SOCS3 Drives Proteasomal Degradation of TBK1 and Negatively Regulates Antiviral Innate Immunity


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Running Title: SOCS3 inhibits TBK1
TANK-binding kinase 1 (TBK1)-mediated induction of type I interferon plays critical role in host antiviral responses and immune homeostasis. The negative regulation of TBK1 activity is largely unknown. We report that suppressor of cytokine signaling 3 (SOCS3) inhibits IFN-β signaling pathway by promoting proteasomal degradation of TBK1. Overexpression and knockdown experiments indicated that SOCS3 is a negative regulator of IRF3 phosphorylation and IFN-β transcription. Moreover, SOCS3 directly associates with TBK1, and they co-localize in the cytoplasm. SOCS3 catalyzes K48-linked polyubiquitination of TBK1 at Lys341 and Lys344 and promotes subsequent TBK1 degradation. On the contrary, SOCS3 knockdown markedly increases the abundance of TBK1. Interestingly, both the BOX domain of SOCS3 and the Ser172 phosphorylation of TBK1 are indispensable for the process of ubiquitination and degradation. Ectopic expression of SOCS3 significantly inhibits vesicular stomatitis virus (VSV) and influenza A virus strain A/WSN/33 (WSN)-induced IRF3 phosphorylation and facilitates the replication of WSN virus by detecting the transcription of its vRNAs. Knockdown of SOCS3 represses WSN replication. Collectively, these results demonstrated that SOCS3 acts as a negative regulator of IFN-β signal by ubiquitinating and degrading TBK1, which shed light on the understanding of antiviral innate immunity and provide potential target for developing antiviral agents.
Introduction

Innate immunity is the first barrier to protect host cells from microbial infection, which is great threat to health and even lives of human. RIG-I-like receptors (RLRs), such as RIG-I and MDA5, can bind to viral RNA through its C-terminal regulatory domain (RD) and interact with the CARD domain of the mitochondrial adaptor protein MAVS by its N-terminal CARD domains (1-4). Subsequently, MAVS activates downstream kinases TBK1/IKKε and IKKα/β, leading to the activation of transcription factors IRF3/7 and NF-κB, respectively. As a consequence, these factors translocate into nucleus and initiate transcription of type I interferon and other proinflammatory cytokines (5). IFN-α/β bind to the cell surface receptors and activate Janus tyrosine kinase (JAK) family, which in turn provokes Janus tyrosine kinase and signal transducer and activator of transcription (JAK/STAT) signal transduction cascades. The ultimate products, IFN-stimulated genes (ISG), antagonize the replication of various pathogens (6).

Suppressor of cytokine signaling (SOCS) is another important protein family stimulated by JAK/STAT. The SOCS proteins comprise eight members, including cytokine-inducible SH2 domain-containing protein (CIS) and SOCS1-7 (7). They share several domains: a central Src homology 2 (SH2) domain participating in the association with substrates through recognition of the phosphorylated tyrosine residues; a C-terminal SOCS BOX domain involved in the formation of the E3 ligase.
complex with elongins B and C, cullin5 and Rbx-1 (8, 9); an N-terminal extended SH2 subdomain (N-ESS) contributing to substrate association (10). Besides, the unique kinase inhibitory region (KIR) owned by SOCS1 and SOCS3 inhibits Janus tyrosine kinases (JAKs) by acting as their pseudosubstrate (11, 12). SOCS3 binds to and inhibits JAKs through its SH2 domain and N-terminal KIR (11).

In addition to the feedback inhibition of JAK/STAT signaling, it has been reported that SOCS3 could be induced by the infection of several viruses (13-16) and represses innate immune responses including NF-κB (17) and interferon signal pathway (13, 14). For example, SOCS3 is upregulated by influenza virus (13, 14), HSV type I (15) or HIV (16) and blocks the expression of type I interferon, resulting in the augment of virus replication; SOCS3 interacts with TRAF6 and attenuates its ubiquitination, thus hindering the formation of TRAF6/TAK1 complex and reducing the kinase activity of TAK1 (17). However, the molecular mechanism of SOCS3 mediated inhibition of IFN-β expression remains largely unknown.

TANK-binding kinase 1 (TBK1) plays critical roles in RIG-I signaling. The activated TBK1 phosphorylates transcription factor IRF3 to transcript type I interferon following viral stimulation. The activity of TBK1 is regulated in multiple ways, including phosphorylation, ubiquitination, kinase activity modulation and complex disruption (18). It has been reported that many proteins promote or inhibit lys63(K63)-linked polyubiquitination of TBK1, such as Nrdp1 (19), MIB1/2 (20),
CYLD (21) and A20-TAX1BP1-ABIN1 (22). On the other hand, E3 ubiquitin ligases DTX4 and TRAF-interacting protein (TRIP) also target TBK1 for K48-linked ubiquitination and degradation and attenuate IFN-β production (23, 24). However, the negative regulation of TBK1 activity is not well understood.

In this study, we elucidated the mechanism of SOCS3 in negative regulation of IFN-β signal pathway by targeting TBK1 for proteasome dependent degradation. SOCS3 forms complex with TBK1, intensifies K48-linked polyubiquitination of TBK1 at lys341 and lys344. Knockdown of SOCS3 significantly enhances activation of IFN-β signal and abrogates degradation of TBK1. Moreover, the replication of influenza A virus can be effectively augmented by SOCS3. Collectively, SOCS3 drives proteasomal degradation of TBK1 and negatively regulates antiviral innate immune responses.
Materials and Methods

Plasmids and viruses

Flag-TBK1 S172A was kindly provided by Prof. Hongbin Shu (Wuhan University, Wuhan, China). RIG-I, MAVS (also termed VISA), TBK1, TRAF3, IRF3 and ISRE reporter plasmids were gifts from Prof. Xin Ye at Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). Plasmids encoding IKKe and TANK were friendly provided by Prof. Dongyan Jin (The University of Hong Kong, Hong Kong, China). Expression vectors for HA-tagged wildtype Ub, K48-Ub and K63-Ub were from Prof. Feng Shao (NIBS, China). His-tagged WT Ub was shared by Prof. Eli Song at Institute of Biophysics, Academy of Sciences in Beijing of China. TBK1 mutants (KD, KU, ΔULD, CC, K341R, K344R, K323R, K372R, K341/344R and K38A) were generated by cloning corresponding cDNAs into pcDNA3.0-Flag vector (Invitrogen). Similarly, SOCS3 truncations (Δ(N+KIR), ΔBOX, ΔSH2, Δ(ESS+SH2) and Δ(PEST+BOX)) were constructed by cloning respective cDNA sequences into pcDNA4-Myc/His or pGEX4T-1 expression vectors (Invitrogen), and Myc-SOCS3-rescue plasmid was constructed according to the sequence of SOCS3 specific siRNA #1. The reverse genetic system of A/WSN/33 (WSN) virus was provided by Prof. George F. Gao at Institute of Microbiology, Chinese Academy of Sciences. VSV was purchased from National Institutes for Food and Drug Control.
Reagents

The rabbit antibodies against phospho-IRF3 (Ser396; #4947), IRF3 (#4302), p-TBK1 (#5483), TBK1 (#3013) and SOCS3 (#2923) were purchased from Cell Signaling Technology (BOSTON, USA). The mouse antibodies specific for Myc (sc-40), Ubiquitin (sc-8017) and β-actin (47778) were from Santa Cruz Biotechnology (CA, USA). The mouse anti-Flag (F3165) antibody was ordered from Sigma (USA). The rabbit Myc (A00172) and goat HA (A00168) antibodies were bought from Genscript (Nanjing, China). The mouse anti-His Ab was purchased from ZSGB-BIO (Beijing, China). Poly(I:C) was ordered from Sigma and used at a final concentration of 1μg/ml in 293T cells and MEF cells. MG-132, Protein G beads and GST beads were purchased from Santa Cruz Biotechnology and GE Healthcare (USA), respectively. DAPI staining kit (KGA511) was bought from KeyGENBioTECH (Nanjing, China). TNT T7 coupled reticulocyte lysate system and Dual-Luciferase Reporter Assay System were ordered from Promega (Wisconsin, USA). ELISA kit was purchased from USCN life science (SEA222Mu).

Cell culture and transfection

293T, Hela, RAW264.7 and A549 cells were cultured in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) at 37°C in 5% CO₂. Mouse embryonic fibroblasts (MEF) were prepared from embryos at day 15 and
cultured in DMEM supplemented with 10% FBS. Indicated plasmids or si-RNAs were transfected into cells with Entranster-H (Engreen, Beijing, China) or Lipofectamine 2000 (Invitrogen).

**Dual-luciferase reporter assay**

Cells were transfected with plasmid encoding IFN-β or ISRE luciferase reporter gene together with pRL-TK and other plasmids. Twenty-four hours after transfection, collected and lysed cells. Subsequently, luciferase activity was measured with the dual-luciferase reporter assay system (Promega) according to the manufacturer’s protocols. Data were normalized by the ratio of firefly luciferase activity to renilla luciferase activity.

**RNAi and qRT-PCR**

siRNA oligonucleotides were transfected into 293T or Hela cells by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The sequences for SOCS3 small interfering RNAs were si-#1: 5’-GACCCAGUCUGGGACCAAGdTdT-3’, si-#2: 5’-GAAGAGCCUAUUACACUAUCUAdTdT-3’ and si-#3: 5’-CACCUGGACUCCUAUGAGA dTdT-3’. These siRNAs were all synthesized in RIBOBIO (Guangzhou, China).
Total RNA was extracted with TRNzol (TIANGEN, Beijing, China) according to protocols. cDNA was synthesized using the reverse transcription kit (TIANGEN, Beijing, China). SYBR RT-PCR kit (Takara, Dalian, China) was used for quantitative real-time PCR assays. The primers used for RT-PCR were as follows: h-SOCS3 forward 5'-AGCAGATGGAGGGTTCTGCTTTGT-3' and reverse 5'-ATTGGCTGTGTTTGCTCCTTGTG-3'; h-IFN-β forward 5'-GTCAGAGTGGAAATCCTAAG-3' and reverse 5'-ACAGCATCTGCTGGTTGAAG-3'; h-GAPDH forward 5'-GGAGAAACCTGCCAAGTATG-3' and reverse 5'-TTACTCCTTGGAGGCCATGTAG-3'; h-NA forward 5'-ATTCAAGGGGACCTTTAAGGACAG-3' and reverse 5'-CTGACCAAGCAACCGATTCAAACCT-3'; h-NP forward 5'-GATCTGGCACTCCAATTTGAATGAT-3' and reverse 5'-CTAGGGAGGGTTGACCCGTGGTAATG-3'; h-PA forward 5'-AGAGGACCTGAAAATCGAAACAAAC-3' and reverse 5'-TATTGACTCGCTTGTCTGATG-3'; h-M1 forward 5'-ACAGGACTTGAAGATGTCTTTGCA-3' and reverse 5'-CTAAAATCCCCTAGTAGGTGA-3'; 18SrRNA forward 5'-GTAACCCGTTGAACCCATT-3' and reverse 5'-CCATCCAATCGGTAGTAC-3'.
Cells were collected and lysed with 1x lysis buffer (Cell Signaling Technology), and then lysates were incubated with appropriate antibodies and protein G beads (Santa Cruz Biotechnology) at 4°C overnight. The beads were washed three times with IP buffer (50mM Tris-HCl (PH 7.4), 150mM NaCl and 1% Nonidet P40), followed by IB analysis. For the immunoblot, cells were harvested and lysed with 1x RIPA buffer (Cell Signaling Technology), followed by samples were boiled for five minutes together with 2x loading buffer to be used to perform SDS-PAGE. Proteins were further transferred onto nitrocellulose membranes (Bio-Rad). And the membrane was sealed with 5% fat-free milk in TBS-T for 2 hours at room temperature and incubated with appropriate primary antibody at 4°C overnight. Then the membrane was washed three times with TBS-T and incubated with a HRP-conjugated secondary antibody for 2 hours at room temperature. After washing three times with TBS-T, the membrane was flushed with ECL (Applygen, Beijing, China). The bands were detected by X-Ray film (FUJI, JAPAN) exposed to developer and fixing solution.

Ubiquitination assays

Cells were transfected with indicated plasmids, followed by collecting and lysing cells using 1x cell lysis buffer (Cell Signaling Technology). Then cell supernatant was incubated with protein G beads and appropriate antibodies at 4°C overnight. Beads were washed three times with IP buffer (50mM Tris-HCl (PH 7.4), 150mM NaCl and 1% Nonidet P40). The samples were boiled for five minutes together with 2x loading buffer.
buffer and then performed SDS-PAGE, followed by transferred onto nitrocellulose membranes, sealed with 5% milk and incubated with primary and secondary antibodies. The final results were obtained by using ECL (Applygen, Beijing, China) and film exposed to developer and fixing solution.

GST pull-down assay

GST and GST-fused proteins were expressed and purified from E.coli strain (BL21 Star). Indicated plasmids were transfected into 293T cells for 24h, and then collected and lysed the cells by using lysis buffer. The lysates were incubated with prepared GST or GST-fused protein, including GST-fused SOCS3 protein and its mutants proteins, at 4°C overnight. The beads were washed three times with PBS and further boiled for 5 minutes with loading buffer. Prepared samples were analyzed by IB.

In vitro translation assays

Flag-tagged TBK1 truncations (KU and CC) were translated in vitro with TNT T7 coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. The in vitro-translated products were incubated with GST or GST-fused SOCS3 protein at 4°C overnight. Then beads were washed three times and boiled to be used for IB.
Immunofluorescence microscopy

Cells were transfected with Flag-TBK1 and Myc-SOCS3 plasmids for 24h. Following eluted, fixed and blocked with 5% BSA, cells were incubated with primary antibodies (mouse anti-Flag and rabbit anti-Myc Abs) and secondary antibodies (FITC-conjugated anti-mouse IgG Ab and TRITC-conjugated anti-rabbit IgG Ab). The nuclei were stained with DAPI (KeyGENBioTECH). The final result was observed by using laser confocal fluorescence microscopy (Leica TCS SP2, Germany).

Generation and infection of viruses

Generation of influenza A virus was performed as previously described (25). A series of vRNA expression plasmids were first transfected into 293T cells for three days, and then the supernatants were harvested and used to propagate IAV in MDCK cells. For the antiviral assays, two kinds of viruses, including IAV and VSV, were used to infect 293T cells or A549 cells. First of all, the cells were washed three times with PBS, followed by infected with the virus (diluted in DMEM containing 2.5ug/ml trypsin) for one hour. Then the cells were washed three times with PBS and cultured in DMEM containing 2.5ug/ml trypsin for indicated time. Finally, the cells were collected and used to perform IB analysis or qRT-PCR.
ELISA

RAW264.7 or primary MEF cells were stimulated by poly(I:C) at a final concentration of 10μg/ml or 1μg/ml. Then the culture supernatants were harvested and used to measure the concentrations of IFN-β according to the manufacturer’s instructions.

Statistical analysis

All experiments were performed for more than three replicates. And two-tailed Student t test with a P value <0.05 was used to identify the significance of these data.
Results

SOCS3 negatively regulates IFN-β signaling pathway

We performed dual-luciferase reporter screening to identify candidate regulators of RIG-I signal pathway (26), and the result revealed that SOCS3 is an inhibitor of this signal axis (data not shown). In order to further verify the regulation, dual-luciferase reporter assay is performed by transfecting HEK293T human embryonic kidney cells (293T cells) with plasmids encoding IFN-β promoter luciferase reporter, internal control renilla luciferase and RIG-I signaling adaptor (MAVS, TBK1, IKKe or IRF3) in the absence or presence of SOCS3 expression vector. The data showed that SOCS3 overexpression significantly attenuates the activation of IFN-β promoter induced by MAVS, TBK1 and IKKe, but not IRF3 (Fig. 1A). These results suggested that SOCS3 acts upstream of IRF3 and TBK1 is a candidate target. To evaluate whether SOCS3 directly represses IFN-β promoter, we detected the effects of this protein on TBK1-induced activation of interferon-stimulated response element (ISRE) luciferase reporter. The result suggested that MAVS or TBK1 but not IKKe or IRF3 mediated ISRE activation is markedly reduced by SOCS3 overexpression (Fig. 1B). In addition, we found that SOCS3 potently lowers poly(I:C) stimulated IFN-β expression in 293T cells (Fig. 1C).

To reveal the mechanism of SOCS3 mediated inhibition of IFN-β promoter, we
assessed the phosphorylation of IRF3. We transfected SOCS3 expression vector together with MAVS, TBK1 or IKKε plasmid into 293T cells and found that SOCS3 substantially attenuates the IRF3 phosphorylation induced by MAVS and TBK1, but not IKKε (Fig. 1D). These data suggested that SOCS3 negatively regulates type I interferon signal pathway at upstream of IRF3.

To evaluate endogenous p-IRF3 and TBK1 under physiologic condition, poly (I:C) was used to stimulate Hela cells and primary MEF cells for different time, respectively. Within twelve hours, TBK1 and IRF3 phosphorylation expression levels are all upregulated in a time dependent manner after poly(I:C) stimulation (Fig. 1E). And similar result was obtained in primary MEF cells (Fig. 1F).

Knockdown of SOCS3 heightens IFN-β transcription

We next sought to demonstrate whether knockdown of SOCS3 could enhance IFN-β response. Three SOCS3 specific small interfering RNAs (siRNAs, named as si-#1, si-#2 and si-#3) were used to verify the hypothesis. We first evaluated the knockdown efficiency of these siRNAs at mRNA level. As shown in Fig. 2A and 2B, si-#1 and si-#3 significantly reduced the abundance of endogenous SOCS3 mRNA in Hela cells whereas si-#1 markedly reduced SOCS3 expression in 293T cells. In addition, we assessed the effects of these siRNAs by western blotting. We

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co-transfected Flag-SOCS3 plasmid with siRNA control or SOCS3 specific siRNAs into 293T cells, and the result confirmed the knockdown (Fig. 2C). Furthermore, transfection of SOCS3 targeting siRNA resulted in higher IFN-β production at mRNA level (Fig. 2D). Collectively, si-#1 has the best knockdown efficiency in both Hela and 293T cells (Fig. 2A, B and C) and results in the highest IFN-β expression (Fig. 2D). This siRNA was used in the following experiments.

Subsequently, we detected the effects of SOCS3 knockdown on RIG-I signaling. As shown in Fig. 2E and 2F, transfection of SOCS3 siRNA markedly increased TBK1 induced activation of IFN-β promoter and IFN-β transcription. Consistent with these observations, TBK1 mediated ISRE reporter activation is also upregulated in cells transfected with SOCS3 siRNA (Fig. 2G). Furthermore, knockdown of SOCS3 considerably enhances the abundance of IRF3 phosphorylation (Fig. 2H). These results were consistent with the ones of SOCS3 overexpression.

To exclude off target effects and validate RNAi data, we included a rescue control. As shown in Fig. 2I, IRF3 phosphorylation level is significantly upregulated by SOCS3 knockdown and further repressed by co-transfection of SOCS3 rescue plasmid.

In addition, we checked the effect of SOCS3 knockdown in primary cells. Mouse embryo fibroblasts (MEF) were isolated from embryos of BALB/c mice at day 15 and
were cultured in DMEM supplemented with 10% FBS. We transfected prepared MEF cells with control siRNA or SOCS3 specific siRNA. The result suggested that SOCS3 knockdown significantly increased the abundance of TBK1 (Fig. 2J).

These data further confirmed that SOCS3 is a negative regulator of type I interferon signal transduction.

SOCS3 targets TBK1 for degradation

We observed that SOCS3 overexpression reduces the abundance of TBK1 at protein level (Fig. 1A, B and D), and the data from dual luciferase reporter (Fig. 1A and B) and IRF3 phosphorylation (Fig. 1D) assays suggested that SOCS3 acts upstream of IRF3 and downstream of TBK1, so we doubt whether TBK1 is a target of SOCS3. We transfected SOCS3 expression vector together with plasmid encoding RIG-I signal adaptor (MAVS, TRAF3, TBK1, TANK, IKKe or IRF3) into 293T cells. The results indicated that TBK1, but not other adaptors, is considerably decreased by SOCS3 ectopic expression (Fig. 3A). Subsequently, we found that SOCS3 overexpression induced degradation of ectopic and endogenous TBK1 in a dose-dependent manner (Fig. 3B). Besides, the phosphorylation level of endogenous TBK1 was significantly inhibited by SOCS3 overexpression (Fig. 3C). In addition, we performed the time-course experiments of SOCS3 mediated the degradation of TBK1 and the inhibition of IFN-β activity. As shown in Fig. 3D and E, endogenous TBK1
degradation induced by SOCS3 overexpression starts at sixteen hours after transfection, whereas SOCS3 mediated inhibition of TBK1 stimulated IFN-β transcription starts at twelve hours. Furthermore, knockdown of SOCS3 increased the level of ectopic expressed TBK1 protein (Fig. 3F). All these data demonstrated that the stability of TBK1 is regulated by SOCS3.

To further evaluate which domain of SOCS3 is responsible for TBK1 degradation, we generated a series of SOCS3 mutants including Δ(N+KIR), ΔSH2 and ΔBOX (Fig. 3G). Overexpression of wildtype, but not Δ(N+KIR), ΔBOX or ΔSH2 mutated form of SOCS3 reduced the level of ectopic expressed Flag-TBK1 (Fig. 3G). In another assay, compared with wildtype SOCS3, truncations such as Δ(N+KIR) or ΔBOX restored the expression of TBK1 as well as TBK1 induced phosphorylation of IRF3 in 293T cells (Fig. 3H). Interestingly, the dual luciferase assay showed that these SOCS3 mutants failed to repress TBK1 induced activation of IFN-β promoter (Fig. 3I).

Meanwhile, we also checked the effect of SOCS3 wildtype or its mutants on TBK1 phosphorylation by IP and subsequent WB assays. 293T cells were transfected with indicated plasmids (Fig. 3J), and TBK1 protein were purified by IP with anti-Flag antibody, which were evaluated by IB assay with anti-p-TBK1. The result suggested that TBK1 phosphorylation was significantly reduced by wildtype SOCS3, but not its mutants (ΔBOX and Δ(N+KIR)) (Fig. 3J). This result is consistent with the result that wildtype SOCS3 but not its mutants induced the degradation of TBK1 (Fig. 3G).
Interestingly, SOCS3 does not have obvious effects on the stability of TBK1-S172A, a mutated form of TBK1 with autophosphorylation site Serine 172, which is critical for virus-stimulated IFN-β induction and IRF3 activation (27), been mutated to alanine (Fig. 3K). For comparison, SOCS3 promoted the degradation of TBK1 K38A mutant, a kinase inactive mutant in which the ATP binding residue Lys38 was mutated to alanine (Fig. 3K). It is reasonable to conclude that the process of SOCS3 reducing the abundance of TBK1 is dependent on TBK1 Serine 172 but not Lysine 38. It has been reported that some kinases such as Glycogen Synthase Kinase 3β (GSK3β) promote TBK1 self-association and autophosphorylation at Ser172 (29), which may participate in the process of SOCS3 mediated TBK1 degradation.

Taken together, SOCS3 negatively regulates IFN-β signal transduction cascades through targeting TBK1 for degradation, and all fragments detected (N+KIR, SH2 and BOX) are indispensable for this process.

**SOCS3 enhances K48-linked polyubiquitination of TBK1**

Several studies have suggested that SOCS3 functions as an E3 ligase by forming complex with elongins B and C, cullin5 and Rbx-1 in many physiological processes (8, 9). Intriguingly, the accumulation and function of TBK1 is also regulated by ubiquitination, including in K48-linked and K63-linked types. In view of the facts, we detected whether SOCS3 lowers the abundance of TBK1 protein by
ubiquitin-proteasome pathway. Initially, we evaluated whether SOCS3 induced TBK1 degradation is via proteasomal pathway. We transfected 293T cells with indicated plasmids and treated them with dimethyl sulfoxide (DMSO) or MG-132 for 6h. Immunoblot analysis suggested that loss of TBK1 protein induced by SOCS3 was blocked by the proteasome inhibitor MG-132 (Fig. 4A). Furthermore, ubiquitination of endogenous TBK1 is enhanced in 293T cells with co-expression of SOCS3 (Fig. 4B, left two panels). To specify ubiquitination type of TBK1, we further included two ubiquitin mutants K48 and K63 in the experiments, of which all lysine residues were substituted by arginine residues except the site 48 or 63, respectively. The data suggested SOCS3 promotes more K48-linked ubiquitination of TBK1, whereas attenuates its K63-linked ubiquitination (Fig. 4B). As K48-linked polyubiquitination is accompanied by proteasomal degradation of target proteins, this phenomenon is consistent with the result of SOCS3 mediated TBK1 degradation. Besides, we included TBK1 ubiquitination assays after knockdown SOCS3 to validate the result from Fig. 4B. As shown in Fig. 4C, knockdown of SOCS3 significantly attenuated TBK1 ubiquitination. In addition, endogenous TBK1 ubiquitination level was tested with different treatments. We found that SOCS3 overexpression or poly(I:C) stimulation dramatically promoted the endogenous ubiquitination of TBK1(Fig. 4D).

Besides, we evaluated domains of SOCS3 which play crucial roles in the process of ubiquitination and degradation of TBK1. Compared to wildtype SOCS3, ubiquitination level of TBK1 is considerably diminished by ΔBOX truncation, but not
the mutant deleting N-terminal residues and the KIR domain (Fig. 4E). These results indicated that BOX domain of SOCS3 participates in the process of TBK1 ubiquitination and degradation. Finally, we found that BOX domain of SOCS3 is indispensable for SOCS3 to attenuate K63-linked ubiquitination of TBK1 which is important for its activity, and the mechanism remains to be revealed (Fig. 4F). Therefore, SOCS3 reduces the abundance of TBK1 by enhancing its K48-linked ubiquitination.

K341 and K344 of TBK1 are pivotal sites for ubiquitination

To determine the sites responsible for SOCS3 induced polyubiquitination, we constructed three truncations of TBK1 (Fig. 5A), including KD (kinase domain), KU (lacking the coiled-coil domain) and △ULD (lacking the ubiquitin-like domain). Subsequently, these three mutants were separately transfected into 293T cells together with HA-tagged ubiquitin vector in the absence or presence of SOCS3 plasmid. Compared to KU and △ULD, KU truncation harbors highest amount of polyubiquitin after SOCS3 overexpression (Fig. 5B), which indicates that the ubiquitin-like domain (ULD) contains potential ubiquitination sites. In this region, we identified four lysine residues (K323, K341, K344 and K372) which are conserved in human and mouse but not in zebrafish (Fig. 5C). We mutated these sites (K323R, K341R, K344R and K372R) separately, and transfected each of them or wildtype TBK1 into 293T cells with His-Ub, accompanied with or without Myc-SOCS3 co-transfection. Compared to
wildtype TBK1, mutation on K341 or K344 but not K323 or K372 significantly decreases SOCS3 induced TBK1 ubiquitination (Fig. 5D). Consistent with this observation, there are no obvious SOCS3-induced K48-linked polyubiquitination of K341R, K344R or K341/344R (lysine residues of both sites were substituted by arginine) mutants (Fig. 5E). These results demonstrated that Lys341 and Lys344 in TBK1 are two major sites responsible for SOCS3 stimulated ubiquitination. However, the double mutant of TBK1 failed to rescue IFN-β activity inhibited by SOCS3, which indicated that other mechanisms may also involved in SOCS3 mediated TBK1 degradation (Fig. 5F). Meanwhile, co-IP experiment with Flag-tagged and HA-tagged wildtype or the double mutant of TBK1 showed that K341/344R mutation doesn’t interfere the process of TBK1 self-association (Fig. 5G).

It has been suggested that SOCS3 failed to degrade TBK1-S172A, but not TBK1-K38A (Fig. 3J), so we further investigated whether SOCS3 is responsible for their ubiquitination. SOCS3 significantly increases polyubiquitination of wildtype or K38A mutant, but not S172A mutant of TBK1 in 293T cells (Fig. 5H and 5I), indicating that SOCS3 mediated TBK1 ubiquitination and degradation require Serine 172 but not Lysine 38 of TBK1.

Therefore, K341 and K344 of TBK1 are pivotal sites for ubiquitination induced by SOCS3, and the process is dependent on TBK1 Serine 172 but not Lysine 38.
SOCS3 forms complex with TBK1

Our studies suggested that SOCS3 negatively regulates IFN-β activation by enhancing K48-linked ubiquitination and degradation of TBK1. Then we asked whether SOCS3 could directly interact with TBK1. GST-fused SOCS3 protein was expressed and purified from E.coli and incubated with lysates from 293T cells transfected with Flag-tagged TBK1 expression vector. IB assay suggested that SOCS3 directly associates with TBK1 (Fig. 6B). To determine regions of SOCS3 participating in the interaction with TBK1, we expressed and purified GST-fused SOCS3 truncations (termed Δ(N+KIR), ΔSH2, Δ(ESS+SH2), ΔBOX and Δ(PEST+BOX)) from E.coli (Fig. 6A and 6B). Then these GST-fused proteins were incubated with lysates from Flag-TBK1 overexpressed 293T cells. The result of GST pull-down assay showed that there is less association between TBK1 and Δ(ESS+SH2) mutant of SOCS3 among all interactions (Fig. 6B), indicating that the N-terminal ESS domain is responsible for SOCS3 to form protein complex with TBK1.

Reciprocally, we performed GST pull-down assay to determine regions of TBK1 required for its interaction with SOCS3. We incubated GST-fused SOCS3 protein with lysates from 293T cells transfected with Flag-tagged TBK1 truncations (KD, KU and ΔULD), respectively. All these TBK1 mutants harbor the kinase domain, and western blotting revealed that all of them interact with SOCS3 (Fig. 6C). These results suggested the kinase domain of TBK1 is sufficient for the interaction. In addition, we
incubated GST-fused SOCS3 protein with products from in vitro-translated KU or CC proteins using reticulocyte lysate system (Fig. 6D). The result showed that SOCS3 interacts with KU but not CC (Fig. 6D), suggesting that CC domain does not participate in the formation of SOCS3-TBK1 complex. Theoretically, TBK1-KU protein (containing 383 amino acids) is larger than TBK1-CC protein (containing 346 amino acids). In fact, we detected TBK1-CC protein has higher molecular weight than TBK1-KU protein (Fig. 6D), which is consistent with the report of Cui et al. (23). We also performed co-IP in 293T cells transfected with Myc-SOCS3 and Flag-3.0 or Flag-TBK1, which further indicated SOCS3 directly associates with TBK1 (Fig. 6E). To check the endogenous association between TBK1 and SOCS3, we also performed co-IP in 293T cells with IgG or TBK1 antibody. IB analysis suggested that SOCS3 forms complex with TBK1 under the physiological conditions (Fig. 6G). Finally, we performed immunofluorescence microscope assay and confirmed the colocalization of SOCS3 and TBK1 in the cytoplasm (Fig. 6F). Taken together, SOCS3 directly binds to TBK1 to promote ubiquitination and degradation of this protein, and the N-terminal ESS domain of SOCS3 and the kinase domain of TBK1 are indispensable for their physical interaction.

Though IKKα induced IFN-β activity, ISRE activity and IRF3 phosphorylation were less obviously inhibited by ectopic expression of SOCS3 than that induced by TBK1 (Fig. 1 A, B and D), we also checked whether SOCS3 interacts with IKKα, which is structurally similar to TBK1 (another IKK-related kinase). Co-IP and GST
pull-down assays showed that SOCS3 associated with IKKε (Fig. 6H). In addition, we compared whether the ubiquitinated lysine residues are conserved between TBK1 and IKKε. As shown in Fig. 6I, the lysine residue 341 of TBK1 is not only involved in the process of SOCS3 mediated TBK1 polyubiquitination (Fig. 5D and E) but also conserved between the two kinases.

SOCS3 negatively regulates viral replication

Type I interferon is an important barrier to defend against viral infection, thus we further detected the effects of SOCS3 on viral replication. Cells were transfected with TBK1 or SOCS3 for 24 hours, followed by WSN stimulation for 0h, 12h or 16h. Subsequently, qRT-PCR was performed to detect the abundance of vRNAs (NP, NA, PA and M1) in cells. The results showed that the transcription of these vRNAs was significantly inhibited by TBK1 but increased by SOCS3 (Fig. 7A and B). Conversely, knockdown of SOCS3 obviously reduced their abundance (Fig. 7C).

In addition to the transcription of WSN vRNAs, we also investigated the potential role of SOCS3 on RNA virus stimulated downstream signaling cascades. We evaluated IRF3 phosphorylation in 293T cells transfected with Flag-tagged 3.0 or SOCS3, which are subsequently infected with VSV for 0h, 2h, 4h or 8h. As shown in Fig. 7D, phosphorylation of IRF3 is markedly increased in Flag-3.0 transfected cells upon VSV infection, which is significantly reduced by SOCS3 overexpression.
Consistent with the result, ectopic expression of SOCS3 significantly inhibited IRF3 phosphorylation induced by WSN infection for 12h, 16h or 24h (Fig. 7E). Besides, we performed WSN infection experiment in mouse primary MEF cells. As shown in Fig. 7F, WSN virus activated the highest TBK1 protein level at 16 hours after infection, and then the protein abundance was downregulated as the upregulation of SOCS3 protein expression. Furthermore, we performed ELISA assays to check poly(I:C)-induced IFN-β production in Raw264.7 cells and primary MEF cells. And the data indicated that poly(I:C) stimulation resulted in more IFN-β production at protein level (Fig. 7G and H). Collectively, SOCS3 blocks IRF3 signaling and facilitates the replication of virus.
Innate immunity is a forceful weapon to defend against viruses. RIG-I signaling pathway is triggered through recognition of viruses RNA, followed by signal cascades driving expression of type I interferons and other proinflammatory cytokines (5). Subsequently, JAK/STAT signal is further provoked by IFN-α/β to stimulate the transcription of antiviral genes, as well as suppressor of cytokine signaling 3 (SOCS3), which plays an important role in the regulation of NF-κB and JAK/STAT signal pathways (11, 17). Though it has been reported that SOCS3 lowers the expression of IFN-β (13), the molecular mechanism remains largely unknown.

In this study, we found that SOCS3 negatively regulates IFN-β signaling pathway through promoting K48-linked polyubiquitination of TBK1 at Lys341 and Lys344 sites and subsequent its degradation (Fig. 8). It was further identified that ectopic expression of SOCS3 inhibits IFN-β activation induced by multiple adaptors except IRF3, whereas knockdown of SOCS3 enhances IFN-β signaling. It has been reported that SOCS3 BOX domain is involved in the formation of the E3 ligase complex by interaction with elongins B and C, cullin5 and Rbx-1 (8, 9). As a member of the complex, SOCS3 participated in the process of degrading some proteins such as Focal adhesion kinase (FAK) (26) and CD33-related Siglec7 (27). Though (N+KIR) and BOX domains of SOCS3 are indispensable for the degradation of TBK1, they may be involved in different mechanisms. Here, we found BOX domain is responsible for
ubiquitination of TBK1, which results in subsequent its degradation (Fig. 4E).

Traditionally, SOCS3 SH2 domain mediates the association with substrate by recognition of the phosphorylated tyrosine residues (9), whereas N-ESS domain facilitates substrate association (10). In this report, we found that N-ESS domain is required in the process of SOCS3-TBK1 complex formation (Fig. 6B). Our study revealed TBK1 acts as a new target protein of SOCS3, which broadens the function of SOCS3 as an E3 ligase by complex formation with other proteins.

Obviously, SOCS3 is a negative regulator of RIG-I signal. It functions as a member of E3 ligase complex both in NF-κB signaling pathway and IFN-β signaling pathway. It has been reported that SOCS3 suppresses ubiquitination of TRAF6, thus hindering the formation of TRAF6/TAK1 complex and TAK1 kinase activity, which resulted in the inhibition of NF-κB signal (17). Besides, we reported that SOCS3 negatively regulates IFN-β signal by promoting the ubiquitination and degradation of TBK1 (Fig. 8).

TBK1, a key member of IFN-β signal, is activated by mitochondrial adaptor protein MAVS, which further phosphorylates transcription factor IRF3 to transcript type I interferon (28). It has been reported that GSK3β phosphorylates TBK1 and promotes its self-association and autophosphorylation at Ser172 which is important for its activity (29). In addition to phosphorylation, TBK1 can be regulated by K63-linked and K48-linked ubiquitination (19-21, 23, 24, 31, 32). Here, we found
SOCS3 promotes ubiquitination and degradation of TBK1 (Fig. 3A, 3B and 4A, B), which leads to inhibition of IFN-β activation (Fig. 1). TBK1 is degraded in a SOCS3 dose-dependent manner (Fig. 3B), however, it can avoid being ubiquitinated and degraded by SOCS3 if serine on 172 of TBK1 is mutated to alanine (Fig. 3K and 5H). In addition, kinase domain of TBK1 is also responsible for SOCS3-TBK1 complex formation. Consequently, Ser172 of TBK1, as an autophosphorylation site (29), is indispensable for SOCS3-medicated inhibition of IFN-β. Ubiquitin-like domain (ULD) of TBK1 plays important roles in the regulation of IRF3 activation (33). We found ULD is mainly responsible for SOCS3 induced polyubiquitination of TBK1, and two critical ubiquitination sites (K341 and K344) of TBK1 are identified in the process of SOCS3-medicated regulation (Fig. 5D and 5E). Similar to SOCS3, NLRP4 and TRIP also negatively regulate IFN-β signal cascade by targeting TBK1 for Lys48 (K48)-linked polyubiquitination and degradation. However, different from SOCS3 as a member of E3 ligase complex, NLRP4 functions by recruiting the E3 ubiquitin ligase DTX4 to TBK1, whereas TRIP is an E3 ligase (23, 24).

The crosstalk between RIG-I and JAK/STAT signal pathways has been revealed these years. Viruses or cytoplasmic nucleic acids activate RIG-I signaling and recruit STAT6, an important transcription factor of JAK/STAT pathway, to the endoplasmic reticulum, where it is phosphorylated by TBK1 on Ser407 independent of JAKs. The phosphorylated STAT6 then undergoes dimerization and translocates from cytoplasm to the nucleus and initiates the transcription of specific targets responsible for immune
cell homing (34). It still remains to be a mystery how JAK/STAT axis feedback regulates RIG-I signaling. It has been reported that infection of some RNA viruses, such as influenza A and HIV, upregulates SOCS3 expression via JAK/STAT and NF-κB pathways (14, 16). We found that SOCS3 negatively regulates RIG-I signaling by targeting TBK1 for proteasome mediated degradation. As a consequence, ectopic expression of SOCS3 significantly increases the replication of WSN (Fig. 7B), whereas knockdown of SOCS3 greatly lowers vRNA abundance (Fig. 7C).

Taken together, we demonstrated a new mechanism of immunologic escape. SOCS3 negatively regulates cellular antiviral responses through promoting K48-linked ubiquitination and proteasomal degradation of TBK1. These findings provide new insight into antiviral innate immunity and potential target for the development of antiviral drugs.

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RING Finger Protein 11 Targets TBK1/IKKi Kinases to Inhibit Antiviral Signaling. Plos One 8.


FIGURE 1. SOCS3 inhibits IFN-β signaling pathway. (A-C) Luciferase assays reveal SOCS3 mediated suppression of RIG-I signaling. 293T cells were transfected with plasmids encoding IFN-β promoter or ISRE promoter together with indicated plasmids (A and B), or with or without poly (I:C) co-transfection (1μg/ml, C). 24 h after transfection the cells were lysed and the luciferase activities were monitored. (D) SOCS3 negatively regulates induced IRF3 phosphorylation. 293T cells were transfected with Flag-tagged MAVS, TBK1 or IKKe vectors and IRF3 plasmid (50ng/ml) with or without Myc-SOCS3 expression vector. Immunoblots were used to analyze the levels of IRF3 phosphorylation and the expression of total IRF3. The intensity of p-IRF3 of western blotting bands was measured with ImageJ software. (E-F) Immunoblot analysis in Hela cells or primary MEF cells with poly(I:C) treatment at a final concentration of 1μg/ml. α- represents anti-. Error bars indicate SD. *p<0.05, **p<0.01, ***p<0.001.

FIGURE 2. Knockdown of SOCS3 activates IFN-β signaling pathway. (A) qRT-PCR (left) and RT-PCR (right) analysis of SOCS3 knockdown efficiency at mRNA level in Hela cells. (B) qRT-PCR analysis of SOCS3 knockdown efficiency at mRNA level in 293T cells. (C) Immunoblot analysis of the knockdown of exogenous SOCS3 in 293T cells transfected with Flag-tagged SOCS3 plasmid and siRNA control or SOCS3 specific siRNAs. (D) Real-time PCR analysis of IFN-β mRNA in 293T cells.
transfected with control siRNA or si-RNAs against SOCS3. (E) 293T cells were transfected with si-control or si-#1 for 36h, and further transfected with plasmids encoding IFN-β promoter and TK reporter genes with or without TBK1 expression vector. The activity of IFN-β promoter was analyzed by the dual-luciferase assays. (F) Hela cells were transfected with si-control or si-#1, and further transfected with or without TBK1 plasmid. The transcription of IFN-β mRNA was evaluated by real-time PCR. (G) Thirty-six hours after transfection with siRNAs, 293T cells were transfected with indicated plasmids. Luciferase assays was performed to evaluate the activity of ISRE reporter. (H) Immunoblot analysis of IRF3 phosphorylation in 293T cells transfected with IRF3 plasmid (50ng/ml) and si-control or si-#1. (I) Immunoblot analysis of IRF3 phosphorylation in 293T cells transfected with indicated siRNA or SOCS3 rescue plasmid. Overexpressed and endogenous SOCS3 was indicated with arrow. (J) Primary MEF cells were transfected with si-control or si-#1. The expression of TBK1 and SOCS3 were evaluated by IB analysis. Error bars indicate SD. *p<0.05, **p<0.01 and ***p<0.001. ns represents non significant.

FIGURE 3. SOCS3 targets TBK1 for degradation. (A) Plasmids encoding adaptors of RIG-I signal were separately transfected into 293T cells with or without Myc-SOCS3 co-transfection. IB was performed to identify the abundance of these adaptors (Flag-tagged MAVS, TRAF3, TBK1, TANK, IKKε and IRF3). (B-C) 293T or Hela cells were transfected with indicated plasmids. The expression of TBK1 and p-TBK1 (S172) were evaluated by western blot. (D-E) The time-course of SOCS3 mediated

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degradation of TBK1 and inhibition of IFN-β activity were evaluated by western blot and dual-luciferase assay in 293T cells, respectively. (F) 293T cells were transfected with si-control or si-#1 (SOCS3-specific siRNA) using Lipofectamine 2000 for 36h, followed by the transfection of Flag-TBK1. IB analyzed the expression of TBK1 and SOCS3 proteins. (G) Constructs of Myc-tagged wildtype SOCS3 and its truncations (left). Flag-tagged TBK1 and wildtype or mutated Myc-SOCS3 were transfected into 293T cells for 24h, and then IB evaluated TBK1 expression level (right). (H) 293T cells were transfected with IRF3 (50ng/ml), Flag-TBK1 and Myc-SOCS3 wildtype or truncations for 24h, and IRF3 phosphorylation and total IRF3 were analyzed by IB. (I) Dual-luciferase assay was performed to identify the activation of IFN-β reporter in 293T cells transfected with indicated plasmids. (J) 293T cells were transfected with indicated plasmids for 24h. Cells lysates were incubated with protein G beads plus anti-Flag Ab at 4°C overnight, followed by immunoblot analysis with anti-p-TBK1 Ab. (K) 293T cells were transfected with an increasing amounts of SOCS3 plasmid and Flag-TBK1-S172A mutant or Flag-TBK1-K38A mutant for 24h. The expression of the two mutants was evaluated by western blot. Error bars indicate SD. **p<0.01.
anti-TBK1 Ab at 4°C overnight, followed by immunoblot analysis with anti-HA Ab.

(C) 293T cells were transfected with si-control or si-#1 for 24h, followed by further transfection with HA-Ub plasmids for 24h. Cells lysates were used to perform ubiquitination assays. (D) Ubiquitination assays of the endogenous TBK1. 293T cells were transfected with HA-Ub and SOCS3 plasmid or poly(I:C), then the cells lysates were incubated with protein G beads plus anti-TBK1 Ab at 4°C overnight, followed by immunoblot analysis with anti-HA Ab. (E) 293T cells were transfected with plasmids encoding Flag-TBK1, His-Ub and Myc-tagged wildtype or truncated SOCS3 for 24h, followed by immunoprecipitation with protein G beads plus anti-Flag Ab and immunoblot analysis with anti-His Ab. (F) 293T cells were transfected with Flag-TBK1, HA-Ub(K63) and Myc-SOCS3 wildtype or △BOX for 24h, and then lysates were used to perform ubiquitination assays.

FIGURE 5. Identification of ubiquitination sites on TBK1. (A) Constructs of wildtype TBK1 and its truncations. (B) 293T cells were transfected with plasmids encoding HA-Ub and TBK1 truncations with or without Myc-SOCS3 co-transfection, and then cell extracts were used to perform ubiquitination assays. (C) Identification of four lysines in the ULD domain of TBK1. (D) 293T cells were transfected with plasmids encoding His-Ub and TBK1 mutations (K341R, K344R, K372R or K323R) with or without Myc-SOCS3 co-transfection, and then cell extracts were used to perform ubiquitination assays. (E) 293T cells were transfected with plasmids encoding HA-Ub (K48) and TBK1 mutations (K341R, K344R or K341/344R) with or
without Myc-SOCS3 co-transfection, and then extracts were used to perform ubiquitination assays. (F) Dual-luciferase assays of SOCS3 mediated IFN-β activation induced by wildtype or K341/344R mutant of TBK1 and western blot assays of the expression of these proteins. (G) Co-immunoprecipitation checking TBK1 dimerization in 293T cells transfected with different tagged TBK1 wildtype or K341/344R mutant plasmids. (H-I) Lysates of 293T cells transfected with His-Ub and Flag-TBK1 or Flag-TBK1-S172A or Flag-TBK1-K38A with or without Myc-SOCS3 co-transfection were used to perform ubiquitination assays.

**FIGURE 6.** SOCS3 and TBK1 form complex in cells. (A) Constructs of GST-tagged wildtype SOCS3 and its truncations. (B) GST or GST-fused proteins were incubated with lysates of 293T cells transfected with Flag-tagged TBK1 at 4°C overnight. Immunoblot was used to analyze the association between TBK1 and wildtype or mutated SOCS3. (C) GST or GST-fused SOCS3 protein was incubated with lysates of 293T cells transfected with Flag-tagged TBK1 truncations (KU, ΔULD and KD) at 4°C overnight. Immunoblot was used to analyze the association between SOCS3 and TBK1 mutants. (D) GST or GST-fused SOCS3 protein was incubated with in vitro-translated KU or CC protein produced by reticulocyte lysate system, and the interactions between SOCS3 and TBK1 mutants were identified with IB. (E) Co-immunoprecipitation evaluating the association between SOCS3 and TBK1 in 293T cells transfected with Myc-SOCS3 and Flag-3.0 or Flag-TBK1. (F) Immunofluorescence microscopy detecting the colocalization of Flag-TBK1 and
Myc-SOCS3 in 293T cells. (G) co-IP assays of endogenous interaction between SOCS3 and TBK1 with rabbit IgG or TBK1 antibody, followed by IB analysis with anti-SOCS3 Ab. (H) co-IP and GST pull-down analysis of the association between SOCS3 and IKKε. (I) Identification of ubiquitinated lysine residues between TBK1 and IKKε. Scale bars, 10μm. CBS represents coomassie blue staining.

**FIGURE 7.** SOCS3 facilitates viral replication. (A) 293T cells were transfected with or without TBK1 for 24h, followed by WSN infection for 0h, 12h or 16h. The transcription of vRNAs (NP, NA, PA, and M1) was evaluated by qRT-PCR. (B) 293T cells were transfected with or without SOCS3 for 24h, followed by WSN infection for 0h, 12h or 16h. The transcription of vRNAs (NP, NA, PA, and M1) was evaluated by qRT-PCR. (C) Control siRNA or SOCS3-specific siRNA (si-#1) was transfected into A549 cells for 48h, followed by WSN infection for 0h, 12h or 16h. qRT-PCR was performed to detect the expression of vRNAs (NP, NA, PA, and M1). (D) IRF3 phosphorylation was evaluated in 293T cells transfected with IRF3 (50ng/ml) and Flag-3.0 or Flag-SOCS3 for 24h followed by VSV infection for indicated time. (E) 293T cells were transfected with indicated plasmids for 24h, and then cells were infected with WSN for additional 0h, 12h, 16 or 24h. IB analyzed the phosphorylation of IRF3 and total IRF3. (F) Immunoblot analysis of the expression of TBK1 and SOCS3 in primary MEF cells with WSN virus infection. (G-H) ELISA assays evaluating the IFN-β production at protein level in RAW264.7 cells or primary MEF cells with 10μg/ml or 1μg/ml poly(I:C) stimulation. Error bars indicate SD.
FIGURE 8. Model of SOCS3 mediated inhibition of IFN-β signal cascade by promoting the ubiquitination and degradation of TBK1. Upon RNA virus infection, RIG-I recognizes 5'-triphosphate viral RNA and activates MAVS, which further recruits TBK1 and IKKe to phosphorylate IRF3. IRF3 undergoes phosphorylation and dimerization to stimulate the production of IFN-β, which activates downstream signal to suppress viral replication. However, SOCS3 inhibits IFN-β activity by targeting TBK1 for K48-linked ubiquitination on K341 and K344 sites and subsequent degradation, thus facilitates viral replication.
Fig. 4

A

Flag-TBK1

DMSO

MG-132

Myc-SOCS3

IB-α-Flag

IB-α-Myc

IB-β-actin

1 0.64 0.41

C

HA-Ub

sRNA

Con

SOCS3

IP-α-TBK1

IB-α-HA

IB-α-TBK1

IB-α-SOCS3

IB-β-actin

WCL

D

HA-Ub

SOCS3

poly(I:C)

IP-α-TBK1

IB-α-HA

IB-α-TBK1

IB-α-SOCS3

IB-β-actin

WCL

E

His-Ub / Flag-TBK1

ΔN

Myc-SOCS3

IB-α-His

IB-α-Myc

IB-β-actin

WCL

F

HA-K63-Ub / Flag-TBK1

Myc-SOCS3

Myc-SOCS3ΔBOX

IP-α-Flag

IB-α-HA

IB-α-Myc

IB-β-actin

WCL
Fig. 8