



# TSG101 Physically Interacts with Linear Ubiquitin Chain Assembly Complex (LUBAC) and Upregulates the TNF $\alpha$ -Induced NF- $\kappa$ B Activation

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**Linear ubiquitin chain assembly complex (LUBAC) is a ubiquitin E3 ligase complex composed of HOIP, HOIL-1L, and SHARPIN that catalyzes the formation of linear/M1-linked ubiquitin chain. It has been shown to play a pivotal role in the nuclear factor (NF)- $\kappa$ B signaling induced by proinflammatory stimuli. Here, we found that tumor susceptibility gene (TSG101) physically interacts with HOIP, a catalytic component of LUBAC, and potentiates LUBAC activity. Depletion of TSG101 expression by RNA interference decreased TNF $\alpha$ -induced linear ubiquitination and the formation of TNF $\alpha$  receptor 1 signaling complex (TNF-RSC). Furthermore, TSG101 facilitated the TNF $\alpha$ -induced stimulation of the NF- $\kappa$ B pathway. Thus, we suggest that TSG101 functions as a positive modulator of HOIP that mediates TNF $\alpha$ -induced NF- $\kappa$ B signaling pathway.**

**Keywords:** HOIL-1-interacting protein, linear ubiquitin chain assembly complex, nuclear factor- $\kappa$ B, tumor necrosis factor  $\alpha$ , tumor susceptibility gene 101

## INTRODUCTION

Linear ubiquitin chain assembly complex (LUBAC), which is the only known E3 ligase generating the linear (or M1-linked) ubiquitin chains in mammals, is composed of HOIL-1-inter-

acting protein (HOIP, also known as RNF31), heme-oxidized IRP2 ubiquitin ligase-1L (HOIL-1L, also known as RBCK1), and shank-associated RH domain-interacting protein (SHARPIN) (Gerlach et al., 2011; Ikeda et al., 2011; Kirisako et al., 2006; Tokunaga et al., 2011). HOIP is the catalytic component of LUBAC, and it belongs to the family of the RING-between-RING (RBR)-type E3 ligases (Kirisako et al., 2006; Stieglitz et al., 2012). Ubiquitin-conjugated E2 enzyme binds to the RING1 domain of HOIP, followed by the transfer of a donor ubiquitin to the active-site cysteine in the RING2 domain. Subsequently, the C-terminal carboxy group of the donor ubiquitin is conjugated to an acceptor ubiquitin in the linear ubiquitin chain determining domain (LDD) of HOIP, which leads to the formation of linear ubiquitin chains (Lechtenberg et al., 2016; Smit et al., 2012; Stieglitz et al., 2013). HOIL-1L and SHARPIN mediate the stabilization and activation of LUBAC through their interaction with HOIP (Fujita et al., 2018; Liu et al., 2017; Stieglitz et al., 2012). LUBAC has been shown to play a pivotal role in tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) signaling (Haas et al., 2009; Rahighi et al., 2009; Tokunaga et al., 2009). The depletion in any of the LUBAC components attenuates TNF $\alpha$ -induced nuclear factor (NF)- $\kappa$ B signaling cascades (Gerlach et al., 2011; Ikeda et al., 2011; Peltzer et al., 2014; Rickard et al., 2014; Tokunaga et al., 2009).

Tumor susceptibility gene 101 (TSG101, also known as VPS23) was initially discovered as a tumor suppressor, how-

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ever, its role in tumorigenesis has not been unclear yet (Ferraiuolo et al., 2020; Li and Cohen, 1996). Additionally, *TSG101* was found to be a mammalian ortholog of the class E *VPS23* in *Saccharomyces cerevisiae*, and functions as a component of the endosomal sorting complex required for transport I (ESCRT-I) in the endocytic pathway (Babst et al., 2000; Katzmann et al., 2001; Li et al., 1999). TSG101 includes a proline-rich domain (PRD), a coiled-coil (CC) domain, and a steadiness box (S-BOX) domain as well as a ubiquitin E2 variant (UEV) domain lacking a catalytic activity, which has led to the proposition that it may negatively regulate ubiquitination (Feng et al., 2000; Katzmann et al., 2001; Koonin and Abagyan, 1997; Li et al., 2001; Ponting et al., 1997; Pornillos et al., 2002). Although TSG101 has been thought to be associated with numerous intracellular signaling events, its cellular functions remain elusive.

Here, we have found that TSG101 physically interacts with HOIP and positively regulates HOIP-mediated linear ubiquitination. By means of the interaction with HOIP, TSG101 promotes the E3 ligase activity of LUBAC and thereby enhances the TNF $\alpha$ -induced linear ubiquitination in the NF- $\kappa$ B signaling pathway. Thus, our results suggest that TSG101 functions as a positive modulator of the TNF $\alpha$ -induced NF- $\kappa$ B signaling pathway through its action on HOIP-catalyzed LUBAC activity.

## MATERIALS AND METHODS

### Yeast two-hybrid screening

Yeast two-hybrid screening was performed by Panbionet Corp. (Korea), using human HOIP cDNA inserted in a pGBKL bait vector.

### Cell culture and DNA transfection

HEK293T cells and HeLa cells were cultured in DMEM supplemented with 7% fetal bovine serum at 37°C in a humidified incubator with 5% CO<sub>2</sub>. DNA transfection of HEK293T cells was performed with the use of polyethylenimine (Sigma, USA).

### Plasmids

The plasmids pReceiver-M14/Flag-human HOIP, pReceiver-M09/Myc-human HOIL-1L, pReceiver-M07/HA-human SHARPIN, pReceiver-M06/HA-human OTULIN, pGEX-4T-1/GST-HOIP (633-1072), pGEX-4T-1/GST-NEMO (257-346), and pGEX-6P-1/GST-TNF $\alpha$  (77-233) were described previously (Lee et al., 2019). pReceiver-M06/HA-human TSG101 and pReceiver-M06/HA-human SPATA2 were obtained from GeneCopoeia (USA). To generate variants of the plasmid pFlag-CMV5/HOIP (amino acid residues 1-480, 481-632, or 637-1072), each cDNA was amplified by polymerase chain reaction (PCR) using pReceiver-M14/Flag-human HOIP as a template and inserted into a pFlag-CMV5 vector (Sigma-Aldrich, USA). To construct variants of the plasmid pcDNA6/myc-His A-HOIL-1L (amino acid residues 1-129, 130-270, or 271-500), each cDNA was amplified by PCR with pReceiver-M09/Myc-human HOIL-1L as a template and inserted into pcDNA6/myc-His A vector (Invitrogen, USA). The variants of pcDNA3/HA-TSG101 (amino acid residues 1-216 or 271-390) were generated by PCR from pReceiver-M06/HA-hu-

man TSG101 and insertion into a pcDNA3/HA vector. An expression vector encoding His<sub>6</sub>-TSG101 was constructed by subcloning the cDNA of human TSG101 into pET28a/His<sub>6</sub> vector. The vector construct encoding pCMV-3Tag-4A/Myc-TSG101 was constructed by inserting the cDNA of human TSG101 into a pCMV-3Tag-4A vector (Stratagene, USA).

### Antibodies and reagents

Mouse monoclonal antibodies to Myc epitope and to HA epitope were purified from 9E10 and 12CA5 hybridoma cells, respectively. Rabbit monoclonal antibodies to Flag, to HA, to Myc, to phospho-p65 (Ser536), and to p65, rabbit polyclonal antibody to I $\kappa$ B $\alpha$ , and mouse monoclonal antibody to phospho-I $\kappa$ B $\alpha$  (Ser32/36) were from Cell Signaling Technology (USA). Mouse monoclonal antibodies to linear ubiquitin, to RIP1, and to cIAP were from Millipore (USA), BD Transduction Laboratories (USA), and R&D Systems (USA), respectively. Mouse monoclonal antibodies to GAPDH, to HOIL-1L, to TSG101, to NEMO, to TNF-R1, and ubiquitin as well as rabbit polyclonal antibodies to TRAF2, to NEMO, and to GST were from Santa Cruz Biotechnology (USA). Rabbit polyclonal antibody to HOIP was from Abcam (UK). Rabbit polyclonal antibodies to SHARPIN and to TSG101 were from Proteintech Group (USA). Mouse IgG, rabbit IgG, and mouse monoclonal antibodies to Flag were from Sigma. Recombinant murine TNF $\alpha$  was purchased from Peprotech (USA).

### Co-immunoprecipitation and immunoblot analysis

Cells were lysed with NETN lysis buffer (0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 120 mM NaCl) supplemented with 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 2 mg/ml leupeptin. Cell lysates were subjected to immunoprecipitation and immunoblot analysis as previously described (Lee et al., 2019). The intensity of immunoblot bands was quantified using the ImageJ software (NIH, USA).

### Ubiquitination assay

For *in vitro* ubiquitination assay, HEK293T cells were transfected with the indicated expression vectors. Flag-immunoprecipitates prepared from the cells were incubated for 1 h at 37°C with 50 ng of E1 (Boston Biochem, USA), 200 ng of UbcH5c (Boston Biochem), and 2  $\mu$ g of ubiquitin (Sigma) in a 20  $\mu$ l reaction buffer (50 mM Tris-HCl, pH 7.6, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 2 mM ATP). In the case of with the His<sub>6</sub>-TSG101 protein, 1  $\mu$ g of His<sub>6</sub>-TSG101 was also added in the reaction. The reaction was terminated by adding 5 $\times$  SDS sample buffer and boiling the samples at 95°C for 5 min. Samples were then subjected to immunoblot analysis with antibodies to ubiquitin. For *in vitro* ubiquitination assay using GST-fused recombinant HOIP catalytic domain, 2  $\mu$ g of GST-fused HOIP (633-1072) was incubated for 1 h at 37°C in 34  $\mu$ l of reaction buffer containing 50 ng of E1, 200 ng of UbcH5c, and 2  $\mu$ g of ubiquitin in the absence or presence of 2  $\mu$ g of His<sub>6</sub>-TSG101, and then the reaction samples were analyzed by immunoblotting with antibodies to ubiquitin. For the linear ubiquitination assay of NEMO in HeLa cells, the cells were left untreated or treated with 20 ng/ml TNF $\alpha$  for 5 min and lysed with a lysis buffer (1% Triton X-100, 5 mM EGTA, 20 mM Tris-HCl, pH

7.4, 150 mM NaCl, 12 mM  $\beta$ -glycerophosphate, 0.5% sodium deoxycholate, 10 mM N-ethylmaleimide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 2 mg/ml leupeptin). Cell lysates were subjected to immunoprecipitation with rabbit IgG or anti-NEMO antibody, followed by immunoblotting with antibodies to linear ubiquitin.

### RNA interference

HeLa cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA oligonucleotides specific for human TSG101 and GFP control (GenePharma, China) were synthesized and targeted to the sequences 5'-CUAUUGAAG-ACACUAUCUUDtT-3' and 5'-GGCUACGUCCAGGAGC-GCACC-3', respectively. The siRNA specific for human HOIL-1L (Integrated DNA Technologies, USA) was also synthesized and targeted to the sequence 5'-GAGGAUGAUGUCAAU-GAGUUCACCUdTdT-3'.

### Pull-down analysis of endogenous linear ubiquitin conjugates

HeLa cells were lysed with a lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 1% Nonidet P-40, 2 mM EDTA) supplemented with 1 mM dithiothreitol, 5 mM N-ethylmaleimide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 2 mg/ml leupeptin. Lysates were then subjected to pull down with M1-SUB, as previously described (Keusekotten et al., 2013). The pull-down pellets were examined for linear ubiquitination by immunoblot analysis.

### Analysis of TNF-RSC

The TNF $\alpha$  receptor 1 (TNFR1) signaling complex (TNF-RSC) analysis was performed as described previously (Lee et al., 2019). Briefly, HeLa cells were stimulated with 1  $\mu$ g/ml GST-TNF $\alpha$  for the indicated duration and lysed with NETN lysis buffer supplemented with 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 2 mg/ml leupeptin. In the case of untreated control cells, the lysates were mixed with 0.1  $\mu$ g GST-TNF $\alpha$ . All lysates were mixed with glutathione-agarose beads at 4°C for a minimum of 2 h, followed by immunoblotting with the indicated antibodies.

### Reverse transcription followed by quantitative PCR (RT-qPCR)

Total RNA was isolated from HeLa cells using TRIzol (Invitrogen). The RNA (1  $\mu$ g) was reverse-transcribed to cDNA using an oligo (dT) primer (Integrated DNA Technologies) and M-MLV Reverse Transcriptase (Beams Biotechnology, Korea). Quantitative PCR was performed using SYBR Green Supermix (Thermo Fisher Scientific, USA) in an iQ5 thermocycler (Bio-Rad, USA). Relative mRNA expression levels of human interleukin 6 (IL-6) were analyzed in triplicate using the iQ5 optical system software (Bio-Rad). Gene expression levels were normalized to that of GAPDH. The following primers (Integrated DNA Technologies) were used [gene names, forward primer, reverse primer]: human IL-6, 5'-AGCCAGAGCTGTGCAGATGA-3', 5'-GCAGGCTGCATTGTGGTT-3'; human GAPDH, 5'-GGAAGGTGAAGGTCGGAGTC-3', 5'-GAAGGGGT-CATTGATGGCAAC-3'.

### ELISA

HeLa cell culture supernatants were assayed for IL-6 using a human IL-6 ELISA Kit (Invitrogen) according to the manufacturer's instructions.

### Statistical analysis

Quantitative data are presented as the mean  $\pm$  SD. Student's *t*-test was used for statistical analysis on Excel 2010 (Microsoft, USA). *P* values < 0.05 were considered statistically significant.

## RESULTS

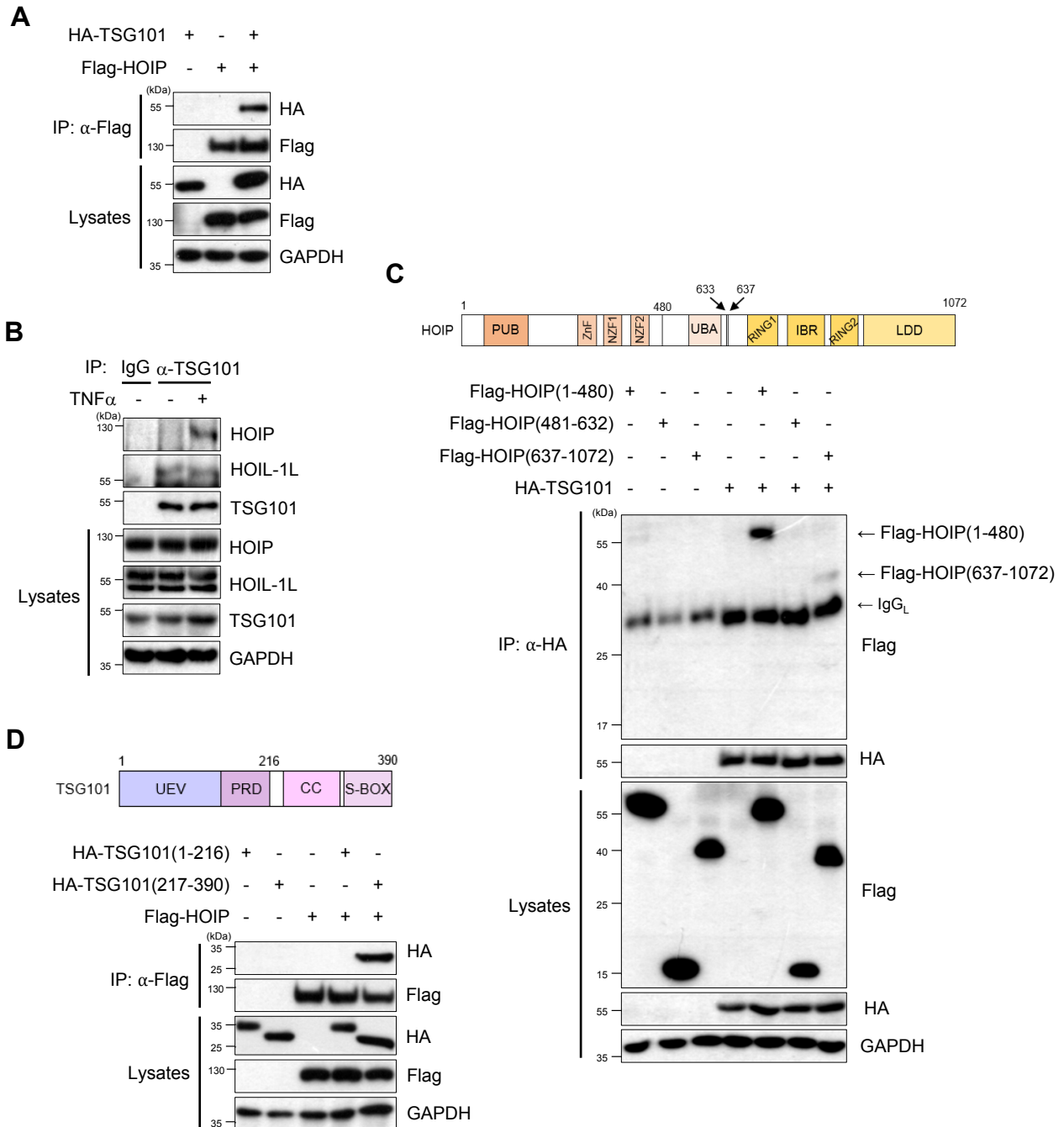
### TSG101 physically associates with LUBAC

To better understand the mechanism underlying the regulation of LUBAC-mediated linear ubiquitination, we searched for the protein candidates that could interact with the RBR-LDD domain of HOIP, the catalytic component of LUBAC, with yeast two-hybrid assays, and were able to identify TSG101 as a HOIP-binding protein.

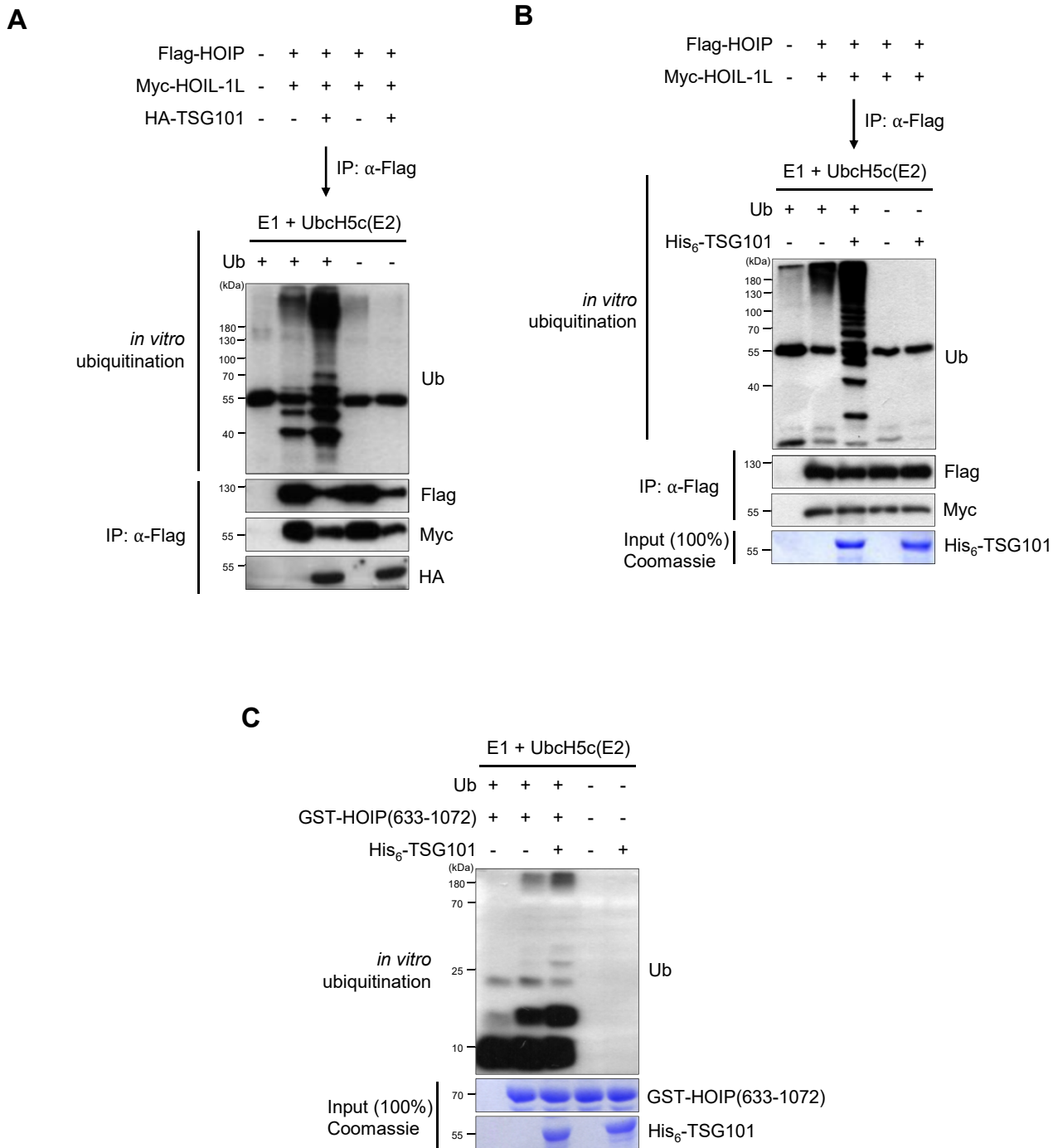
Transfection studies using HEK293 cells also indicated that ectopically expressed TSG101 physically interacted with HOIP in co-immunoprecipitation assays (Fig. 1A). TSG101 also interacted with HOIL-1L, but not with SHARPIN (Supplementary Figs. S1A and S1B). In separate co-immunoprecipitation experiments, we also found that TNF $\alpha$  induced the physical association between endogenous TSG101 and HOIP in HeLa cells (Fig. 1B). In addition, TSG101 interacted with HOIL-1L in HeLa cells regardless of TNF $\alpha$  treatment. Collectively, our data suggested that TSG101 physically associates with LUBAC in intact cells. Given that TSG101 interacts with HOIP in a TNF $\alpha$ -dependent manner, we decided to further investigate a possible action of TSG101 on HOIP activity. First, we sought to identify the regions of HOIP potentially mediating its interaction with TSG101. Our co-immunoprecipitation analysis indicated that TSG101 physically interacted with HOIP (1-480) and HOIP (637-1072), but not with HOIP (481-632) (Fig. 1C). In separate co-immunoprecipitation experiments, TSG101 interacted with HOIL-1L (130-270), but not with HOIL-1L (1-129) or HOIL-1L (271-500) (Supplementary Fig. S1C). Of note, both HOIP and HOIL-1L interacted with TSG101 (217-390), but not with TSG101 (1-216) (Fig. 1D, Supplementary Fig. S1D). TSG101 (217-390) includes the CC domain as well as the S-BOX domain (Feng et al., 2000; Pornillos et al., 2002).

### TSG101 promotes the E3 ligase activity of LUBAC

Given that TSG101 was physically associated with HOIP (Fig. 1), which is the catalytic subunit of the LUBAC complex, we next investigated whether TSG101 might affect the E3 ligase activity of LUBAC. We obtained LUBAC immunocomplex from 293T cells transfected with expression vectors for Flag-tagged HOIP and Myc epitope-tagged HOIL-1L with or without a vector for TSG101, and then performed *in vitro* E3 ligase assays for the linear ubiquitination driven by LUBAC. Our *in vitro* assay revealed that the LUBAC complex mediated the linear ubiquitination, and that the E3 ligase activity of LUBAC was markedly increased by TSG101 (Fig. 2A). Furthermore, in separate experiments, the E3 ligase activity of the LUBAC



**Fig. 1. TSG101 interacts with HOIP.** (A) HEK293T cells were transfected for 72 h with a plasmid vector for HA-TSG101 together with a vector for Flag-HOIP as indicated. Cell lysates were immunoprecipitated with antibodies for Flag epitope. The resulting immunopellets were immunoblotted with the indicated antibodies. Cell lysates were also directly immunoblotted with the indicated antibodies. (B) HeLa cells were left untreated or treated with 20 ng/ml TNF $\alpha$  for 5 min, and lysed. Cell lysates were then immunoprecipitated with rabbit preimmune IgG or anti-TSG101 antibody, and the resulting immunoprecipitates were subjected to immunoblotting with antibodies for HOIP, for HOIL-1L, or for TSG101. (C) HEK293T cells were transfected for 72 h with a vector for HA-TSG101 together with indicated combinations of Flag-tagged HOIP variants (1-480, 481-632, or 637-1072). Cell lysates were immunoprecipitated with anti-HA antibody, then the resulting precipitates were immunoblotted with the indicated antibodies. (D) HEK293T cells were transfected for 72 h with plasmid vectors for HA-tagged variants of TSG101 (1-216 or 217-390) together with a vector for Flag-HOIP. Cell lysates were immunoprecipitated with antibodies to Flag and the resulting precipitates were immunoblotted with the indicated antibodies. In panels of (C) and (D), schematic domain regions of HOIP (C), or TSG101 (D) are also shown. HOIP: PUB, PNGase/UBA or UBX-containing proteins; ZnF, zinc finger; NZF, Npl4-zinc finger; UBA, ubiquitin-associated; RING, really interesting new gene; IBR, in-between RING; LDD, linear ubiquitin chain determining domain. TSG101: UEV, ubiquitin E2 variant; PRD, proline-rich domain; CC, coiled-coil; S-BOX, steadiness box.



**Fig. 2. TSG101 enhances the E3 ligase activity of LUBAC.** *In vitro* linear ubiquitination assay for LUBAC activity. (A) HEK293T cells were transfected for 72 h with the indicated combinations of vectors for Flag-HOIP, Myc-HOIL-1L, and HA-TSG101. Cell lysates were immunoprecipitated with anti-Flag antibody, and the resulting precipitates were examined for linear ubiquitination-promoting activity by *in vitro* assay with E1, E2, and ubiquitin. (B) HEK293T cells were transfected for 72 h with vectors for Flag-HOIP and Myc-HOIL-1L, as indicated, and then immunoprecipitated with anti-Flag antibody. The resulting Flag precipitates were examined for linear ubiquitination-promoting activity by *in vitro* assay in the absence or presence of His<sub>6</sub>-TSG101. (C) GST-fused recombinant HOIP catalytic domain was subjected to *in vitro* ubiquitination reaction in the absence or presence of His<sub>6</sub>-TSG101. The input of the HOIP catalytic domain (C) and His<sub>6</sub>-TSG101 (B and C) was visualized by Coomassie brilliant blue staining.



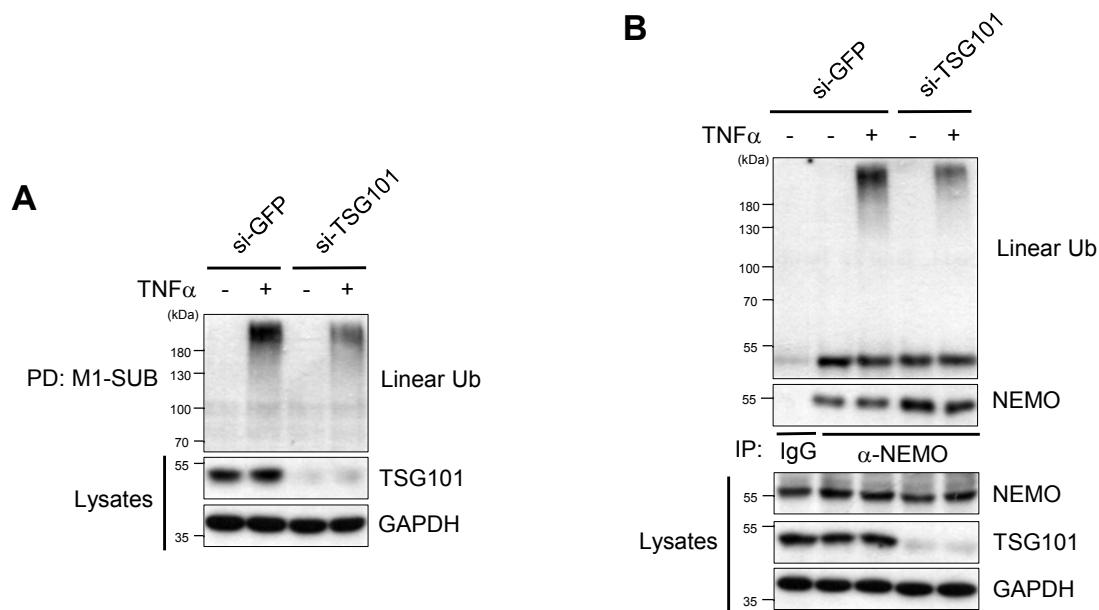
complex was enhanced by addition of the purified recombinant TSG101 protein in *in vitro* assays (Fig. 2B). Moreover, TSG101 also increased the linear ubiquitination promoting activity of HOIP (633-1072), which contains the RBR-LDD (catalytic) domain (Fig. 2C). Thus, these results suggested that TSG101 could enhance the E3 ligase activity of LUBAC complex through directly interacting with HOIP.

Next, given that TSG101 increases the E3 ligase activity of HOIP *in vitro* (Fig. 2), we examined whether TSG101 affects the linear ubiquitination promoting activity of LUBAC in intact cells after treatment with TNF $\alpha$ , which can initiate the activation of LUBAC and consequently induces the NF- $\kappa$ B signaling events (Haas et al., 2009; Rahighi et al., 2009; Tokunaga et al., 2009). Exposure of HeLa cells to TNF $\alpha$  increased the linear ubiquitination of total proteins (Fig. 3A) and NEMO (Fig. 3B), and these increases were reduced by siRNA-mediated depletion of TSG101 (Fig. 3). These results thus suggested that TSG101 would enhance the TNF $\alpha$ -induced linear ubiquitination promoting activity of HOIP in intact cells. Next, given that TSG101 also interacts with HOIL-1L (Supplementary Figs. S1A and S1B), we assessed whether HOIL-1L might affect the action of TSG101 on the TNF $\alpha$ -induced linear ubiquitination in HeLa cells. As expected, compared with the control cells, siRNA-mediated depletion of HOIL-1L mitigated the TNF $\alpha$ -induced linear ubiquitination of total proteins (Supplementary Fig. S1E), which confirms that HOIL-1L is a crucial regulatory component of LUBAC. Interestingly, siRNA-mediated depletion of TSG101 did not further reduce the levels of linear ubiquitination in HOIL-1L-knockdown cells, suggesting that HOIL-1L might be also involved in the mechanism by

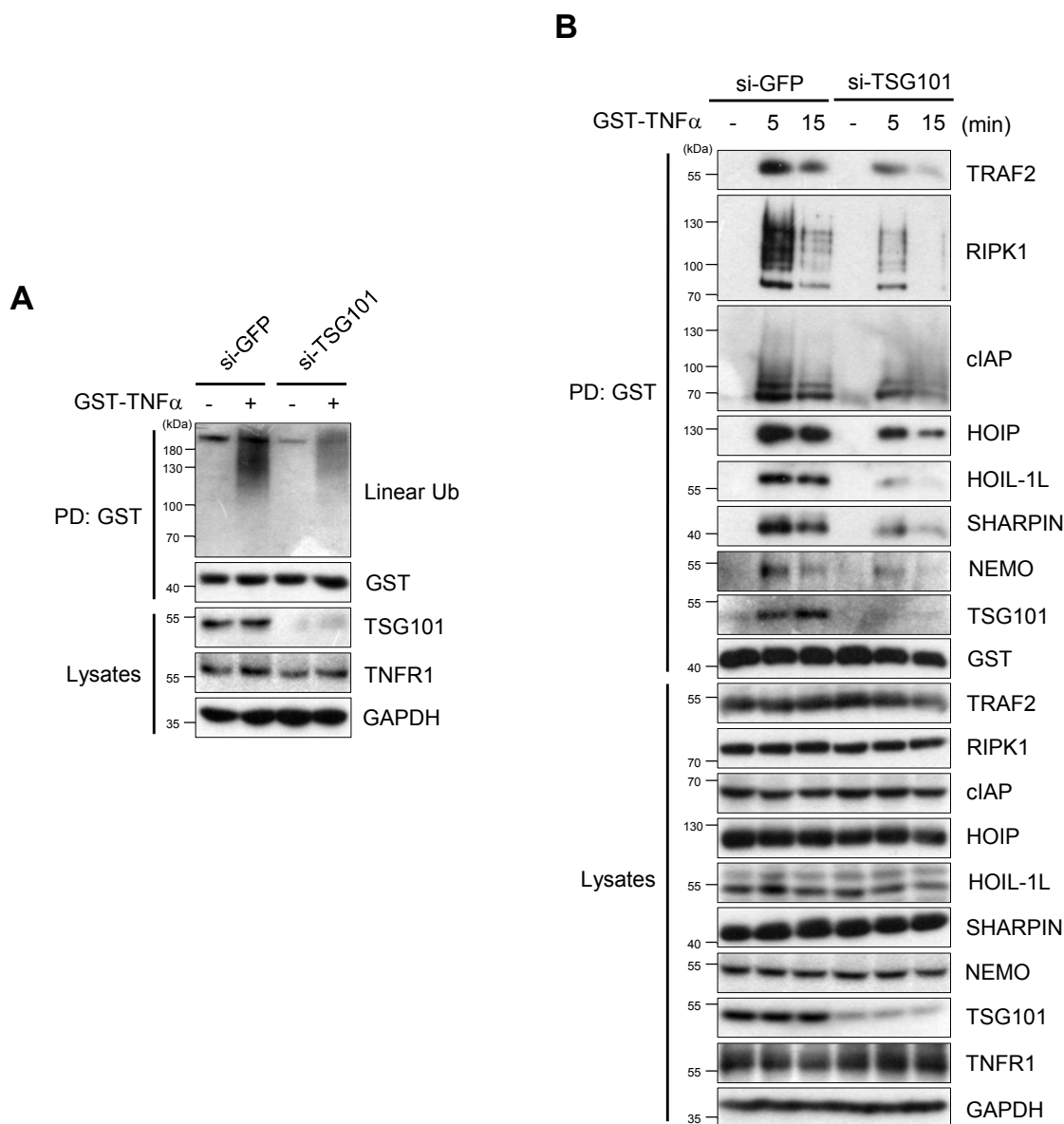
which TSG101 promotes LUBAC activity.

Next, we decided to further investigate a possible mechanism by which TSG101 potentiates the TNF $\alpha$ -induced E3 ligase activity of HOIP in intact cells. TNF $\alpha$  has been shown to induce the E3 ligase activity of LUBAC through promoting the translocation of HOIP as well as other components of the LUBAC to the TNF-RSC (Gerlach et al., 2011; Haas et al., 2009). We, therefore, examined a possible action of TSG101 on the TNF $\alpha$ -induced linear ubiquitination in TNF-RSC. TNF $\alpha$ -treatment resulted in an increase in the linear ubiquitination in TNF-RSC, and this increase was reduced by siRNA-mediated depletion of TSG101 (Fig. 4A), suggesting that TSG101 potentiates the TNF $\alpha$ -induced linear ubiquitination in TNF-RSC. We also found that siRNA-mediated depletion of TSG101 reduced the TNF $\alpha$ -induced translocation of LUBAC components, including HOIP, HOIL-1L, and SHARPIN, as well as TRAF2, RIPK1, cIAP, and NEMO into TNF-RSC (Fig. 4B). It is noteworthy that TRAF2, RIPK1, and cIAP have been shown to increase the stability of TNF-RSC (Hsu et al., 1996; Shu et al., 1996; Varfolomeev et al., 2008; Vince et al., 2009) and the activation of LUBAC (Haas et al., 2009) in TNF-RSC, while NEMO is one of the major substrates of LUBAC (Tokunaga et al., 2009). Thus, our results suggest that TSG101 upregulates the TNF $\alpha$ -induced stimulation of the linear ubiquitination promoting activity of LUBAC in TNF-RSC.

We also examined whether TSG101 could upregulate the physical interaction of HOIP with the other components of LUBAC, i.e., HOIL-1L and SHARPIN. Our co-immunoprecipitation data revealed that TSG101 did not affect the binding of HOIP to HOIL-1L or SHARPIN in intact cells under either basal



**Fig. 3. Depletion of TSG101 by siRNA mitigates the TNF $\alpha$ -induced E3 ligase activity of LUBAC.** (A and B) HeLa cells were transfected for 48 h with control (si-GFP) or *TSG101* (si-TSG101) siRNA, and were then left untreated or treated with 20 ng/ml TNF $\alpha$  for 5 min. Cell lysates were subjected to pull-down using GST-fused M1-SUB (A) or to immunoprecipitation with rabbit preimmune IgG or anti-NEMO antibody (B). The resulting pellets were immunoblotted with antibodies to linear ubiquitin chains (A and B) or to NEMO (B). The lysates were also immunoblotted with indicated antibodies.



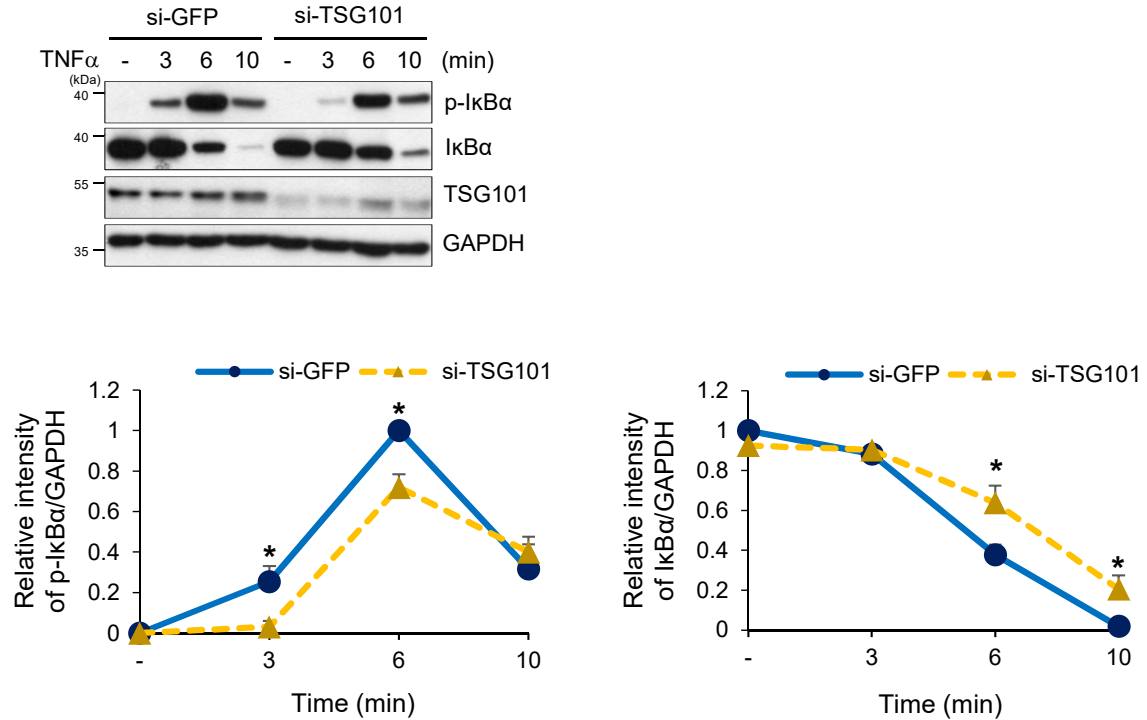
**Fig. 4. Effect of siRNA-mediated TSG101 depletion on the E3 activity of LUBAC in TNF-RSC.** HeLa cells were transfected for 48 h with control (si-GFP) or *TSG101* siRNA (si-TSG101), and were then left untreated or treated with 1  $\mu$ g/ml GST-TNF $\alpha$  for 5 min (A) or for the indicated durations (B). Cell lysates were pulled down with glutathione-agarose beads, and the bead-bound proteins were analyzed by immunoblotting with indicated antibodies.

state or after TNF $\alpha$  stimulation (Supplementary Fig. S2A). It is also noteworthy that TSG101 did not change the binding of HOIP to either the deubiquitinase (DUB) OTULIN (Supplementary Fig. S2B) or the adaptor protein SPATA2 (Supplementary Fig. S2C). OTULIN and SPATA2 have been shown to interact with HOIP and thereby modulate the E3 ligase activity of LUBAC (Elliott et al., 2014; 2016; Kupka et al., 2016; Schaefer et al., 2014; Takiuchi et al., 2014; Wagner et al., 2016).

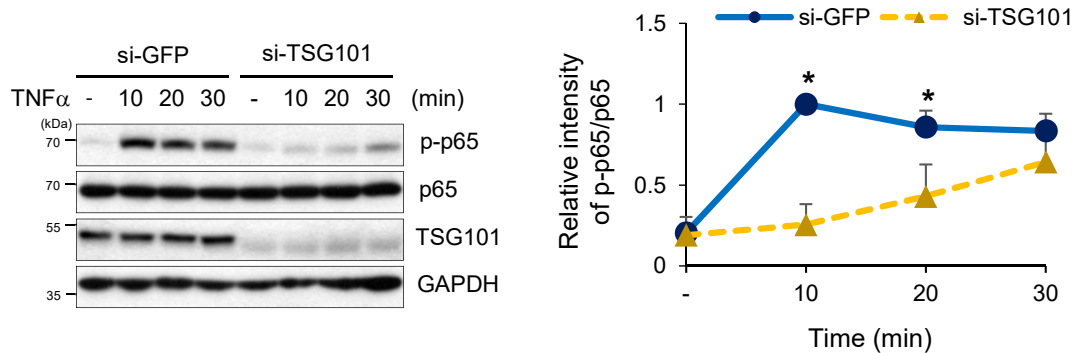
#### TSG101 positively regulates the TNF $\alpha$ -induced stimulation of the NF- $\kappa$ B signaling pathway

Given that TSG101 enhances the TNF $\alpha$ -induced stimulation of the E3 ligase activity of LUBAC (Figs. 3 and 4), we examined whether TSG101 might potentiate the TNF $\alpha$ -induced stimulation of NF- $\kappa$ B signaling. TNF $\alpha$  treatment increased the phosphorylation of I $\kappa$ B $\alpha$  and decreased the cellular levels of I $\kappa$ B $\alpha$  in HeLa cells, and these effects of TNF $\alpha$  were reduced by siRNA-mediated depletion of TSG101 (Fig. 5A). Furthermore, siRNA-mediated depletion of TSG101 mitigated the TNF $\alpha$ -induced phosphorylation (activation) of NF- $\kappa$ B p65

**A**



**B**



**Fig. 5. TSG101 promotes TNF $\alpha$ -induced NF- $\kappa$ B activation.** HeLa cells were transfected for 48 h with control or *TSG101* siRNA, after which the cells were left untreated or treated with 20 ng/ml TNF $\alpha$  for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. The band intensity of phospho-IκB $\alpha$  or IκB $\alpha$  relative to that of GAPDH (A) or the band intensity of phospho-p65 relative to that of p65 (B) was quantified. Quantitative data are presented as the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

(Fig. 5B). The gene knockdown of TSG101 also reduced the TNF $\alpha$ -induced transcription (Fig. 6A) and protein synthesis (Fig. 6B) of IL-6, an NF- $\kappa$ B target gene, in HeLa cells. Together, these results suggest that TSG101 enhances the TNF $\alpha$ -induced activation of the NF- $\kappa$ B signaling events.

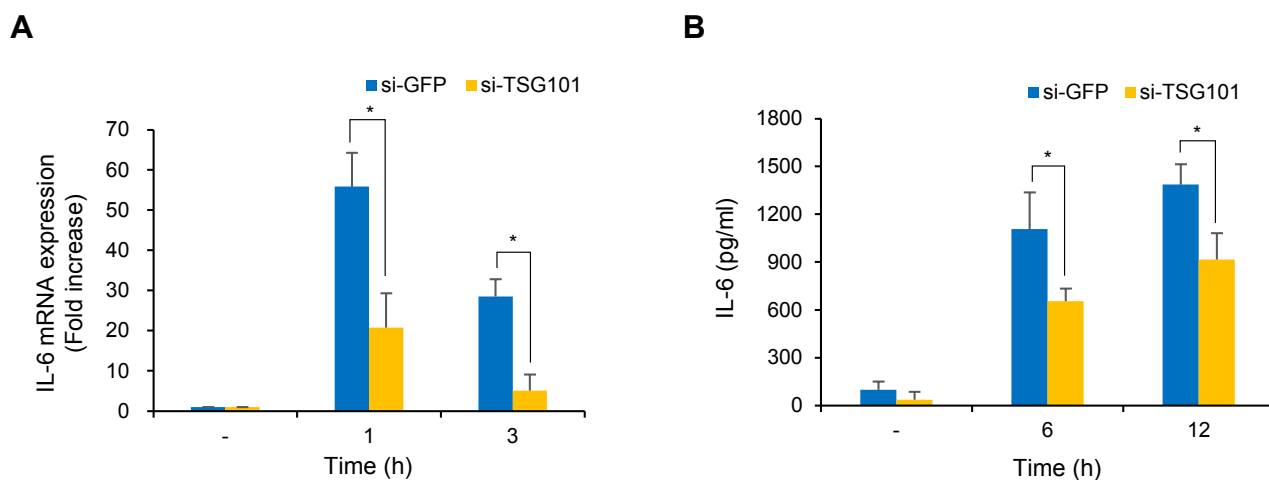
## DISCUSSION

Our findings indicate that TSG101 functions as a new regu-

lator of the LUBAC. TSG101 physically interacts with HOIP, the catalytic component of the linear ubiquitin-producing E3 ligase complex, and thereby promotes the LUBAC-mediated NF- $\kappa$ B signaling pathway.

In this study, we initially found that TSG101 is a binding partner of HOIP by performing yeast-two hybrid assays using RBR-LDD (the catalytic region) of HOIP as a bait, and then confirmed that TSG101 physically associates with HOIP in intact cells by co-immunoprecipitation assays. Furthermore,





**Fig. 6. TSG101 deficiency impairs TNF $\alpha$ -induced gene induction.** HeLa cells were transfected for 48 h with control or *TSG101* siRNA, and were then left untreated or treated with 20 ng/ml TNF $\alpha$  for the indicated times. (A) The cells were examined by RT-qPCR for *IL-6* gene expression. (B) Culture media were collected and examined by ELISA for IL-6. Data are presented as the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

TSG101 was able to potentiate the catalytic activity of HOIP. Interestingly, TSG101 did not affect the binding of HOIP to HOIL-1L or SHARPIN, the other components of LUBAC, suggesting that the TSG101-mediated increase in LUBAC activity is not mediated through the interactions among the components of LUBAC.

Our co-immunoprecipitation analyses indicated that TSG101 interacts not only with protein fragments containing RBR and LDD domains, but also with those containing PUB, ZnF, and NZF domains. The PUB domain of HOIP, in particular, has been shown to be involved in its interaction with the deubiquitinating enzymes, OTULIN or CYLD. OTULIN binds directly to HOIP, and CYLD interacts with HOIP through the adaptor protein, SPATA2 (Elliott et al., 2014; 2016; Kupka et al., 2016; Schaeffer et al., 2014; Takiuchi et al., 2014; Wagner et al., 2016). Our binding data, however, indicated that TSG101 did not affect the binding of HOIP to either OTULIN or SPATA2, implying that the modulation of the interaction with DUB should not be a primary mechanism for the TSG101-mediated potentiation of HOIP activity.

LUBAC is a component of TNF-RSC, and E3 ligase activity therein plays an important role in TNF $\alpha$ /TNFR1-mediated NF- $\kappa$ B signaling cascade (Gerlach et al., 2011; Haas et al., 2009). We observed that TSG101 is present in TNF-RSC in TNF $\alpha$ -treated cells, suggesting that it might be a regulatory component of TNF-RSC, and that it promotes the activity of LUBAC in TNF-RSC. As LUBAC is essential for maintaining the stability of TNF-RSC (Haas et al., 2009), it is possible that TSG101 might contribute to the stability of TNF-RSC.

TSG101 has been shown to function as a component of ESCRT-I in pathways such as endosomal trafficking (Lu et al., 2003; Pomillos et al., 2002). Intriguingly, TSG101 has the catalytically inactive UEV domain, so it can physically interact with ubiquitin without E2 enzymatic activity (Koonin and Abagyan, 1997; Ponting et al., 1997). One may propose that the ubiquitin-interacting capability of TSG101 could be asso-

ciated with the molecular mechanism by which TSG101 positively regulates LUBAC/HOIP-mediated linear ubiquitination. Given that the LUBAC/NF- $\kappa$ B pathway plays a pivotal role in proinflammatory events, our findings may provide an insight into a novel function of TSG101 in the inflammation and other related immune responses.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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## AUTHOR CONTRIBUTIONS

E.K., H.C., G.L., H.B., and I.Y.L. conducted the experiments. E.K., H.C., I.Y.L., and E.-J.C. designed the experiments. E.K. and E.-J.C. wrote the paper.

## CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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