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Ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 inhibits inflammation by regulating Nur77 stability

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ABSTRACT

Nur77 (NR4A1) plays an important role in various inflammatory responses. Nur77 is rapidly degraded in cells and its protein level is critically controlled. Although few E3 ligases regulating the Nur77 protein have been defined, the deubiquitinase (DUB) responsible for Nur77 stability has not been reported to date. We identified ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) as a DUB that stabilizes Nur77 by preventing its proteasomal degradation. We found that OTUB1 interacted with Nur77 to deubiquitinate it, thereby stabilizing Nur77 in an Asp88-dependent manner. This suggests that OTUB1 targets Nur77 for deubiquitination *via* a non-canonical mechanism. Functionally, OTUB1 inhibited TNFα-induced IL-6 production by promoting Nur77 protein stability. OTUB1 modulated the stability of Nur77 as a counterpart of tripartite motif 13 (Trim13). That is, OTUB1 reduced the ubiquitination and degradation of Nur77 potentiated by Trim13. In addition, this DUB also inhibited IL-6 production, which was further amplified by Trim13 in TNFα-induced responses. These findings suggest that OTUB1 is an important regulator of Nur77 stability and plays a role in controlling the inflammatory response.

1. Introduction

Nur77, an orphan nuclear receptor whose ligand is unknown, is a transcription factor belonging to the nuclear receptor subfamily 4 group A member (NR4A) family. Nur77 (NR4A1) is the same family as Nurr1 (NR4A2) and Nor-1 (NR4A3). This family has common functional domains, consisting of an N-terminal activation function domain, a central DNA-binding domain, and a C-terminal ligand-binding domain [1,2]. Although the cellular level of Nur77 is very low, it is rapidly induced by certain stimuli, such as lipopolysaccharides (LPS) or cytokines [3]. Nur77 is widely expressed in myeloid [4], lymphoid [5], and epithelial cells [6] and is an important coordinator for the differentiation of regulatory T-cells [7] and Ly6C^{low} monocytes [8,9]. Extracellular stimuli regulate Nur77 expression and activity; however, post-translational modifications such as phosphorylation are also important for controlling Nur77 function [10,11]. Nur77 forms homodimers or heterodimers with other types of transcription factors to exert transcriptional activity [12]. In addition, the Nur77 protein is known to bind to several proteins, including E3 ligases [13], but how these binding proteins regulate Nur77 is not well understood.

The regulation of apoptosis by Bcl-2 binding is one of the most important roles of Nur77 [14]. Additionally, Nur77 plays an important role in steroid hormone synthesis [15], metabolism [16,17], and vascular remodeling [18]. Nur77 has been reported to be a potent suppressor of inflammation. Nur77 reduced airway inflammation in mice through the inhibition of nuclear factor κB (NF-κB) activity [6]. Nur77 has been reported to regulate TRAF6-mediated NF-κB activity, thus exhibiting protective effect against inflammatory bowel disease [19]. Furthermore, Nur77 suppressed LPS-induced inflammation by regulating its relationship with p38 mitogen-activated protein kinase [20].

Abbreviations: NR4A, nuclear receptor subfamily 4 group A member; LPS, lipopolysaccharide; Trim13, tripartite motif containing 13; DUB, deubiquitinase; OTUB1, ovarian tumor domain-containing ubiquitin aldehyde binding protein 1; MDM2, mouse double minute 2 homolog; (sgRNA), synthetic guide RNA; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; CHX, cycloheximide; IL-6, interleukin-6; TNFα, tumor necrosis factor α; TRAF, TNF receptor associated factor; USP, ubiquitin specific peptidase

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The mechanism of Nur77 is mainly influenced by transcriptional activity to exert anti-inflammatory effects. Recently, however, the level of Nur77 protein has been considered to be important for its anti-inflammatory effect. We have previously reported that the degradation of Nur77 protein by tripartite motif 13 (Trim13) amplified TNFα-induced inflammatory responses [21]. It has been also reported that Nur77, triggered by sumoylation, is ubiquitinated and degraded by ring finger protein 4 (RNF4) and eventually regulated macrophage cell death [13]. Ubiquitination and deubiquitination in cells are reversible reactions, which are important in the regulation of protein stability. However, the regulation of Nur77 deubiquitination remains largely unclear. Therefore, identifying the deubiquitinase (DUB) responsible for the stability of Nur77 is essential.

Ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) was first identified as a DUB-mediating lymphocyte antigen [22]. Subsequently, OTUB1 was reported as a DUB that regulated the stability and transcriptional activity of estrogen receptor α by deubiquitination [23] or modulated the anti-viral response by deubiquitination of TRAF3/6 in virus-triggered cell signaling [24]. The key function of OTUB1 is to regulate protein stability. Proteins stabilized by OTUB1 include Snail that promotes metastasis of esophageal cancer [25], DEP domain-containing mTOR-interacting protein (DEPTOR) that inhibits mammalian target of rapamycin (mTOR) complex 1 activity [26], Smad2/3 that facilitates transforming growth factor β signaling [27], and c-inhibitor of apoptosis (c-IAP) protein that regulates TNF receptor-mediated signaling [28]. As described, the target proteins of OTUB1 are very diverse and have a wide range of functions. Another important role of OTUB1 is to counteract the role of E3 ligases. For example, OTUB1 acted as a partner of mouse double minute 2 homolog (MDM2) to stabilize p53, which promoted cell apoptosis and inhibited cell proliferation [29]. Although MDM2 has been the only E3 ligase that has been identified to counteract OTUB1, there may be more E3 ligases that compete with OTUB1 for a specific target protein.

Here, we demonstrated that OTUB1 catalyzed deubiquitination at Asp88 after binding to Nur77, thus stabilizing these proteins. Furthermore, OTUB1 was involved in the anti-inflammatory effect of Nur77 by delaying its degradation in the TNFα-induced IL-6 production. OTUB1 also counteracted DUB in the ubiquitination and degradation of Nur77 by Trim13. These results suggested that OTUB1 and Trim13 serve as a counterbalance in the regulation of Nur77 stabilization and may be used as an axis controlling the inflammatory response.

2. Materials and methods

2.1. DNA constructs

OTUB1 and its mutants were cloned into pFLAG-CMV2 or pCMV2-Myc vectors and verified by DNA sequencing. The OTUB1 mutants were generated by targeting Asp88, Cys91, or His265 of OTUB1 by mutagenesis. Full-length Nur77 cDNA was cloned into the pFLAG-CMV2 vector. For Myc-tagged Nur77, *Nur77* was amplified from pCMV2 Flag-tagged Nur77 by PCR and inserted into the *Eco*RI/BamHI sites. Full-length Trim13 cDNA was also cloned into the pEGFP-C1 vector. Ubiquitin and all ubiquitin mutants were cloned into the pCMV-HA-N vector (Clontech, Mountain View, CA).

2.2. Cell culture and transfection

HeLa cells (human epithelial cell line) and U937 (human macrophage-like cell line) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and split sub-confluent cultures (70–80%) 1:3 to seeding using 0.25% Trypsin/EDTA. Cultured cells were transfected using the Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA) for cDNA, Lipofectamine RNAiMAX transfection reagent (Invitrogen) for siRNA and synthetic guide RNA (sgRNA). *OTUB2* siRNA (sc-76016) was purchased from Santa Cruz Biotechnology (Dallas, TX).

2.3. Antibodies and immunoblotting

The following antibodies were used in this study: a polyclonal anti-OTUB1 antibody (Abcam, Cambridge, UK); a polyclonal anti-Nur77 antibody (Cell Signaling Technology, Beverly, MA or Proteintech, Rosemont, IL); an anti-Flag-M2 antibody (Sigma-Aldrich, St. Louis, MO); an anti-Myc antibody, an anti-HA antibody, and an anti-GFP antibody (Santa Cruz Biotechnology); and the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Cell extracts were prepared in Nonidet P-40 (NP-40) buffer (50 mM Tris-HCl [pH 8.0], 105 mM NaCl, 1% NP-40, 1% SDS, and protease inhibitor cocktail). Proteins were resolved by SDS-PAGE and transferred onto an Amersham Protran NC membrane (GE Healthcare Life science, Freiburg, Germany). Immunoblots were visualized using the LAS-3000 system (GE Healthcare Life science). Densitometric analysis was performed using the LAS-3000 Image Reader and MultiGauge 3.0 software.

2.4. CRISPR-Cas9-mediated gene editing

For the generation of an OTUB1 knockout cell line, a CRISPR-Cas9 construct from Santa Cruz Biotechnology (sc-407665-Nic) was used. sgRNA (25 nM) expression vector containing GFP was transfected into HeLa cells using Lipofectamine RNAiMAX transfection reagent. After 72 h, direct cells were confirmed by observations for GFP and seeded in 96-well plates for single colony isolation. The OTUB1 knockout clones were selected by immunoblot analysis using OTUB1 antibody.

2.5. Site-directed mutagenesis

OTUB1 mutants were cloned into a pFlag-CMV2 expression vector with *Eco*RI/BamHI restriction enzyme sites. Glutamate, serine, or alanine substitutions were introduced into the Nur77 Flag-CMV2 construct at Asp88 (D88E), Cys91 (C91S), and His265 (H265A) using the QuikChange site-directed mutagenesis kit (Stratagene). All mutations were confirmed by DNA sequencing.

2.6. Reverse transcription (RT) and polymerase chain reaction (PCR)

Confluent HeLa cells were transfected with Myc-tagged OTUB1 construct for 36 h before RNA isolation. Total RNA from cultured cells was isolated using the RNEasy mini-kit (Qiagen, Valencia, CA), and concentration was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RT was performed on 1 μg RNA using AMV RTase XL kit (TaKaRa, Japan). PCR was performed using TaKaRa Ex Taq kit on a PCR platform (TaKaRa) and qPCR was performed using LightCycler FastStart DNA master SYBR Green I kit (Roche) on a LightCycler 1.5 PCR platform (Roche). Target gene (Nur77) expression was normalized by dividing by the geometric mean of β-actin or β2m housekeeping gene expression.

2.7. Deubiquitination assay

For the deubiquitination assay, cells were transfected with vectors expressing each gene as indicated. At 36 h after the transfection, the cells were treated with the proteasome inhibitor MG132 (10 μM) for 4 h. Next, the cells were collected in PBS, and 5% of the cell suspension was stored as the input sample. The remainder of the cell suspension was centrifuged for 5 min, and the cells were then lysed in 700 μl of ubiquitination buffer (0.5% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM *N*-ethylmaleimide, and protease inhibitor cocktail). The lysates were then centrifuged at 12,000 × *g* for 10 min, and then incubated with 1.5% SDS and 1 mM dithiothreitol at

65 °C for 20 min in order to denature the proteins, after which they were diluted 10-fold with lysis buffer. The denatured lysates were incubated overnight with the indicated antibodies and protein A/G PLUS agarose (Santa Cruz Biotechnology, Dallas, TX), resolved by SDS-PAGE, and immunoblotted with the indicated antibodies.

2.8. Protein half-life analysis

Comparison of protein stability in cells achieved by cycloheximide (CHX) chase experiment. Cells were treated with CHX (20 μg/ml) for various times, and Nur77 level was detected by immunoblotting. The density of the bands corresponding to Nur77 or β-actin were measured. The amount of the Nur77 protein was quantified by normalization with the level of β-actin protein, and the percentage of the remaining Nur77 was plotted.

2.9. Quantification of IL-6 production by enzyme immunoassay (EIA)

After cell stimulation, the concentrations of IL-6 in culture supernatants were measured by EIA kit (R&D systems, Minneapolis, MN). The supernatants from the cell cultures with or without exposure to the indicated stimulant were collected at various time points. EIA for quantifying IL-6 was performed according to the manufacturer's instructions.

2.10. Immunoprecipitation (IP)

For IP, whole cell extracts were collected 36 h after transfection and were lysed in IP buffer containing 1% NP-40, 20 mM HEPES, pH 7.5, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl, 10 μM NaF, and a protease inhibitor mixture. Cell lysates were incubated with the appropriate antibody overnight and subsequently incubated with protein A-Sepharose beads for 1 h. Immnunoprecipitates were eluted by boiling with 2× SDS sample buffer. For western blot analysis, immunoprecipitates or whole cell lysates were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted using the appropriate antibodies.

2.11. Statistical analysis

Optical intensity was measured using the AlphaEasy program (Version 5.1; Alpha Innotech, San Leandro, CA). Data were analyzed using GraphPad Prism Version 4 (GraphPad Software Inc., San Diego, CA). All numerical values are presented as mean ± SD. Statistical significance was determined using two-tailed Student's *t*-test.

3. Results

3.1. OTUB1 stabilizes the Nur77 protein

To identify OTUB1, which might regulate Nur77 protein stability, we transfected Myc-tagged OTUB1 plasmids into HeLa cells and analyzed the Nur77 protein levels. We found that overexpression of OTUB1 significantly increase the Nur77 protein level. Other transcription factors, FoxO1 or STAT3, did not change the level (Fig. 1A). These results suggest that OTUB1 specifically regulates the level of Nur77. Considering that Nur77 is an important regulator of inflammation, we studied OTUB1 for its potential to regulate Nur77 deubiquitination and stabilization. Meanwhile, we simultaneously examined the mRNA level of Nur77 and found that overexpression of OTUB1 did not affect Nur77 mRNA levels (Fig. 1B). To further validate the observation that OTUB1 regulates Nur77 protein stability, we established OTUB1-knockout HeLa cells using the CRISPR-Cas9 system. The knockout of OTUB1 lead an attenuation of Nur77 protein despite the absence of changes in the Nur77 mRNA level (Figs. 1C & D). These results indicated that OTUB1 could facilitate the accumulation of Nur77 protein without affecting mRNA expression. To confirm the stability of Nur77 by OTUB1, we performed a CHX chase assay. The plasmid containing Nur77 was co-transfected with the vector containing OTUB1 into the cells, and then the half-life of the Nur77 protein was analyzed. Our data showed that Nur77 protein degradation was dramatically delayed by overexpression of OTUB1, whereas knockout of OTUB1 affected Nur77 degradation faster (Fig. 1E & F).

3.2. OTUB1 binds Nur77 and reverses its ubiquitination

To further investigate the mechanism of OTUB1-mediated Nur77 stabilization, we examined the interaction between OTUB1 and Nur77. Flag-tagged Nur77 was transfected into HeLa cells and Nur77 was immunoprecipitated. The results revealed that the endogenous OTUB1 protein was co-immunoprecipitated by Nur77 (Fig. 2A). To confirm their interaction, OTUB1 and Flag-tagged Nur77 were co-transfected, and Nur77 was immunoprecipitated with the same method. The result also revealed that the OTUB1 was co-immunoprecipitated by Nur77 (Fig. 2B). A reciprocal co-immunoprecipitation assay was then performed by co-transfection of Nur77 and Flag-OTUB1 into HeLa cells, and the result further validated the interaction of Nur77 and OTUB1 (Fig. 2C). These results suggest that Nur77 physically interacts with OTUB1. Since OTUB1 is a DUB, we assumed that OTUB1 mediated the ubiquitination status by regulating the Nur77 protein level. We thus examined the effect of OTUB1 on Nur77 ubiquitination. OTUB1 significantly reduced Nur77 polyubiquitination, whereas knockout of OTUB1 potentiated Nur77 polyubiquitination (Fig. 2D & E). Upon overexpression of OTUB1, the deubiquitination of Nur77 was promoted in HeLa cells transfected with wild-type (WT) ubiquitin or its K48 ubiquitin mutant (K48) (Fig. 2F). These results confirmed that OTUB1 reverses K48-linked ubiquitination of Nur77.

3.3. Asp88 is require for OTUB1 to regulate Nur77 stability

We examined whether the deubiquitination of Nur77 by OTUB1 was dependent on its catalytic activity. D88, C91, and H265 of the OTU domain form the catalytic center of this DUB. For this, we generated OTUB1 mutants by targeting D88, C91, or H265 *via* mutagenesis. Among the three mutants, the D88E mutant lost most of its ability to stabilize Nur77 when compared to WT OTUB1 (Fig. 3A). Our data showed that the D88E mutant lost the ability to deubiquitinate Nur77. In contrast, the C91S and H265A mutants could still deubiquitinate Nur77 as efficiently as the WT OTUB1 (Fig. 3B), indicating that Cys91 and His265 were not required for OTUB1 to deubiquitinate Nur77. We further validated whether OTUB1 mutants could regulate Nur77 stability using a CHX chase assay. The expression of Nur77 was co-transfected with OTUB1 or each mutant into cells. We found that Nur77 protein degradation was still delayed by overexpression of C91S or H265A. However, the ability of WT OTUB1 to delay the Nur77 degradation was lost in D88E overexpression (Fig. 3C). Together, these results indicated that Asp88 was critical for OTUB1 to participate in the deubiquitination of Nur77 and suggested that OTUB1 targeted Nur77 for deubiquitination *via* a deubiquitinase activity-independent non-canonical mechanism.

3.4. OTUB2 stabilizes the Nur77 protein in a manner similar to OTUB1

OTUB1 and OTUB2 have high sequence homology and belong to the same OTU family. We compared the effects of OTUB1 and OTUB2 on the regulation of Nur77 stability. When the Flag-tagged OTUB1 or OTUB2 was transfected unto HeLa cells, the levels of Nur77 protein was similarly increased (Fig. 4A). Overexpression of OTUB1 or OTUB2 deubiquitinated the Nur77 to a similar extent (Fig. 4B). The effect of increasing the Nur77 stability by the two DUBs was confirmed by the CHX chase experiment. That is, OTUB1 or OTUB2 suppressed Nur77 degradation which was increased by CHX treatment (Fig. 4C). We also

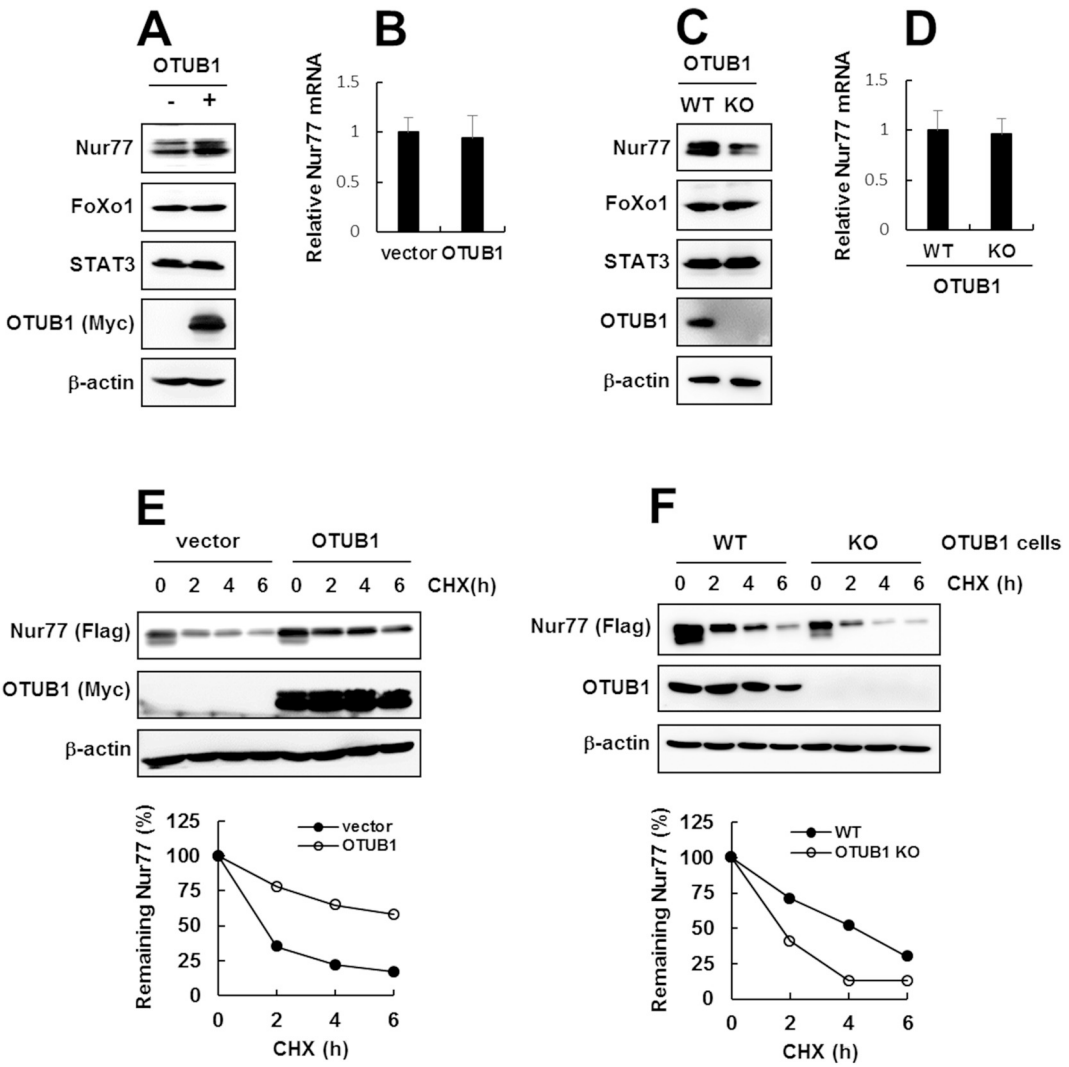

Fig. 1. OTUB1 stabilizes Nur77 protein. (A) HeLa cells were transfected with Myc-tagged OTUB1 for 36 h. The cell lysates were analyzed by immunoblotting as indicated. (B) Cells were transfected with either control vector or Myc-tagged OTUB1. Nur77 mRNA was measured by real-time PCR. (C-D) OTUB1-knockout (OTUB1-KO) cells were generated by CRISPR-Cas9. Wild-type (WT) or OTUB1-KO cells were cultured and the levels of Nur77 protein were analyzed by immunoblotting and levels of Nur77 mRNA were analyzed by real-time PCR. (E) Cells were co-transfected with Flag-Nur77 combined with either control vector or OTUB1, and then treated with CHX (50 μg/ml) for the indicated times, following which lysates were analyzed by immunoblotting. Data were quantified with GraphPad Prism Version 4 software. (F) WT or OTUB1-KO cells were transfected with Nur77 and then treated with CHX for the indicated times, following which the lysates were analyzed by immunoblotting. The results of the CHX assay (D & E) are representative of four independent experiments.

observed that OTUB2 also bind to Nur77 (Fig. 4D). Next, the effect of OTUB2 on the action of OTUB1 was tested. OTUB1 still maintained Nur77 stabilization after OTUB2 knock-down (Fig. 4E). These results suggest that OTUB1 and OTUB2 independently regulate the stabilization of Nur77.

3.5. *OTUB1 inhibits TNFα-induced IL-6 production through the stabilization of Nur77*

Nur77 is an important regulator of inflammation and has been reported to be related to protective effects against inflammatory diseases [17,18,20,30,31]. To elucidate the physiological function of OTUB1 in cells, we investigated the effect of OTUB1 on inflammatory response. TNFα was used as a stimulus for the inflammatory response in the cells since we previously confirmed a relationship between Nur77 stability and IL-6 production in TNFα-stimulated cells. TNFα treatment of HeLa cells induced Nur77 expression, which peaked at 2 h, and then rapidly degraded as shown in the previous reports [21]. Because Nur77 induction by TNFα treatment is enormous, the stability of basal Nur77 by OTUB1 overexpression appeared to be relatively weak. Nevertheless, the overexpression of OTUB1 delayed the degradation of Nur77 induced by TNFα treatment (Fig. 5A). In addition, TNFα induced the production of the inflammatory cytokine IL-6, but its production was attenuated by OTUB1 overexpression (Fig. 5B). Overexpression of OTUB1 inhibited mRNA levels of chemokine MCP-1 as well as IL-6 (Fig. 5C). Next, we performed the above experiment again on OTUB1-knockout cells. TNFα treatment further accelerated the degradation of Nur77 in OTUB1-knockout cells compared to WT cells (Fig. 5D). IL-6 production by TNFα stimulation was also increased in OTUB1-knockout cells compared to WT cells (Fig. 5E). The mRNA levels of IL-6 and MCP-1 were also increased in OTUB1-knockout cells (Fig. 5F). OTUB1 also inhibited TNFα-induced IL-6 gene and protein expression in U937 cells, a human macrophage-like cell line (Fig. 5G & H). These results suggest that stabilization of Nur77 protein by OTUB1 inhibits the inflammatory response caused by TNFα.

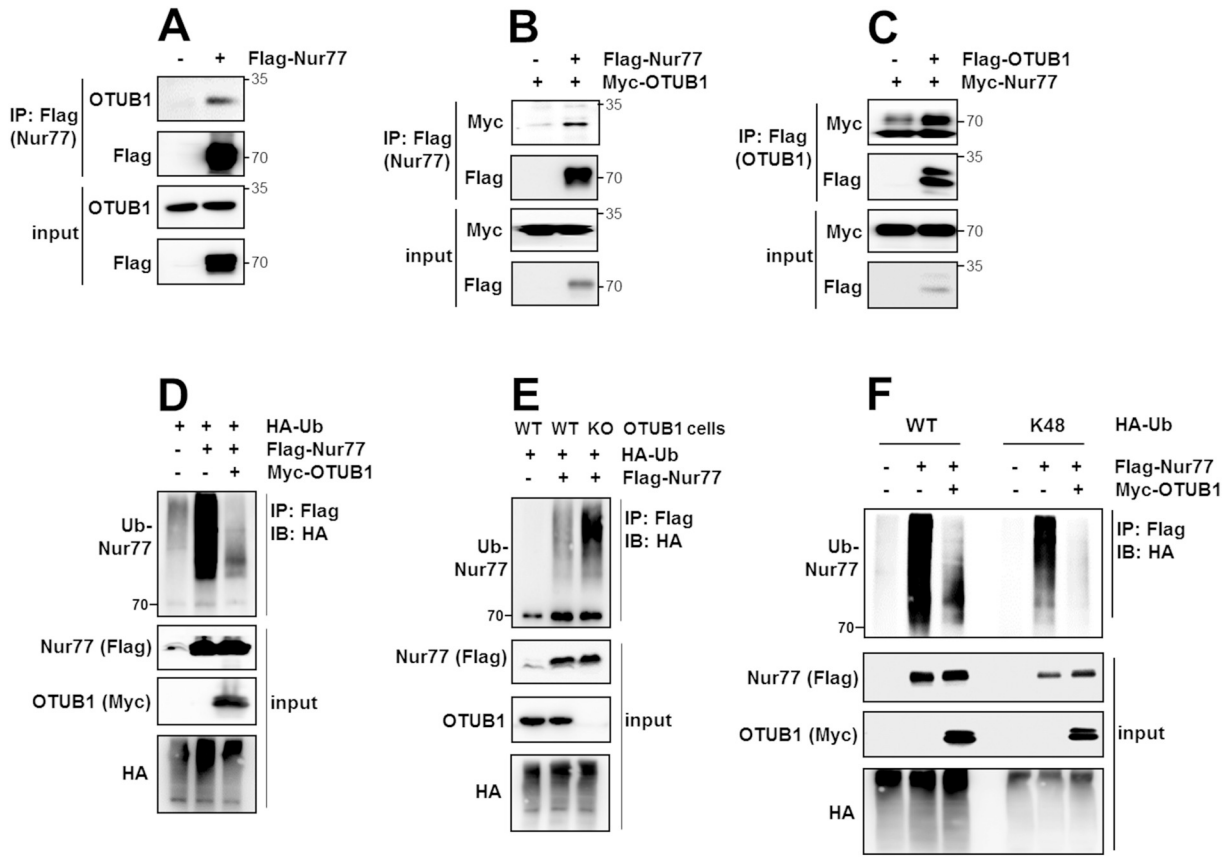

Fig. 2. OTUB1 interacts and deubiquitinates Nur77. (A) HeLa cells were transfected with either control vector or Flag-Nur77 for 36 h. The cell lysates were immunoprecipitated (IP) as shown, and the samples were subjected to electrophoresis and analyzed by immunoblotting. Five percent of the IP sample was analyzed by immunoblotting (input). (B) HeLa cells were transfected with Myc-OTUB1 in the absence or presence of Flag-Nur77. The cell lysates were IP as shown. (C) HeLa cells were transfected with Myc-Nur77 in the absence or presence of Flag-OTUB1. The cell lysates were IP as shown. (D) HeLa cells were transfected with a combination of Nur77 and OTUB1 to assess deubiquitination and analyzed by immunoprecipitation and immunoblotting. Five percent of the IP sample was analyzed by immunoblotting (input). Ub-Nur77, polyubiquitin chains of Nur77. (E) WT or OTUB1-KO cells were transfected with Flag-Nur77 to assess deubiquitination and analyzed by immunoprecipitation and immunoblotting. (F) HeLa cells were transfected with wild-type (WT) ubiquitin or its mutant (K48 ubiquitin) along with a combination of Flag-Nur77 or Myc-OTUB1. A deubiquitination assay was performed after 36 h, and the expression of constructs was determined by immunoblotting.

3.6. OTUB1 acts as a counterpart of Trim13 in regulation of Nur77 stability

Previously, we identified Trim13, an ubiquitin E3 ligase that promoted Nur77 ubiquitination and degradation [21]. To demonstrate that OTUB1 was indeed able to deubiquitinates Nur77 and thereby antagonizes Trim13, we performed a Nur77 stability and ubiquitination assay as well as a CHX chase assay. As expected, the overexpression of Trim13 reduced the stability of Nur77, but co-transfection of OTUB1 with Trim13 restored the stability of Nur77 (Fig. 6A). In addition, the ubiquitination of Nur77 increased by Trim13 was also decreased by OTUB1 (Fig. 6B). We confirmed the above results using the CHX chase assay. Overexpression of Trim13 further weakened the stability of Nur77. CHX treatment reduced the stability of the overexpressed Trim13 protein. Nevertheless, the overexpression of OTUB1 rescued the instability of Nur77 by Trim13 (Fig. 6C). These results suggest that OTUB1 can act as a DUB in reversing the action of Trim13, and that the balance between the two proteins may be important in regulating the stability of Nur77. Next, we tested whether OTUB1 is a partner of Trim13 in the IL-6 production. Similar to the previous results, Trim13 further amplified IL-6 production by TNFα stimulation. As shown in the Fig. 6D, OTUB1 overexpression reduced IL-6 production by TNFα. Most importantly, OTUB1 overexpression also inhibited Trim13-amplified IL-6 production. The above results were confirmed in OTUB1-knockout cells. The increase of IL-6 production by Trim13 was greater in OTUB1-knockout cells than in WT cells (Fig. 6E). OTUB2 also inhibited the ubiquitination of Nur77 and IL-6 production by TNFα stimulation, which were increased by Trim13, similar to that of OTUB1 (Fig. 6F & G). These results suggest that OTUB1 or OTUB2 acts as a counterpart for Trim13 to regulate IL-6 production in the inflammatory response.

4. Discussion

Nur77, a transcription factor and orphan nuclear receptor that plays an important role in a variety of cellular activities, but dysregulation of Nur77 plays a role in a variety of human diseases such as inflammation [17,18,20,30,31] and cancer [32]. Therefore, precise regulation of Nur77 is a necessary process to maintain normal physiological function or to prevent related diseases. However, due to the absence of core ligands, the precise regulation of Nur77 was relatively unclear. Instead, the process of regulating Nur77 has been mostly studied at the transcriptional level. In particular, some of the factors that regulate the transcription of Nur77 have been identified, demonstrating the importance of this protein [3]. Post-transcriptional processes, including phosphorylation, are also important in the regulation of Nur77 [10,11]. Nur77 has been predicted to phosphorylate > 10 sites, which are mainly serine sites. In addition, Nur77 has been suggested to be involved in ubiquitination as well, since the ubiquitination of proteins have been linked to stability. Currently, E3 ligases that directly ubiquitinates Nur77 include RNF4 [13], Trim13 [21], TRAF2 [33], and Smurf1 [34]. The ubiquitination of Nur77 is sometimes linked to other types of post-transcriptional modifications. For example, it has been reported that phosphorylation is involved in the ubiquitination of

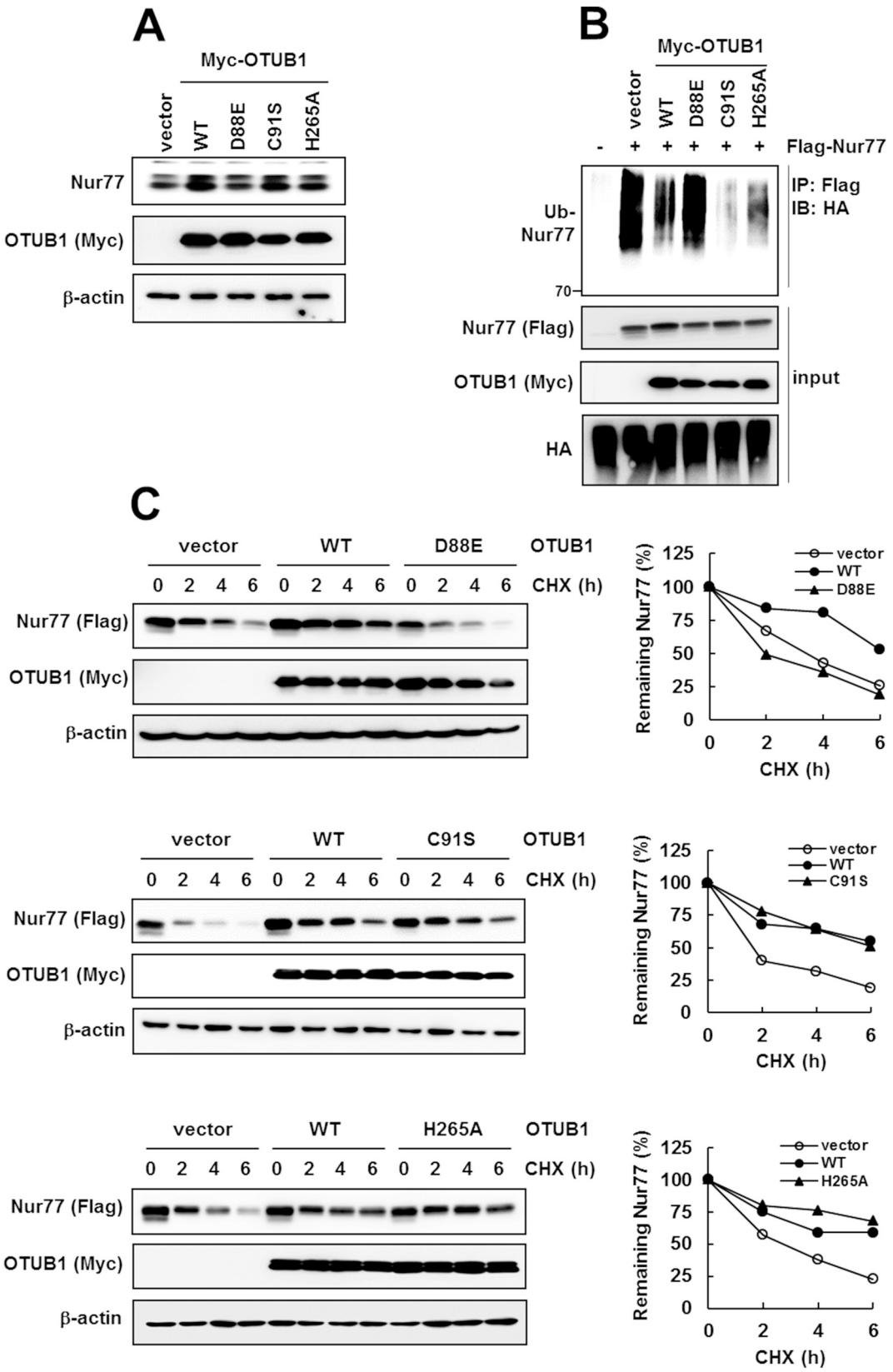

Fig. 3. Asp88 is critical for OTUB1 to deubiquitinate Nur77. (A) HeLa cells were transfected with either wild-type (WT) OTUB1 or its point mutants as indicated, and the lysates were analyzed by immunoblotting. (B) Nur77 was co-transfected with either WT OTUB1 or its point mutants into cells. A deubiquitination assay was performed after 36 h, and the expressions of the constructs were determined by immunoblotting. (C) Nur77 was co-transfected with either WT OTUB1 or each point mutant, and then treated with CHX for the indicated times, following which lysates were analyzed by immunoblotting. Data were quantified with GraphPad Prism Version 4 software. The results of the CHX assay are representative of three independent experiments.

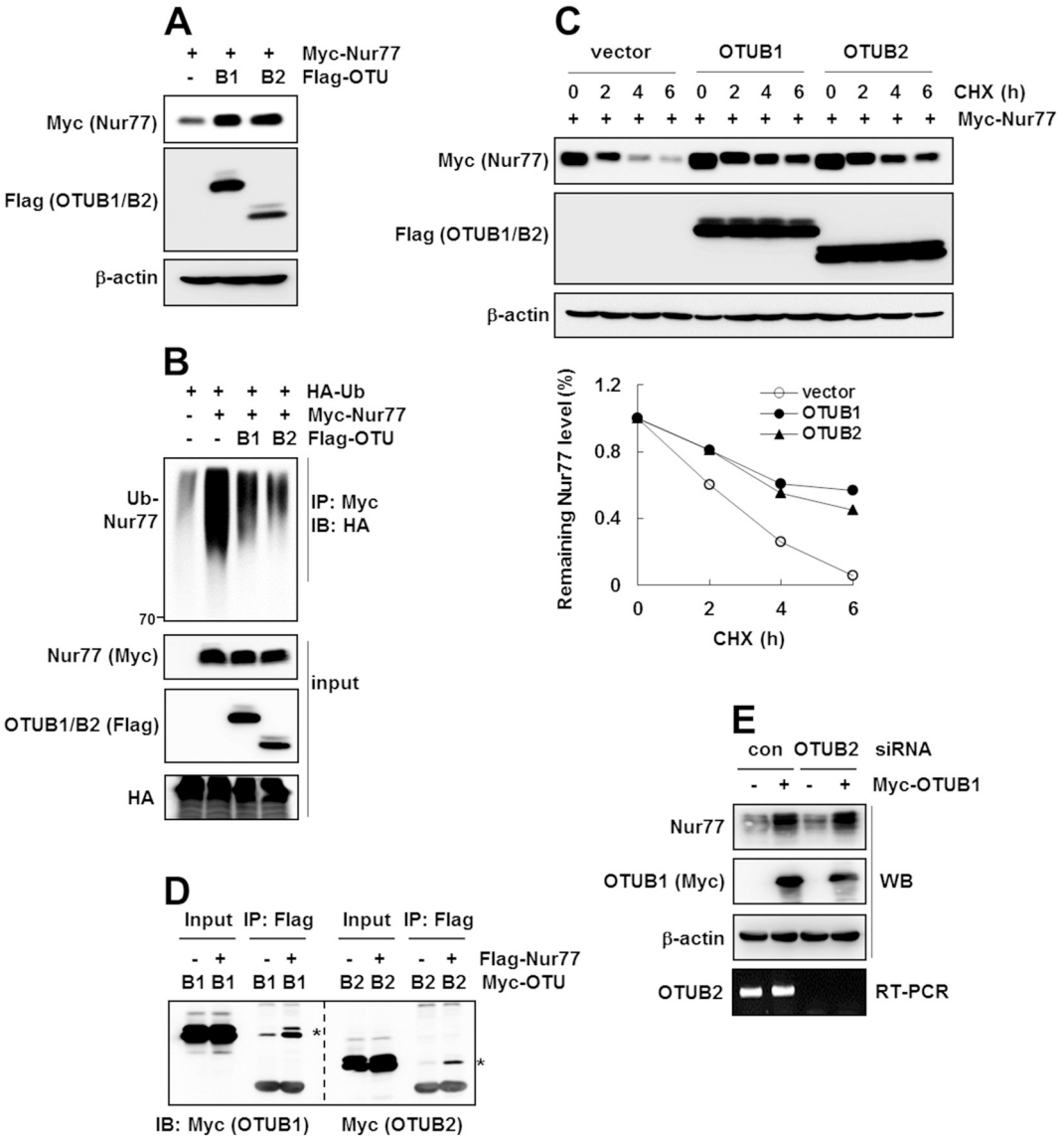

Fig. 4. OTUB2 stabilizes Nur77 protein in a manner similar to OTUB1. (A–B) Nur77 was co-transfected with either the OTUB1 or OTUB2 constructs into cells. The cell lysates were analyzed by immunoblotting as indicated (A). The cell lysates were immunoprecipitated with a Myc antibody as shown. A deubiquitination assay was performed after 36 h, and the expression of constructs was determined by immunoblotting (B). (C) Nur77 was co-transfected with either OTUB1 or OTUB2, and then treated with CHX for the indicated times, following which lysates were analyzed by immunoblotting. Data were quantified with GraphPad Prism Version 4 software. The results of the CHX assay are representative of three independent experiments. (D) HeLa cells were transfected with the Flag-Nur77 construct with OTUB1 or OTUB2. The cell lysates were immunoprecipitated as shown, and the samples were subjected to electrophoresis, and analyzed by immunoblotting. Five percent of the immunoprecipitate or the input was analyzed by IB. (E) HeLa cells were transfected with either control siRNA or OTUB2 siRNA, and then the change of Nur77 level by OTUB1 overexpression was tested by immunoblotting.

Nur77 by Trim13 [21] or Smurf1 [34], and sumoylation is involved in the ubiquitination of Nur77 by RNF4 [13]. As described above, ubiquitination by E3 ligase has been recognized as one of the key processes for controlling Nur77 activity. The ubiquitination of proteins is a reversible reaction. However, the deubiquitination of Nur77 remains obscure. Therefore, the identification of the DUB that is responsible for Nur77 deubiquitination is of great significance. While OTUB1 has already been known to stabilize various other proteins in cells, in this study, we revealed for the first time that OTUB1 stabilizes Nur77. The OTUB1 gene is located on chromosome 11q13.1 and encodes an ovarian tumor protease (OTU) domain-containing cysteine protease that preferentially cleaves K48-linked di-ubiquitin *in vitro* [29]. OTUB1 disassembled K48-linked polyubiquitin chains from c-IAP in the TWEAK receptor signaling complex [28]. It is also known that OTUB1 catalyzes the cleavage of K48-specific ubiquitin linkage in the deubiquitination of FOXM1 [35]. Our result also showed that deubiquitination of Nur77 by OTUB1 occurs in the K48-linked polyubiquitin. Cleavage of K48-linked polyubiquitin by OTUB1 is expected to contribute to the stabilization of Nur77.

We have shown that OTUB1 interacts with Nur77 to stabilize this protein. For the catalytic center of the OTUB1 enzyme, Asp88, Cys91, and His265 located in the OTU domain have been demonstrated to be important, and Cys91 has been identified an essential catalytic site for the deubiquitination enzymatic activity. Nevertheless, a mutation at Cys91 did not remove the ability of OTUB1 to deubiquitinate and stabilize Nur77. Interestingly, our results showed the importance of Asp88 in the function of OTUB1. There have been previous reports demonstrating that OTUB1 functions in a non-canonical mechanism [36,37]. Recently, the importance of Asp88 in OTUB1 has also been reported in the stability control of Snail [25] and DEPTOR [26]. Furthermore,

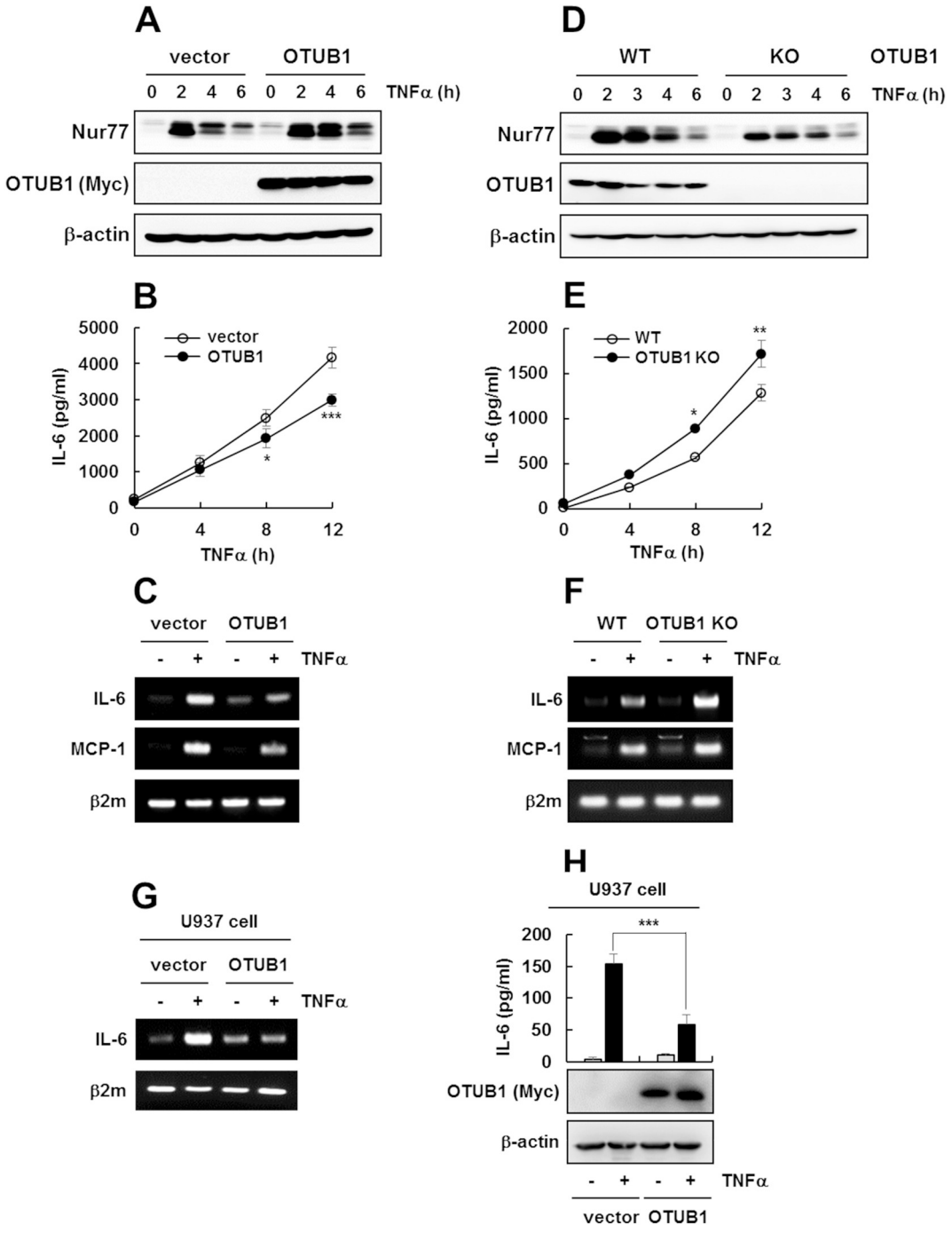

Fig. 5. OTUB1 inhibits TNFα-induced IL-6 production through Nur77 stabilization. (A-B) HeLa cells were transfected with either control vector or OTUB1 and then treated with TNFα (2 ng/ml) for the indicated times. The cell lysates were analyzed for Nur77 and OTUB1 expression by immunoblotting, and the supernatants were assayed for IL-6 using enzyme immunoassay. (C) Two types of transfected cells were treated with TNFα for 1 h and then the levels of mRNA were analyzed by conventional RT-PCR. (D-E) Wild-type (WT) or OTUB1-knockout HeLa cells were cultured and then treated with TNFα for the indicated times. The cell lysates were analyzed for Nur77 and OTUB1 expression by immunoblotting, and the supernatants were assayed for IL-6 using EIA. (F) WT or OTUB1 cells were treated with TNFα for 1 h and then the levels of mRNA were analyzed by conventional RT-PCR. (G) Vector or OTUB1 transfected U937 cells were treated with TNFα for 1 h and then the levels of mRNA were analyzed by conventional RT-PCR. (H) U937 cells were transfected with either control vector or OTUB1 and then treated with TNFα. The supernatants were assayed for IL-6 using enzyme immunoassay. Data on B, E, and H are shown as means ± SD ($n = 3$ for B, E, and H). p values were calculated using two-tailed Student's t-test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

OTUB1 stabilizes p53, a transcription factor, by inhibiting the ubiquitination of p53 mediated by MDM2; this was also proved to occur by a non-canonical mechanism involving Asp88. Our observation was also consistent with the non-canonical mechanism by which OTUB1 suppresses chromatin ubiquitination by DNA damage [37].

DUBs are all cysteine proteases except for the JAMM family of DUBs. DUBs are divided into the following six groups according to the active site and catalytic mechanism: ubiquitin C-terminal hydrolases, ubiquitin-specific proteases (USPs), Machado-Josephin domain proteases, OTUs, Jab1/Mov34/Mpr1 Pad1 N-terminal + domain proteases, and monocyte chemotactic protein-induced proteins. OTUB1 and OTUB2 are DUBs belonging to the OTU family [38]. OTUB1 and OTUB2

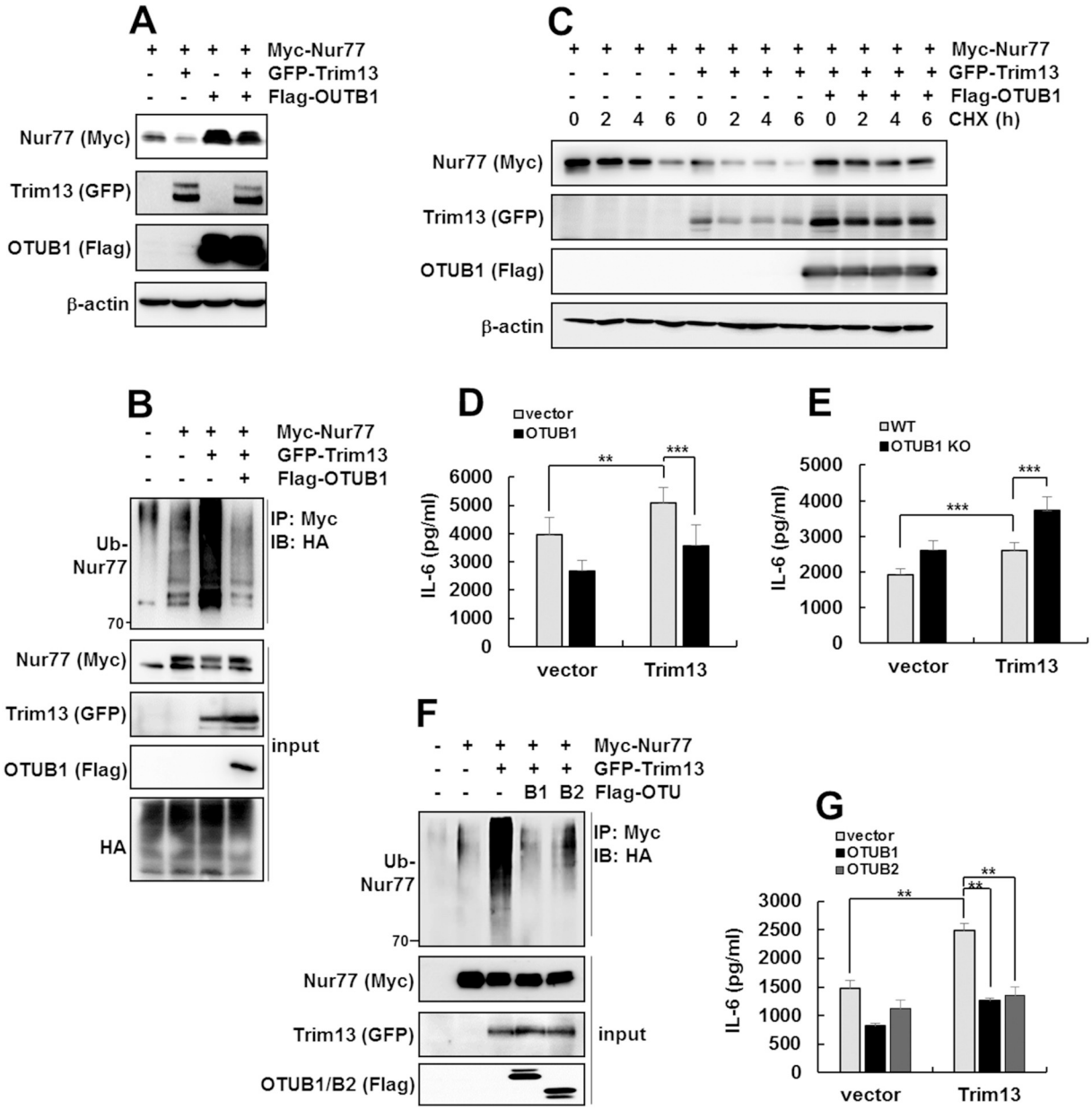

Fig. 6. OTUB1 acts to counteract Trim13 in stabilizing Nur77 and the production of IL-6. (A) Nur77 was co-transfected with Trim13 or OTUB1 constructs into HeLa cells. The cell lysates were analyzed by immunoblotting as indicated. (B) Nur77 was co-transfected with a combination of Trim13 or OTUB1 constructs into HeLa cells to assess deubiquitination and analyzed by immunoprecipitation and immunoblotting. (C) HeLa cells were transfected with a combination of Nur77, Trim13, and OTUB1 constructs and then treated with CHX for the indicated times, following which lysates were analyzed by immunoblotting. The results of the CHX assay are representative of three independent experiments. (D) Cells were transfected with a combination of OTUB1 and Trim13, and then treated with TNFα for 12 h. The cell supernatants were assayed for IL-6 using an enzyme immunoassay. (E) WT or OTUB1 knockout (KO) cells were transfected with either vector or Trim13, and then treated with TNFα for 12 h. The cell supernatants were assayed for IL-6 using enzyme immunoassay. (F) Nur77 and Trim13 were co-transfected with a combination of OTUB1 or OTUB2 constructs into cells to assess deubiquitination and analyzed by immunoprecipitation and immunoblotting. (G) Cells were transfected with a combination of OTUB1 or OTUB2 and Trim13, and then treated with TNFα for 12 h. The cell supernatants were assayed for IL-6 using an enzyme immunoassay. Data are shown as means ± SD (n = 4 for D & E, n = 3 for G). p values were calculated using two-tailed Student's t-test. $^{**}P < .01$, $^{***}P < .001$.

may exhibit similar effects on the stabilization of the protein. Both DUBs showed a similar effect when TRAF3/6 was deubiquitinated by virus in the production of type I interferon [24]. In our observations, OTUB1 and OTUB2 stabilized Nur77 in a similar manner. That is, OTUB2 also bound Nur77 and was shown to increase the stability of Nur77 as shown by the CHX chase experiment. Unlike our results, the two DUBs had opposite effects on p53 stability. OTUB1 participated in the stabilization of p53 through deubiquitination, but OTUB2 did not [29]. Despite the fact that two DUBs belong to the same family with high sequence homology, it is unknown why the functions of OTUB1 and OTUB2 are different depending on the substrate protein.

We concluded that Trim13 plays a role in Nur77 ubiquitination, while OTUB1 plays a role in Nur77 deubiquitination. Furthermore, it is well known that E3 ligase and DUB cooperate to control the stability of certain proteins. A representative example is the transcription factor p53 protein. MDM2 and USP7 act as an axis for the stability of p53 protein, thereby regulating various cellular responses [39]. In particular, USP7 acts as a DUB for MDM2, which is involved in the ubiquitination of p53. These results suggest that USP7 may be a key regulator of p53 rather than MDM2. Based on this possibility, USP7

inhibitors are being developed to treat p53-related diseases such as cancer [40]. It has been speculated that the stability of the transcription factor Nur77 is also dependent on both E3 ligase and DUB, which may change the inflammatory response. Additionally, it has been reported that Trim13 modulated TLR2- or TNFα-mediated inflammatory responses [21,41]. We observed that Trim13 itself was also degraded by the proteasomal degradation mechanism, and OTUB1 promoted deubiquitination not only for Nur77 but also for Trim13 (data not shown). These observations suggest that OTUB1 may regulate the stability of E3 ligase acting as a partner as well as its substrate. Further studies are needed to confirm this conclusion. Nevertheless, these results suggest that Trim13 and OTUB1 may act as an axis to control the stability of the Nur77 protein, and thus the inflammatory response may be affected.

5. Conclusions

We first identified the role of OTUB1 in the stabilization of the transcription factor Nur77. The stabilization of Nur77 by OTUB1 was achieved by interaction between the two proteins, and the mechanism was mediated by a non-canonical pathway independent of DUB activity. We observed that OTUB1 inhibited IL-6 production by TNFα stimulation. One of the most important discoveries in this study was that OTUB1 was a counterpart of Trim13, an E3 ligase, in the regulation of Nur77 stability. OTUB1 acts as a counterpart of Trim13 to stabilize Nur77, and eventually suppressed the production of IL-6 amplified by Trim13.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Han Zhong Pei and Bin Huang performed the experiments and interpreted data.

Hyeun-Wook Chang interpreted data and contributed the writing paper.

Suk-Hwan Baek designed the research and wrote the paper.

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