Molecular mechanisms of novel regulators in cytokine signal transduction

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Chapter 3

UBE2O negatively regulates TRAF6-mediated NF-κB activation by inhibiting TRAF6 polyubiquitination

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Abstract

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a key regulator of the activation of transcription factor NF-κB by the Interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR). Recruitment of TRAF6 to the receptor-associated IRAK1-IRAK4-MyD88 adaptor protein complex induces lysine 63 (K63) auto-polyubiquitination of TRAF6, which triggers NF-κB activation by association with its regulators, such as TAB2/3 and TAK1. Here, we identified the putative E2 ubiquitin-conjugating enzyme UBE2O as a novel negative regulator of TRAF6-dependent NF-κB signaling. We found UBE2O to bind to TRAF6, to inhibit its K63 polyubiquitination, and to prevent the activation of NF-κB by IL-1β and lipopolysacharide. We further show that the inhibitory effect of UBE2O is independent of its carboxy-terminal ubiquitin-conjugating (UBC) domain. In contrast, we found UBE2O to disrupt the IL-1β inducible association of TRAF6 with MyD88. These results provide novel clues on the regulation of signaling by IL-1R/TLR and TRAF6.

Keywords: UBE2O, TRAF6, NF-κB signaling, polyubiquitination

Introduction

The interleukin-1 receptor (IL-1R) receptor/Toll-like receptor (TLR) plays a pivotal role in the innate immunity and inflammation. It receives and transduces signals from cytokines, such as IL-1β and LPS, and thereby activates the transcription factors nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and activator protein (AP)-1 (Akira and Takeda, 2004; Medzhitov, 2001). Upon ligand stimulation, IL-1R or TLR recruits the adaptor proteins Myeloid differentiation primary response gene (MyD)88 and interleukin-1 receptor-associated kinase (IRAK)1/4, which subsequently form a complex with the mediator protein tumor necrosis factor associating factor (TRAF)6 to trigger lysine 63 (K63) auto-polyubiquitination of TRAF6 with the help of ubiquitin conjugating enzyme (UBC)13/ Uev1a (Cao et al., 1996; Deng et al., 2000; Martin and Wesche, 2002; Medzhitov et al., 1998; Wesche, 1999; Wesche et al., 1997). K63 auto-polyubiquitinated TRAF6 forms a recognition signal for recruitment of TGF-β activated kinase (TAK)1 binding protein (TAB)2/3 and activation of the TAB2-associated kinase TAK1 (Wang et al., 2001), which results in phosphorylation and degradation of the NF-κB inhibitor IκBα, by the IκB kinase complex IKKα/β/γ (Takaesu et al., 2000). This cascade of events enables the NF-κB complex to translocate to the nucleus and initiate transcriptional responses.

TRAF6 is a member of the TNF receptor-associated factors that mediate TNFR intracellular signaling, but unlike other TRAFs, TRAF6 also mediates IL-1R/TLR signaling (Chung et al., 2002). TRAF6 contains an amino (N)-terminal RING finger domain, followed by several zinc-finger domains and a conserved carboxy (C)-terminal TRAF domain (Wu and Arron, 2003). The N-terminal RING finger domain is responsible for binding of the E2 enyzme 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-κB signaling has been demonstrated by the fact that TRAF6 knockout cells failed to response to IL-1β/LPS (Lomaga et al., 1999). Control of TRAF6 activity depends for a large extent on K63 auto-polyubiquitination and several deubiquitinases have been described to regulate NF-κB signaling by removing ubiquitin chains from TRAF6 (Boone et al., 2004; Jin et al., 2008; Liang et al., 2010; Xiao et al., 2012; Zhou et al., 2012). Hence, modulation of TRAF6 polyubiquitination is an important way to control NF- κB signaling.

The putative ubiquitin conjugating enzyme UBE2O, together with BRUCE (Bartke et al., 2004; Klemperer et al., 1989), constitute the two large E2 enzymes. UBE2O was first purified from rabbit reticulocytes and named E2-230K (Klemperer et al., 1989). It is ubiquitously expressed, but preferentially in brain, skeletal muscle and heart tissues (Yokota et al., 2001). In erythroid cells, UBE2O is upregulated during the reticulocyte stage of erythroid differentiation (Haldeman et al., 1995; Wefes et al., 1995). The function of UBE2O remains mostly unknown. By using yeast two-hybrid screens, Markson and co-workers identified more than hundred E3 ubiquitin ligases interacting with UBE2O, including TRAF6 (Markson et al., 2009). 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 ubiquitin conjugating domain and that UBE2O disrupts the formation of the MyD88-TRAF6 protein complex. As far as we know, this is the first paper to show a function for UBE2O.

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-1β, lipopolysaccharides (LPS) and MG132 were purchased from Sigma. Poly(I:C) was purchased from Invivogen. Full-length hUBE2O (Yokota et al., 2001) 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 a 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-κB and AP-1 reporters have been described previously (Munoz et al., 1994; Zhou et al., 2012). Flag-TLR4 was was a gift of Bruce Beutler (addgene plasmid 27148) (Poltorak et al., 1998). Flag-IRAK1, Flag-IRAK4, MyD88-Myc was kindly given by Prof. Thomas Miethke (Cirl et al., 2008). TRIF-HA was provided by Dr. Luke A. J. O'Neill (Doyle et al., 2012) and Flag-TLR3 was a gift of Saumen Sarkar (addgene plasmid 32712) (Zhu et al., 2010).

Immunoblotting and immunoprecipitation

Cells were lysed with TNE-lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40) plus protease inhibitors cocktail (Roche) for 10 min on ice. After centrifugation at 13200 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 hours. The precipitates were washed three times with TNE buffer, bound proteins removed by boiling with 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 β-actin (A5441) from Sigma, UBE2O (NBP1-03336) from Novus Biologicals, and IκBα (4814), phospho-IκBα (9246), p65 (3034), phospho-p65 (3033),

IKKβ (2678), phospho-IKKα/β (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 hours with IL-1β (10 ng/ml) or LPS (1 μg/ml) when indicated, and harvested 36 hours after transfection. Luciferase activity was measured using the luciferase reporter assay system from Promega by a Perkin Elmer luminometer. *LacZ* expression plasmid (25 ng/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 100mm plates.

Lentiviral vectors were produced in HEK293T cells with the helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV as described before (Zhang et al., 2012). Cell supernatants were harvested 48 hours post transfection. For stable infection, cells were treated for 24 hours with the lentivirus-containing supernatants in the presence of 5 μg/ml of polybrene (Sigma). For western blot or quantitative real-time PCR analysis cells were re-seeded under puromycin selection (HeLa cells, 1 μg/ml; RAW246.7 cells, 3 μg/ml; MEF cells, 4 μg/ml) in 6-well plates. Lentiviral vectors expressing specific shRNAs were obtained from Sigma (MISSION® shRNA). Five shRNAs were tested, and two efficient shRNAs were chosen for experiments. For human UBE2O, TRCN0000004587 (sh1), and TRCN0000004589 (sh2) were used. For mouse UBE2O, TRCN0000095042 (sh1), TRCN0000095041 (sh2) were used in this study.

Ubiquitination assays

Cells were treated with MG132 for 4 hours before harvesting. For *in vivo* ubiquitination analysis via immunoprecipitation (Zhou et al., 2012), 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 13200 rpm/ 4°C for 10 min. Supernatants were incubated with Flag-Resin for 2 hours, or with TRAF6 antibody and protein A/G-Sepharose (GE healthcare) for 3 hours, at 4°C. After 3 times washing with RIPA buffer, bound proteins were removed by boiling with 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 (Tatham et al., 2009). 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 Na2HPO4, 0.1 M NaH2PO4, 10 mM Tris-HCl, 10 mM imidazole, 10 mM β-mercaptoethanol). Lysates were centrifuged at 4000 rpm for 10 min at room temperature (RT) and incubated with Nickel beads at RT for 2 hours. The beads were washed 3 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® RNA II (MACHEREY-NAGEL). Equal amounts of RNA were retro-transcribed using RevertAidTM First Strand cDNA Synthesis Kits (Fermentas), and real time reverse transcription-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.

Results

UBE2O interacts with TRAF6 and inhibits its transcription stimulatory activity

Figure 1

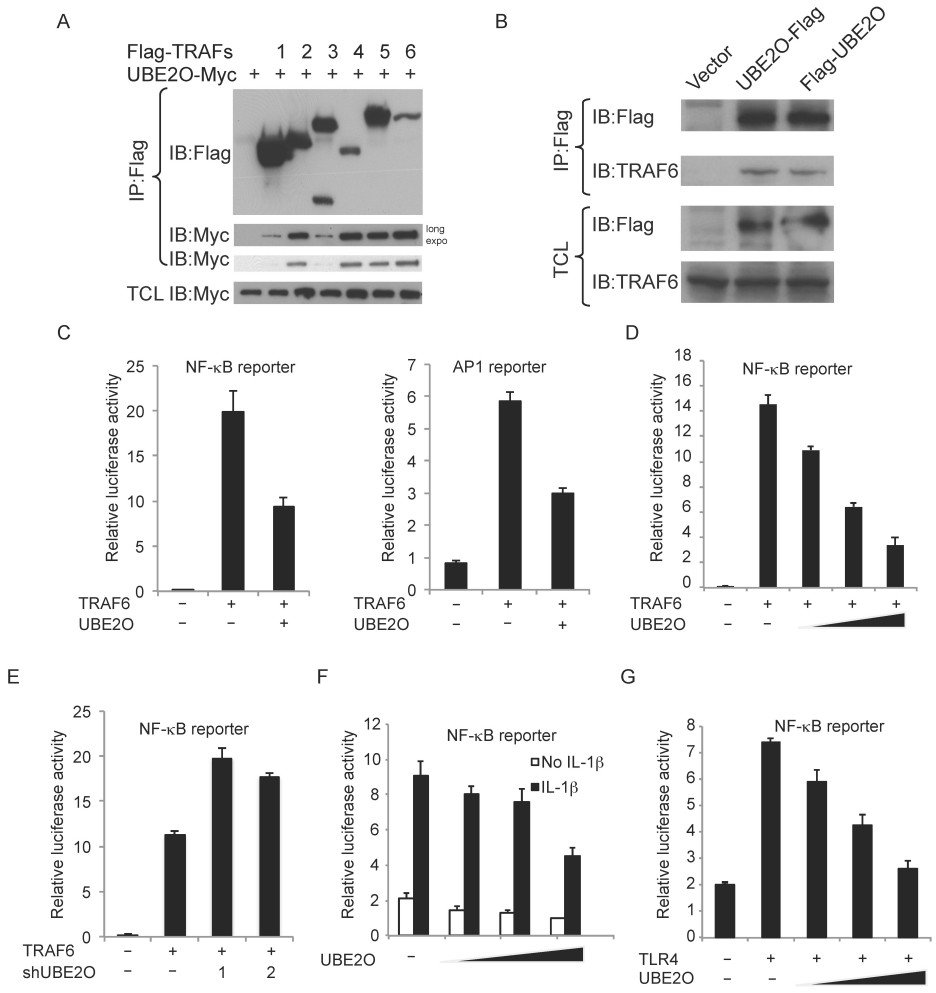

Figure 1 UBE2O interacts with TRAF6 and inhibits its transcription stimulatory activity. (**A**) HEK293T

cells were transiently transfected with UBE2O-Myc and Flag-TRAF expression vectors and analyzed by immunoprecipitation (IP) and/or immunoblotting (IB) as indicated. Myc antibody was used for detection of TRAF-associated UBE2O. TCL: total cell lysate. (**B**) Primary mouse embryonic fibroblast (MEF) cells were infected with the indicated UBE2O vectors and analyzed by immunoprecipitation (IP) and/or immunoblotting (IB) to detect UBE2O-associated endogenous TRAF6. (**C, D**) HEK293T cells were co-transfected with NF-κB or AP-1 transcriptional luciferase reporter constructs, together with TRAF6 and (increasing amounts of) UBE2O vectors as indicated. Luciferase activity was measured 36 hours after transfection. (**E**) HEK293T cells were co-transfected with NF-κB reporter, TRAF6 vector and two independent UBE2O shRNA vectors as indicated. (**F**) HEK293T cells were co-transfected with NF-κB reporter, and increasing amounts of UBE2O vector. 24 hours after transfection, cells were treated with 10 ng/ml IL-1β for 12 hours. (**G**) HEK293T cells were co-transfected with NF-κB 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.

The putative E2 enzyme UBE2O has previously been reported to interact with the signaling mediators TRAF4, TRAF5 and TRAF6 in yeast two-hybrid assays (Markson et al., 2009). 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 strongly interact with TRAF5 and 6. We next analyzed the effect of UBE2O on TRAF-regulated NF-κB reporter gene activity. UBE2O had the strongest effects on TRAF6-induced activation (Supplementary information, Figure S1C and Supplementary information, S1D), and we therefore further focused on the effects of UBE2O on TRAF6-mediated signaling. Endogenous TRAF6 was found also to interact with ectopic UBE2O, both in primary mouse fibroblasts (MEF) and HEK293T cells (Figure 1B and Supplementary information, Figure S1B). Moreover, UBE2O inhibited the transcription stimulatory function of TRAF6 both on NF-κB and AP1 reporter genes (Figure 1C), and in a UBE2O dose-dependent manner (Figure 1D and Supplementary information Figure S1E). A control reporter lacking the three NF-κB 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 activating ability of TRAF6 on the NF-κB reporter was potentiated by two independent shRNAs targeting UBE2O (Figure 1E and Supplementary information, Figure S1F). UBE2O also dose-dependently inhibited IL-1β or TLR4-induced NF-κB reporter activation (Figure 1F, 1G and Supplementary information, Figure S1G). We next analyzed the specificity of the inhibitory effect of UBE2O on NF-κB signaling, by stimulating with poly (I:C) or TIR-domain-containing adapter-inducing interferon-β (TRIF). Importantly, UBE2O had no effect on poly (I:C)- or TRIF- induced NF-κB reporter activity (Supplementary information, Figure S2A and Supplementary information, Figure S2B). Moreover, UBE2O inhibited NF-κB 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-κB signaling cascade. In conclusion, UBE2O appears to be a potent regulator of TRAF6.

UBE2O impairs NF-κB activation in multiple cell types

Figure 2

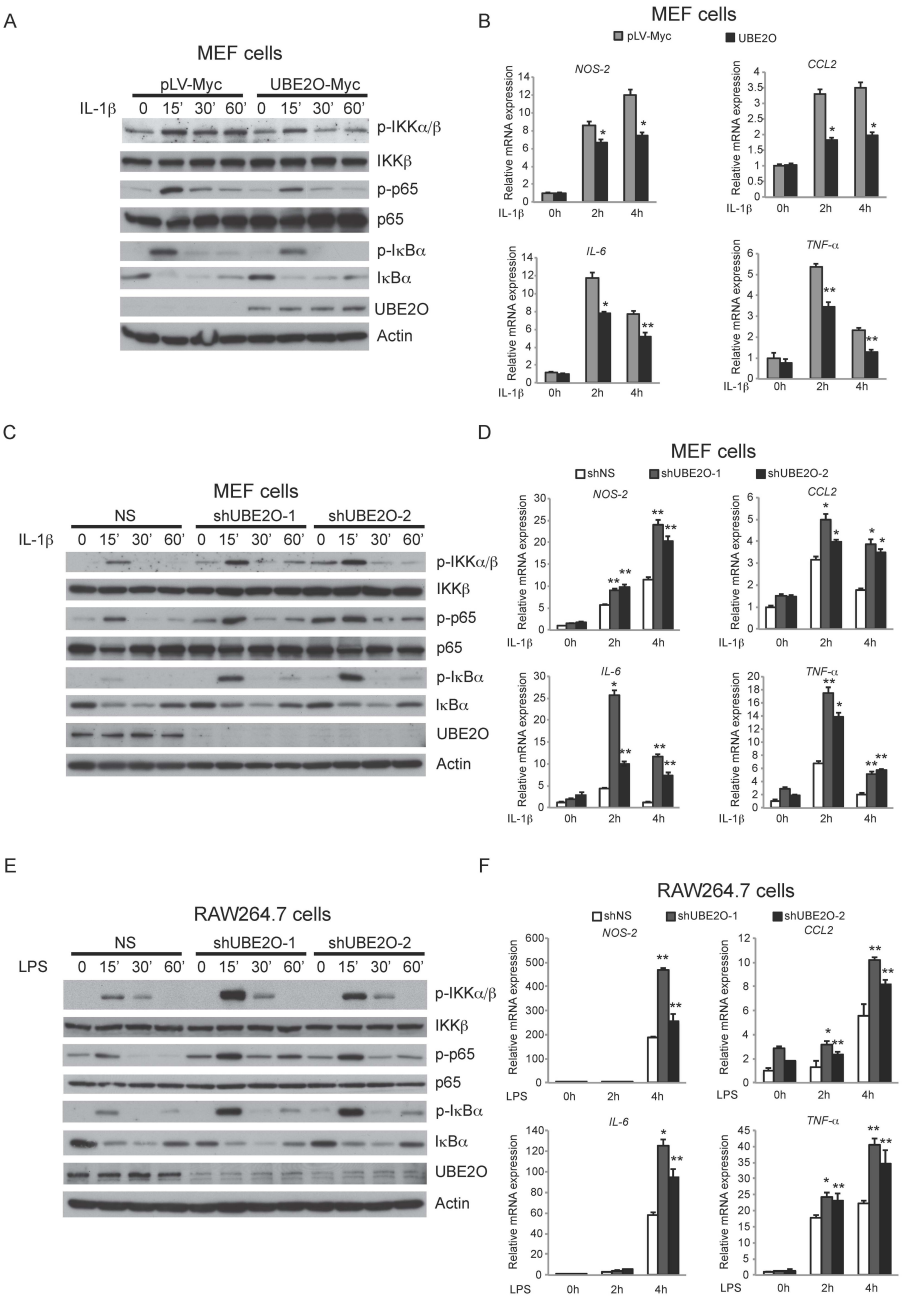

Figure 2 UBE2O impairs IL-1β/LPS-induced NF-κB activation. (**A, B**) Primary MEF cells were infected with control (pLV-Myc) or UBE2O-Myc lentiviral vectors, selected for puromycin-resistance, treated with 10 ng/ml IL-1β for the indicated time points and cell lysates were analyzed for expression levels of selected (phosphor) proteins by immunoblotting with the indicated antibodies (A), or of selected genes by real-time PCR mRNA detection (B). Actin was used as loading control (A). GAPDH was used for normalization (B). * indicates $p<0.05$

and ** indicates p<0.01 (UBE2O overexpression was compared with control vector). (C-F) Primary MEF cells (C, D) or macrophage Raw264.7 cells (E, F) infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (shUBE2O-1, -2) were stimulated with 10 ng/ml IL-1β or 1 μg/ml LPS for indicated time points and analyzed by immunoblotting (C , E) or real-time PCR mRNA detection (D, F). Actin was included as a loading control (C, E). GAPDH was used for normalization (D, F). Values and error bars represent the mean SD of triplicates. * indicates p<0.05 and ** indicates p<0.01 (shUBE2Os were compared with non-specific control shRNA).

To examine whether UBE2O is relevant for the activation of endogenous NF-κB, we analyzed the NF-κB signaling components IκBα, IKKα/β and p65 in primary MEF cells. As shown in Figure 2A, lentivirus-mediated expression of UBE2O-Myc was found to reduce the IL-1β-induced phosphorylation of IκBα, IKKα/β and p65. Also IL-1β-induced phosphorylation of p38 or JNK was reduced by UBE2O overexpression (Supplementary information, Figure S3A). Since IκBα, IKKα/β and p65 phosphorylation is essential for the translocation of the NF-κB complex into the nucleus and to initiate transcription, we next analyzed NF-κB target gene expression. As shown by qPCR assays in Figure 2B, the four target genes examined (*NOS2, CCL2, IL-6* and *TNF-α*) were all significantly inhibited by UBE2O. In line with this, knockdown of UBE2O enhanced NF-κB activation by IL-1β or LPS. IL-1β-induced phosphorylation of IκBα, IKKα/β 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-1β- or LPS-induced p38 or JNK phosphorylation was also increased in UBE2O knockdown cells (Supplementary information, Figure S3B and Supplementary information, Figure S3C). Moreover, the IL-1β- or LPS-induced expression of NF-κB target genes was significantly increased when UBE2O was depleted in these cells (Figure 2D and Figure 2F). Thus, UBE2O functions as an inhibitor of NF-κB activation by IL-1R/TLR in multiple cell type.

UBE2O decreases TRAF6 polyubiquitination

TRAF6 activity is regulated by K63 polyubiquitination, which is induced by upstream receptors upon ligand binding (Deng et al., 2000). To investigate whether TRAF6 inhibition by the putative E2 ubiquitin-conjugating 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-1β stimulation. Strikingly, forced expression of UBE2O strongly inhibited this polyubiquitination both in the presence and absence of IL-1β stimulation. Similar inhibition of TRAF6 polyubiquitination by UBE2O was obtained with nickel pull down assays and co-transfection of Myc-His-ubiquitin (Figure 3B). Moreover, IL-1β-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. In contrast to TRAF6, no inhibition of polyubiquitination of other TRAFs was observed. 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 type of conjugations (Figure 3D and Figure 3E). In summary, these results suggest that UBE2O-mediated inhibition of TRAF6-induced signaling involves inhibition of TRAF6 K63 polyubiquitination.

Figure 3

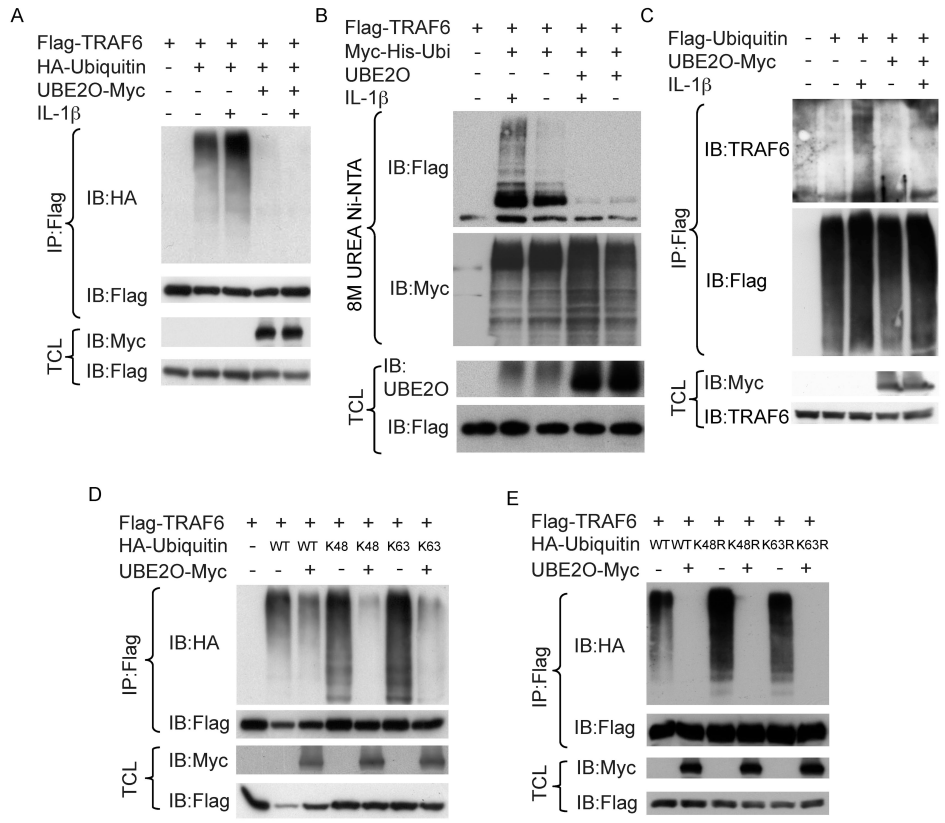

Figure 3 UBE2O decreases polyubiquitination of TRAF6. (**A**) HeLa cells were co-transfected with HA-ubiquitin, Flag-TRAF6 and UBE2O-Myc expression vectors as indicated. 44 hours post transfection the proteasome inhibitor MG132 was added for 4 hours. When indicated cells were stimulated with IL-1β for the last 30 min, and harvested for immunoprecipitation (IP) and immunoblot (IB) analysis. HA antibody was used to detect polyubiquitinated TRAF6. TCL: total cell lysate. (**B**) HeLa cells were co-transfected with Myc-His-ubiquitin, Flag-TRAF6 and UBE2O vectors as indicated, treated with MG132 and IL-1β as described above and harvested for nickel pull down (Ni-NTA) and IB analysis. (**C**) HeLa cells were co-transfected with Flag-ubiquitin and UBE2O-Myc vectors, treated with MG132 and IL-1β, and harvested for IP and IB analysis. (**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 analysis.

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

treated with IL-1β or LPS, as reported previously (Zhou et al., 2012). Importantly, loss of UBE2O by two distinct shRNAs enhanced IL-1β- or LPS-induced polyubiquitination of TRAF6 in these cells (Figure 4A and 4B). These results indicate that endogenous UBE2O negatively controls NF-κB 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 was increased upon UBE2O knockdown (Figure 4C), although the increase on K63 polyubiquitination is more obvious.

Figure 4

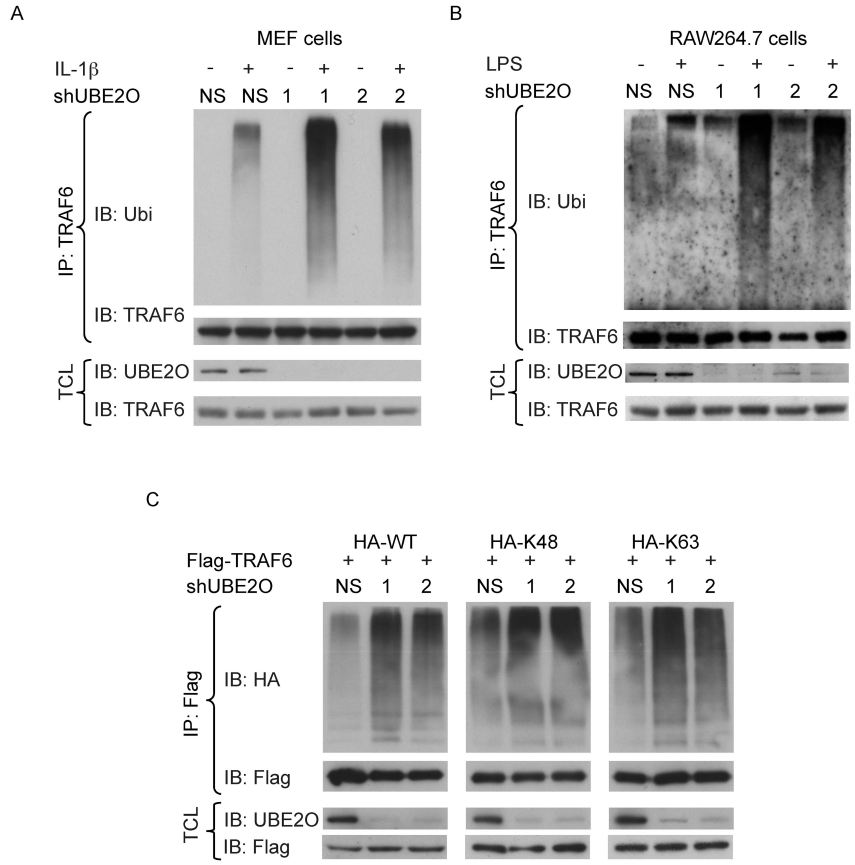

Figure 4 Depletion of UBE2O increases polyubiquitination of TRAF6. (**A, B**) Primary MEF cells (A) or macrophage Raw264.7 cells (B) stably infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (1, 2) were treated with MG132 for 4h, stimulated with IL-1β (A) or LPS (B) for 30 min and analyzed by immunoprecipitation (IP) and immunoblotting (IB). TCL: total cell lysate. 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 (1, 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 4h. HA antibody was used to detect polyubiquitinated TRAF6.

The UBC domain of UBE2O is not required for inhibition of TRAF6

Figure 5

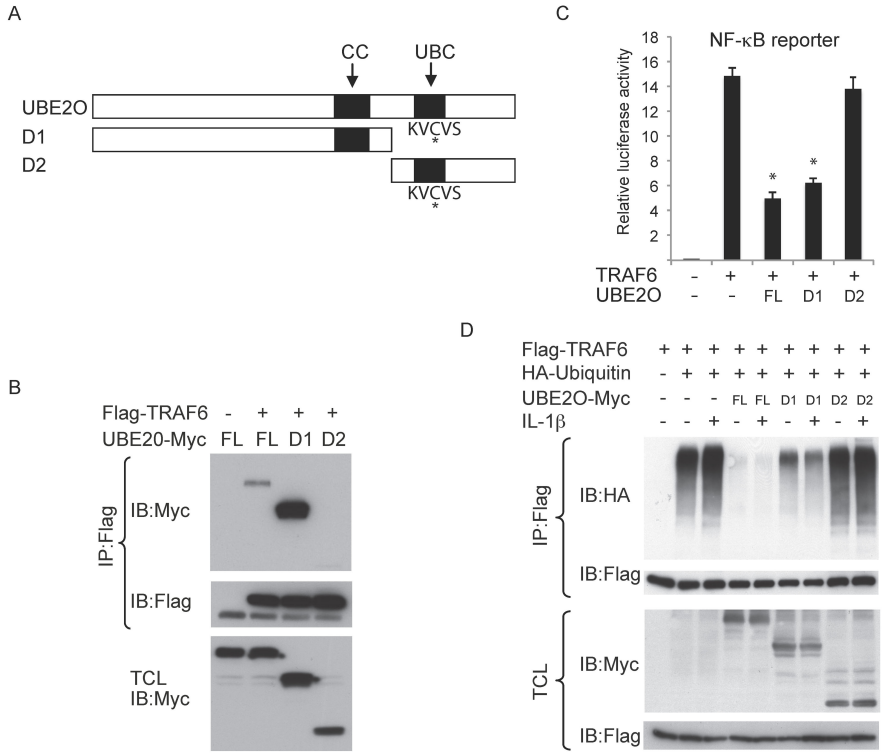

Figure 5 The UBC domain of UBE2O is not required for TRAF6 inhibition. (**A**) Conserved domains present in wild type UBE2O and UBE2O deletion mutants D1 and D2. CC: coiled-coil domain, UBC: ubiquitin-conjugating domain. KVCVS: amino acids in the E2 active site. Asterisk indicates E2 active site of UBE2O. (**B**) HEK293T cells were transfected with Flag-TRAF6 and full length (FL) or deleted (D1, D2) UBE2O-Myc expression vectors as indicated, and analyzed by immunoprecipitation (IP) and immunoblotting (IB). TCL, total cell lysate. (**C**) HEK293T cells were co-transfected with NF-κB reporter, TRAF6 and full length (FL) or deleted (D1, D2) UBE2O-Myc expression vectors as indicated. Luciferase activity was measured 36 hours after transfection. * indicates $p<0.05$. (**D**) Hela cells were co-transfected with the indicated expression vectors. 44 hours post transfection the proteasome inhibitor MG132 was added for 4 hours. When indicated cells were stimulated with IL-1β for the last 30 min, and harvested for immunoprecipitation (IP) and immunoblot (IB) analysis. HA antibody was used to detect polyubiquitinated TRAF6. TCL: total cell lysate.

Since 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 an ubiquitin-conjugating (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 construct D1, which lacks the UBC domain, but still contains the CC domain (Figure 5A), efficiently interacts with TRAF6 (Figure 5B). However, the N-terminal deletion construct D2, lacking the CC domain, but still containing the UBC domain did not (Figure 5B). Importantly, the UBC domain-deleted mutant D1 also could still inhibit

TRAF6-induced NF-κB reporter activity, in contrast to the N-terminal and CC-deleted 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

Figure 6

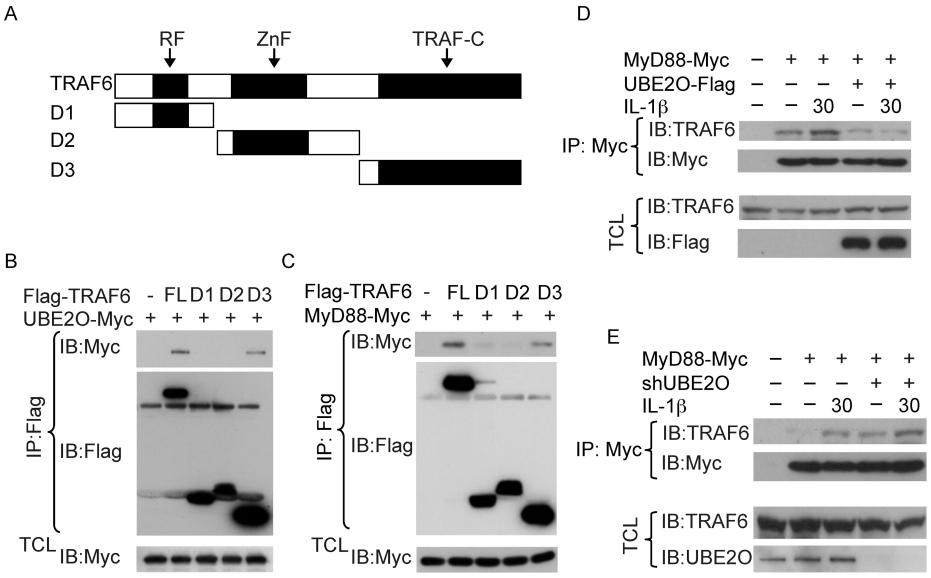

Figure 6 UBE2O disrupts the interaction between TRAF6 and MyD88. (**A**) Conserved domains present in wild type TRAF6 and the TRAF6 deletion mutants D1, D2, and D3. RF: RING finger domain; ZnF: Zinc finger domains; TRAF-C: TRAF domain. (**B, C**) HEK293T cells were transfected with full-length (FL) Flag-TRAF6, 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. TCL: total cell lysate. (**D**) HeLa cells were transfected with MyD88-Myc and UBE2O-Flag as indicated. 48 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for IP and IB analysis. 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 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for IP and IB analysis. Immunoprecipitates were blotted for MyD88 associated endogenous TRAF6 by using TRAF6 antibody.

Next, we investigated which domains of TRAF6 are required for the interaction with UBE2O. Three TRAF6 deletion constructs (D1-3) were used, containing either the N-terminal RING finger domain (RF), several zinc-finger domains (ZnF) located in the center, or the C-terminal TRAF domain (TRAF-C) (Wu and Arron, 2003). 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. 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-1β stimulation, but inhibited by forced expression of full length UBE2O or the D1 deletion mutant (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 and the interaction between MyD88 and IRAK4 (Supplementary information, Figure S5C and Supplementary information, Figure 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 its upstream regulator MyD88, and thereby counteracts TRAF6-polyubiquitination and activation of downstream signaling components.

Discussion

UBE2O was first isolated from rabbit reticulocytes, and classified as a putative E2 enzyme since it has a conserved UBC domain (Klemperer et al., 1989). However, the function of UBE2O is as yet unknown. Recently, more than hundred E3 ubiquitin ligases were found to interact with UBE2O in yeast two-hybrid screens (Markson et al., 2009), including TRAF6, a mediator of TLR/IL-1R-induced NF-κB activation. As an important part of the host defense system, TLR/IL-1R induced signaling needs to be tightly controlled to sustain immune homeostasis and avoid 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 (Akira and Takeda, 2004; Medzhitov, 2001). To avoid uncontrolled activation multiple negative regulators are engaged to serve this purpose. In this article, we demonstrate that UBE2O is involved in TRAF6-mediated NF-κB activation. Forced expression of UBE2O suppresses IL-1β and TRAF6-induced signaling and depletion of UBE2O enhances TRAF6-induced NF-κB activation. Importantly, we found that poly (I:C)- or TRIF-induced NF-κB activation is not affected by UBE2O, indicating that UBE2O specifically affects MyD88/TRAF6-mediated NF-κB activation. Furthermore, knockdown of UBE2O 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 UBE2O is a physiological negative regulator of TRAF6-mediated NF-κB signaling and JNK/p38 activation. However, our data do not rule out 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 overexpressed UBE2O also weakly reduce NF-κB reporter activity induced by some other TRAFs and TNF-α (Supplementary information, Figure S1D and data not shown). Therefore, the role of UBE2O on other TRAFs and TNF-α induced signaling need to be investigated further.

Depletion of TRAF6 revealed a determining role for TRAF6 in IL-1R/TLR signaling, since without TRAF6 cells failed to response to IL-1β and LPS (Lomaga et al., 1999). To activate NF-κB signaling, TRAF6 needs to be polyubiquitinated by 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 deubiquitnases A20, CYLD, MCPIP1, USP4 or USP2a (Boone et al., 2004; He et al., 2012; Jin et al., 2008; Liang et al., 2010; Xiao et al., 2012; Zhou et al., 2012). In our study, we found UBE2O to bind endogenous TRAF6 and to inhibit 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 when coexpressed with UBE2O. Therefore, it remains to be established whether the change in K48 polyubiquitination has any effects on TRAF6 function.

Figure 7

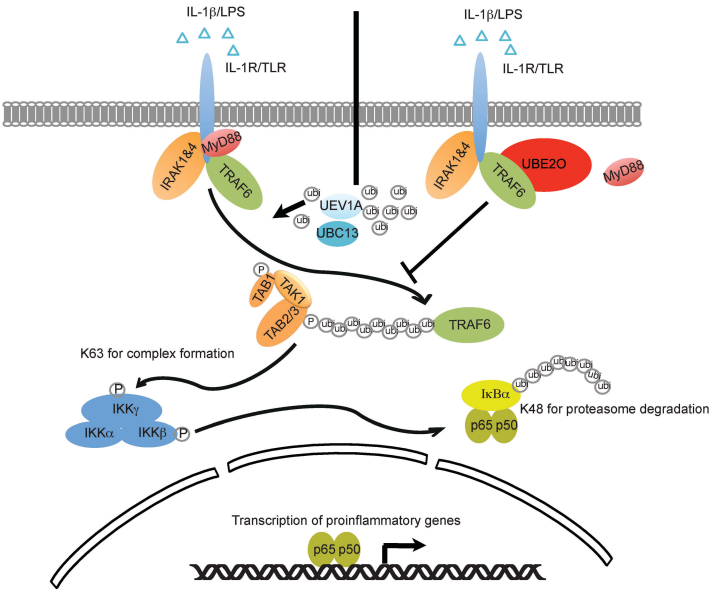

Figure 7 Schematic representation on how UBE2O interferes with TRAF6 mediated NF-κB signaling. This figure is adapted from a figure in a review (Shembade and Harhaj, 2012).

We found a C-terminal deletion mutant of UBE2O, lacking the UBC domain but still containing the coiled-coil domain, to interact 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, suggested that UBE2O disrupts the interaction between TRAF6 and one or more of its upstream regulators. Indeed, UBE2O interferes with the interaction of TRAF6 and MyD88, but not with the interaction between IRAK1, IRAK4 or MyD88, nor interferes with the interaction between IRAK1 and TRAF6 or the interaction between IRAK4 and MyD88. Moreover, we showed that

only the C-terminal deletion mutant of UBE2O could 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-signaling. Moreover, our results show that a putative E2 enzyme can act as scaffold protein rather than as an ubiquitin conjugation enzyme to restrict of TRAF6-mediated NF-κB activation. This helps to understand the molecular regulation of TRAF6-dependent processes and the functions of E2 enzymes.

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Conflict of interest:

The authors declare no conflicts of interest.

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Supplementary Information

Supplementary Figures

Figure S1

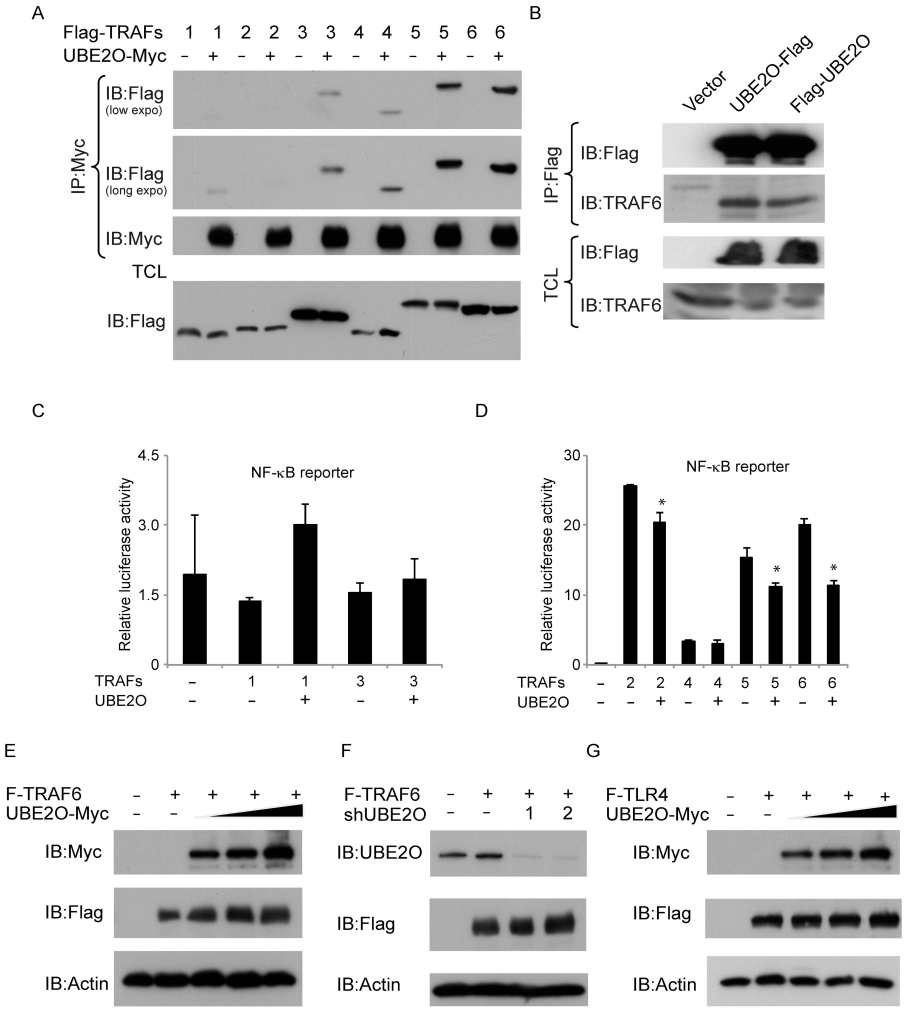

Figure S1 UBE2O interacts with and inhibits TRAF6-induced NF-κB reporter activity. (A) HEK293T cells were transiently transfected with UBE2O-Myc and Flag-TRAFs expression vectors and analyzed by immunoprecipitation (IP) and/or immunoblotting (IB) as indicated. Anti-c-Myc agarose affinity gel was used to immuoprecipitate UBE2O. Flag antibody was used for detection of UBE2O-associated TRAFs. TCL: total cell lysate. (**B**) HEK293T cells were transfected with the indicated UBE2O vectors and analyzed by immunoprecipitation (IP) and/or immunoblotting (IB) to detect UBE2O-associated endogenous TRAF6. (**C**) and (**D**) HEK293T cells were co-transfected with NF-κB reporter and Flag-TRAFs (1, 2, 3, 4, 5, 6) in the absence or presence of UBE2O. Luciferase activity was measured after 36 hours transfection. For all the luciferase assays, *LacZ* expression plasmid was co-transfected as internal reference. Each experiment above was performed in triplicate. * indicates $p<0.05$. (**E**), (**F**) and (**G**) Cell lysates from Figure 1D, Figure 1E and Figure 1G were used to analyze expression of Flag-TRAF6, UBE2O-Myc and Flag-TLR4. Actin was served as internal reference.

Figure S2

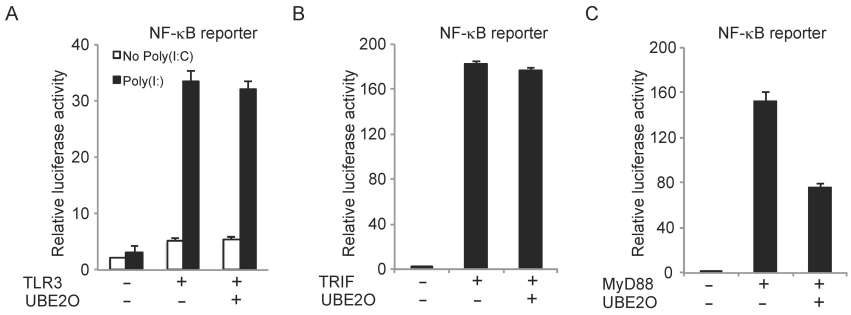

Figure S2 UBE2O has no effects on TLR3 mediated NF-ɤB signal activation. (A) HEK293T cells co-transfected with NF-ɤB reporter, TLR3 or/and UBE2O were treated with poly (I:C) as indicated. **(B)** HEK293T cells were co-transfected with NF-ɤB reporter, together with TRIF and UBE2O vectors as indicated. **(C)** HEK293T cells were co-transfected with NF-ɤB reporter, together with MyD88 and UBE2O vectors as indicated. Luciferase activity was measured after 36 hours transfection. For all the luciferase assays, *LacZ* expression plasmid was co-transfected as internal reference. Each experiment above was performed in triplicate.

Figure S3

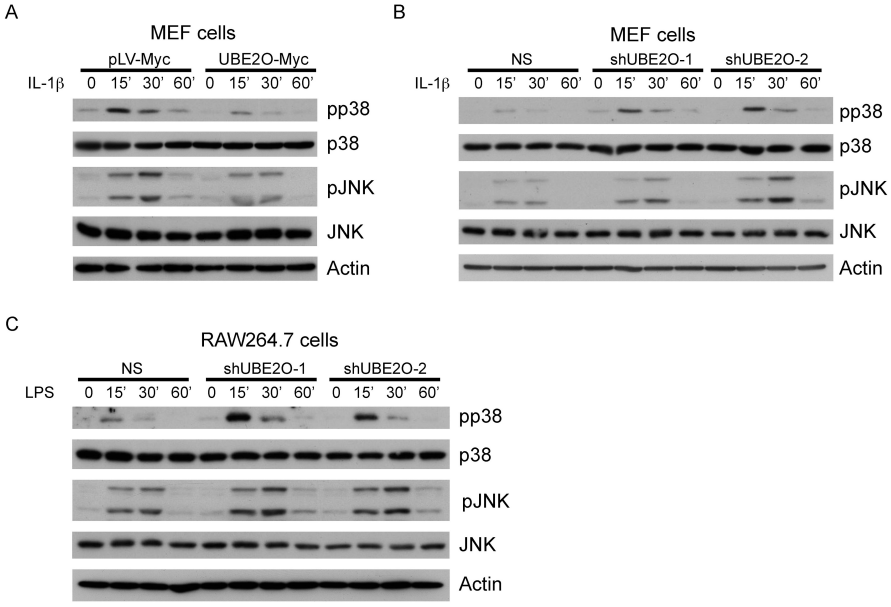

Figure S3 UBE2O impairs IL-1β/LPS-induced MAPK activation. (**A**) Primary MEF cells were infected with control (pLV-Myc) or UBE2O-Myc lentiviral vectors, selected for puromycin-resistance, treated with 10 ng/ml IL-1β for the indicated time points and analyzed by immunoblotting. Same protein samples as used in Figure 2A. (**B**) and (**C**) Primary MEF cells (**B**) or macrophage Raw264.7 cells (**C**) infected with lentiviral vectors expressing non-specific control shRNA (NS) or UBE2O shRNAs (shUBE2O-1, -2) were stimulated with 10 ng/ml IL-1β (**B**) or 1 μg/ml LPS (C) for indicated time points and analyzed by immunoblotting. Same protein samples as used in Figure 2C and Figure 2E.

Figure S4

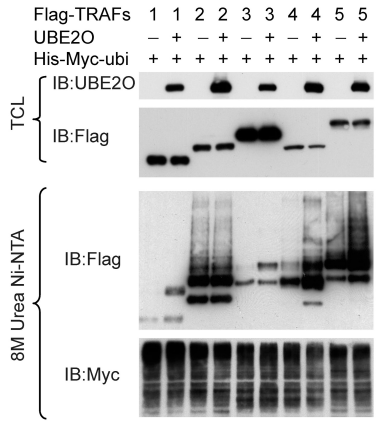

Figure S4 Effects of UBE2O on TRAF ubiquitination. HeLa cells were co-transfected with Myc-His-ubiquitin, Flag-TRAFs with or without UBE2O as indicated. 44 hours post transfection the proteasome inhibitor MG132 was added for 4 hours, total cell lysates (TCL) were harvested for nickel pull down (Ni-NTA) and immunoblotting (IB) analysis.

Figure S5

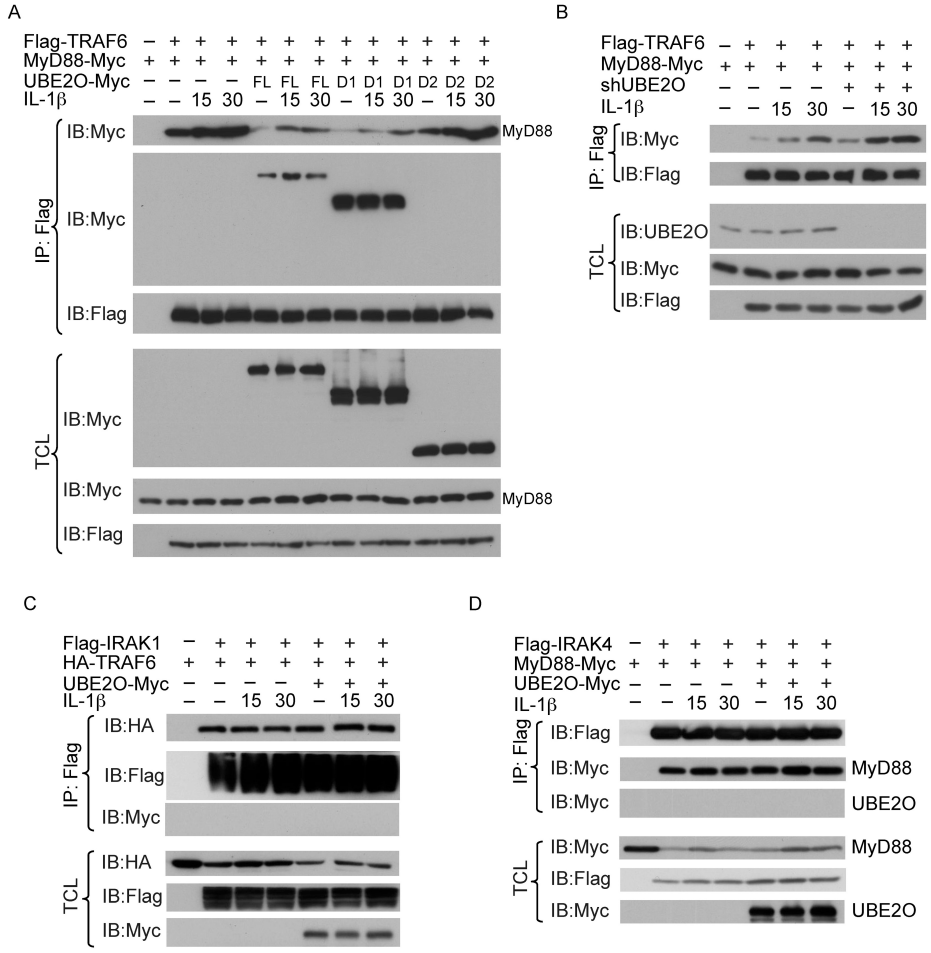

Figure S5 UBE2O disrupts the interaction between TRAF6 and MyD88, but not the TRAF6-IRAK1 or MyD88-IRAK4 complex. (**A**) HEK293T cells were transfected with Flag-TRAF6, MyD88-Myc and full length (FL) or deleted (D1, D2) UBE2O-Myc expression vectors as indicated. 48 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for immunoprecipitation (IP) and immunoblotting (IB) analysis. (**B**) HEK293T cells stably expressing UBE2O shRNAs (shUBE2O-1+shUBE2O-2) were transfected with Flag-TRAF6 and MyD88-Myc as indicated. 48 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for IP and IB analysis. (**C**) HEK293T cells were transfected with Flag-IRAK1, HA-TRAF6 and UBE2O-Myc as indicated. 48 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for IP and IB analysis. Immunoprecipitates were blotted for IRAK1-associated TRAF6 by using HA antibody. (**D**) HEK293T cells were transfected with Flag-IRAK4, MyD88-Myc and UBE2O-Myc as indicated. 48 hours after transfection cells were treated with IL-1β for the indicate minutes and lysed for IP and IB analysis. Immunoprecipitates were blotted for IRAK4-associated MyD88 by using Myc antibody.