Ubiquitin-Specific Protease 13 Regulates IFN Signaling by Stabilizing STAT1

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The IFN immune system comprises type I, II, and III IFNs, signals through the JAK-STAT pathway, and plays central roles in host defense against viral infection. Posttranslational modifications such as ubiquitination regulate diverse molecules in the IFN pathway. To search for the deubiquitinating enzymes (DUBs) involved in the antiviral activity of IFN, we used RNA interference screening to identify a human DUB, ubiquitin-specific protease (USP) 13, whose expression modulates the antiviral activity of IFN-α against dengue virus serotype 2 (DEN-2). The signaling events and anti–DEN-2 activities of IFN-α and IFN-γ were reduced in cells with USP13 knockdown but enhanced with USP13 overexpression. USP13 may regulate STAT1 protein because the protein level and stability of STAT1 were increased with USP13 overexpression. Furthermore, STAT1 ubiquitination was reduced in cells with USP13 overexpression and increased with USP13 knockdown regardless of with or without IFN-α treatment. Thus, USP13 positively regulates type I and type II IFN signaling by deubiquitinating and stabilizing STAT1 protein. Overall, to our knowledge, USP13 is the first DUB identified to modulate STAT1 and play a role in the antiviral activity of IFN against DEN-2 replication.

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The IFN pathway is tightly regulated by several posttranslational mechanisms such as phosphorylation, acetylation, and ubiquitination (6–8). Ubiquitin, a 76-aa polypeptide, can be covalently attached to cellular proteins by an enzymatic cascade involving three classes of enzymes, E1, E2, and E3 (9). Ubiquitin forms an isopeptide linkage with substrate protein via the C-terminal glycine residue of ubiquitin and the lysine residue on the substrate protein. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63); each can form an isopeptide bond with the C-terminal glycine residue of the following ubiquitin. Monoubiquitination refers to only one ubiquitin conjugating to the lysine of the substrate protein. However, ubiquitins are often conjugated to substrates by forming a polyubiquitin chain, such as K48-linked polyubiquitination, which marks proteins for proteasome degradation, and K63-linked polyubiquitination, which can regulate signal transduction and DNA repair processes (10).

Several components of the IFN signaling pathway are modified and controlled by ubiquitination. For example, a cullin-based E3 ligase facilitates the ubiquitination and degradation of type I IFN receptor IFNAR1 (11), and suppressor of cytokine signaling 1, a component of an E3 ligase, promotes JAK2 polyubiquitination and degradation (12, 13). STAT1 could also be regulated by ubiquitination, and IFN-γ triggers STAT1 phosphorylation and ubiquitin-mediated protein degradation (14). A nuclear E3 ligase called STAT-interacting LIM (SLIM) protein (also known as PDLIM2) causes STAT1 and STAT4 ubiquitination and degradation (15, 16). Another E3 ligase, Smad ubiquitination regulating factor 1 (Smurf1), was recently found to catalyze K48 polyubiquitination and proteasomal degradation of STAT1 (17). Overexpression of SLIM or Smurfl attenuates IFN-γ-mediated STAT1 activation, whereas knockdown of SLIM or Smurfl enhances IFN-γ-mediated STAT1 activation and the antiviral immune response (15, 17).

Ubiquitination is a reversible process and the human genome contains ~100 deubiquitinating enzymes (DUBs) (18, 19). Similar to phosphorylation and dephosphorylation, deubiquitination provides an important layer of regulation for ubiquitination by re-
moving ubiquitin from their substrates. Several DUBs negatively regulate immune responses. CYLD specifically removes K63-linked polyubiquitin chains from TNFR-associated factors (TRAFs), thus leading to IkB kinase inhibition in the NF-κB activation pathway (20). A20 is a dual-function enzyme with DUB activity, which preferentially cleaves K63-linked polyubiquitin chains, and E3 ligase activity, which builds K48-linked polyubiquitin chains (21, 22). Thus, A20 inhibits NF-κB activation upstream of IkB kinase by removing K63-linked polyubiquitin chains and promoting K48-linked polyubiquitination and degradation of receptor-interacting protein 1 (23–25). Additionally, ovarian tumor (OTU) B1 and OTUB2 inhibit virus-triggered IFN-β expression by deubiquitinating TRAF3 and TRAF6 (26). Furthermore, ubiquitin-specific protease (USP) 17 (also called DUB3) positively regulates virus-induced type I IFN signaling through interaction and deubiquitination of RIG-I and MDA5 (27). However, research into deubiquitination lags behind that of ubiquitination, and not many DUBs with a role in cellular immunity have been revealed.

To gain more insight into the role of cellular DUBs in host defense, we performed RNA interference (RNAi) screening to search for DUBs involved in the antiviral activity of type I IFN against dengue virus (DEN) infection. DEN belongs to the Flavivirus genus of the family Flaviviridae and has emerged as the most important vector-borne viral disease in tropical and subtropical areas (28). DEN has four serotypes, and people infected with DEN may develop mild dengue fever or severe dengue hemorrhagic fever and dengue shock syndrome. The magnitude of viral replication is thought to be one of the determining factors of DEN pathogenesis. DEN replication is sensitive to IFN in cell-based assays and in experimental animals (29, 30). Thus, understanding the antiviral actions of IFN against dengue virus (DEN) infection is of great importance. Recent studies have revealed that several DUBs involved in the antiviral activity of type I IFN against dengue virus were reduced in cells with USP13 overexpression. Thus, USP13, a cellular DUB, may deubiquitinate protein degradation of STAT1 were reduced in cells with USP13 overexpression. Thus, understanding the antiviral actions of IFN against dengue virus (DEN) infection is of great importance.

**Materials and Methods**

**Virus and cell lines**

DEN serotype 2 (DEN-2) PL46 strain (31) was propagated in mosquito C6/36 cells with RPMI 1640 medium supplemented with 5% FBS (Life Technologies) and 2 mM t-glutamine (Life Technologies). Human lung carcinoma A549 and human embryonic kidney (HEK293T) cells were cultured in 10% FBS and 2 mM t-glutamine containing F-12 and DMEM medium (both Life Technologies), respectively. Baby hamster kidney fibroblasts (BHK-21) were cultured in RPMI 1640 medium containing 5% FBS and 2 mM t-glutamine. For viral infection, cells were absorbed with virus at the indicated multiplicity of infection (MOI) for 2 h at 37°C. The unbound virus was removed by gently washing with HBSS (HyClone), and then cells were cultured at 37°C. At the indicated times postinfection, the culture supernatants were collected and sequentially diluted for plaque-forming assays on BHK-21 cells as previously described (31).

**DUB screening**

The human deubiquitinating enzyme set (C6-2, v1.0; Taiwan National RNAi Core Facility) contains the individual vesicular stomatitis virus-G-pseudotyped lentivirus targeting 84 human DUBs with at least five short hairpin RNAs (shRNAs) for each DUB (Supplemental Table I). A549 cells seeded in 96-well plates were transduced with lentivirus expressing shRNA targeting the individual human DUBs. Cells were selected with puromycin (10 μg/ml) for 3 d, treated with 1000 U/ml IFN-α-2a (Roferon-A) for 16 h, then infected with DEN-2 (MOI of 1) for 2 d. The degree of DEN infection was detected by immunofluorescence assay with anti-dengue NS3 Ab and quantified by use of Cellomics ArrayScan HT image reader (Thermo Scientific). We selected the candidate gene USP13 for further study based on the criteria that at least two clones show a >2-fold induction of DEN infection as compared with the luciferase-targeted shRNA (shLuc) control and the clones have a live cell number not <40% of the shLuc control.

**Generation of recombinant lentivirus**

The lentiviral vectors expressing shRNAs targeting USP13 (G9, 5′-GCA-GATAAAGAAGTGACTTT-3′ [TRCN0000007248] and B10, 5′-CGAT- TTAATAGGCGACGTATT-3′ [TRCN0000007251]) were from the Taiwan National RNAi Core Facility. Lentivirus was prepared by cotransfection of USP13-targeted shRNA (shUSP13/pLKO.1-paromomycin with helper plasmids, pCMVꞌRS.91 and pMD.G, into HEK293T cells with use of Lipofectamine 2000 (Invitrogen). The culture supernatants were harvested 24, 36, and 48 h after transfection and further concentrated by centrifuging at 20,000 × g for 3 h at 4°C.

**Plasmid construction**

The human USP13 cDNA with or without hemagglutinin (HA) tag was PCR amplified from Flag-HA-USP13 (Addgene, plasmid 22568) with the primer pair sequences for HA-USP13, 5′-CCCAAGCATTATGCACCGACGACTCTGCTTACGATGTCACA-3′ and 5′-AACGGGCCCTTTAGTGGTATGTTGC-3′, and USP13, 5′-TAAAGCTTATAGGCAGCAGGCGGCGCTTGGG-3′ and 5′-AATGCGGGCCACGCTTGGCCTTG-3′ (the restriction enzyme recognition sequences are in italics and USP13 sequences are in bold). The HA-USP13 and USP13 cDNAs were subcloned into pCR3.1 and pcDNA3.1, respectively. To generate RNAi-resistant USP13 (USP13res), we introduced two point mutations for USP13-shRNA targeting sequences by the single-primer mutagenesis method (32) with the primer sequences for shUSP13-G9, 5′-CTGAATCTTGTAGTGACAAGGCTCAAGAATCTTGGTATGC-3′ and shUSP13-B10, 5′-AATGCGGGCCACGCTTGGCCTTG-3′, and USP13, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′ with the primer sequences for C345A, 5′-ACCCTGGCAAGGCTCAAGAATCTTGGTATGC-3′ and B10, 5′-TGACGATGTCACAATGTCATATGCA-3′.
STAT1, anti-pSTAT1 (Tyr701), and anti-USP13 Abs, respectively. To determine the interaction of endogenous STAT1 and USP13, lysates of HEK293T cells with or without IFN-α treatment were immunoprecipitated with anti-USP13 Ab (HPA004827; Sigma-Aldrich), anti-STAT1 Ab (9172; Cell Signaling Technology), or anti-pSTAT1 (Tyr701) Ab (9171; Cell Signaling Technology). The immune complexes bound to Protein A Mag Sepharose Xira (28-9670-56; GE Healthcare Life Sciences) were washed three times with protein lysis buffer plus 0.1% Nonidet P-40. The immunoprecipitated proteins were then subjected to Western blot analysis with the indicated Abs.

To detect STAT1 ubiquitination, HEK293T cells were cotransfected with STAT1-Flag plus the wild-type (WT) HA-Ub or mutated HA-Ub, respectively. The immune complex bound to Protein G Mag Sepharose beads (28-9670-70; GE Healthcare Life Sciences) was washed five times with RIPA lysis buffer (0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5], and 5 mM EDTA) at 4°C for 30 min. The immunoprecipitated proteins were then separated by SDS-PAGE and detected by Western blot analysis.

Reporter assay

The type I IFN reporter (pISRE-driven luciferase [pISRE-Luc], 0.5 μg) or type II IFN reporter (pGAS-driven luciferase [pGAS-Luc], 0.5 μg) plus the internal control pRL-TK (0.1 μg) were co-transfected with STAT1-Flag or STAT1-Flag plus WT or mutated HA-Ub. Cell lysates were immunoprecipitated with mouse anti-Flag M2 Ab (F3165; Sigma-Aldrich) overnight. The immune complex was washed four times with protein lysis buffer, eluted with elution buffer, and subjected to Western blot analysis with anti-Flag Ab (M2, Sigma-Aldrich) or anti-USP13 Ab (HPA004827; Sigma-Aldrich), anti-STAT1 Ab (9172; Cell Signaling Technology), or anti-pSTAT1 (Tyr701) Ab (9171; Cell Signaling Technology). The immunoprecipitated proteins were then subjected to Western blot analysis with the indicated Abs.

RNA extraction and quantitative RT-PCR

Total cellular RNA was extracted by the RNeasy Total RNA kit (Qiagen), and cDNA was reverse transcribed from 1 μg RNA by the Transcriptome RT-PCR system (Invitrogen). PCR quantification involved use of the specific primers that was provided by the Taiwan National RNAi Core Facility. A549 cells transduced with these DUB-targeting lentiviruses were selected with puromycin for 3 d and treated with IFN-α overnight. The levels of viral replication were determined by immunofluorescence assay with Ab against DEN-2 NS3 protein and measured by a Cellomics ArrayScan HT image reader. IFN-α greatly reduced DEN-2 viral NS3 protein expression in control cells transduced with lentivirus expressing shLuc (Fig. 1B, 1Bb), but its anti–DEN-2 activity was repressed in cells transduced with lentivirus expressing shUSP13 (Fig. 1Bc).

Knockdown of USP13 hampers the anti–DEN-2 effect of IFN-α

To verify the screening data, we established two stable USP13-knockdown A549 cell lines by transduction with lentiviruses expressing shRNAs targeting two different regions of USP13, shUSP13-G9 and shUSP13-B10. DEN-2 NS3 protein expression measured by Western blotting and viral progeny production measured by plaque-forming assay were higher in both USP13-knockdown cell lines with IFN-α treatment as compared with shLuc control cells (Fig. 2A). We then rescued the USP13 protein expression by overexpressing USP13-res generated by introducing two wobble mutations into the targeting sequences of each shUSP13 (Fig. 2B). The enhanced DEN-2 replication in IFN-α–treated USP13-knockdown cells was significantly lost in cells with USP13-res but not GFP overexpression (Fig. 2B).

Ex vivo DUB assay

HEK293T cells were transfected with HA-GFP, HA-USP13, or HA-USP13-M664/739E (1.5 μg) or with STAT1-Flag (1 μg) plus HA-Ub (1 μg) in the presence of MG-132 (2 μM) for 16 h. The reporters were also cotransfected with HA-USP13 or GFP control (1 μg) to A549 cells for 16 h. Before harvesting for dual luciferase assay (Promega), cells were treated with 1000 U/ml IFN-α or IFN-γ (CYT-402; ProSpec) for 6 h. Firefly luciferase activity was normalized to that of Renilla luciferase.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X-100. Cells were blocked with skim milk in PBS with 0.1% Tween 20 and then incubated with mouse anti-DEN-2 NS3 Ab (31) and Alexa Fluor 488–conjugated secondary Ab (Invitrogen). DAPI was used to stain nuclei. DEN-2 NS3 content was measured by use of the Cellomics ArrayScan HT image reader.
USP13 positively regulates type I and type II IFN signaling pathways

To address whether USP13 modulates the IFN-α–triggered JAK-STAT signaling pathway, we measured IFN-α activation levels by using pSRE-Luc. The basal and IFN-α–triggered ISRE-Luc activities were lower in cells with USP13 knockdown (Fig. 3A), whereas overexpression of USP13 enhanced the ISRE-Luc activities (Fig. 3B), which suggests that USP13 positively regulates the JAK-STAT signaling pathway. To identify the molecules targeted by USP13, we detected the expression and activation of STAT proteins. Interestingly, levels of total STAT1 and pSTAT1 but not much of STAT2 and STAT3 were reduced in cells with USP13 knockdown (Fig. 3C). Furthermore, the levels of total STAT1 and pSTAT1 protein were recovered in the USP13-rescued shUSP13 cells (Fig. 3C). Because the STAT1 transcript levels were not significantly different in cells with altered USP13 expression (Fig. 3D), the effect of USP13 on STAT1 likely occurred at the protein but not RNA level.

STAT1 is involved in both type I and type II IFN signaling pathways, so we explored whether USP13 also plays a role in type II IFN signaling. In measuring the GAS-driven reporter activity (pGAS-Luc), USP13 knockdown reduced and USP13 overexpression augmented the reporter activity of IFN-γ–triggered GAS-Luc (Fig. 4A, 4B). Furthermore, the anti–DEN-2 activity of IFN-γ was also reduced with USP13 knockdown and rescued by USP13 overexpression in shUSP13 cells (Fig. 4C, 4D). Thus, USP13 may target STAT1 protein in both type I and type II IFN signaling events.

USP13 stabilizes STAT1 protein level

To address how USP13 regulates STAT1 protein levels, we traced the protein stability of STAT1 in cells treated with the protein synthesis inhibitor cycloheximide with and without USP13 overexpression. Endogenous STAT1 protein levels were higher in HEK293T cells transfected with USP13-expressing plasmid than in control GFP-transfected cells (Fig. 5A) at all of the time points tested. The transfected STAT1-Flag protein levels were also increased in cells with USP13 overexpression (Fig. 5B). Thus, ectopic overexpression of USP13 prolonged the half-life of STAT1 protein (Fig. 5C) and increased STAT1 expression.

USP13 physically interacts with STAT1

To determine how USP13 modulates STAT1 protein, we tested whether USP13 physically interacts with STAT1. By immunoprecipitation (IP)-immunoblotting (IB) assay, anti-Flag Ab brought down not only Flag-tagged STAT1 but also USP13 with or without IFN-α treatment (Fig. 6A, lanes 5 and 6). Furthermore, anti-HA Ab coprecipitated HA-tagged USP13 with STAT1 but barely with phosphorylated STAT1 (Fig. 6B, lanes 5 and 6). We further tested whether the endogenous STAT1 and USP13 proteins interact with each other. Similar to the data of overexpressed proteins, anti-STAT1 but not anti-pSTAT1 Ab coprecipitated STAT1 with USP13 in cell lysates with or without IFN-α treatment (Fig. 6C). Furthermore, anti-USP13 Ab brought down USP13 with STAT1 but little with phosphorylated STAT1 (Fig. 6D). Thus, USP13 interacts with STAT1 but not much with activated STAT1.

USP13 decreases the ubiquitination of STAT1

To address whether USP13 affects the ubiquitination of STAT1, we first determined the ubiquitination pattern of STAT1 in cells cotransfected with plasmids expressing STAT1-Flag and HA-Ub. By IP-IB assay with anti-Flag and anti-HA Abs, smeared protein reactivity with a molecular mass larger than the expected size of STAT1 protein indicated polyubiquitination and/or multiple monoubiquitination of STAT1 (Fig. 7A, lane 1). To determine whether the two lysine residues most commonly used in polyubiquitination, K48 and K63, contributes to STAT1 polyubiquitination, we cotransfected cells with STAT1-Flag and HA-tagged ubiquitin with K48R, K63R, or K48/63R mutation. The levels of STAT1 ubiquitination were slightly reduced most commonly used in polyubiquitination, K48 and K63, contributes to STAT1 polyubiquitination, we cotransfected cells with STAT1-Flag and HA-tagged ubiquitin with K48R, K63R, or K48/63R mutation. The levels of STAT1 ubiquitination were slightly reduced with K48R and K48/63R mutants, but not much with the K63R mutant, as compared with WT ubiquitin (Fig. 7A, lanes 3–5). Thus, USP13 decreased the ubiquitination of STAT1 in cells cotransfected with plasmids expressing STAT1-Flag and HA-Ub.
uitin, with which neither polyubiquitination nor monoubiquitination can occur, greatly reduced STAT1 ubiquitination (Fig. 7A, lane 2). Previously, both K48-linkage polyubiquitination (17) and monoubiquitination (36) have been reported for STAT1 protein. Our results thus suggest that polyubiquitination through K48 and the other five less used lysines (K6, K11, K27, K29, and K33) as well as monoubiquitination may occur on STAT1 protein.

We then determined whether USP13 affects STAT1 ubiquitination. Interestingly, STAT1 ubiquitination was greatly reduced in cells with USP13 overexpression as compared with unrelated LacZ-V5 overexpression (Fig. 7B). We further tested whether the ubiquitination of endogenous STAT1 can be downregulated by the ubiquitin–proteasome system (14). As outlined in Fig. 9, USP13 can reduce the ubiquitination levels of STAT1, stabilize its protein level, and result in a stronger IFN antiviral response. Thus, to our knowledge, USP13 is the first cellular DUB involved in deubiquitinating and stabilizing STAT1 protein in both type I and type II IFN signaling.

To demonstrate the ability of USP13 to deubiquitinate STAT1, we determined the sequences required for USP13 to stabilize STAT1 protein. Both the cysteine catalytic motif (37) and ubiquitin-associated (UBA) domain (38) have been implicated in USP13 function, so we constructed two USP13 mutants, C345A with mutated cysteine catalytic motif and M664/739E with mutated UBA domain. The ability of USP13 to increase STAT1 protein level was reduced by either C345A or M664/739E mutation (Fig. 8A), confirming the importance of the cysteine catalytic motif and UBA domain of USP13 on its function. We then performed an ex vivo DUB experiment to directly demonstrate that USP13 is able to deubiquitinate STAT1. The ubiquitination level of STAT1 protein was reduced by incubation with USP13, but not with M664/739E-mutated USP13, when compared with the GFP control (Fig. 8B). Thus, our results suggest that USP13 can deubiquitinate STAT1 and increase the stability of STAT1 protein.

**Discussion**

By RNAi screening, we identified USP13 as a positive regulator of the IFN signaling pathway. STAT1 is involved in both type I and type II IFN signaling and is targeted by the ubiquitin–proteasome system (14). As outlined in Fig. 9, USP13 can reduce the ubiquitination levels of STAT1, stabilize its protein level, and result in a stronger IFN antiviral response. Thus, to our knowledge, USP13 is the first cellular DUB involved in deubiquitinating and stabilizing STAT1 protein in both type I and type II IFN signaling. To address whether IFN and/or viral infection can modulate USP13 expression to regulate STAT1 and the IFN system, we determined the protein expression level of endogenous USP13 by Western blotting. Similar USP13 protein levels were noted in mock- and DEN-infected cells with or without IFN-α treatment (Supplemental Fig. 1), indicating that USP13 protein expression is not modulated by type I IFN or DEN infection. Recently, accumulating evidence suggests that the activity of DUBs can be reversibly inhibited by oxidation mediated by reactive oxygen species (39). Thus, whether the activity of USP13 could be blocked by reactive oxygen species in DEN-
infected cells that then results in less STAT1 protein to benefit viral replication remains elusive.

The human genome encodes ~95 DUBs categorized into five major families, including USP, ubiquitin C-terminal hydrolase, OTU domain containing protease, Machado–Joseph disease protease, and JAB1/MPN/Mov34 metalloenzyme (18, 40). USP represents the largest family of DUBs, with >50 members that might have specific substrates regulating distinct cellular processes (18, 41). USPs are Cys-dependent proteases containing a catalytic core domain including an N-terminal Cys-box and a C-terminal His-box, which comprise the catalytic Cys and His residues, respectively. USPs also contain other functional ubiquitin-binding domains, such as the zinc finger ubiquitin-specific protease (ZnF-UBP) domain, the ubiquitin-interacting motif, and the UBA domain

FIGURE 4. USP13 enhances the signaling and anti–DEN-2 activity of IFN-γ. (A) shLacZ control and shUSP13-A549 cells were cotransfected with pGAS-Luc (0.5 μg) and pRL-TK (0.1 μg) for 16 h and then treated with IFN-γ (1000 U/ml) for 6 h. Cells were analyzed as in Fig. 3A. (B) A549 cells were cotransfected with the reporter plasmids (0.5 μg pGAS-Luc and 0.1 μg pRL-TK) plus GFP control or USP13 expression plasmid (1 μg) for 16 h and then treated with IFN-γ (1000 U/ml) for 6 h. Cells were analyzed as in (A). (C) Western blot analysis of DEN-2 NS3, USP13, and actin protein levels in cells pretreated with IFN-γ (1000 U/ml) overnight and infected with DEN-2 (MOI of 1) for 2 d. (D) Plaque-forming assay of viral titration in culture supernatants. Data are means ± SD virus titers (PFUs/ml) from two independent experiments analyzed by a two-tailed Student t test.

FIGURE 5. STAT1 protein levels are increased by USP13. Western blot analysis of protein levels in HEK293T cells transfected with (A) HA-Ub plus GFP or USP13 plasmid or (B) HA-Ub and STAT1-Flag plus GFP or USP13 overnight and then treated with cycloheximide (CHX, 50 μg/ml) for the indicated times. (C) The protein band density was quantified with use of ImageJ and the level of STAT1 was normalized to that of actin. The protein levels of STAT1 relative to that at time 0 of each group were calculated and are shown.
flanking or inserting in the core domain (40, 41). The UBA and ubiquitin-interacting motif domains can facilitate substrates binding onto enzymes, and the ZnF-UBP domain might stimulate the hydrolysis reaction or regulate other biological functions (42, 43).

USP13, also called isopeptidase T3, shares \( \sim \)80% sequence similarity with USP5. Both proteins have four ubiquitin-binding sites, including two UBA domains, a ZnF-UBP domain, and a catalytic domain (44). The ZnF-UBP domain of USP5 recognizes and recycles the unanchored polyubiquitination chains to keep the free ubiquitin pool stable (45, 46). The ZnF-UBP domain of USP13 shares a similar fold with that of USP5, but it cannot bind ubiquitin, and USP13 does not function similar to USP5 in recycling ubiquitin (44). As compared with USP5, USP13 shows weaker deubiquitination activity, probably through its tandem UBA domains binding with ubiquitin (44).

A couple of proteins involved in diverse cellular functions have been reported as targets of USP13. For example, USP13 appears to be responsible for the protein stability of microphthalmia-associated transcription factor, which is essential for melanocyte development (37). USP13 can reduce the ubiquitination and degradation of

FIGURE 6. USP13 mainly interacts with the nonactivated form of STAT1. IP-IB analysis with control IgG, anti-Flag affinity gel, or anti-HA affinity gel (A, B) in HEK293T cells cotransfected with plasmids expressing HA-USP13 and STAT1-Flag for 18 h and then treated with or without IFN-\( \alpha \) (1000 U/ml) for 30 min. IP-IB analysis of protein levels of USP13, STAT1, and pSTAT1 in the immune complex and in total cell lysates is shown. (C and D) The interaction of endogenous USP13 and STAT1 was investigated by IP-IB analysis. Immunoprecipitation of HEK293 cells with or without IFN-\( \alpha \) treatment was carried out with control IgG, anti-STAT1, anti-pSTAT1, or anti-USP13 Abs as indicated. The immunoprecipitated proteins were then analyzed by Western blot with anti-USP13, anti-STAT1, and anti-pSTAT1 Abs, respectively.

FIGURE 7. STAT1 ubiquitination is reduced by USP13. (A) IP-IB and IB analysis with the indicated Abs in HEK293T cells cotransfected with STAT1-Flag plus the WT or mutated GG/AA, K48R, K63R, or K48/63R HA-Ub in the presence of MG-132 (2 \( \mu \)M) for 16 h. (B) IP-IB and IB analysis with the indicated Abs in HEK293T cells transfected with STAT1-Flag plus USP13 or LacZ-V5 as well as HA-Ub or HA-Ub-GG/AA with MG-132 (2 \( \mu \)M) for 16 h. (C) IP-IB and IB analysis with the indicated Abs in HEK293T cells with control LacZ knockdown or USP13 knockdown with or without IFN-\( \alpha \) treatment (1000 U/ml, 30 min).
Sky2, the F-box adaptor of the E3 ubiquitin ligase SCF<sub>Skp2</sub> (47). It can bind to and stabilize another E3 ligase, the RING finger E3 ubiquitin ligase Siah2, even though it reduces the ubiquitin ligase activity of Siah2 (38). USP13 interacts with and deubiquitinates Beclin 1, a tumor suppressor and autophagy inducer (48). Furthermore, interaction with Beclin 1 can enhance the DUB activity of USP13. USP10, known as a DUB of p53 (49), is another substrate of USP13. USP10 also interacts with USP13 and increases its DUB activity (48). Additionally, USP13 interacts with some components of the P97/VCP complex, a key chaperone in endoplasmic reticulum–associated degradation (19) and increases the protein level of CD3<i>d</i>, an endoplasmic reticulum–associated degradation substrate (44). In this study, we add STAT1 to the growing list of USP13 substrates by demonstrating that USP13 can remove STAT1 ubiquitination and increase STAT1 protein stability. Because STAT1 is involved in type I, II, and III IFN signaling, USP13 thus also regulates the innate immunity and host defense mechanism.

Two E3 ligases, SLIM and Smurf1, have been reported to contribute to STAT1 ubiquitination (15–17). SLIM, a nuclear E3 ligase, can interact with phosphorylated STAT4 and promote the ubiquitination and degradation of STAT4 protein (15). SLIM also promotes the ubiquitination of STAT1 and inhibits STAT1-mediated gene expression (15). Smurf1, a HECT-type E3 ligase, catalyzes K48-linked polyubiquitination of STAT1 and promotes STAT1 degradation, independent of STAT1 phosphorylation (17). We also detected STAT1 polyubiquitination in cells transfected with plasmids expressing STAT1 and ubiquitin (Fig. 7). Furthermore, ubiquitin with K48R but not K63R mutation could be conjugated to STAT1 protein to a level lower than that of WT ubiquitin but higher than that of the GG/AA mutant. These results suggest that STAT1 might be monoubiquitinated as previously reported (36) and/or polyubiquitinated with K48 linkage as well as linkage through other lysines such as K6, K11, K27, K29, and/or K33. However, regardless of the types of ubiquitin linkage, STAT1 ubiquitination was greatly reduced in cells with USP13 overexpression. USP13 has been reported to elicit cleavage of both K48- and K63-linked polyubiquitin chains (44, 47), and USP5 can hydrolyze the K63-, K48-, K11-, and K29-linked and linear polyubiquitination chains (50, 51). Thus, USP13 might possess broad DUB activity against several types of ubiquitin linkages and warrants further study to reveal its enzymatic activity.

The incidence of DEN infection has grown around the world, and according to the World Health Organization, dengue now threatens >40% of the world’s population. Despite the clinical significance of the infection, no specific treatment or vaccine is available for DEN infection. DEN infection may cause a wide spectrum of illnesses ranging from mild dengue fever to severe dengue hemorrhagic fever and dengue shock syndrome. The pathogenesis mechanisms for DEN-related diseases are not clear, but high viral replication might be a risk factor for the severe form of DEN infection. DEN replication was found sensitive to type I and type II IFNs in cell-based assays and in infected animals (29, 30). Different DEN strains may...

![Figure 8](http://www.jimmunol.org/)  
**Figure 8.** USP13 deubiquitinates STAT1. (A) Western blot analysis of protein levels in HEK293T cells cotransfected with STAT1-Flag plus WT USP13, USP13-C345A, USP13-M664/739E, or GFP control for 20 h. (B) STAT1 proteins were purified from HEK293T cells cotransfected with STAT1-Flag and HA-Ub in the presence of MG-132 (2 μM) by anti-Flag beads and then were incubated with HA-GFP, HA-USP13, or HA-USP13 (M664/739E) mutant proteins immunoprecipitated by anti-HA affinity gel in vitro at 37°C for 2 h with deubiquitination buffer. The products of deubiquitination reaction were analyzed by immunoblotting with anti-ubiquitin Ab.

![Figure 9](http://www.jimmunol.org/)  
**Figure 9.** USP13 reduces STAT1 protein ubiquitination and enhances the signaling and antiviral activity of type I and type II IFNs. (A) STAT1 is an essential molecule in the signaling cascades of type I and type II IFNs, which leads to gene activation mediated by promoters containing ISRE and GAS, respectively. STAT1 proteins are ubiquitinated and targeted for protein degradation. USP13 can reduce the STAT1 ubiquitination and increase its protein level. (B) USP13 regulates the outcomes of IFN antiviral activity by controlling the protein levels of STAT1. Increased USP13 expression may stabilize STAT1 proteins, enhance the IFN response, and inhibit virus replication. In contrast, reduced USP13 level may reduce the STAT1 protein level, weaken the IFN response, and increase virus replication.
regulate the IFN pathway differently (52), but the determining factors controlling the levels of DEN replication in infected patients are largely unclear. Therefore, more understanding of the antiviral mechanism, such as the cellular proteins involved in IFN antiviral activity against DEN replication, will shed light on the overall interplay between DEN and host immunity. Our study revealed a cellular protein USP13 that participates in the antiviral action of type I and II IFN against DEN infection and paves the way to a new direction in research into IFN signaling.

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Disclosures
The authors have no financial conflicts of interest.

References