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IL-37-induced activation of glycogen synthase kinase 3β promotes IL-1R8/Sigirr phosphorylation, internalization, and degradation in lung epithelial cells

Lian Li[1,#], **Jianxin Wei**[2,#], **Tomeka L. Suber**[2], **Qinmao Ye**[1], **Jiaxing Miao**[1], **Shuang Li**[2], **Sarah J. Taleb**[1], **Kevin C. Tran**[1], **Arya S. Tamaskar**[1], **Jing Zhao**[1,3], **Yutong Zhao**[1,3,*]

[1]Department of Physiology and Cell Biology, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH

[2]Department of Medicine, The University of Pittsburgh, Pittsburgh, PA

[3]Department of Internal Medicine, The Ohio State University, Columbus, OH

Abstract

Interleukin (IL)-37 diminishes a variety of inflammatory responses through ligation to its receptor IL-1R8/Sigirr. Sigirr is a Toll like receptor (TLR)/IL-1R family member. We have shown that Sigirr is not stable in response to IL-37 treatment, IL-37-induced Sigirr degradation is mediated by the ubiquitin-proteasome system, and that the process is reversed by a deubiquitinase, USP13. However, the molecular mechanisms by which USP13 regulates Sigirr stability have not been revealed. In this study, we investigate the roles of glycogen synthesis kinase 3β (GSK3β) in Sigirr phosphorylation and stability. IL-37 stimulation induced Sigirr phosphorylation and degradation, as well as activation of GSK3β. Inhibition of GSK3β attenuated IL-37-induced Sigirr phosphorylation, while exogenous expressed GSK3β promoted Sigirr phosphorylation at threonine (T)372 residue. Sigirr association with GSK3β was detected. Amino acid residues 51–101 in GSK3β were identified as the Sigirr binding domain. These data indicate that GSK3β mediates IL-37-induced threonine phosphorylation of Sigirr. Further, we investigated the role of GSK3β-mediated phosphorylation of Sigirr in Sigirr degradation. Inhibition of GSK3β attenuated IL-37-induced Sigirr degradation, while T372 mutant of Sigirr was resistant to IL-37-mediated degradation. Furthermore, inhibition of Sigirr phosphorylation prevented Sigirr internalization and association with USP13, suggesting GSK3β promotes Sigirr degradation through disrupting Sigirr association with USP13.

Keywords

IL-37; GSK3β; IL-1R8/Sigirr; phosphorylation; ubiquitination

[*]Address Correspondence to: Yutong Zhao, MD, PhD, Department of Physiology and Cell Biology, The Ohio State University, 333 10th Avenue, Graves Hall 2166D, Columbus, OH, United States, 43065, Yutong.zhao@osumc.edu; Tel.: 614-685-0360.

[#]Both authors contributed equally to this manuscript.

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Introduction

Interleukin (IL)-1 family cytokines play a central role in immune regulation and inflammatory responses, producing both pro- and anti-inflammatory effects. IL-1α, IL-1β, and IL-33 regulate pro-inflammatory responses in variety of cell types including macrophages and neutrophil (Baker, Houston, & Brint, 2019; Mantovani, Dinarello, Molgora, & Garlanda, 2019; Sims & Smith, 2010). Among the cytokines, IL-37 has attracted attention in recent years. IL-37 has been shown to play anti-inflammatory responses in both innate and acquired immunity (Abulkhir et al., 2017; Boraschi et al., 2011; Cavalli & Dinarello, 2018; Ding et al., 2017). The effect of IL-37 is achieved through both its nuclear translocation and release into the extracellular space (Abulkhir et al., 2017; Boraschi et al., 2011; Cavalli & Dinarello, 2018; Ding et al., 2017). Most studies have focused on secreted IL-37 and revealed that secreted IL-37 binds to a membrane receptor IL-1R8/Sigirr. Sigirr is a Toll like receptor (TLR)/IL-1R family member and contains a short intracellular tail (Liu et al., 2019; Lunding et al., 2015; Nold-Petry et al., 2015). Sigirr contributes to IL-37-mediated anti-inflammatory responses. It also attenuates IL-1R and TLRs-mediated pro-inflammatory responses as well (Huang, Hazlett, Du, & Barrett, 2006; Leemans et al., 2012; Qin, Qian, Yao, Grace, & Li, 2005; Riva et al., 2012; Veliz Rodriguez et al., 2012; Zhang, Wu, Zhao, Deng, & Qian, 2011). Overexpression of Sigirr diminished IL-1β- and lipopolysaccharide (LPS)-induced cytokine release (Huang et al., 2006; Qin et al., 2005; Veliz Rodriguez et al., 2012; Zhang et al., 2011). Though the anti-inflammatory effects of Sigirr have been well demonstrated, its molecular regulation has not been well studied. The ubiquitin-proteasome system plays a critical role in regulation of cellular protein homeostasis. A disbalance in the homeostasis of plasma membrane receptors affects extracellular signaling transfer inside cells, therefore altering cellular responses (Bedford, Lowe, Dick, Mayer, & Brownell, 2011; Weathington, Sznajder, & Mallampalli, 2013). We have revealed that Sigirr is degraded by the ubiquitin-proteasome system in response to IL-37 ligation (L. Li et al., 2019). The ubiquitination and degradation of Sigirr is negatively regulated by a deubiquitinase, USP13 (L. Li et al., 2019).

Protein ubiquitination is regulated by other post-translational modifications, such as phosphorylation and acetylation. Phosphorylation has been considered as a degron signal, which may promote substrates binding to ubiquitin E3 ligases (Skaar, Pagan, & Pagano, 2013). Several kinases, such as AKT (Choppara, Malonia, Sankaran, Green, & Santra, 2018; Tang et al., 2007), CDK (Montagnoli et al., 1999; Watanabe et al., 2005), and GSK3β (Kumar et al., 2016; Xu, Kim, & Gumbiner, 2009) have been identified to promote substrate phosphorylation and degradation. Among them, phosphorylation by GSK3β has been well demonstrated to promote intracellular and membrane protein degradation (Xu et al., 2009). GSK3β is ubiquitously expressed in most cell types [27]. SCF^{FBXW7} E3 ligase-mediated c-Myc and c-Myb ubiquitination and degradation are dependent on GSK3β-catalyzed phosphorylation (Kitagawa et al., 2009; Yada et al., 2004). We have shown that GSK3β phosphorylates IL-33 and IL-22 receptors and facilitates their ubiquitination and degradation (Weathington et al., 2014; Zhao et al., 2012). GSK3β phosphorylates the first S/T in a GSK3β phosphorylation consensus sequence, (S/T)XXX p(S/T) (Farghaian, Turnley, Sutherland, & Cole, 2011).

Plasma membrane receptor internalization and ubiquitination regulates receptor desensitization and the magnitude of their intracellular signaling and cellular responses (Huangfu & Fuchs, 2010; Rajagopal & Shenoy, 2018). Receptor phosphorylation in response to ligand ligation triggers receptor internalization and intracellular trafficking (Sorkin & Duex, 2010). We have shown that inhibition of GSK3β attenuates IL-33-induced ST2L internalization (Zhao et al., 2015). In the current study, we found that GSK3β phosphorylates IL-1R8/Sigirr and increases IL-1R8/Sigirr internalization and degradation.

Materials and Methods

Cells and reagents.

Mouse lung epithelial cells (MLE12) and Human monocyte-like cell line (THP1) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Recombinant human IL-37 was from R&D Systems (Minneapolis, MN, USA). Cycloheximide, leupeptin, and LPS were purchased from Sigma Aldrich (St Louis, MO, USA). MG-132 was purchased from EMD Chemicals (Gibbstown, NJ, USA). Immobilized protein A/G beads and control IgG and anti-Sigirr (for mouse, rat, and human origin) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-USP13 was purchased from Protein Tech (Chicago, IL, USA) and Bethyl Laboratories (Montgomery, TX, USA). Antibodies against HA tag, GSK3β, pY216GSK3β, phospho-threonine, and ubiquitin were purchased from Cell Signaling (Beverly, MA, USA). Anti-V5, mammalian expression plasmid pcDNA3.1/His-V5-topo, *Escherichia coli* Top10 competent cells were purchased from Invitrogen and Thermo life technology (Gaithersburg, Maryland, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All commercially available materials used were of the highest quality.

Plasmid transfection.

The cDNA encoding human *Sigirr*, mutants, GSK3β were synthesized by PCR and ligated into pCDNA3.1/V5-His-Topo mammalian expressing vector (Invitrogen, Carlsbad, CA, USA). Mammalian expression plasmids were transfected into MLE12 cells by a Lonza electroporation protocol.

Immunostaining.

MLE12 cells were cultured in glass bottom D35-dishes for immunofluorescence staining. Briefly, cells were fixed with 3.7% formaldehyde for 20 min, followed by blocking with 1% BSA in TBST (25 mM Tris HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20) for 30 min. Indicated antibodies were added for 1 h. Cells were then washed with PBS three times, followed by incubation with fluorescent-conjugated secondary antibodies. Images were captured by a Nikon A1R HD25 inverted microscope.

Western blotting analysis.

After treatment, cells were quickly rinsed with cold PBS (1 ml). Cells were collected by a rubber scraper in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and

1 μg/ml pepstatin. The cells were destroyed by sonication on ice for 12 s. Cell debris were removed by centrifugation at 4 °C at 5000 rpm for 10 min. Protein concentrations were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of samples were loaded to SDS-PAGE gel and transferred to membrane. Blots were blocked with 1% of BSA in TBST and immunoblotted with primary antibodies for 2 h (room temperature) or overnight (4 °C). The membranes were then washed for 10 min x 3 times with TBST. Secondary antibodies were added for a further 1 h. Immunosignals were stimulated by an Enhanced Chemiluminescence Detection Kit (Thermo Fisher Scientific, Waltham, MA) and recorded with an Azure c600 imaging system.

Co-immunoprecipitation and in vivo ubiquitination assay.

MLE12 cells were rinsed with cold PBS and collected in cell lysis buffer as described above. 1 mg of protein in cell lysates were incubated with the indicated antibodies or control IgG overnight. 40 μl of agarose beads were added for additional 1 h, followed by washing with PBS and lysis buffer 3 times. Immunoprecipitated complexes were separated from beads by boiling for 5 minutes. For the *in vivo* ubiquitination assay, a modified protocol under denaturing conditions was used. Briefly, cells were collected in PBS. After centrifugation at 1000 rpm for 5 min, 1 μl of ubiquitin aldehyde was added to cell pellets along with 1 μl of NEM, in 50–80 μl of 2% SDS lysis buffer. The cell lysates were then boiled at 100°C for 10 min after sonication. The lysates were then combined with 500–800 μl of 1×TBS, followed by immunoprecipitation as described above.

Quantification and statistical analysis.

Immunoblot and immunostaining intensities were analyzed by ImageJ software (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA). Comparisons were analyzed by t-tests. Data were expressed as mean ± standard error of the mean of triplicate measurements from three independent immunoblotting. $p<0.05$ was considered statistically significant.

Results

IL-1R8/Sigirr is degraded in the proteasome.

Sigirr is a transmembrane receptor for IL-37. We have previously reported its proteasomal degradation in response to IL-37 ligation (L. Li et al., 2019). Consistent with our previous findings, we found that Sigirr is not stable and that it degrades in a time-dependent manner in the presence of protein synthesis inhibitor cycloheximide (CHX) (Fig. 1A). The degradation is prevented when the cells were pre-incubated with proteasome inhibitor (MG-132), but not lysosome inhibitor (leupeptin) (Fig. 1B), indicating that Sigirr is degraded in the proteasome system. We have shown that Sigirr can be ubiquitinated. To confirm that ubiquitination leads to Sigirr degradation, we increased intracellular ubiquitin levels in the cells by transfecting cells with HA-tagged ubiquitin (HA-Ubi) and examined the effect of exogenous HA-Ubi on Sigirr abundance. As shown in Fig. 1C and 1D, both overexpressed Sigirr-V5 and endogenous Sigirr abundance were decreased while ubiquitin levels increased proportionally, suggesting that Sigirr degradation is mediated by the ubiquitin-proteasome system.

GSK3β regulates IL-1R8/Sigirr threonine phosphorylation.

Receptors are commonly phosphorylated in response to ligands [36]. Sigirr phosphorylation has not been reported. We found that IL-37 treatment triggers threonine phosphorylation of Sigirr (Fig. 2A) as well as tyrosine phosphorylation of GSK3β (Fig. 2B). Further, inhibition of GSK3β by TWS119 significantly reduced endogenous Sigirr phosphorylation (Fig. 2C), as well as IL-37-indcued Sigirr-V5 phosphorylation (Fig. 2D), suggesting that IL-37-activated GSK3β regulates IL-1R8/Sigirr threonine phosphorylation.

GSK3β is associated with IL-1R8/Sigirr.

Serine residue 9 phosphorylation in GSK3β causes its inactivation. A mutation in serine residue 9 to alanine (S9A) of GSK3β increases its activation [37]. To investigate whether the effect of GSK3β on Sigirr phosphorylation is direct or not, we determined the association of GSK3β and Sigirr by co-immunoprecipitation and co-immunofluorescence staining. MLE12 cells were transfected with Sigirr-V5 with or without GSK3βS9A plasmid. Active form of GSK3β was detected in the Sigirr-V5-immunoprecipitated complex (Fig. 3A), indicating that GSK3β is associated with Sigirr. Further, co-localization of exogenous GSK3β and Sigirr was detected in the plasma membrane (Fig. 3B), supporting that GSK3β targets Sigirr for its phosphorylation. Co-immunoprecipitation experiment confirmed the association between exogenous GSK3β and Sigirr (Fig. 3C). To identify the Sigirr binding domain on GSK3β, we constructed a series of V5-tagged deletion mutants of GSK3β and examined their association with Sigirr. The wild type GSK3β, C-terminal deletion mutants, and N-terminal 1–51 deletion mutant were detected in the Sigirr immunoprecipiated complex, while N-terminal 1–101 deletion mutant failed to associate with Sigirr (Fig. 3D), suggesting that Sigirr binding site occurs in the domain between residues 51–101 on GSK3β.

Activation of GSK3β promotes Sigirr degradation in the proteasome.

To further investigate the effect of GSK3β on Sigirr degradation, we transfected cells with the constitutively active form GSK3βS9A or treated cells with GSK3β inhibitor TWS119. Overexpression of GSK3βS9A reduced exogenous Sigirr levels (Fig. 4A), and the effect was attenuated by MG-132, suggesting that activation of GSK3β promotes Sigirr proteasomal degradation. This conclusion was further confirmed by inhibition of GSK3β. Consistent with our previous findings, IL-37 reduced Sigirr abundance in a time-dependent manner, while TWS119 significantly attenuated IL-37-induced Sigirr degradation (Fig. 4B). Taken together, this data indicates that IL-37-induced GSK3β activation promotes Sigirr proteasomal degradation.

Threonine residue 372 is the phosphorylation site on Sigirr.

A conserved GSK3β phosphorylation consensus sequence T372SXXS occurs on Sigirr (Fig. 5A). To determine if T372 is the GSK3β-mediated phosphorylation site on Sigirr, we generated a V5-tagged SigirrT372A mutant expressing plasmid. Compared to Sigirr wild type, SigirrT372A failed to be phosphorylated by IL-37 (Fig. 5B). Further, the degradation rate of SigirrT372A was significantly lower than Sigirr wild type (Fig. 5C), indicating that phosphorylation of Sigirr at T372 residue plays a critical role in Sigirr degradation.

GSK3β-mediated phosphorylation regulates IL-1R8/Sigirr internalization.

Receptor internalization is an important process for receptor desensitization [33]. Phosphorylation of receptors plays a critical role in the regulation of receptor internalization. To determine if GSK3β-mediated phosphorylation regulates Sigirr internalization, MLE12 cells were transfected with Sigirr-V5 plasmid for 48 h, then treated with TWS119 prior to IL-37 stimulation. Sigirr-V5 is primarily localized on the plasma membrane and IL-37 treatment triggered its internalization into the cytoplasm. TWS119 pretreatment attenuates IL-37-induced Sigirr internalization (Fig. 6A). Similar to wild type, SigirrT372A-V5 was localized on the plasma membrane in the untreated cells. IL-37 induced Sigirr wild type internalization into the cytoplasm, but SigirrT372A-V5 failed to be internalized in response to IL-37 treatment (Fig. 6B). Taken together, IL-37-induced Sigirr internalization is dependent on phosphorylation of Sigirr in T372 residue by GSK3β.

GSK3β-mediated phosphorylation reduces the association between IL-1R8/Sigirr and USP13.

We have shown that deubiquitination of Sigirr by USP13 increased Sigirr half-life (L. Li et al., 2019). To determine molecular mechanisms by which GSK3β promotes Sigirr degradation, we examined the effect of GSK3β-mediated phosphorylation on the association between Sigirr and USP13. SigirrT372A increased the association with USP13 compared to Sigirr wild type (Fig. 7A). Overexpression of GSK3β reduced the association between Sigirr and USP13 (Fig. 7B), suggesting that GSK3β-mediated phosphorylation prevents Sigirr degradation from Sigirr interaction with its stabilizer, USP13.

Discussion

Most members of IL-1R/TLR family exhibit pro-inflammatory properties, while IL-1R8/Sigirr plays anti-inflammatory effects in both innate and adaptive immune responses. The effects of IL-1R8/Sigirr are not only dependent on activation by its ligand, IL-37; it restrains IL-1R/TLRs-mediated signaling and pro-inflammatory responses by competing interaction with Myd88 (Huang et al., 2006; Liu et al., 2019; Lunding et al., 2015; Qin et al., 2005; Riva et al., 2012; Veliz Rodriguez et al., 2012; Zhang et al., 2011). Though the cellular functions of IL-1R8/Sigirr have been gaining attention recently, its post-translational modifications have not been well studied. We have shown that IL-1R8/Sigirr abundance is regulated by the ubiquitin-proteasome system (L. Li et al., 2019). USP13 deubiquitnates and stabilizes IL-1R8/Sigirr (L. Li et al., 2019). In this current study, we further investigate molecular regulation of IL-1R8/Sigirr ubiquitination and degradation, focusing especially on the role of phosphorylation in IL-1R8/Sigirr ubiquitination, internalization, degradation, and association with USP13 (Fig. 8C).

GSK3β targets and phosphorylates multiple proteins and plays various cellular functions (Augello et al., 2020; Wadhwa, Jain, & Jadhav, 2020). Phosphorylation is one of the priming signals for protein ubiquitination and degradation (Skaar et al., 2013). It has been previously demonstrated that ubiquitination is dependent on protein pre-phosphorylation. The role of GSK3β-mediated phosphorylation in the regulation of protein ubiquitination and turnover has been reported. Ligand-induced ubiquitination and degradation of IL-22 receptor or

IL-33 receptor is dependent on GSK3β-mediated phosphorylation. GSK3β phosphorylates IL-22 and IL-33 receptors and facilitates their ubiquitination and degradation (Weathington et al., 2014; Zhao et al., 2012). This study reveals that IL-1R8/Sigirr phosphorylation is catalyzed by GSK3β (L. Li et al., 2019) and that T372 is the phosphorylation site on human IL-1R8/Sigirr. GSK3β targets the first S/T site in the conserved phosphorylation consensus sequence, while the process needs the second S/T site to receive a priming phosphorylation. Thus, the S376 (the second S/T site of the conserved sequence on the Sigirr) phosphorylation is an essential for GSK3β targeting of IL-1R8/Sigirr. The protein kinase for S376 phosphorylation and its role in IL-1R8/Sigirr ubiquitination and degradation remain unknown.

GSK3β activation is regulated by phosphorylation at different residues. GSK3β kinase activity is increased once tyrosine (Y)-216 on GSK3β is phosphorylated, while serine-9 phosphorylation of GSK3β deceases its ability to bind substrates (Augello et al., 2020; Wadhwa et al., 2020). We have shown that FAK phosphorylates Y216 on GSK3β, therefore the activated GSK3β triggers IL-33 receptor internalization and degradation (Zhao et al., 2015). In this study, we reveal that IL-37 induces Y216 phosphorylation of GSK3β, indicating that is activated by IL-37 stimulation; however, whether the phosphorylation and activation of GSK3β are mediated by FAK in response to IL-37 is not clear. GSK3β ubiquitination and degradation have been reported to be regulated by SCF^{FBXO17} E3 ligase (Suber et al., 2017). The role of SCF^{FBXO17} in the regulation of IL-1R8/Sigirr stability and degradation needs to be explored in the future.

We have shown that phosphorylation of a G protein-coupled receptor, LPA1, enhances its binding to its ubiquitin E3 ligase and interrupts its association with its deubiquitinase (Zhao et al., 2016). In this current study, we reveal that GSK3β-mediated T372 phosphorylation interrupts the association between IL-R8/Sigirr with USP13. The fragment between amino acids 290–374 on IL-1R8/Sigirr is the binding domain for USP13 (L. Li et al., 2019). The T372 phosphorylation site is within the USP13 binding domain, suggesting that T372 phosphorylation may modify the conformation to prevent USP13 interaction. The ubiquitin E3 ligase for IL-1R8/Sigirr has not been identified. The effect of the T372 phosphorylation on IL-1R8/Sigirr interaction with its E3 ligase is worth further investigation. Another possibility is that phosphorylation of IL-1R8/Sigirr-triggered receptor internalization may interrupt its co-localization with USP13.

In addition to phosphorylation of substrates, deubiquitinase can be phosphorylated. Phosphorylation of USP20 by protein kinase A has been reported to regulate β2 adrenergic receptors intracellular trafficking (Yu et al., 2019). We have shown that GSK3β phosphorylates USP48, ultimately resulting increases in deubiquitinase activity (S. Li et al., 2018). In this study, we focus on investigating the role of GSK3β in the phosphorylation of substrates, while USP13 contains several conserved GSK3β phosphorylation censuses sequences; thus it is possible that GSK3β may phosphorylate and regulate USP13 activation and its interaction with IL-1R8/Sigirr.

Understanding molecular regulation of IL-1R8/Sigirr is important for development of anti-inflammatory molecules to increase IL-1R8/Sigirr abundance and impede IL-1R8/TLRs-

mediated inflammatory responses. In this study, we report that GSK3β is a downstream molecule of IL-37/IL-R8/Sigirr. Activation of GSK3β plays a role of feedback control of IL-1R8/Sigirr abundance by promoting its phosphorylation, ubiquitination, internalization, and degradation. Interestingly, we reveal that the phosphorylation interrupts IL-1R8/Sigirr interaction with USP13. The biological function of this phosphorylation will be our future focus.

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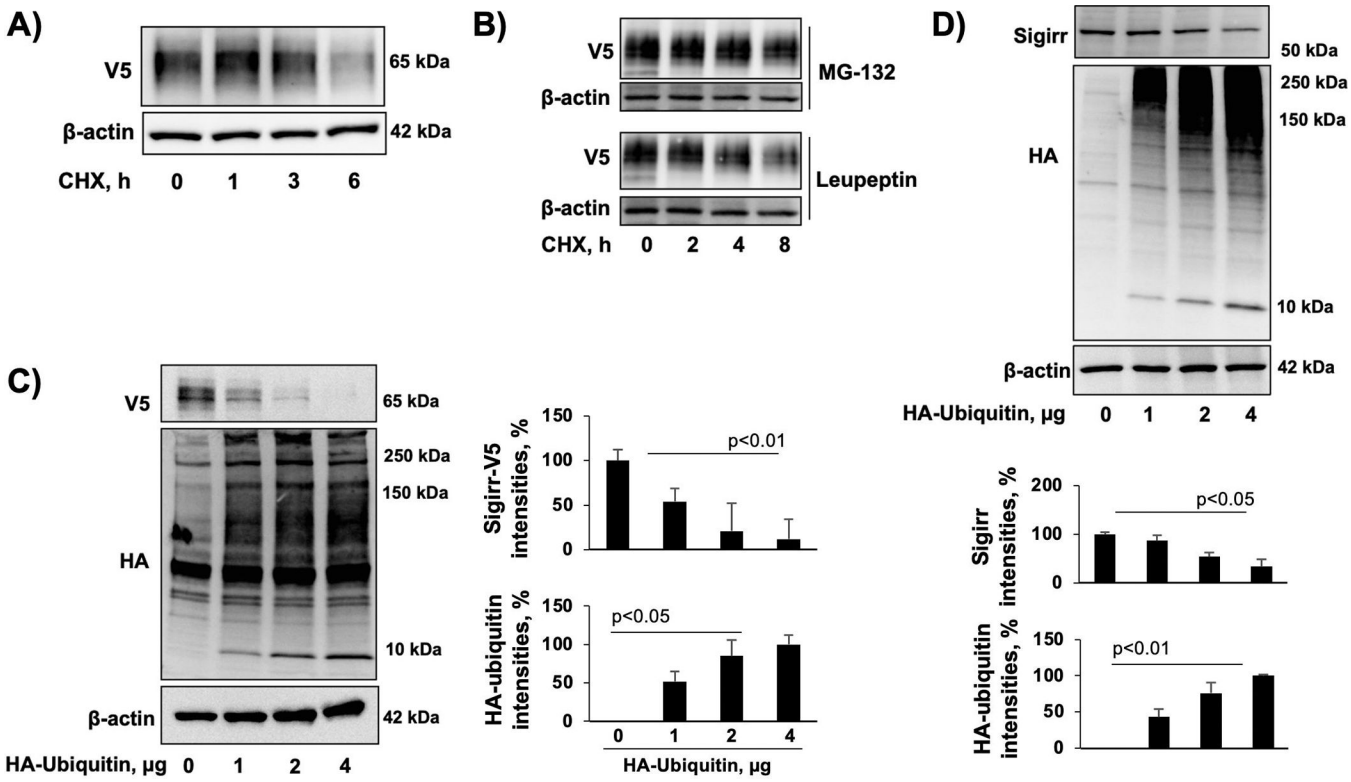

Figure 1. Sigirr degradation is regulated by the ubiquitin-proteasome system.

A, B. MLE12 cells were transfected with Sigirr-V5 plasmid for 48 h, and then cells were treated with cycloheximide (CHX, 20 μg/ml, 0, 1, 3, and 6 h) with or without MG-132 (20 μM) or leupetin (100 μM) pretreatment (1 h). Cell lysates were analyzed by immunoblotting with V5 tag and β-actin antibodies. **C.** MLE12 cells were transfected with 1 μg of Sigirr-V5 plasmid with increasing doses of HA-Ubiquitin (Ubi) plasmid for 48 h. Cell lysates were analyzed by immunoblotting with V5 tag, HA tag, and β-actin antibodies. Sigirr-V5 and HA-ubiquitin levels were analyzed and quantified. **D.** MLE12 cells were transfected with increasing doses of HA-Ubiquitin (Ubi) plasmid for 48 h. Cell lysates were analyzed by immunoblotting with Sigirr, HA tag, and β-actin antibodies. Sigirr and HA-ubiquitin levels were analyzed and quantified. All the immunoblots are representatives from three independent experiments.

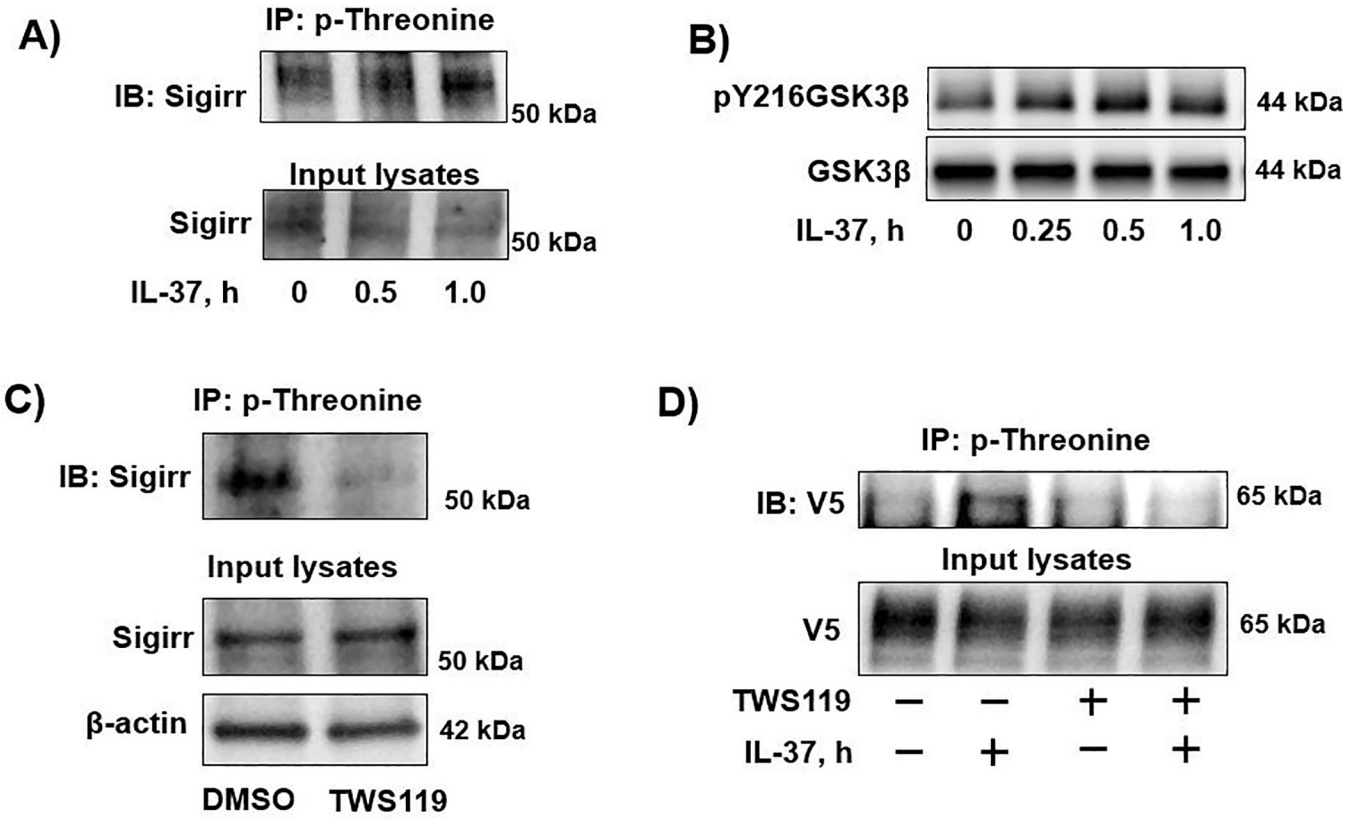

Figure 2. IL-37-induced GSK3β activation mediates Sigirr threonine phosphorylation.

A. MLE12 cells were treated with IL-37 (50 ng/ml) for 0, 0.5, and 1 h. Cell lysates were subjected to immunoprecipitation with a phospho-threonine (p-Thr) antibody, followed by immunoblotting with a Sigirr antibody. Input lysates were analyzed by Sigirr immunoblotting. **B.** THP1 cells were treated with IL-37 (50 ng/ml) for 0, 0.25, 0.5, and 1 h. Cell lysates were analyzed by immunoblotting with pY216GSK3β and GSK3β antibodies. **C.** MLE12 cells were treated with DMSO or TWS119 (10 μM) for 3 h, and then cell lysates were subjected to immunoprecipitation with a p-Thr antibody, followed by immunoblotting with a Sigirr antibody. Input lysates were analyzed by Sigirr and β-actin immunoblotting. **D.** MLE12 cells were transfected with Sigirr-V5 plasmid for 48 h, and then cells were treated with TWS119 (20 μM, 1 h) prior to IL-37 (50 ng/ml, 0.5 h) treatment. Cell lysates were subjected to immunoprecipitation with a p-Thr antibody, followed by immunoblotting with a V5 tag antibody. Input lysates were analyzed by V5 and β-actin immunoblotting. All the immunoblots are representatives from three independent experiments.

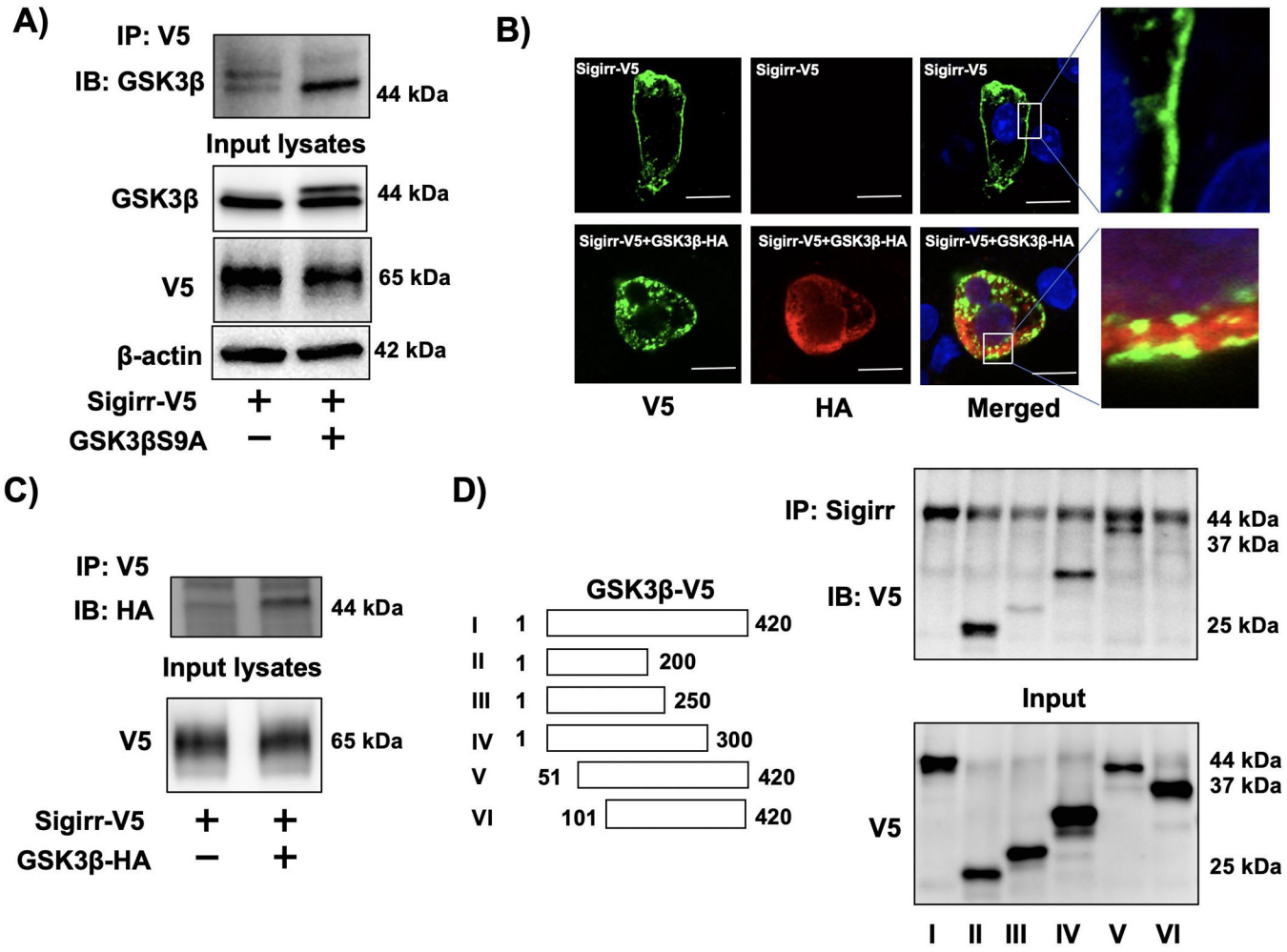

Figure 3. GSK3β is associated with Sigirr.

A. MLE12 cells were transfected with Sigirr-V5 plasmid with or without GSK3βS9A plasmid for 48 h. Cell lysates were subjected to immunoprecipitation with a V5 tag antibody, followed by immunoblotting with a GSK3β antibody. Input lysates were analyzed by GSK3β, V5, and β-actin immunoblotting. **B.** MLE12 cells were transfected with Sigirr-V5 plasmid with or without GSK3β-HA plasmid for 48 h. Co-immunofluorescence staining were performed with V5 and HA tag antibodies. Sigirr-V5 (green), GSK3β-HA (red), nuclei (DAPI). **C.** MLE12 cells were transfected with Sigirr-V5 plasmid with or without GSK3βS9A plasmid for 48 h. Cell lysates were subjected to immunoprecipitation with a V5 tag antibody, followed by immunoblotting with a HA antibody. Input lysates were analyzed by V5 immunoblotting. All the immunoblots and images are representatives from three independent experiments. **D.** V5-tagged GSK3β deletion mutants (II-VI) were generated based on GSK3β-V5 wild type (I) by site-directed mutagenesis kit. MLE12 cells were transfected with GSK3β-V5 and its mutants for 48 h, and then cell lysates were subjected to immunoprecipitation with a Sigirr antibody, followed by immunoblotting with a V5 antibody. Input lysates were analyzed by V5 immunoblotting.

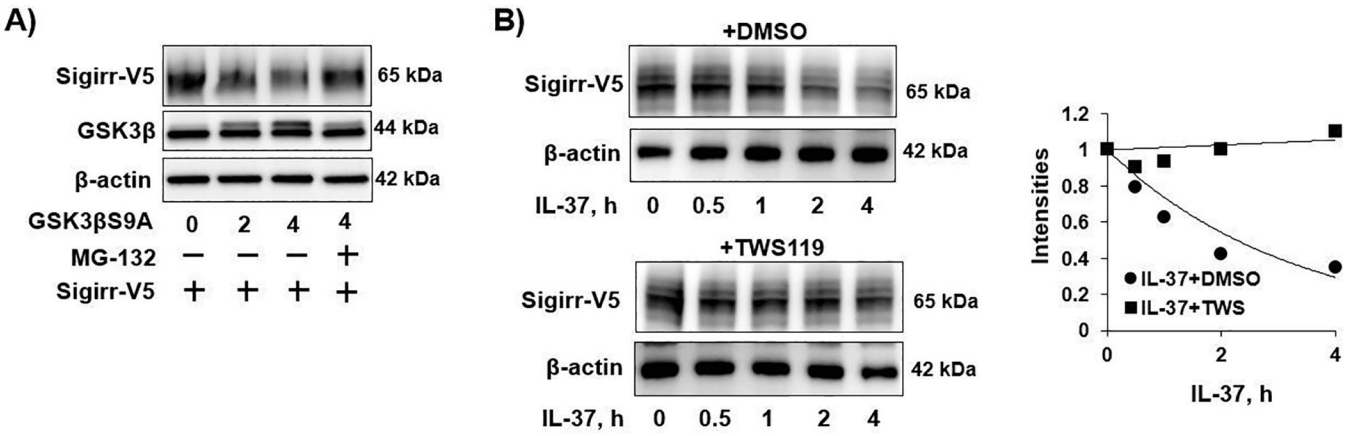

Figure 4. GSK3β regulates Sigirr stability.

A. MLE12 cells were transfected with Sigirr-V5 plasmid with or without GSK3βS9A (2 and 4 μg) for 24h, and then cells were treated with MG-132 (20 μM) for 12 h. Cell lysates were analyzed by immunoblotting with V5, GSK3β, and β-actin antibodies. **B.** MLE12 cells were transfected with Sigirr-V5 plasmid for 48 h, and then cells were treated with IL-37 (50 ng/ml) for 0, 0.5, 1, 2, and 4 h. Cell lysates were analyzed by immunoblotting with V5 tag and β-actin antibodies. Sigirr-V5 immunoblots were quantified by ImageJ software. All the immunoblots are representatives from three independent experiments.

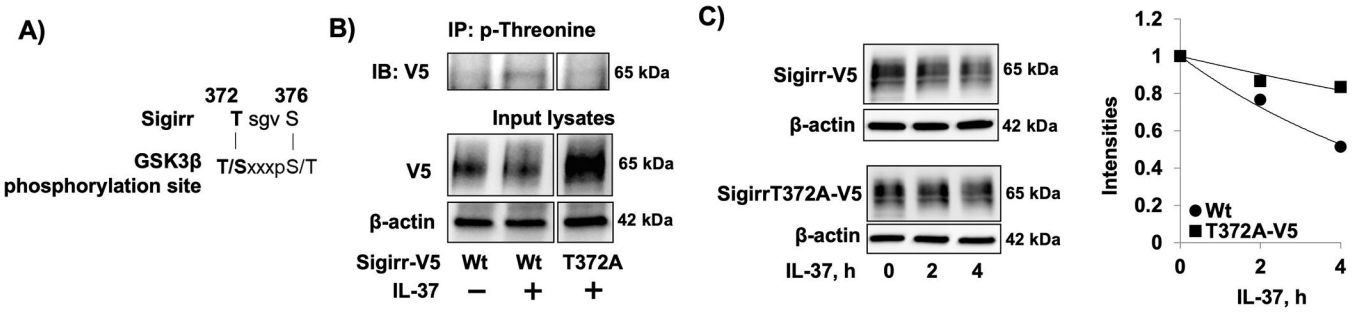

Figure 5. T372 is the phosphorylation site on Sigirr.

A. Alignment of Sigirr sequence (372-TSQVS-376) with a conserved GSK3β phosphorylation sequence. **B.** MLE12 cells were transfected with Sigirr (wild type)-V5 or SigirrT372A-V5 plasmid for 48 h, and then cells were treated with IL-37 (50 ng/ml) for 0.5 h. Cell lysates were subjected to immunoprecipitation with a p-Thr antibody, followed by immunoblotting with a V5 tag antibody. Input lysates were analyzed by V5 and β-actin immunoblotting. Blots were from different parts of the same gels. **C.** The cells from (B) were treated with IL-37 (50 ng/ml) for 0, 2, and 4 h. Cell lysates were analyzed by immunoblotting with V5 tag and β-actin antibodies. Sigirr-V5 and SigirrT372A-V5 immunoblots were quantified by ImageJ software. All the immunoblots are representatives from two or three independent experiments.

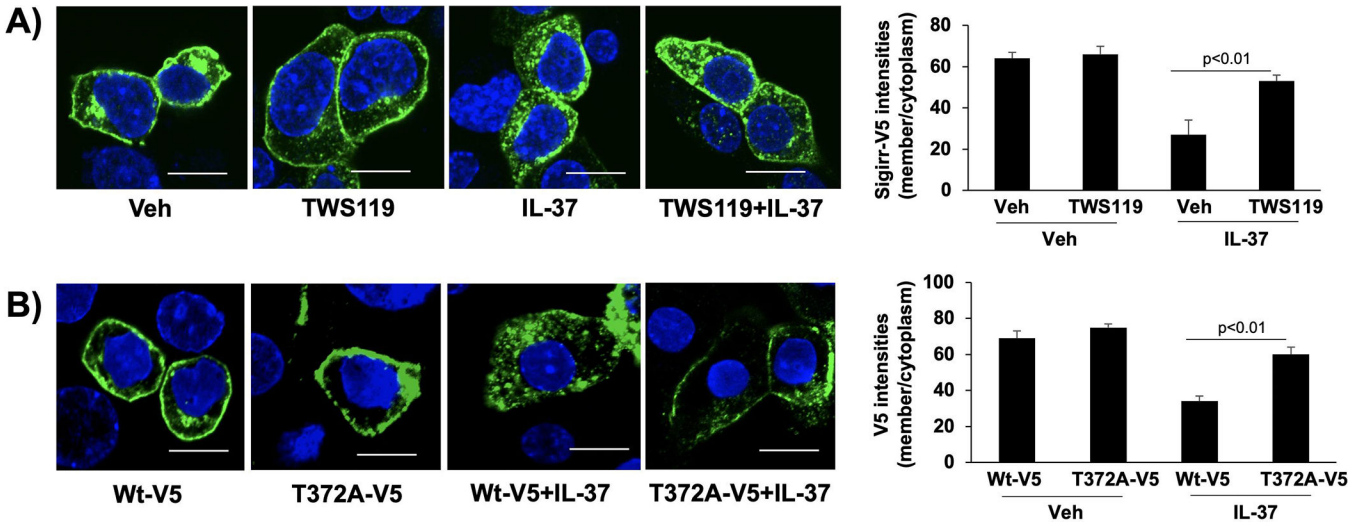

Figure 6. GSK3β-induced phosphorylation regulates Sigirr internalization.

A. MLE12 cells were transfected with Sigirr-V5 plasmid for 48 h, and then cells were treated with TWS119 (10 μM, 1 h) prior to IL-37 (50 ng/ml, 1 h) treatment. Cells were immunofluorescence stained with a V5 antibody. Sigirr-V5, green; nuclei, blue. **B.** MLE12 cells were transfected with Sigirr-V5 or SigirrT372A-V5 plasmid for 48 h, and then cells were treated with IL-37 (50 ng/ml, 1 h). Cells were immunofluorescence stained with a V5 antibody. Sigirr-V5, green; nuclei, blue. Fluorescence signals on the plasma membrane and cytoplasm were quantified. All the images are representatives from two or three independent experiments.

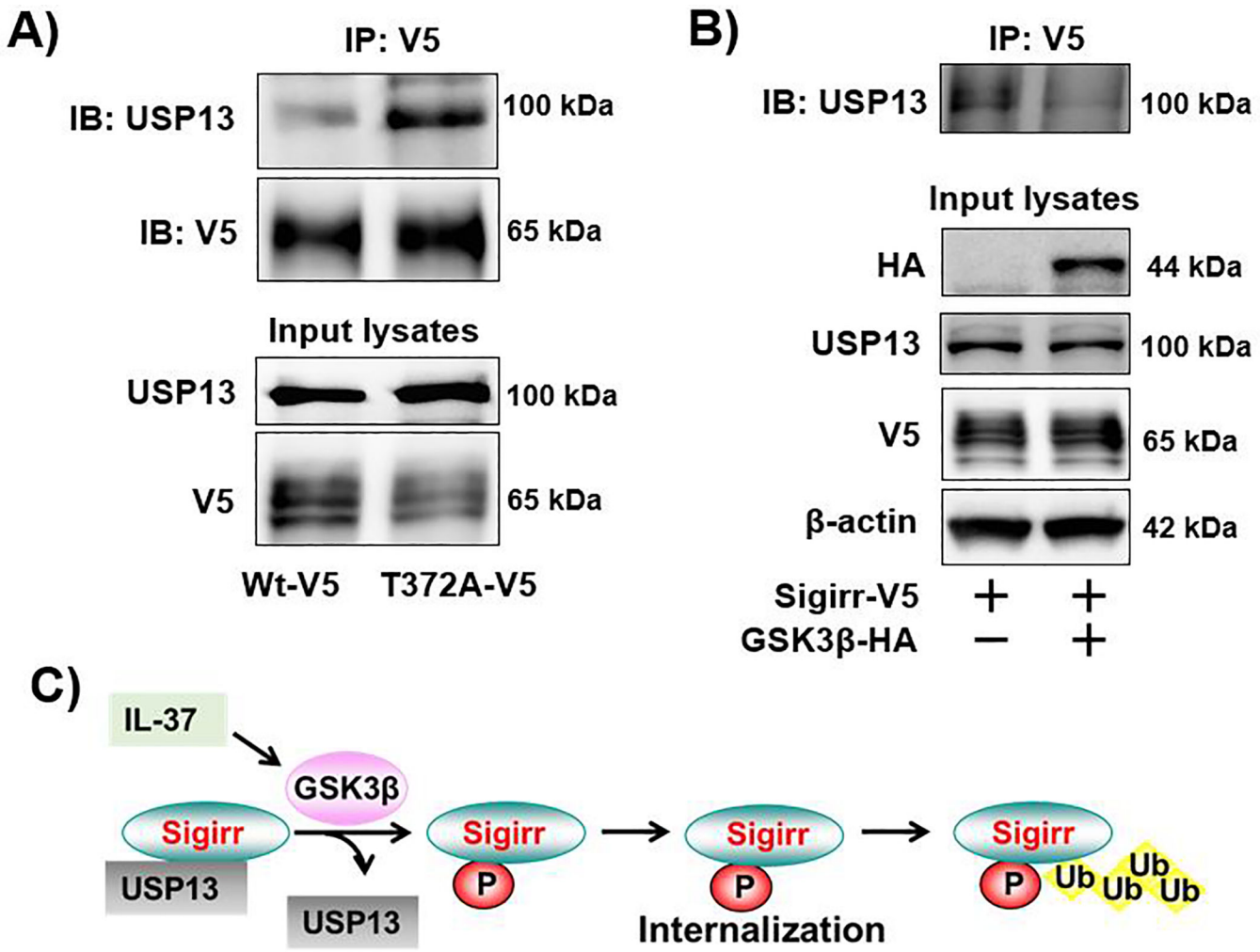

Figure 7. GSK3β-induced phosphorylation reduces Sigirr association with USP13.

A. MLE12 cells were transfected with Sigirr wild type-V5 and SigirrT372A-V5 plasmids for 48 h, and then cell lysates were subjected to immunoprecipitation with a V5 antibody, followed by immunoblotting with USP13 and V5 tag antibodies. Input lysates were analyzed by USP13 and V5 immunoblotting. **B.** MLE12 cells were transfected with Sigirr-V5 plasmid with or without GSK3β-HA plasmid for 24 h, and then cells were treated with MG-132 (20 μM, 12 h). Cell lysates were subjected to immunoprecipitation with a V5 antibody, followed by immunoblotting with a USP13 antibody. Input lysates were analyzed by HA, USP13, V5 and β-actin immunoblotting. All the immunoblots are representatives from three independent experiments. **C.** Schema shows that GSK3β-induced phosphorylation promotes Sigirr internalization and ubiquitination.