UBIQUITIN-SPECIFIC PROTEASE 4 (USP4) TARGETS TRAF2 AND TRAF6 FOR DEUBIQUITINATION AND INHIBITS TNFα-INDUCED CANCER CELL MIGRATION

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INTRODUCTION

Protein ubiquitination is an essential post-translational modification with critical roles in various biological functions, such as cell growth, apoptosis, DNA damage repair, immune responses and neuron degeneration [1–3]. It is a process of covalently attaching one or more ubiquitins to the lysine residues of the targeted proteins triggered by an enzymatic cascade [4]. Ubiquitin is a highly conserved polypeptide of 76 amino acids with seven lysine residues (Lys5, Lys6, Lys7, Lys11, Lys12, Lys16 and Lys27). Most protein ubiquitination can be divided into mono-ubiquitination, and Lys48-linked and Lys63-linked polyubiquitination based on the length and linkage of ubiquitin chains. Lys48-linked polyubiquitination, where ubiquitin is attached to another ubiquitin on its Lys48 residue, is thought to target proteins for 26S proteasome-dependent degradation. Lys63-linked polyubiquitination mainly plays a non-proteolytic role in protein trafficking, DNA damage repair and activation of signalling pathways [4,5]. Three distinct classes of enzymes including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) are involved in the protein ubiquitination process. E3 ubiquitin ligases containing a substrate recognition motif compose a large protein family with over 600 members and are responsible for determining the substrate specificity [6,7].

Ubiquitination is a reversible process by a family of DUBs (deubiquitinases). The ubiquitin can be directly removed from the substrate specificity [6,7]. Currently, nearly 100 DUBs have been identified. According to their enzyme features, DUBs can be divided into two families: a metalloprotease family and a cysteine protease family. The cysteine protease family can be further identified. According to their enzyme features, DUBs can be divided into two families: a metalloprotease family and a cysteine protease family. The cysteine protease family can be further divided into four subclasses based on their ubiquitin protease domains: USPs (ubiquitin-specific proteases), UCHs (ubiquitin C-terminal hydrolases), OTUs (otubain proteases), and MJDs (Machado–Joseph disease proteases). The USPs compose the biggest subfamily with more than 50 members [8]. However, the function of most DUBs remains unknown.

Ubiquitination/deubiquitination plays a critical role in the activation of the NF-κB (nuclear factor κB) signalling pathway, which has multiple functions in regulating cell proliferation, apoptosis and immune responses [9]. For example, both Lys63-linked polyubiquitination of IKKγ [IkB (inhibitor of NF-κB) kinase γ] and Lys63-linked polyubiquitination of IκBα are important to NF-κB activation [10]. Polyubiquitination of TRAF TNFR [TNF (tumour necrosis factor) receptor]-associated factor 6 is critical to its activity towards downstream targets to mediate IL-1β-induced NF-κB activation [11]. TRAF2 possesses an S1P (sphingosine 1-phosphate)-dependent E3 ubiquitin ligase activity [12] and its polyubiquitination has also been shown to be critical to TNFα-induced NF-κB activation [13]. Deubiquitination of TRAF2 or TRAF6 by DUBs markedly inhibits cytokines, such as TNFα- and IL-1β-mediated NF-κB activation. For example, CYLD, A20 and USP20 have been substrates by DUBs [8].

We found that USP4 specifically interacts with TRAF2 and TRAF6, but not TRAF3. Moreover, USP4 associates with TRAF6 both in vitro and in vivo, independent of its deubiquitinase activity. The USP domain is responsible for USP4 to interact with TRAF6. Ectopic expression of USP4 inhibits the TRAF2- and TRAF6-stimulated NF-κB reporter gene and negatively regulates the TNFα-induced IκBα degradation and TNFα-induced NF-κB activation. Knockdown of USP4 significantly increased TNFα-induced cytokine expression. Furthermore, we found that USP4 deubiquitinates both TRAF2 and TRAF6 in vitro and in vivo in a deubiquitinase activity-dependent manner. Importantly, the results of the present study showed that USP4 is a negative regulator of TNFα- and IL-1β-induced cancer cell migration. Taken together, the present study provides a novel insight into the regulation of the NF-κB signalling pathway and uncovers a previously unknown function of USP4 in cancer.

Key words: migration, nuclear factor κB (NF-κB), tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), ubiquitination, ubiquitin-specific protease 4 (USP4).
reported to negatively regulate NF-κB signalling, at least partially, through deubiquitinating TRAF2 and/or TRAF6 [14–17]. In the present study, we identified USP4 as a novel deubiquitinate for both TRAF2 and TRAF6. We found that USP4 interacts with and deubiquitinitates TRAF2 and TRAF6, and consequently inhibits TNFα- and IL-1β-induced NF-κB activation. Importantly, we found that USP4 negatively regulates TNFα-mediated migration of lung cancer cells.

**EXPERIMENTAL**

**Cell culture and transfection**

HEK (human embryonic kidney)-293T cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and cells from the human lung adenocarcinoma epithelial cell line A549 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. The cells were incubated at 37°C in 5% CO₂. Transfections were performed using calcium phosphate/DNA co-precipitation (for HEK-293T cells) and Lipofectamine™ 2000 (for A549 cells) according to the manufacturer’s instructions. siRNA (small interfering RNA) oligonucleotides were transfected using Lipofectamine™ 2000.

**Plasmids and siRNA**

USP4, cIAP (cellular inhibitor of apoptosis) and TRAF2 were amplified from HEK-293T cells by RT (reverse transcription)–PCR and cloned into pcDNA3.1 vectors with a HA (haemagglutinin), FLAG or GFP (green fluorescent protein) tag at the N-terminus. HEK-293T cells were lysed in ice-cold 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40 and a cocktail of protease inhibitors] containing protease inhibitor cocktail and NEM (N-ethylmaleimide). The cell lysates were incubated with FLAG M2 beads overnight with rotation at 4°C. The supernatant in the presence of 4 mg/ml polybrene. After incubation for 48 h, the cells were sorted for stable cell line analysis by flow cytometry.

**GST pull-down assay**

HEK-293T cells stably expressing USP4 were lysed and cleared by centrifugation (12000 g for 15 min at 4°C). The cell lysates were incubated with cell lysates for 1 h and the GST proteins were purified using glutathione–Sepharose 4B (Amersham Biosciences). The bound USP4 was detected by immunoblotting.

**Luciferase assay**

The indicated plasmids were transiently transfected into HEK-293T cells in the presence of NF-κB-dependent firefly luciferase and Renilla luciferase plasmids. The cells were harvested after 36 h and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity. Results represent three independent experiments performed in duplicate.

**Deubiquitination assay**

For in vivo deubiquitination, HEK-293T cells were transfected with His–ubiquitin in the presence of the plasmids as indicated. The transfected cells were lysed by denaturing buffer (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄ and 10 mM imidazole), followed by nickel bead purification. Ubiquitination was detected by immunoblot analysis. For the in vitro deubiquitination assay, FLAG–TRAF6 or Flag–TRAF2 was co-transfected with His–ubiquitin into HEK-293T cells. At 24 h later, the transfected cells were stimulated with IL-1β or TNFα for 15 min. The cells were harvested using RIPA buffer [100 mM Tris/HCl (pH 7.4), 30 mM NaCl, 2.5% sodium deoxycholate, 2 mM EDTA and 2% Nonidet P40] containing protease inhibitor cocktail and N (N-ethylmaleimide). The cell lysates were incubated with FLAG M2 beads overnight with rotation at 4°C. After extensive washing with TBS [Tris-buffered saline (25 mM Tris/HCl, pH 7.4, 150 mM NaCl and 3 mM KCl)], FLAG–TRAF6 or FLAG–TRAF2 was eluted with elution buffer [1× PBS (pH 7.4)] containing 3× FLAG peptide. Then 15 μl of the eluted FLAG–TRAF6 or FLAG–TRAF2 was incubated with purified GST–USP4 in DUB assay buffer [50 mM Hepes/NaOH (pH 8.0), 10% glycerol and 3 mM DTT (dithiothreitol)] at 37°C for 4 h. Ubiquitination was analysed by Western blotting using an anti-His, anti-FLAG or anti-USP4 antibody.

**RNA isolation and real-time RT–PCR**

Total RNA was isolated from cells by using RNAiso Plus reagent (TaKaRa) as described in the manufacturer’s protocol. For mRNA
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Migration assay
A cell migration assay was performed using Transwell® migration chambers (8 μm pore size; Millipore) according to the manufacturer’s instructions. For each experiment, the number of cells in five random fields on the underside of the filter was counted, and three independent filters were analysed. For the wound healing assay, A549 cells were seeded and scratched with micropipette tips when the cells had grown to near confluency (∼90 %), and images were captured at 0 and 14 h after wounding. Cells were treated with mitomycin C (10 mg/ml; Sigma) to inhibit cell proliferation.

Live cell imaging
A549 cells were plated in 35 cm dishes and transfected as described above. At 48 h post-infection, images were captured every 5 min for 10 h using a Leica TCS SP5 confocal microscope and analysed using ImageJ (NIH) software.

RESULTS
USP4 associates with TRAF2 and TRAF6

To identify the novel regulator of TRAF6 E3 ubiquitin ligase, we used a co-immunoprecipitation assay to search for potential binding partners. Among the proteins (including USP8, USP25, USP28 and USP33) we examined, the DUB USP4 (also known as UnpEL/Unph) was found to specifically interact with TRAF2 and TRAF6 in HEK-293T cells (Figure 1A). Moreover, TRAF2, another essential regulator of the NF-κB signalling pathway, could also bind to USP4. In contrast, USP4 failed to interact with TRAF3 under the same conditions, indicating that USP4 specifically binds to TRAF2 and TRAF6 (Figure 1A). The catalytically inactive mutant USP4 C311S [19] interacted with TRAF6 as well as wild-type USP4, indicating that the interaction between USP4 and TRAF6 is independent of the USP4 DUB activity (Figure 1B). To examine the interaction between USP4 and TRAF6 under more physiological conditions, endogenous USP4 was immunoprecipitated with an anti-USP4 antibody from HEK-293T cells and the associated TRAF6 was detected using an anti-TRAF6 antibody. As shown in Figure 1(C), endogenous TRAF6 was readily detected in USP4, but not in IgG, immunoprecipitates.

In addition, endogenous USP4 was able to bind to TRAF6 in HEK-293T cells (Figure 1D). A GST pull-down assay showed that USP4 could bind to the purified GST–TRAF6 in vitro (Figure 1E). Taken together, these results indicate that USP4 is a novel binding partner of TRAF6.

USP4 contains a DUB domain present in USP domain and an USP domain. The USP domain is conserved in most USP members and essential for its DUB activity [19]. To identify the domains of USP4 responsible for its interaction with TRAF6, we constructed serial deletion mutants of USP4 (Figure 2A). These deletion mutants were co-transfected with FLAG–TRAF6 into HEK-293T cells with empty vector or FLAG–TRAF2, FLAG–TRAF3 or FLAG–TRAF6. Transfected cells were harvested and immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates and the original whole-cell extracts were analysed by immunoblotting using anti-HA or anti-FLAG antibodies. (B) USP4 associated with TRAF6-independent DUB activity. Wild-type HA–USP4 (WT) or C311S HA–USP4 (C/S) was transfected into HEK-293T cells with empty vector or FLAG–TRAF6. Binding was measured by immunoprecipitation assay. * indicates a non-specific band. (C) An endogenous interaction between USP4 and TRAF6. Endogenous USP4 was immunoprecipitated from HEK-293T cells using an anti-USP4 antibody, and the immunoprecipitates and original cell lysates were subjected to immunoblotting using anti-TRAF6 or anti-USP4 antibodies. (D) HEK-293T cells transfected with FLAG–TRAF6 were harvested and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates and whole-cell extracts were immunoblotted with anti-USP4 or anti-FLAG antibodies. (E) GST or GST–TRAF6 proteins were purified from E. coli and incubated with the cell lysates stably expressing USP4. The products of GST pull-down were analysed by immunoblotting using anti-USP4 or anti-GST antibodies. The molecular mass in kDa is indicated on the right-hand side. IB, immunoblot; IP, immunoprecipitate; WCE, whole-cell extract; WT, wild-type.

Figure 1 USP4 associates with TRAF2 and TRAF6

(A) USP4 specifically interacted with TRAF2 and TRAF6. HA–USP4 (wild-type) was transfected into HEK-293T cells with empty vector or FLAG–TRAF2, FLAG–TRAF3 or FLAG–TRAF6. Transfected cells were harvested and immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates and the original whole-cell extracts were analysed by immunoblotting using anti-HA or anti-FLAG antibodies. (B) USP4 associated with TRAF6-independent DUB activity. Wild-type HA–USP4 (WT) or C311S HA–USP4 (C/S) was transfected into HEK-293T cells with empty vector or FLAG–TRAF6. Binding was measured by immunoprecipitation assay. * indicates a non-specific band. (C) An endogenous interaction between USP4 and TRAF6. Endogenous USP4 was immunoprecipitated from HEK-293T cells using an anti-USP4 antibody, and the immunoprecipitates and original cell lysates were subjected to immunoblotting using anti-TRAF6 or anti-USP4 antibodies. (D) HEK-293T cells transfected with FLAG–TRAF6 were harvested and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates and whole-cell extracts were immunoblotted with anti-USP4 or anti-FLAG antibodies. (E) GST or GST–TRAF6 proteins were purified from E. coli and incubated with the cell lysates stably expressing USP4. The products of GST pull-down were analysed by immunoblotting using anti-USP4 or anti-GST antibodies. The molecular mass in kDa is indicated on the right-hand side. IB, immunoblot; IP, immunoprecipitate; WCE, whole-cell extract; WT, wild-type.
USP4 inhibits NF-κB activation

Given the pivotal role for TRAF2 and TRAF6 in the NF-κB signalling pathway, we investigated whether USP4 is a negative regulator of TRAF2- and TRAF6-mediated NF-κB activation using an NF-κB-dependent luciferase reporter gene assay. As shown in Figures 3(A) and 3(B), co-expression of wild-type USP4 significantly suppressed TRAF6- and TRAF2-mediated NF-κB activation. In contrast, the USP4 C311S mutant without DUB activity reduced the inhibitory effect on NF-κB activation. These results suggest that DUB activity is responsible for the suppression of TRAF6- and TRAF2-mediated NF-κB activation by USP4. It has been reported previously that TRAF6 is involved in IL-1β-mediated NF-κB activation and that TRAF2 is involved in TNFα-mediated NF-κB activation [16]. Thus, we examined whether USP4 can suppress TNFα- and IL-1β-induced NF-κB activation. As shown in Figure 3(C), ectopic expression of wild-type, but not the C311S mutant, USP4 suppressed both TNFα- and IL-1β-induced NF-κB activation. Previous studies indicate that TRAF2 is required for both TNFR1- and TNFR2-induced NF-κB activation [21]. Our results showed that USP4 could suppress both TNFR1- and TNFR2-mediated NF-κB activation (Figure 3D).
knockdown of USP4 had little effect on the stability of TRAF2 and TRAF6. Taken together, our results demonstrated that USP4 is a negative regulator of the NF-κB signalling pathway.

**USP4 deubiquitinates TRAF2 and TRAF6**

The enzymatic activity of TRAF6 requires its Lys63-linked polyubiquitination [11,16]. Therefore we investigated whether USP4 acts as a DUB targeting TRAF6 to inhibit NF-κB activation. FLAG–TRAF6 was transfected into HEK-293T cells with His-ubiquitin in the presence or absence of wild-type or C311S HA–USP4. The ubiquitinated TRAF6 was detected using an in vivo ubiquitination assay. As shown in Figure 4(A), wild-type USP4 markedly reduced the ubiquitination level of TRAF6, whereas the USP4 C311S mutant failed to do so. A similar inhibitory effect was also observed on TRAF2 (Figure 4A). In addition, we used an in vitro deubiquitination assay to confirm these results. In this assay, the FLAG–TRAF6 or FLAG–TRAF2 purified from HEK-293T cells were incubated with purified GST–USP4 and the level of ubiquitinated TRAF6 or TRAF2 was detected by Western blotting. We found that the presence of USP4 significantly reduced the ubiquitination of TRAF6 and TRAF2 (Figure 4B). The effect of endogenous USP4 on the ubiquitination of TRAF6 was also tested. In this assay, we found that knockdown of endogenous USP4 significantly increased the ubiquitination level of FLAG–TRAF6 (Figure 4C). Taken together, our results demonstrate that USP4 is a novel DUB that targets TRAF6 and TRAF2 for deubiquitination.

Evidence has shown that both TRAF2 and TRAF6 can be degraded by the ubiquitin–proteasome pathway [23,24]. Given that USP4 can deubiquitinate TRAF2 and TRAF6, we examined whether USP4 has any effect on their protein stability. Our results showed that neither overexpression nor knockdown of USP4 affected TRAF2 and TRAF6 protein stability (Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/441/bj4410979add.htm). A previous study showed that cIAP is the major E3 ubiquitin ligase to degrade TRAF2 in the TNFR2 signalling pathway [23]. Therefore we examined whether USP4 affects cIAP-mediated TRAF2 degradation. Our results showed that USP4 has little effect on cIAP-mediated TRAF2 degradation (Supplementary Figure S1C). Taken together, the results suggest that USP4 regulates TRAF2- and TRAF6-mediated NF-κB activation mainly through the regulation of their polyubiquitination.

**USP4 negatively regulates TNFα-induced gene expression**

Given that USP4 is a negative regulator of TNFα- and TRAF2/TRA6-mediated NF-κB activation, we investigated whether USP4 can regulate the TNFα-mediated biological function. Activation of NF-κB by TNFα can induce the expression of various genes, such as IL6 and IL8. Thus we asked whether USP4 is involved in TNFα-induced gene expression. USP4 has been suggested to be an oncoprotein which is elevated in several cancers, including small cell lung carcinomas and adenocarcinomas [25,26]. Our results have also shown that USP4 could be readily detected in cells of two lung cancer cell lines A549 and H1299 (results not shown). Specific siRNAs of USP4 could efficiently inhibit the expression of endogenous USP4 in A549 cells (Figure 5A). Moreover, knockdown of USP4 enhanced the TNFα-induced IkBα degradation in A549 cells, indicating that USP4 also negatively regulates TNFα-induced NF-κB activation in A549 cells (Figure 5B). To determine further the role of USP4 on NF-κB target gene expression, we extracted total RNAs from the control and USP4-knockdown A549 cells treated with TNFα for the indicated time points and performed quantitative RT–PCR to examine the effect of knockdown of USP4 on TNFα-induced IL6, IL8 and COX2. As shown in Figures 5(C)–5(E), knockdown of USP4 significantly enhanced the TNFα-induced expression of IL6, IL8 and COX2. Taken together, these results suggest that USP4 negatively regulates NF-κB target gene expression in cancer cells.

**USP4 inhibits TNFα-induced cancer cell migration**

Previous results have shown that TNFα can promote the migration of breast cancer cells [27]. Interestingly, we found that TNFα could also significantly increase the migration of A549 cells, measured using a Transwell® migration assay (Figure 6A). Thus we asked whether USP4 affects TNFα-induced A549 cell migration. To answer this question, endogenous USP4 was knocked down by two siRNAs of USP4 and A549 cell migration was examined using a Transwell® migration assay. In this assay, we found that knockdown of USP4 markedly promoted TNFα-induced cell migration (Figure 6A). As expected, knockdown of USP4 also significantly increased IL-1β-stimulated cell migration.
Figure 5 USP4 negatively regulates TNF-α-mediated gene expression

(A) Knockdown efficiency in A549 cells was examined by quantitative RT–PCR. (B) Knockdown of USP4 promoted the degradation of TNF-α-induced IκBα. A549 cells were transfected with control or USP4 siRNA and stimulated with TNF-α (10 ng/ml) before harvesting. The cell lysates were analysed by immunoblotting using anti-IκBα and anti-actin antibodies respectively. (C) A549 cells were transfected with control siRNA (siNC) or siRNA against USP4 (siUSP4). At 48 h later, the A549 cells were either untreated or treated with TNF-α (10 ng/ml) for the time points indicated. Total RNAs from these cells were harvested. IL6 (C), IL8 (D) and COX2 (E) transcript levels in the siNC and two siUSP4 A549 cells were measured using quantitative RT–PCR and normalized to GAPDH. The data are presented as the mean ± S.D. of three separate experiments.

(Figure 6B). This result was confirmed further using a wound healing assay. As shown in Figure 6(C), knockdown of USP4 promoted TNF-α-induced wound healing in A549 cells. The effect of USP4 knockdown on A549 cell motility was also examined by time-lapse microscopy in the absence or presence of TNF-α. We observed that knockdown of USP4 significantly enhanced both basal and TNF-α-induced cell motility of A549 cells (Figure 6D). Taken together, our results indicate that USP4 is a negative regulator of TNF-α-induced cell migration.

DISCUSSION

In the present study, we identified USP4 as a novel DUB targeting TRAF2/TRAF6. We provide the first evidence to show that USP4 directly binds to the TRAF domain to inhibit the TRAF2/TRAF6 activity by deubiquitinating in a DUB activity-dependent manner. In agreement with a previous study showing that USP4 down-regulates the NF-κB signalling pathway by targeting TAK1 [TGF (transforming growth factor)-β-activated kinase] [19], the present study also demonstrates that USP4 negatively regulates IL-1β- and TNF-α-induced NF-κB activation. Thus, together with the previous study [19], we conclude that USP4 may negatively regulate the NF-κB signalling pathway.
by targeting multiple signalling molecules, including TRAF2, TRAF6 and TAK1. Although the precise mechanism underlying the regulation of NF-κB by USP4 under diverse physiological and pathological conditions is still unclear, targeting multiple signalling molecules suggests that USP4 is an important regulator of the NF-κB signalling pathway.

The present study demonstrates that USP4 is a negative regulator of TNFα-induced cell migration. TNFα is a key cytokine involved in inflammation, immunity and cellular homeostasis. Increasing evidence also links the TNFα signalling pathway to tumorigenesis, including tumour transformation, cell proliferation, angiogenesis, invasion and metastasis in many cancers [28,29]. In the present study, we found TNFα can increase the migration of A549 cells, a human lung adenocarcinoma cell line. Importantly, our results showed that knockdown of endogenous USP4 promotes TNFα-induced cell migration (Figure 6A), indicating that USP4 is a negative regulator of cell migration. To our knowledge, this is the first report that a member of the USP DUB family is involved in TNFα-induced cancer cell migration.

The mechanism by which USP4 negatively regulates cell migration needs to be investigated further. A previous study has shown that TNFα may promote breast cancer cell migration by regulating the stability of Snail, a key regulator of EMT (epithelial–mesenchymal transition), via the NF-κB signalling pathway [27]. Whether the same mechanism is employed in A549 cells remains unknown. However, our unpublished results have shown that USP4 can promote the degradation of Snail (H. Li, N. Xiao and P. Wang, unpublished work). Thus whether USP4 regulates cell migration via promoting EMT is currently being investigated.

Growing evidence has shown that USP4 may be a potential oncoprotein [26]. For example, USP4 mRNA is elevated in various types of cancers, such as colon, thyroid and urinary cancer [26]. USP4 can promote tumorigenesis when overexpressed in mice [30]. It may inhibit the tumour suppressor p53 by stabilizing the E3 ubiquitin ligase ARF-BP1 [26]. However, reports have also shown the USP4 protein level to be decreased in lung cancer cell lines, and it is not elevated in breast and pancreatic cancer [26,31]. Moreover, USP4 is demonstrated as a negative regulator of the Wnt signalling pathway [32], which has tumorigenic activity [33]. The results of the present study suggest that USP4 is a negative regulator of cell migration of cancer cells. Cell migration is critical to cancer metastasis [34]. Thus further investigation is needed to examine the role of USP4 in tumorigenesis and metastasis.

Although TRAF6 is a critical E3 ubiquitin ligase to regulate the NF-κB signalling pathway, a variety of NF-κB-independent functions are also being uncovered. For example, TRAF6 can regulate TLR4 (Toll-like receptor 4)-induced autophagy by promoting the Lys63-linked ubiquitination of Beclin-1 [35]. It is also involved in TGFβ-induced activation of JNK (c-Jun N-terminal kinase) and p38 [36]. A recent study links TRAF6 to Huntington’s disease by promoting the atypical ubiquitination of huntingtin protein [37]. Ubiquitination by TRAF6 is critical to membrane targeting and activation of Akt [38]. The present study shows that USP4 is a potential negative regulator of TRAF6 activity. Thus it will be of great interest to test whether USP4 is also involved in the NF-κB-independent function of TRAF6 by regulating the ubiquitination of other TRAF6 substrate targets.

It is still unclear whether USP4 activity is under the regulation of an extracellular signal to target TRAF2/TRAF6 and/or TAK1. Our results suggest that USP4 may constitutively associate with TRAF6 in vivo. Moreover, our unpublished results indicated that TRAF6 can promote the polyubiquitination of USP4 (H. Li, N. Xiao and P. Wang, unpublished work). Accumulating evidence has shown that Lys63-linked polyubiquitination can modulate the biological function of its target substrates, such as their activity and subcellular localization etc. [5,39]. Thus there is a possible model that USP4 may be activated by TRAF6-mediated ubiquitination and provides a negative-feedback loop to regulate TRAF2/TRAF6 and/or TAK1 ubiquitination.

In summary, in the present study we provide the first evidence that USP4 is a novel binding partner of TRAF2 and TRAF6, and acts as an essential DUB to inhibit NF-κB activation. Considering the results of the present study, and results of previous studies [10,11,13,16], we propose a working model (Figure 7), in which cytokines such as TNFα or IL-1β induce NF-κB activation, and the polyubiquitination of TRAF2/TRAF6 is critical to NF-κB activation. USP4 associates with TRAF2/TRAF6 and acts as a DUB to inhibit the ubiquitination of TRAF2/TRAF6 and consequently inhibits NF-κB activation.

ACKNOWLEDGEMENTS
We thank Dr Gang Pei and Dr Justin McCarthy for providing reagents. We thank Dongmei Liu, Yunfei Chen, Taiqi Chen, Su Yu, Yingcong Wang and other members of the Wang laboratory for their assistance.

FUNDING
This work was supported, in part, by the National Basic Research Program of China (973 programme; grant numbers 2010CB529704 and 2012CB910400), and the National Natural Science Foundation of China [grant numbers 30800587, 30971521 and 31171338]. P.W. is a scholar of the Shanghai Rising-Star Program from the Science and Technology Commission of Shanghai Municipality (grant number 09QA1401900).
SUPPLEMENTARY ONLINE DATA

Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNFα-induced cancer cell migration

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Figure S1  USP4 has little effect on the protein stability of TRAF2 and TRAF6

(A) Empty vector or expression vectors encoding wild-type USP4 were transfected into HEK-293T cells. At 24 h after transfection, the cells stimulated with or without TNFα (10 ng/ml) were treated with cycloheximide (CHX) (50 μg/ml) for the indicated times. The cell lysates were subjected to Western blot analysis to detect the protein level of TRAF2/TRAF6 and USP4. (B) The effect of knockdown of USP4 on the protein stability of protein stability. Control siRNA (siNC) or siRNA against USP4 (siUSP4) was transfected into HEK-293T cells. At 48 h later, the cell lysates were subjected to Western blot analysis to detect TRAF2/TRAF6 expression. (C) Effect of USP4 on cIAP-mediated TRAF2 degradation. Flag–TRAF2 was transfected into HEK-293T cells with empty vector, HA–USP4 and GFP–cIAP. Transfected cells were harvested after 24 h. The cell lysates were subjected to Western blot analysis. Con, control.

Received 27 July 2011/22 September 2011; accepted 27 October 2011
Published as BJ Immediate Publication 27 October 2011, doi:10.1042/BJ20111358

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