate the NF- $\kappa$ B reporter was potentiated by two independent shRNAs targeting UBE2O (Figure 1E and Supplementary information, Figure S1F). UBE2O also dose dependently inhibited IL-1 $\beta$ - or TLR4-induced NF- $\kappa$ B reporter activation (Figure 1F, 1G and Supplementary information, Figure S1G). We next analyzed the specificity of the inhibitory effect of UBE2O on NF- $\kappa$ B signaling by examining cells stimulated with poly (I:C) or overexpressing TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF). Importantly, UBE2O had no effect on poly (I:C)- or TRIF-induced NF- $\kappa$ B reporter activity (Supplementary information, Figure S2A and S2B). Moreover, UBE2O inhibited NF- $\kappa$ B reporter activity induced by MyD88, but not by IKKs or p65 (Supplementary information, Figure S2C and data not shown), indicating that UBE2O functions upstream in the TRAF6-NF- $\kappa$ B signaling cascade. In conclusion, UBE2O appears to be a potent regulator of TRAF6.

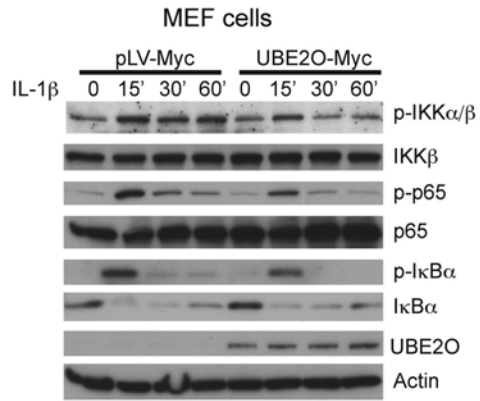
### *UBE2O impairs NF- $\kappa$ B activation in multiple cell types*

To examine whether UBE2O is relevant for the activation of the endogenous NF- $\kappa$ B pathway, we analyzed the NF- $\kappa$ B signaling components I $\kappa$ B $\alpha$ , IKK $\alpha$ / $\beta$  and p65 in primary MEF cells. As shown in Figure 2A, lentivirus-mediated expression of UBE2O-Myc was found to reduce the IL-1 $\beta$ -induced phosphorylation of I $\kappa$ B $\alpha$ , IKK $\alpha$ / $\beta$  and p65. IL-1 $\beta$ -induced phosphorylation of p38 or JNK was also reduced by UBE2O overexpression (Supplementary information, Figure S3A). As the phosphorylation of I $\kappa$ B $\alpha$ , IKK $\alpha$ / $\beta$  and p65 is essential for the translocation of the NF- $\kappa$ B complex into the nucleus to initiate transcription, we next analyzed NF- $\kappa$ B target gene expression. As shown by qPCR assays in Figure 2B, the expression of four target genes examined (*NOS2*, *CCL2*, *IL-6* and *TNF- $\alpha$* ) was all significantly inhibited by UBE2O. In line with this, knockdown of UBE2O enhanced NF- $\kappa$ B activation by IL-1 $\beta$  or LPS (Figure 2C–2F). IL-1 $\beta$ -induced phosphorylation of I $\kappa$ B $\alpha$ , IKK $\alpha$ / $\beta$  or p65 was increased when UBE2O was depleted by two independent shRNAs in primary MEF cells (Figure 2C). Similar results were obtained in macrophage RAW264.7 cells treated with LPS (Figure 2E). Consistently, IL-1 $\beta$ - or LPS-induced p38 or JNK phosphorylation was also increased in UBE2O knockdown cells (Supplementary information, Figure S3B and S3C). Moreover, the IL-1 $\beta$ - or LPS-induced expression of NF- $\kappa$ B target genes was significantly increased when UBE2O was depleted in these cells (Figure 2D and 2F). Thus, UBE2O functions

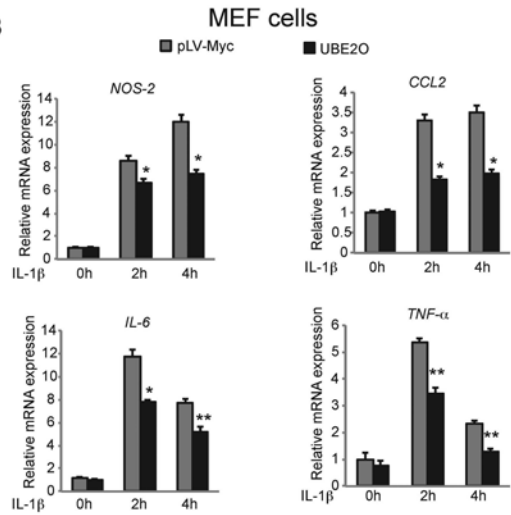


**Figure 1** UBE2O interacts with TRAF6 and inhibits its transcription stimulatory activity. **(A)** HEK293T cells were transiently transfected with UBE2O-Myc and Flag-TRAFs expression vectors, and analyzed by immunoprecipitation (IP) and/or immunoblotting (IB) as indicated. Myc antibody was used for the detection of TRAF-associated UBE2O. TCL: total cell lysate. **(B)** MEF cells were infected with the indicated UBE2O vectors and analyzed by IP and/or IB to detect UBE2O-associated endogenous TRAF6. **(C, D)** HEK293T cells were co-transfected with NF- $\kappa$ B or AP-1 transcriptional luciferase reporter constructs, together with TRAF6 and (increasing amounts of) UBE2O vectors as indicated. Luciferase activity was measured 36 h after transfection. **(E)** HEK293T cells were co-transfected with NF- $\kappa$ B reporter, TRAF6 vector and two independent UBE2O shRNA vectors as indicated. **(F)** HEK293T cells were co-transfected with NF- $\kappa$ B reporter, and increasing amounts of UBE2O vector. 24 h after transfection, cells were treated with 10 ng/ml IL-1 $\beta$  for 12 h. **(G)** HEK293T cells were co-transfected with NF- $\kappa$ B reporter, TLR4 expression vector, and increasing amounts of UBE2O vector. For all luciferase reporter assays, *LacZ* expression plasmid was co-transfected as internal reference. Each experiment was performed in triplicate.

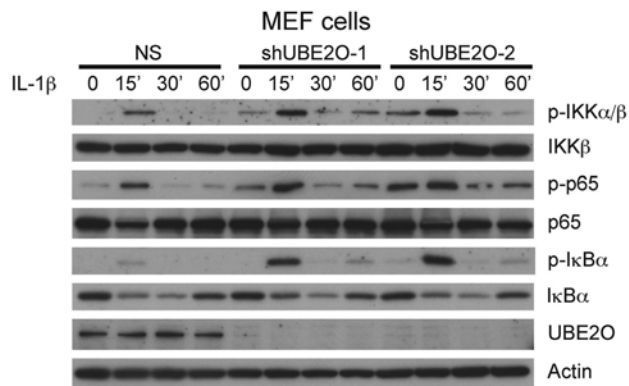
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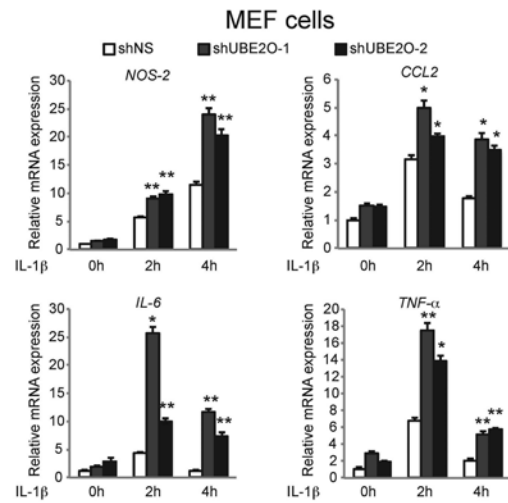
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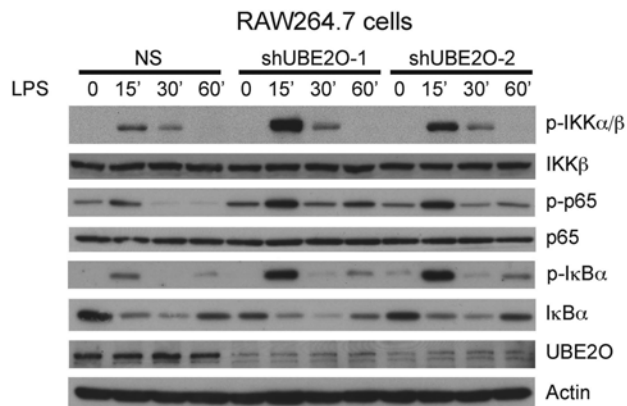
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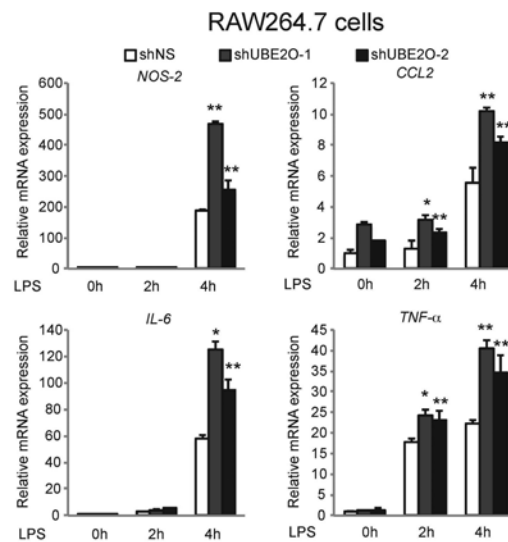
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**Figure 2** UBE2O impairs IL-1 $\beta$ - or LPS-induced NF- $\kappa$ B activation. **(A, B)** Primary MEF cells were infected with lentivirus-carrying control (pLV-Myc) or UBE2O-Myc vectors, selected for puromycin resistance, and treated with 10 ng/ml IL-1 $\beta$  for the indicated time points. Cell lysates were analyzed for the expression levels of selected (phosphor) proteins by immunoblotting with the indicated antibodies **(A)**, or of selected genes by real-time PCR **(B)**. Actin was used as a loading control in **A**. GAPDH was used for normalization in **B**. \* indicates  $P < 0.05$  and \*\* indicates  $P < 0.01$  (groups with UBE2O overexpression were compared with control group). **(C-F)** Primary MEF cells **(C, D)** or macrophage Raw264.7 cells **(E, F)** were infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (shUBE2O-1 or -2). Cells were then stimulated with 10 ng/ml IL-1 $\beta$  or 1  $\mu$ g/ml LPS for the indicated time points and analyzed by IB **(C, E)** or real-time PCR **(D, F)**. Actin was included as a loading control in **C** and **E**. GAPDH was used for normalization in **D** and **F**. The data are presented as mean  $\pm$  SD ( $n = 3$ ). \* indicates  $P < 0.05$  and \*\* indicates  $P < 0.01$  (shUBE2O groups were compared with non-specific control shRNA group).

as an inhibitor of NF- $\kappa$ B activation by IL-1R/TLR in multiple cell types.

#### *UBE2O decreases TRAF6 polyubiquitination*

TRAF6 activity is regulated by K63-polyubiquitination, which is induced by upstream receptors upon ligand binding [8]. To investigate whether TRAF6 inhibition by the putative E2 UBC enzyme UBE2O involves changes in TRAF6 polyubiquitination, we performed TRAF6 ubiquitination assays. As shown in Figure 3A, polyubiquitination of Flag-TRAF6 by co-transfected HA-tagged ubiquitin was easily detected and slightly enhanced by IL-1 $\beta$  stimulation. Strikingly, forced expression of UBE2O strongly inhibited TRAF6 polyubiquitination both in the presence and absence of IL-1 $\beta$  stimulation. Similar inhibition of TRAF6 polyubiquitination by UBE2O was observed in nickel pull down assays with co-transfection of Myc-His-ubiquitin (Figure 3B). Moreover, IL-1 $\beta$ -induced polyubiquitination of endogenous TRAF6 was inhibited by UBE2O as well (Figure 3C). We also checked the effects of UBE2O on ubiquitination of other TRAFs. Different from TRAF6, polyubiquitination of other TRAFs was not inhibited by UBE2O. In contrast, monoubiquitination of TRAF1 and 3, and polyubiquitination of TRAF4 and 5 were increased upon UBE2O overexpression (Supplementary information, Figure S4). Next, we examined whether UBE2O specifically affects K63-polyubiquitination of TRAF6. Transfection of conjugation-specific ubiquitin mutants showed that polyubiquitination of TRAF6 involves both K48 and K63 chains, and that UBE2O inhibits both types of conjugations (Figure 3D and 3E). In summary, these results suggest that UBE2O-mediated inhibition of TRAF6-induced signaling involves inhibition of TRAF6 K63-polyubiquitination.

#### *Knockdown of UBE2O enhances TRAF6 polyubiquitination*

We next examined whether polyubiquitination of TRAF6 is also affected by depletion of UBE2O. In MEF

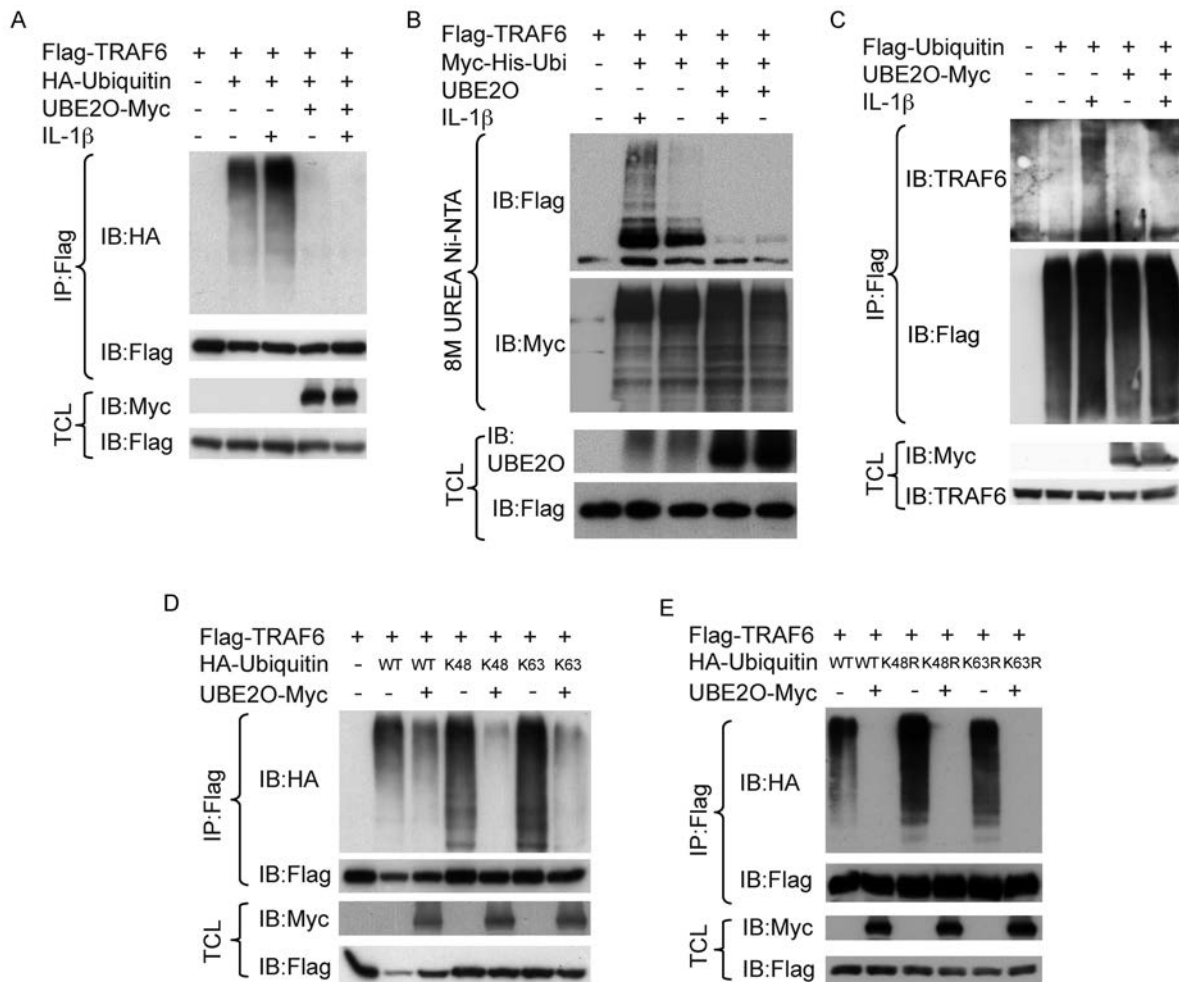
and Raw264.7 cells, polyubiquitination of TRAF6 was increased after IL-1 $\beta$  or LPS treatment, as reported previously [15]. Importantly, depletion of UBE2O by two independent shRNAs enhanced IL-1 $\beta$ - or LPS-induced polyubiquitination of TRAF6 in these cells (Figure 4A and 4B). These results support that endogenous UBE2O negatively controls NF- $\kappa$ B activation via inhibition of TRAF6 polyubiquitination. Moreover, in line with the results shown in Figure 3D and 3E, both K48- and K63-polyubiquitination of TRAF6 were increased upon UBE2O knockdown (Figure 4C), although the increase of K63-polyubiquitination is more obvious.

#### *The UBC domain of UBE2O is not required for the inhibition of TRAF6*

As UBE2O is a putative E2 conjugating enzyme, we next examined whether its inhibitory effects on TRAF6-mediated signaling are associated with its ubiquitin-conjugating potential. UBE2O contains both a coiled-coil (CC) interaction domain and a UBC domain, including C885 as a critical residue in the putative E2 active site (Figure 5A). Co-immunoprecipitation assays showed that the C-terminal deletion mutant D1, which lacks the UBC domain but still contains the CC domain (Figure 5A), efficiently interacted with TRAF6 (Figure 5B). However, the N-terminal deletion construct D2, lacking the CC domain but still containing the UBC domain, did not interact with TRAF6 (Figure 5B). Importantly, the UBC domain-deleted mutant D1 also could still inhibit TRAF6-induced NF- $\kappa$ B reporter activity in contrast to the N-terminal deletion mutant D2 (Figure 5C). Moreover, the UBC domain-deleted mutant D1 was able to inhibit TRAF6-polyubiquitination, whereas mutant D2 containing the UBC domain did not (Figure 5D).

#### *UBE2O disrupts the interaction between TRAF6 and MyD88*

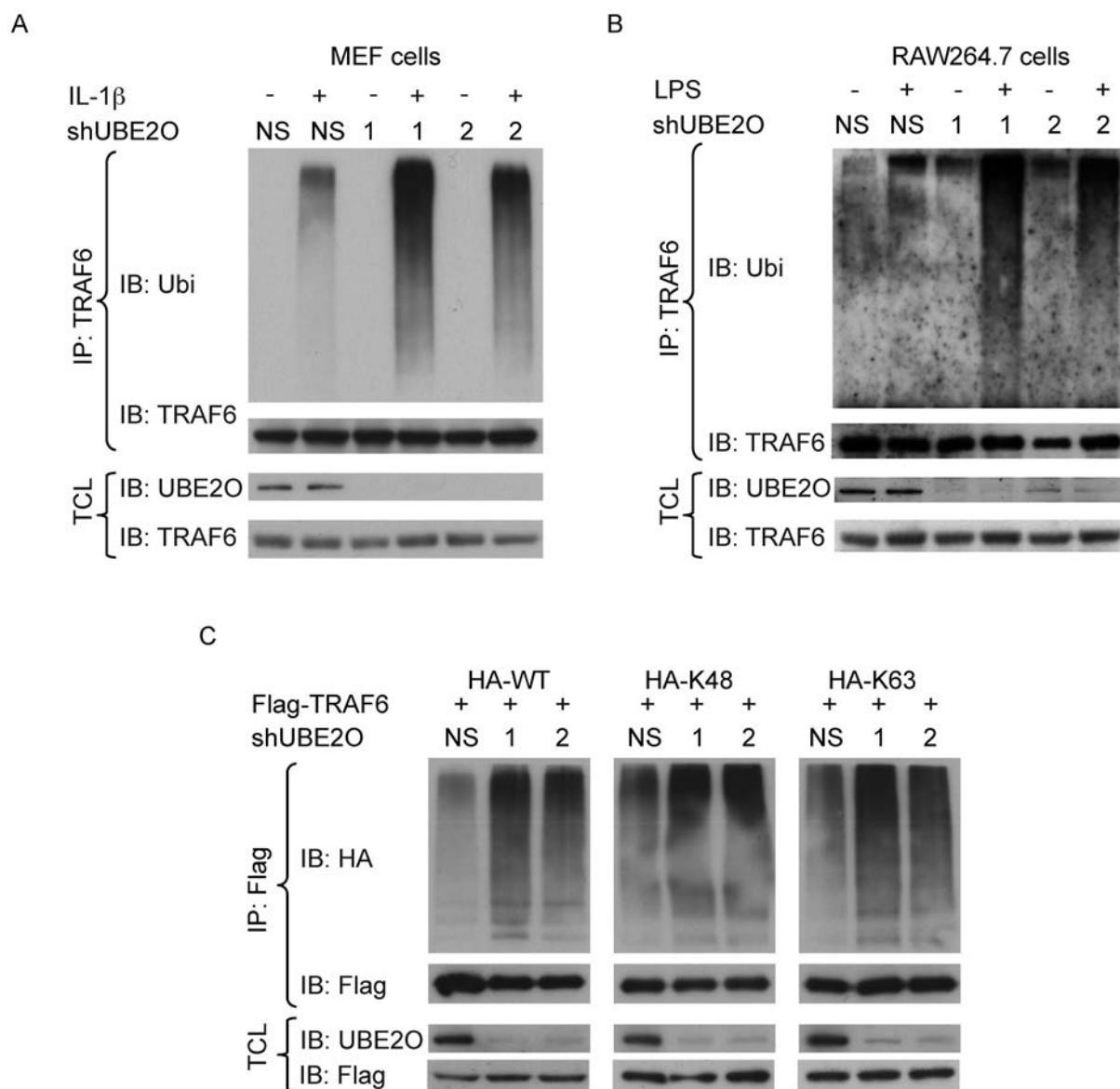
Next, we investigated which domains of TRAF6 are required for the interaction with UBE2O. Three TRAF6 deletion constructs (D1-D3) were used, containing ei-



**Figure 3** UBE2O decreases polyubiquitination of TRAF6. **(A)** HeLa cells were co-transfected with HA-ubiquitin, Flag-TRAF6 and UBE2O-Myc expression vectors as indicated. 44 h post transfection, the proteasome inhibitor MG132 was added for 4 h. After the indicated cells were stimulated with IL-1 $\beta$  for the last 30 min, cells were harvested for IP and IB analyses. HA antibody was used to detect polyubiquitinated TRAF6. **(B)** HeLa cells were co-transfected with Myc-His-ubiquitin, Flag-TRAF6 and UBE2O vectors as indicated, treated with MG132 and IL-1 $\beta$  as described above, and harvested for nickel pull down (Ni-NTA) and IB analyses. **(C)** HeLa cells were co-transfected with Flag-ubiquitin and UBE2O-Myc vectors, treated with MG132 and IL-1 $\beta$ , and harvested for IP and IB analyses. **(D, E)** HeLa cells were transfected with the indicated expression vectors, including K48 and K63 conjugation-specific ubiquitin mutants, treated with MG132 and harvested for IP and IB analyses.

ther the N-terminal RING finger domain (RF), several zinc-finger domains (ZnF) located in the center or the C-terminal TRAF domain (TRAF-C) [12]. Co-immunoprecipitation analysis showed that only the TRAF domain construct D3 could interact with UBE2O (Figure 6B). Interestingly, the TRAF-C domain also mediates the interaction of TRAF6 with upstream receptors and signaling proteins [12]. In line with this, we found that the interaction between TRAF6 and MyD88 mainly depended on the TRAF-C domain (Figure 6C). Importantly, this interaction between TRAF6 and MyD88 was increased by IL-1 $\beta$  stimulation, but inhibited by forced expression of full-

length UBE2O or the UBE2O deletion mutant D1 (Figure 6D and Supplementary information, Figure S5A). In line with this, depletion of UBE2O potentiated the interaction between TRAF6 and MyD88 (Figure 6E and Supplementary information, Figure S5B). In contrast, UBE2O did not interfere with the interaction between TRAF6 and IRAK1, nor the interaction between MyD88 and IRAK4 (Supplementary information, Figure S5C and S5D). Moreover, we did not observe a detectable interaction between MyD88, IRAK1, IRAK4 and UBE2O (data not shown). We conclude from these results that UBE2O specifically disrupts the interaction between TRAF6 and



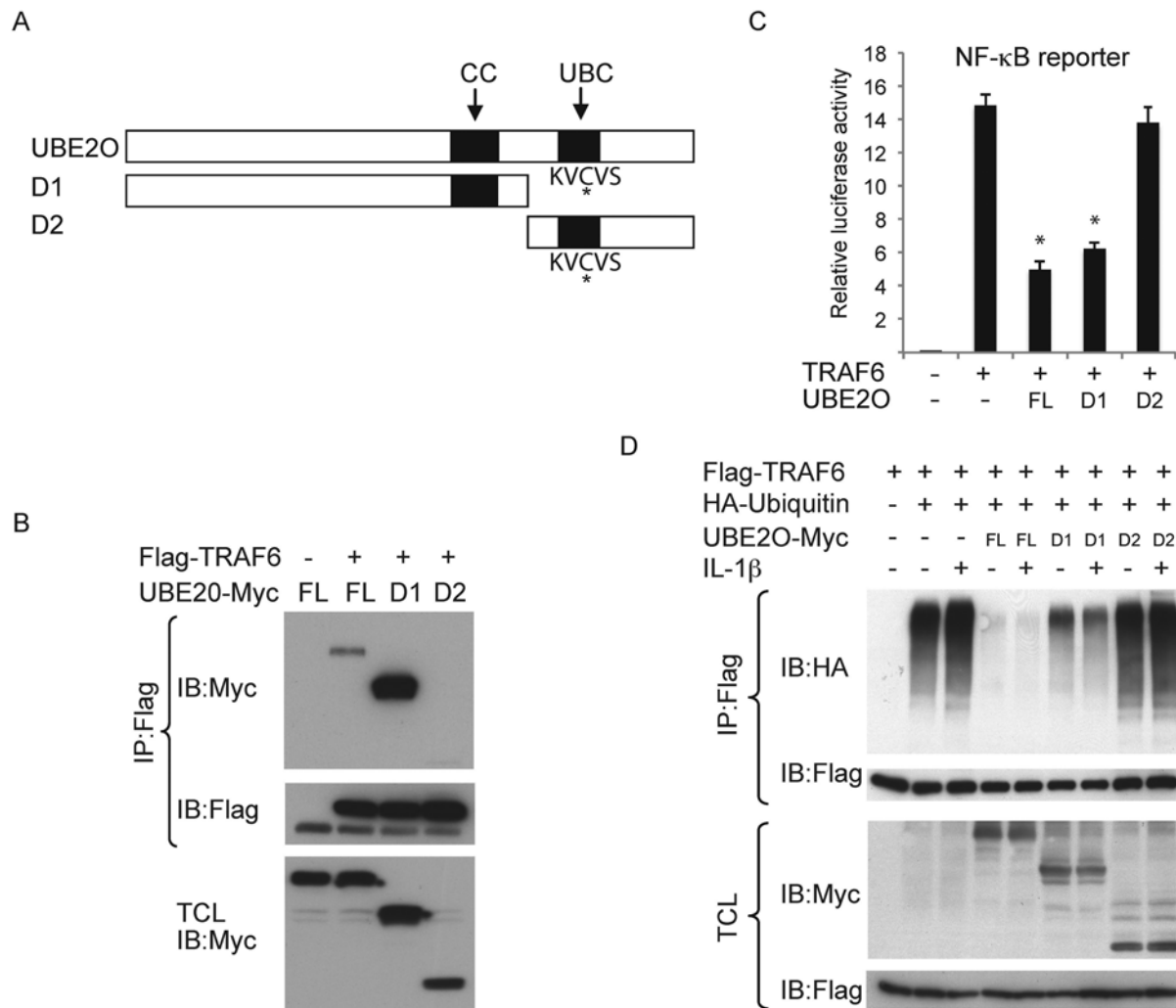
**Figure 4** Depletion of UBE2O increases polyubiquitination of TRAF6. **(A, B)** Primary MEF cells **(A)**, or macrophage Raw264.7 cells **(B)**, stably infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (shUBE2O-1 or -2) were treated with MG132 for 4 h, stimulated with IL-1 $\beta$  **(A)**, or LPS **(B)** for 30 min and analyzed by IP and IB. Ubiquitin antibody was used to detect endogenous polyubiquitinated TRAF6. **(C)** HeLa cells stably infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (shUBE2O-1 or -2) were co-transfected with expression vectors for Flag-TRAF6 and wild-type, or K48, or K63 conjugation-specific HA-ubiquitin (HA-WT, HA-K48, HA-K63) as indicated, and treated with MG132 for 4 h. HA antibody was used to detect polyubiquitinated TRAF6.

its upstream regulator MyD88, and thereby counteracts TRAF6-polyubiquitination and the activation of downstream signaling components.

## Discussion

UBE2O was first isolated from rabbit reticulocytes, and classified as a putative E2 enzyme as it has a con-

served UBC domain [19]. However, the function of UBE2O was previously unknown. Recently, more than one hundred E3 ubiquitin ligases were found to interact with UBE2O in yeast two-hybrid screens [24], including TRAF6, a mediator of TLR/IL-1R-induced NF- $\kappa$ B activation. As an important part of the host defense system, TLR/IL-1R-induced signaling needs to be tightly controlled to maintain immune homeostasis and avoid

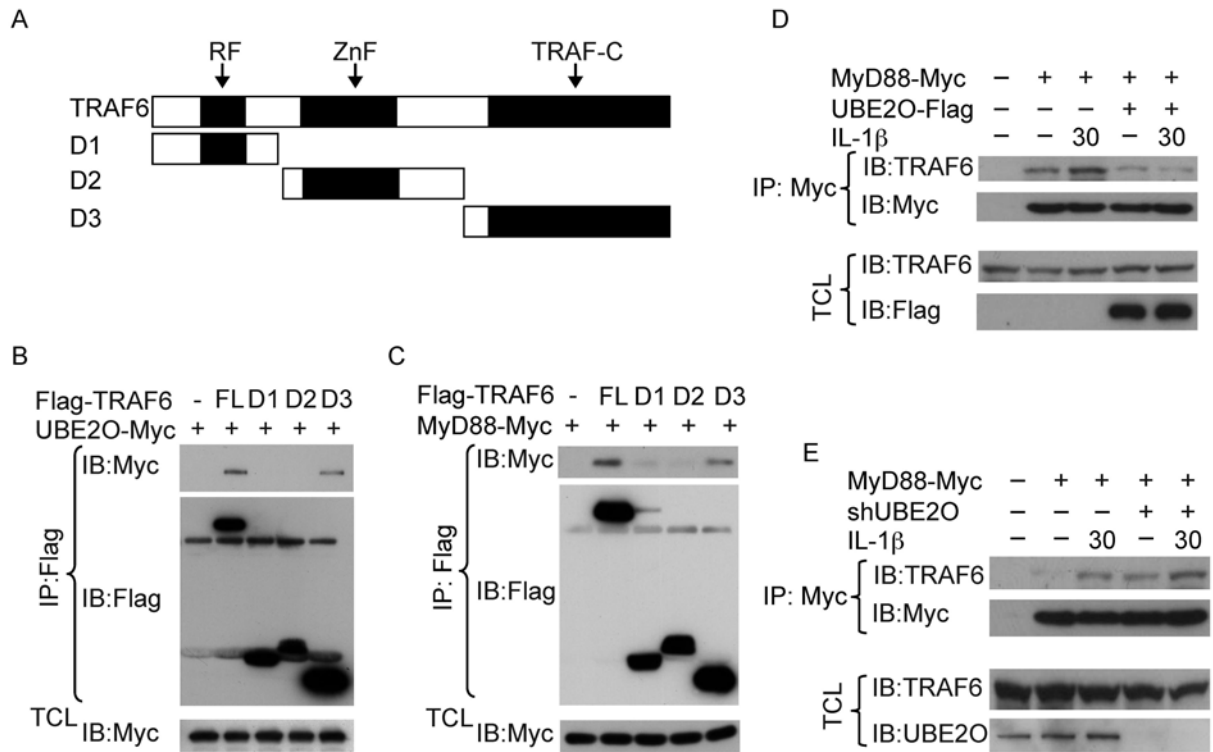


**Figure 5** The UBC domain of UBE20 is not required for TRAF6 inhibition. **(A)** Conserved domains present in wild-type UBE20 and UBE20 deletion mutants D1 and D2. CC: coiled-coil domain, UBC: ubiquitin-conjugating domain. KVCVS: amino acids in the E2 active site. Asterisk indicates the E2 active site of UBE20. **(B)** HEK293T cells were transfected with Flag-TRAF6 and full-length (FL) or deleted (D1, D2) UBE20-Myc expression vectors as indicated, and analyzed by IP and IB. **(C)** HEK293T cells were co-transfected with NF-κB reporter, TRAF6 and FL or deleted (D1, D2) UBE20-Myc expression vectors as indicated. Luciferase activity was measured 36 h after transfection. \* indicates  $P < 0.05$ . **(D)** HeLa cells were co-transfected with the indicated expression vectors. 44 h post transfection, the proteasome inhibitor MG132 was added for 4 h. After the indicated, cells were stimulated with IL-1β for the last 30 min, cells were harvested for IP and IB analyses. HA antibody was used to detect polyubiquitinated TRAF6.

detrimental responses. Inappropriate activation of NF-κB leads to unrestrained innate immune responses and a wide range of human diseases, such as septic shock and rheumatoid arthritis [1, 2]. Multiple negative regulators are thus employed to avoid uncontrolled NF-κB activation [14-18, 25]. In this article, we demonstrate that UBE20 is involved in regulating TRAF6-mediated NF-κB activation. Forced expression of UBE20 suppresses IL-1β- and TRAF6-induced signaling, and depletion of UBE20 enhances TRAF6-induced NF-κB activation.

Importantly, we found that poly (I:C)- or TRIF-induced NF-κB activation is not affected by UBE20, indicating that UBE20 specifically affects MyD88/TRAF6-mediated NF-κB activation. Furthermore, knockdown of UBE20 enhances IL-1β- and LPS-induced phosphorylation of several key components of the NF-κB pathway, as well as NF-κB target gene activation. Our data therefore suggest that UBE20 is a physiological negative regulator of TRAF6-mediated NF-κB signaling and JNK/p38 activation. However, our data do not rule out





**Figure 6** UBE2O disrupts the interaction between TRAF6 and MyD88. **(A)** Conserved domains present in wild-type TRAF6 and the TRAF6 deletion mutants D1, D2 and D3. RF: RING-finger domain; ZnF: zinc-finger domains; TRAF-C: TRAF domain. **(B, C)** HEK293T cells were transfected with FL Flag-TRAF6, TRAF6 deletion constructs (D1-D3), UBE2O-Myc **(B)**, or MyD88-Myc **(C)** vectors as indicated. Flag-resin immunoprecipitates were blotted with Myc antibody to detect UBE2O **(B)**, or MyD88 **(C)** associated with TRAF6. **(D)** HeLa cells were transfected with MyD88-Myc and UBE2O-Flag as indicated. 48 h post transfection, cells were treated with IL-1β for 30 min and lysed for IP and IB analyses. Immunoprecipitates were blotted for MyD88-associated endogenous TRAF6 by using TRAF6 antibody. **(E)** HeLa cells stably expressing UBE2O shRNAs (shUBE2O-1 + shUBE2O-2) were transfected with MyD88-Myc as indicated. 48 h post transfection, cells were treated with IL-1β for 30 min and lysed for IP and IB analyses. Immunoprecipitates were blotted for MyD88-associated endogenous TRAF6 by using TRAF6 antibody.

the possibility that UBE2O may target other TRAFs or regulators involved in NF-κB signaling, as we found that UBE2O overexpression can enhance the ubiquitination of TRAF1, 3, 4 and 5 (Supplementary information, Figure S4). Moreover, we found that the overexpression of UBE2O also weakly reduced NF-κB reporter activity induced by some other TRAFs and TNF-α (Supplementary information, Figure S1D and data not shown). Therefore, the roles of UBE2O in other TRAFs- and TNF-α-induced signaling need to be further investigated.

TRAF6 depletion studies revealed a determining role of TRAF6 in IL-1R/TLR signaling, as without TRAF6, cells failed to respond to IL-1β and LPS [13]. To activate NF-κB signaling, TRAF6 needs to be polyubiquitinated under the assistance of the E2 enzyme Uev1a/Ubc13. Activated TRAF6 functions as an adaptor protein to activate downstream transcription factors. It has been shown that K63 auto-polyubiquitination of TRAF6 is

essential for its activation, and is counteracted by the deubiquitinases A20, CYLD, MCPIP1, USP4 or USP2a [14-18, 25]. In our study, we found that UBE2O binds endogenous TRAF6 and inhibits both K48- and K63-polyubiquitination of TRAF6. In spite of the reduction in K48-polyubiquitination, we did not observe any change in TRAF6 protein levels upon the co-expression of UBE2O. Therefore, it remains to be established whether the change in K48-polyubiquitination has any effect on TRAF6 function.

We found that a C-terminal deletion mutant of UBE2O, lacking the UBC domain but still containing the CC domain, interacted efficiently with TRAF6. Importantly, this mutant also inhibited the activity and polyubiquitination of TRAF6. The fact that both UBE2O and MyD88 interact with TRAF6 via its TRAF-C domain, suggests that UBE2O likely acts to disrupt the interaction between TRAF6 and one or more of its upstream

regulators. Indeed, UBE2O interferes with the interaction between TRAF6 and MyD88, but not the interaction between IRAK1 and TRAF6, nor the interaction between IRAK4 and MyD88 (Supplementary information, Figure S5C and S5D). Moreover, we showed that the C-terminal deletion mutant of UBE2O could also interfere with the interaction between TRAF6 and MyD88. This indicates that UBE2O specifically interacts with part of the TRAF6-activation complex (Figure 7).

In summary, we have uncovered a novel mechanism for the regulation of IL-1R/TLR mediated-signaling. Moreover, our results show that a putative E2 enzyme can act as a scaffold protein rather than a UBC enzyme to restrict TRAF6-mediated NF- $\kappa$ B activation. Our study helps to expand our understanding of the molecular regulation of TRAF6-dependent processes and the functions of E2 enzymes.

## Materials and Methods

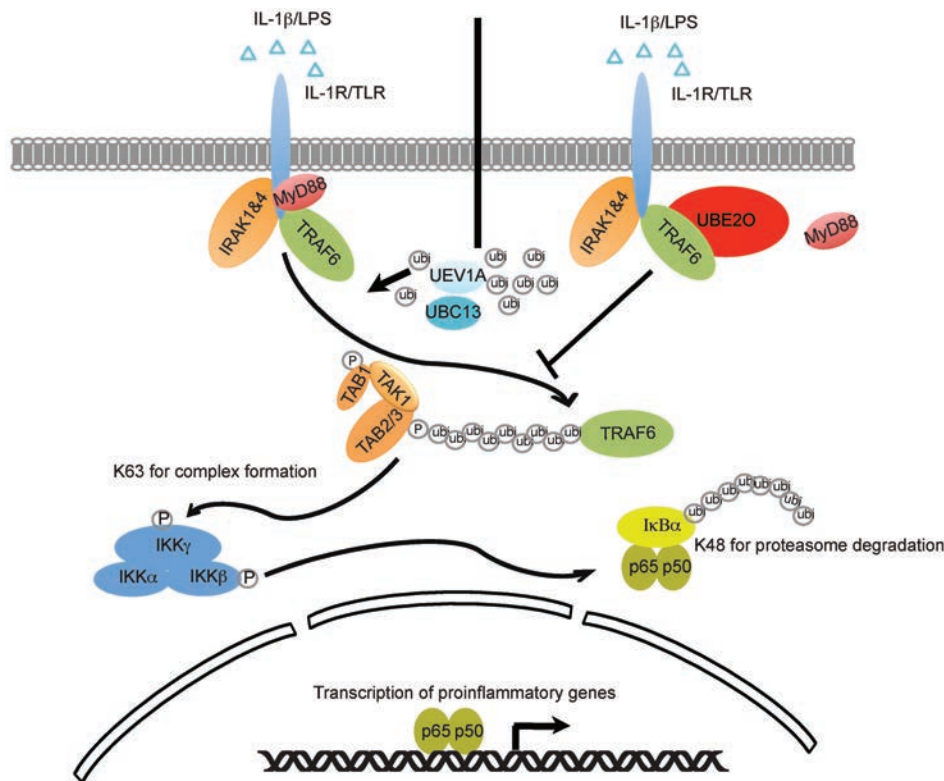
### Cell culture and plasmids

HEK293T and primary MEFs were cultured in Dulbecco's modified Eagle's (Thermo) supplemented with 10% FBS (HyClone), 100 U/ml penicillin/streptomycin (Invitrogen). HeLa and

macrophage RAW246.7 cell lines were cultured in RPMI-1640 (Thermo) supplemented with 10% FBS. IL-1 $\beta$ , LPS and MG132 were purchased from Sigma. Poly (I:C) was purchased from Invitrogen. Full-length hUBE2O [21] from cDNA of HEK293T was cloned into pCR3.1-Myc/Flag or the pLV-bc-CMV-puro lentivirus vector pLV-Myc/Flag. Full-length hTRAF6 was cloned into a pcDNA3 vector with an N-terminal Flag tag (constructed by E Meulmeester). For the deletion constructs of UBE2O and TRAF6, the indicated regions were cloned into C-terminal Myc-tagged pLV and N-terminal Flag-tagged pCR3.1 vector, respectively. The constructs were confirmed by DNA sequencing. The expression constructs HA-Ub-WT, HA-Ub-K48R (only lysine 48 mutated to arginine), HA-Ub-K63R (only lysine 63 mutated to arginine), HA-Ub-K48 (all lysines except lysine 48 mutated to arginines), HA-Ub-K63 (all lysines except lysine 63 mutated to arginines), and the NF- $\kappa$ B and AP-1 reporters have been described previously [15, 26]. Flag-TLR4 (Addgene plasmid 27148) was a gift of Bruce Beutler (UT Southwestern Medical Center, USA) [27]. Flag-IRAK1, Flag-IRAK4 and MyD88-Myc were kindly given by Professor Thomas Miethke (Technische Universität München, Germany) [28]. TRIF-HA was provided by Dr Luke AJ O'Neill (Trinity College Dublin, Ireland) [29] and Flag-TLR3 (Addgene plasmid 32712) was a gift of Saumen Sarkar (University of Pittsburgh Cancer Institute, USA) [30].

### Immunoblotting and immunoprecipitation

Cells were lysed with TNE-lysis buffer (50 mM Tris-HCl, pH



**Figure 7** Schematic representation on how UBE2O interferes with TRAF6-mediated NF- $\kappa$ B signaling. This figure is adapted from a figure in a review [33].

7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40) plus protease inhibitors cocktail (Roche) for 10 min on ice. After centrifugation at 13 200 rpm/4 °C for 10 min, protein concentration was measured (DC protein assay, Bio-Rad) and equal amounts of lysates were used for immunoblotting analysis. For immunoprecipitation, supernatants were incubated with Flag-Resin (A2220, Sigma) or Myc-Resin (A7470, Sigma) at 4 °C for 2 h. The precipitates were washed three times with TNE buffer, bound proteins were recovered by boiling in loading buffer for 5 min, and separated with SDS-PAGE. Western blotting was performed by the Bio-Rad mini-gel running system. Antibodies used were: c-Myc (a-14, sc-789), HA (Y-11, sc-805), TRAF6 (H-274), p38 (C-20, sc-535) and JNK (C-17, sc-474) from Santa Cruz, Flag (F3165), and  $\beta$ -actin (A5441) from Sigma, UBE2O (NBP1-03336) from Novus Biologicals, and I $\kappa$ B $\alpha$  (4814), phospho-I $\kappa$ B $\alpha$  (9246), p65 (3034), phospho-p65 (3033), IKK $\beta$  (2678), phospho-IKK $\alpha/\beta$  (2697), phospho-p38 (4631) and phospho-JNK (9255) from Cell Signaling.

### Transfection and viral infection

Cells were transfected with poly-ethylenimine (PEI, Sigma). For luciferase reporter assays, cells in 24-well plates were stimulated for 12 h with IL-1 $\beta$  (10 ng/ml) or LPS (1  $\mu$ g/ml) when indicated, and harvested 36 h after transfection. Luciferase activity was measured using the luciferase reporter assay system from Promega by a Perkin Elmer luminometer. *LacZ* expression plasmid (25 ng per well) was used for normalization. Each transfection mixture was equalized with empty vector when necessary and every experiment was performed in triplicate. For ubiquitination assays, cells were transfected in 100 mm plates.

Lentiviral vectors were produced in HEK293T cells with the helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol) and pRSV-REV as described before [31]. Cell supernatants were harvested 48 h post transfection. For stable infection, cells were treated for 24 h with the lentivirus-containing supernatants in the presence of 5  $\mu$ g/ml of polybrene (Sigma). For western blot or quantitative real-time PCR analysis, cells were re-seeded under puromycin selection (HeLa cells, 1  $\mu$ g/ml; RAW246.7 cells, 3  $\mu$ g/ml; MEF cells, 4  $\mu$ g/ml) in 6-well plates. Lentiviral vectors expressing specific shRNAs were obtained from Sigma (MISSION<sup>®</sup> shRNA). Five shRNAs were tested, and two effective shRNAs were chosen for experiments. For human UBE2O, TRCN0000004587 (sh1), and TRCN0000004589 (sh2) were used. For mouse UBE2O, TRCN0000095042 (sh1) and TRCN0000095041 (sh2) were used in this study.

### Ubiquitination assays

Cells were treated with MG132 for 4 h before harvesting. For *in vivo* ubiquitination analysis via immunoprecipitation [15], cells were washed twice in cold PBS with 10 mM N-Ethylmaleimide (NEM) and lysed in 1% SDS-RIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Sodium-deoxycholate and 1% SDS) supplemented with protease inhibitors and 10 mM NEM. Lysates were sonicated, boiled for 5 min, diluted to 0.1% SDS by RIPA buffer and centrifuged at 13 200 rpm at 4 °C for 10 min. Supernatants were incubated with Flag-Resin for 2 h, or with TRAF6 antibody and protein A/G-Sepharose (GE healthcare) for 3 h, at 4 °C. After three times washing with RIPA buffer, bound proteins were recovered by boiling in loading buffer for 5 min, and separated with SDS-PAGE. For *in vivo* ubiquitination analysis by nickel pull down (Ni-

NTA), a modified method was used [32]. Cells were washed two times in cold PBS with 10 mM NEM, and lysed in 6 ml 8 M urea buffer (8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 10 mM imidazole, 10 mM  $\beta$ -mercaptoethanol). Lysates were centrifuged at 4 000 rpm for 10 min at room temperature (RT) and incubated with nickel beads at RT for 2 h. The beads were washed three times with 8 M urea buffer, and boiled with loading buffer for 5 min before analysis with SDS-PAGE.

### Quantitative Real-time RT-PCR

RNA extraction was performed using NucleoSpin<sup>®</sup> RNA II (MACHEREY-NAGEL). Equal amounts of RNA were retrotranscribed using RevertAid<sup>™</sup> First Strand cDNA Synthesis Kits (Fermentas), and real time RT-PCR experiments were performed using SYBR Green (Bio-Rad) and a Bio-Rad machine. Primers used in this paper are available on request. Student's *t*-test was used for statistical analysis and *P* < 0.05 was considered to be statistically significant.

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