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SUMOylation Attenuates Human β-Arrestin 2 Inhibition of IL-1R/TRAF6 Signaling*

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Background: It was unknown whether human β-arrestin 2 could be SUMOylated.
Results: Human β-arrestin 2 is SUMOylated on Lys-295. SUMOylation attenuates β-arrestin 2 inhibition of IL-1R/TRAF6 signaling.
Conclusion: SUMOylation attenuates human β-arrestin 2 inhibition of TRAF6 and IL-1R signaling.
Significance: We show SUMOylation as a novel mechanism in regulation of β-arrestin 2-mediated IL-1R-TRAF6 signaling.

β-Arrestin 2 as an adaptor plays a role in the regulation of receptor desensitization, trafficking, and signaling. Bovine β-arrestin 2 has been shown to be SUMOylated on the lysine 400 residue, which links it to the endocytosis of the β_2-adrenergic receptor. Here we identify a major SUMOylation site, lysine 295, on human β-arrestin 2. SUMOylation on this site attenuates β-arrestin 2 binding to TRAF6, then enhances TRAF6 oligomerization and autoubiquitination, and consequently leads to the increase of TRAF6-mediated NF-κB/AP-1 activation. We further determine SENP1 as a specific de-SUMOylation protease that can reverse the SUMOylation of β-arrestin 2-mediated processes. Our study reveals SUMOylation as a novel mechanism in the regulation of β-arrestin 2-mediated IL-1R/TRAF6 signaling.

IL-1 signaling is a key player in the regulation of inflammatory processes. IL-1 stimulates IL-1R and downstream signaling molecules and subsequently activates the transcription factors NF-κB and AP-1, which control the expression of key immunoregulatory genes (1–3). TRAF6 is a critical mediator for the Toll-like/interleukin-1 receptor superfamily (4, 5). As a RING domain containing E3 ubiquitin ligase, TRAF6 is recruited to the receptor complexes and forms oligomers upon signaling activation and then leads to Lys-63-linked polyubiquitination of itself and downstream signaling molecules (6–8). Lys-63 ubiquitin-conjugated TRAF6 recruits TAB2 and activates the TAB2-associated TAK1 kinase, which subsequently phosphorylates and activates IκB kinases. IκB kinase then phosphorylates IκBα, leading to degradation of IκBα and, consequently activation of NF-κB. In addition, TAK1 can also activate the JNK and p38 MAPK family members, then triggering AP-1 activation (8–10). Both of oligomerization and autoubiquitination are critical for TRAF6 activity toward downstream targets to mediate IL-1β- or LPS-induced NF-κB/AP-1 activation.

β-Arrestin 2 (also known as arrestin 3), along with β-arrestin 1 (arrestin 2) and visual rod (arrestin 1) and cone (arrestin 4) arrestins, is part of a small family of cytosolic adaptor proteins functioning as crucial adaptors/mediators in the regulation of a variety of cell surface receptor desensitization, trafficking, and signaling activities (11–13). β-Arrestins have been shown to be modified by phosphorylation, ubiquitination, and nitrosylation (14–16). These modifications have linked β-arrestins to G protein-coupled receptor (GPCR)[3] internalization, trafficking and signal transduction (13). Additionally, β-arrestin 2 directly interacts with TRAF6 after TLR/IL-1R activation, leading to prevention of autoubiquitination of TRAF6 and activation of NF-κB and AP-1 (17).

Recently, bovine β-arrestin 2 has been reported as a SUMOylated protein. The SUMOylation site Lys-400 locates within the C tail of bovine β-arrestin 2, which mediates GPCR endocytosis, and the SUMOylation of bovine β-arrestin 2 has been shown to promote β-arrestin 2-mediated β_2-adrenergic receptor internalization (18). Here we also show human/murine β-arrestin 2 as a SUMOylated target. However, lysine 295, not lysine 400, has been identified as a major SUMO-conjugated site on human β-arrestin 2. We further found that SUMOylation decreases β-arrestin 2 inhibition of TRAF6 oligomerization and autoubiquitination and, consequently, promotes TRAF6-mediated NF-κB/AP-1 activation. Our study reveals SUMOylation as a novel mechanism in the regulation of β-arrestin 2-mediated IL-1R/TRAF6 signaling.

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; SUMO, small ubiquitin-like modifier; MEF, mouse embryonic fibroblast; WB, Western blot.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—The plasmids β-arrestin 2-HA, FLAG-TRAF6, and His-ubiquitin were provided by Dr. Ping Wang (Institute of Biomedical and Science, East China Normal University, China). FLAG-SUMO1, FLAG-SENP1, HA-SUMO1, RGS-SENP1, and RGS-SENP1-mu, a SENP1 catalytic mutant, have been described previously (19–21). β-Arrestin 2 point mutants (K295R and K400R) were generated using site-directed mutagenesis. SENP1-GFP, SENP1mu-GFP, and GFP-TRAF6 were generated using standard cloning procedures and PCR-based mutagenesis (Vazyme Biotech Co., Ltd). Antibodies against FLAG M2 and HA were from Sigma; GFP from Eptomics; RGS-his from Qiagen; and β-arrestin 2, IκBα, SUMO1, ERK, and p-ERK from CST.

Immunoprecipitation and Immunoblotting—Transfected cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, and a mixture of protease inhibitors) and cleared by centrifugation. Cleared cell lysates were incubated with 10 μl of anti-FLAG M2-agarose affinity gel (Sigma) or 10 μl of anti-HA-agarose affinity gel (Sigma) for 2 h. To perform an endogenous assay, MEF cells were lysed in ice-cold radioimmune precipitation assay buffer. Cleared cell lysates were incubated with anti-SUMO-1 antibody (1:200) and 15–20 μl of protein A/G beads (Santa Cruz Biotechnology) for 4 h at 4 °C. After extensive washing, beads were boiled at 100 °C for 10 min. Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore), followed by immunoblotting using corresponding antibodies according to the instructions of the manufacturer. Immunoblots were analyzed using the LAS-4000 system (Fujifilm).

Ubiquitination Assay—HEK293T cells were transfected with His-ubiquitin and FLAG-TRAF6. The transfected cells were lysed by denatured buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, and 10 mM imidazole), followed by Talon bead purification. The ubiquitination was detected by Western blot analysis.

Luciferase Assay—NF-κB- or AP-1-dependent firefly luciferase plasmids were transiently transfected into HEK293T cells with *Renilla* luciferase plasmids and others. The cells were harvested 36 h after transfection, and luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega). The relative luciferase activity was normalized on the basis of the *Renilla* luciferase activity. Data represent three independent experiments performed in duplicate.

Stable Cell Line—β-Arrestin 2 WT or β-arrestin 2 K295R mutant (K295R) lentiviral plasmids were transfected into HEK293T cells with lentivirus packaging vectors by calcium phosphate-DNA coprecipitation method. Viral supernatants were collected 48 h after transfection. MCF-7 cells were infected by lentiviral supernatant in the presence of 10 μg/ml Polybrene for 12 h. 48–72 h later, the cells were sorted for stable cell lines by flow cytometry.

RNA Isolation and Real-time RT-PCR—Total RNA was isolated from cells by using Tripure isolation reagent (Roche). For mRNA analysis, an aliquot containing 2 μg of total RNA was reverse-transcribed using the cDNA synthesis kit (Takara). Real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) and detected by the ABI Prism 7500 sequence detection system (Applied Biosystems). The primers for real-time RT-PCR were as follows: GAPDH, 5′-GAGCTGAACGGGAAGCTCACTG-3′ (sense) and 5′-TGGTGCTCAGTGTAGCCCAGGA-3′ (antisense); TNFα, 5′-CCCTCTGGCCCAGGCAGTCA-3′ (sense) and 5′-ATGGGTGGAGGGGCAGCCTT-3′ (antisense).

ELISA Assay—After serum starvation for 12 h, MCF-7 cells were cultured for 24 h with recombinant human IL-1β (20 ng/ml) (Bioworld Technology). The concentration of TNFα in culture supernatants was determined with a human-specific ELISA kit (ExCell Bio), followed by analysis with a SYNERGY microplate reader (BioTek).

RESULTS

SUMO Conjugates Human β-Arrestin 2 on Lys-295—Wyatt *et al.* (18) have reported previously that bovine β-arrestin 2 is conjugated by SUMO on residue Lys-400. However, when aligning the bovine, human, and murine β-arrestin 2 sequences, we noticed that the Lys-400 residue in human/murine β-arrestin 2 is not in a conserved SUMO consensus motif, ΨK*X*E (Fig. 1*A*), which raised the question of whether human/murine as well as bovine β-arrestin 2 could be SUMOylated. To test this, we performed an *in vivo* SUMOylation assay in HEK293T cells by cotransfecting human β-arrestin 2-HA and FLAG-SUMO1. As shown in Fig. 1, *B* and *C*, the SUMOylated β-arrestin 2 band was readily detected in cells transfected with β-arrestin 2-HA and FLAG-SUMO1. We also observed that endogenous human/mouse β-arrestin 2 was modified by endogenous SUMO1 in HEK293T cells and MEFs (Fig. 1*D* and Fig. 3*C*). These results showed that human/murine β-arrestin 2 could be also conjugated by SUMO similarly as bovine β-arrestin 2.

We mutated Lys-400 on human β-arrestin 2 to test whether this residue was a SUMO conjugation site, as bovine β-arrestin 2. As shown in Fig. 1*E*, mutating the Lys-400 residue had no significant effect on human β-arrestin 2 SUMOylation. However, when mutating Lys-295, a lysine residue located in a conserved SUMO consensus motif, LKHE (ΨK*X*E), human β-arrestin 2 SUMOylation was significantly abolished, suggesting that Lys-295, but not Lys-400, on human β-arrestin 2 is the major residue to accept SUMO.

SUMOylation Attenuates Human β-Arrestin 2 Inhibition of TRAF6 Activation—Lys-400 resides within the C tail of bovine β-arrestin 2, which mediates GPCR endocytosis. SUMOylation on this site has been shown to promote β-arrestin 2-mediated β_2-adrenergic receptor internalization (18). However, Lys-295 on human β-arrestin 2 locates in the TRAF6-binding domain. β-Arrestin 2 binding to TRAF6 has been shown to inhibit autoubiquitination of TRAF6 and activation of NF-κB and AP-1 responding to TLR-IL-1R signaling (17). We therefore proposed that SUMOylation on Lys-295 might regulate β-arrestin 2 interacting with TRAF6 and then modulate IL-1R/TRAF6 signaling. We first tested whether β-arrestin 2 SUMOylation is related to IL-1β stimulation. We showed that IL-1β induced human β-arrestin 2 SUMOylation (Fig. 2*A*). We then determined whether SUMOylation of human β-arrestin 2 could affect TRAF6 ubiquitination. As shown in Fig. 2*B*, the

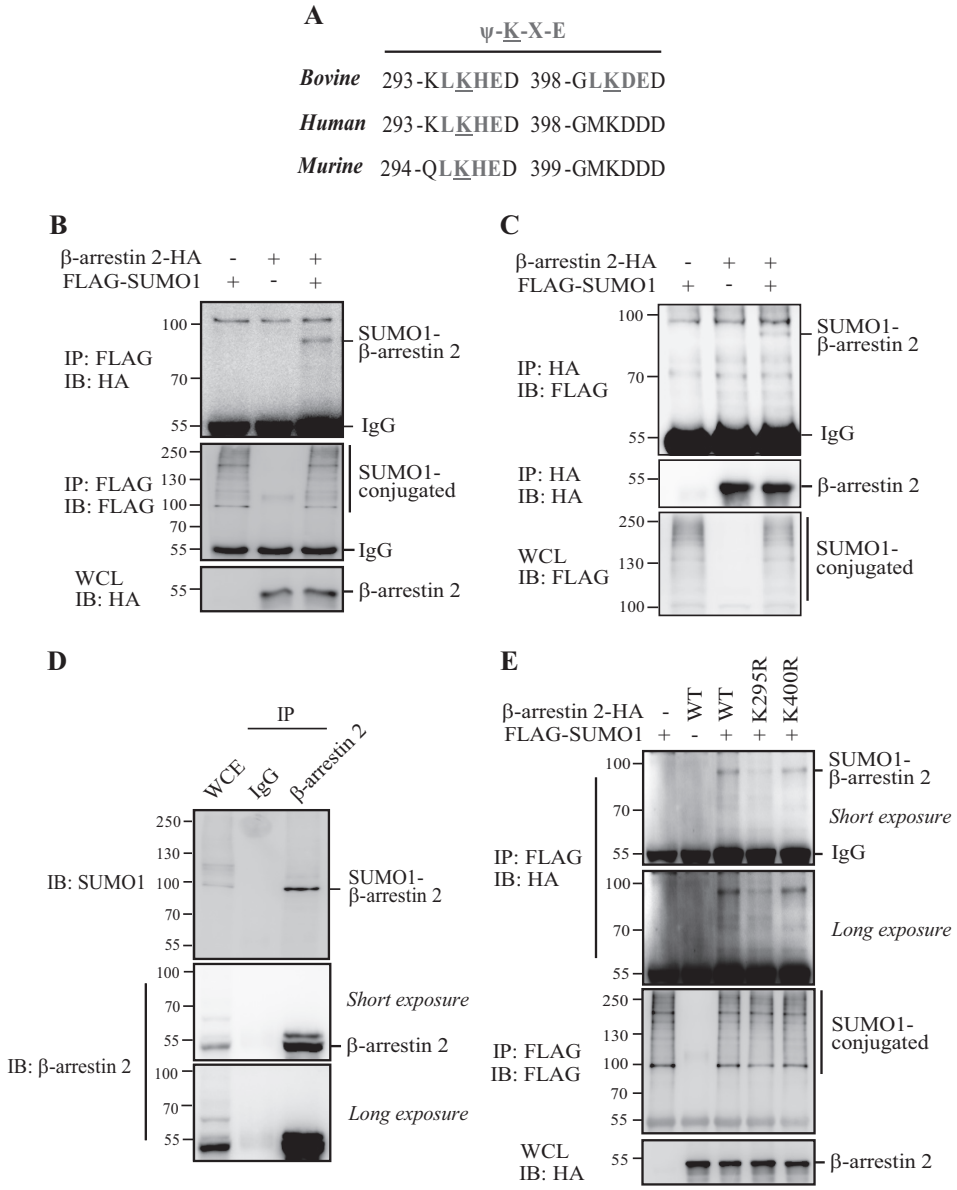


FIGURE 1. **SUMO conjugates human β-arrestin 2 on Lys-295.** *A*, the alignment of bovine/human/murine β-arrestin 2 sequences sowing the SUMO consensus motif. *B* and *C*, human β-arrestin 2 is SUMOylated *in vivo*. HEK293T cells were transfected with the indicated plasmids, and the transfected cells were harvested 36 h after transfection and immunoprecipitated with anti-FLAG M2-agarose beads or anti-HA beads. The immunoprecipitates (*IP*) and the original whole-cell lysates (*WCL*) were analyzed by immunoblotting (*IB*) with anti-HA or anti-FLAG antibodies. *D*, HEK293T cells lysates were immunoprecipitated with anti-β-arrestin 2 antibody or control IgG. The immunoprecipitates and the whole-cell lysates were analyzed by immunoblotting with anti-SUMO1 or anti-β-arrestin 2 antibodies. *E*, Lys-295 is the major SUMOylation site of human β-arrestin 2. HEK293T cells were transfected with the indicated plasmids, and the transfected cell lysates were immunoprecipitated with anti-FLAG M2-agarose beads, followed by Western blot analysis by using anti-HA or anti-FLAG antibodies.

expression of β-arrestin 2 markedly reduced TRAF6 ubiquitination (*lane 3 versus lane 2*). Interestingly, the expression of SUMO1/Ubc9 could almost restore TRAF6 ubiquitination (Fig. 2*B*, *lane 4 versus lane 3*) but had no effect on β-arrestin 2 K295R-mutant (Fig. 2*B*, *lane 5 versus lane 4*). Mutating Lys-400 on human β-arrestin 2 did not affect TRAF6 ubiquitination (Fig. 2*B*, *lane 6 versus lanes 3* and *4*), suggesting that SUMOylation on Lys-295 reduces β-arrestin 2 inhibition of TRAF6 ubiquitination. We further tested whether SUMOylation of β-arrestin 2 had a similar effect on TRAF6-mediated NF-κB and AP-1 activation as on TRAF6 ubiquitination. HEK293T cells were transfected with an NF-κB- or AP-1-dependent luciferase reporter plus TRAF6 and with or without SUMO1/Ubc9. A luciferase assay from these transfected cells showed that SUMOylation on Lys-295 also attenuates β-arrestin 2 inhibition of NF-κB and AP-1 activation (Fig. 2*C*).

We generated human β-arrestin 2 wild-type, K295R-mutant, or K400R-mutant stably transfected MCF-7, a relatively low endogenous β-arrestin 2 cell line (data not shown), to further determine the role of SUMOylation in β-arrestin 2-mediated IL-1R signaling. As shown in Fig. 2*D*, IL-1β-induced TRAF6 ubiquitination was significantly lower in β-arrestin 2 (K295R)-MCF-7 cells than in β-arrestin 2 (WT)-MCF-7 or β-arrestin 2 (K400R)-MCF-7 cells. Similarly, IL-1β-induced NF-κB or AP-1 activation was also significantly lower in β-arrestin 2 (K295R)-MCF-7 cells than that in β-arrestin 2 (WT)-MCF-7 or β-arres-

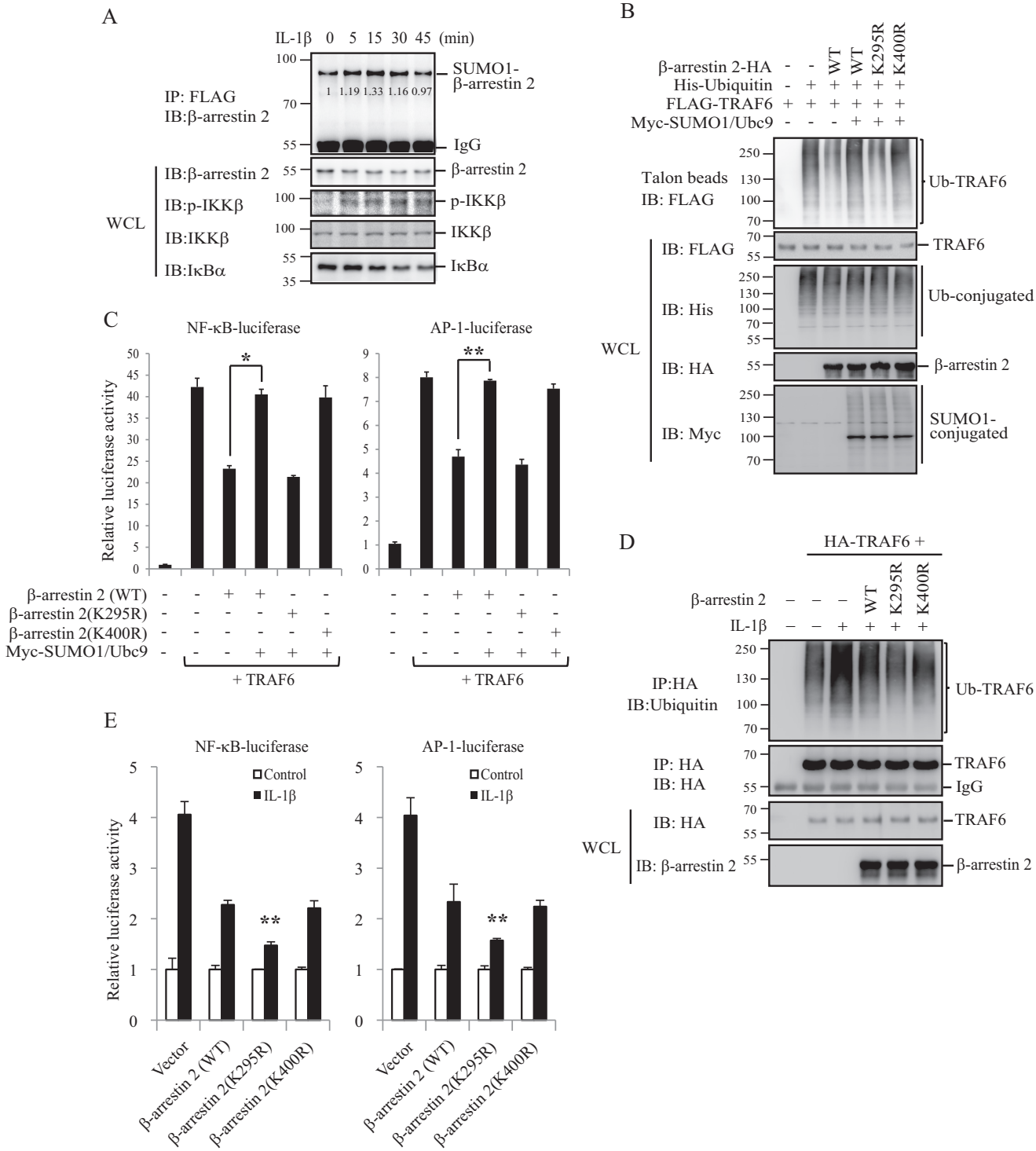


FIGURE 2. **SUMOylation attenuates human β-arrestin 2 inhibition of TRAF6 activation.** *A*, SUMOylation of β-arrestin 2 is induced by IL-1β. HEK293T cells were transfected with FLAG-SUMO1 and β-arrestin 2-HA. The transfected cells were treated with IL-1β (20 ng/ml) for the indicated time before harvest. The cell lysates were immunoprecipitated (*IP*) with anti-FLAG M2-agarose beads and analyzed by immunoblotting (*IB*) with anti-β-arrestin 2, anti-p-IKKβ, anti-IKKβ, or anti-IκBα antibodies. IKKβ phosphorylation and IκBα degradation were used as indicators for the validity of IL-1β stimuli. The relative gray scale determination was analyzed using ImageJ software. *WCL*, whole-cell lysate. *B*, SUMOylation reduces β-arrestin 2 inhibition of TRAF6 autoubiquitination. The indicated plasmids were transfected into HEK293T cells. The Talon bead precipitates and cell lysates were analyzed by immunoblotting with anti-FLAG, anti-HA, anti-His, or anti-Myc antibodies. *Ub*, ubiquitin. *C*, SUMOylation attenuates β-arrestin 2 inhibition of TRAF6-mediated NF-κB and AP-1 activation. An NF-κB- or AP-1-dependent firefly luciferase reporter and a *Renilla* luciferase reporter plus the indicated plasmids were cotransfected into HEK293T cells. The relative luciferase activity was measured 36 h after transfection and normalized on the basis of *Renilla* luciferase activity. *, $p < 0.05$; **, $p < 0.01$. *D*, β-arrestin 2 WT-, β-arrestin 2 K295R-, or β-arrestin 2 K400R-MCF-7 stable cells were transfected with HA-TRAF6 or not as indicated. The cells were treated with IL-1β. The cell lysates were immunoprecipitated with anti-HA-agarose beads, and the immunoprecipitates and the cell lysates were analyzed by immunoblotting with anti-ubiquitin, anti-HA, or anti-β-arrestin 2 antibodies. *E*, β-arrestin 2 WT-, β-arrestin 2 K295R-, or β-arrestin 2 K400R-MCF-7 stable cells were transfected with an NF-κB- or AP-1-dependent firefly luciferase reporter and a *Renilla* luciferase reporter. The relative luciferase activity was measured 36 h after transfection and normalized on the basis of *Renilla* luciferase activity. *, $p < 0.05$; **, $p < 0.01$.

tin 2 (K400R)-MCF-7 cells (Fig. 2*E*). These results suggest that SUMOylation on Lys-295 could attenuate β-arrestin 2 inhibition of TRAF6 activation and TRAF6-mediated IL-1R signaling.

SENP1 De-SUMOylates β-Arrestin 2—We observed that SUMO-specific protease 1 (SENP1) could bind to β-arrestin 2 (Fig. 3*A*). Therefore, we reasoned that SENP1 could be a deconjugation protease for SUMOylated β-arrestin 2. To test this possibility, we performed a de-SUMOylation assay in the transfected cells. As shown in Fig. 3*B*, the expression of SENP1 wild-type but not the SENP1 catalytic mutant made the SUMOylated β-arrestin 2 band disappear. We further confirmed SENP1 as a specific de-SUMOylation protease of β-arrestin 2 by observing the accumulation of the SUMOylated β-arrestin 2 in *Senp1*$^{-/-}$ MEFs (Fig. 3*C*).

Because SUMOylation reduces β-arrestin 2 inhibition of TRAF6 autoubiquitination and TRAF6-mediated NF-κB/AP-1 activation (Fig. 2), we speculated that SENP1 could enhance human β-arrestin 2 inhibition of TRAF6 ubiquitination and activation through de-SUMOylation. To test this, we performed an ubiquitination assay in HEK293T cells that were cotransfected with FLAG-TRAF6 and His-Ubiquitin plus β-arrestin 2-HA in the presence of SENP1-GFP wild-type or the catalytic mutant SENP1mu-GFP. As shown in Fig. 3*D*, the coexpression of SENP1 wild-type, not the catalytic mutant, and β-arrestin 2 decreased TRAF6 ubiquitination more than expression of β-arrestin 2 alone. Interestingly, SENP1-reduced TRAF6 ubiquitination depended on β-arrestin 2 because SENP1 action on TRAF6 ubiquitination could not be detected without the coexpression of β-arrestin 2. We also assessed the effect of SENP1 on TRAF6-mediated NF-κB and AP-1 activation by using an NF-κB- or AP-1-dependent luciferase assay. Similarly, SENP1 wild-type, not the catalytic mutant, can enhance β-arrestin 2 inhibition of TRAF6-mediated NF-κB or AP-1 activation (Fig. 3*E*).

We further determined the role of SENP1 in IL-1R signaling by using *Senp1*$^{-/-}$ MEFs. As shown in Fig. 3*F*, IL-1β induced more TRAF6 ubiquitination in *Senp1*$^{-/-}$ MEFs than that in wild-type MEFs. We also observed that IL-1β-induced more TRAF6 ubiquitination in SENP1 knockdown β-arrestin 2 stably transfected MCF-7 cells (Fig. 3*G*). More interestingly, there was no effect on β-arrestin 2 (K295R)-MCF-7 cells (Fig. 3*G*, *lane 2 versus lane 4*), indicating that SENP1 negative regulation of TRAF6 ubiquitination is dependent on de-SUMOylation of β-arrestin 2. Similarly, IL-1β induced more IκBα phosphorylation and ERK phosphorylation in *Senp1*$^{-/-}$ MEFs than that in wild-type MEFs (Fig. 3*H*). These data suggest that SENP1 enhances β-arrestin 2 inhibition of TRAF6-mediated signaling through de-SUMOylation of β-arrestin 2.

SUMOylation Decreases β-Arrestin 2 Binding to TRAF6—β-Arrestin 2 has been reported to directly interact with TRAF6 after TLR-IL-1R activation, leading to prevention of TRAF6 oligomerization and autoubiquitination (17). Because SUMOylation on Lys-295 attenuates β-arrestin 2 inhibition of TRAF6 autoubiquitination and activation (Fig. 2), we reasoned that SUMOylation might disrupt β-arrestin 2-TRAF6 interaction, which reduces β-arrestin 2 inhibition of TRAF6. To test this, we showed that the coexpression of SUMO1/Ubc9, which promotes β-arrestin 2 SUMOylation, could decrease both β-arrestin 2 wild-type and β-arrestin 2 K400R-mutant binding to TRAF6 (Fig. 4*A*, *lanes 3* and *5 versus lane 2*) but has no effect on β-arrestin 2 K295R-mutant (Fig. 4*A*, *lane 4 versus lane 2*). Furthermore, we assessed β-arrestin 2-TRAF6 interaction in wild-type and *Senp1*$^{-/-}$ MEFs. As shown in Fig. 4*B*, TRAF6 was able to bind to β-arrestin 2 in *Senp1*$^{-/-}$ MEFs much less than that in wild-type MEFs. These results suggest that SUMOylation on Lys-295 could attenuate β-arrestin 2 binding to TRAF6.

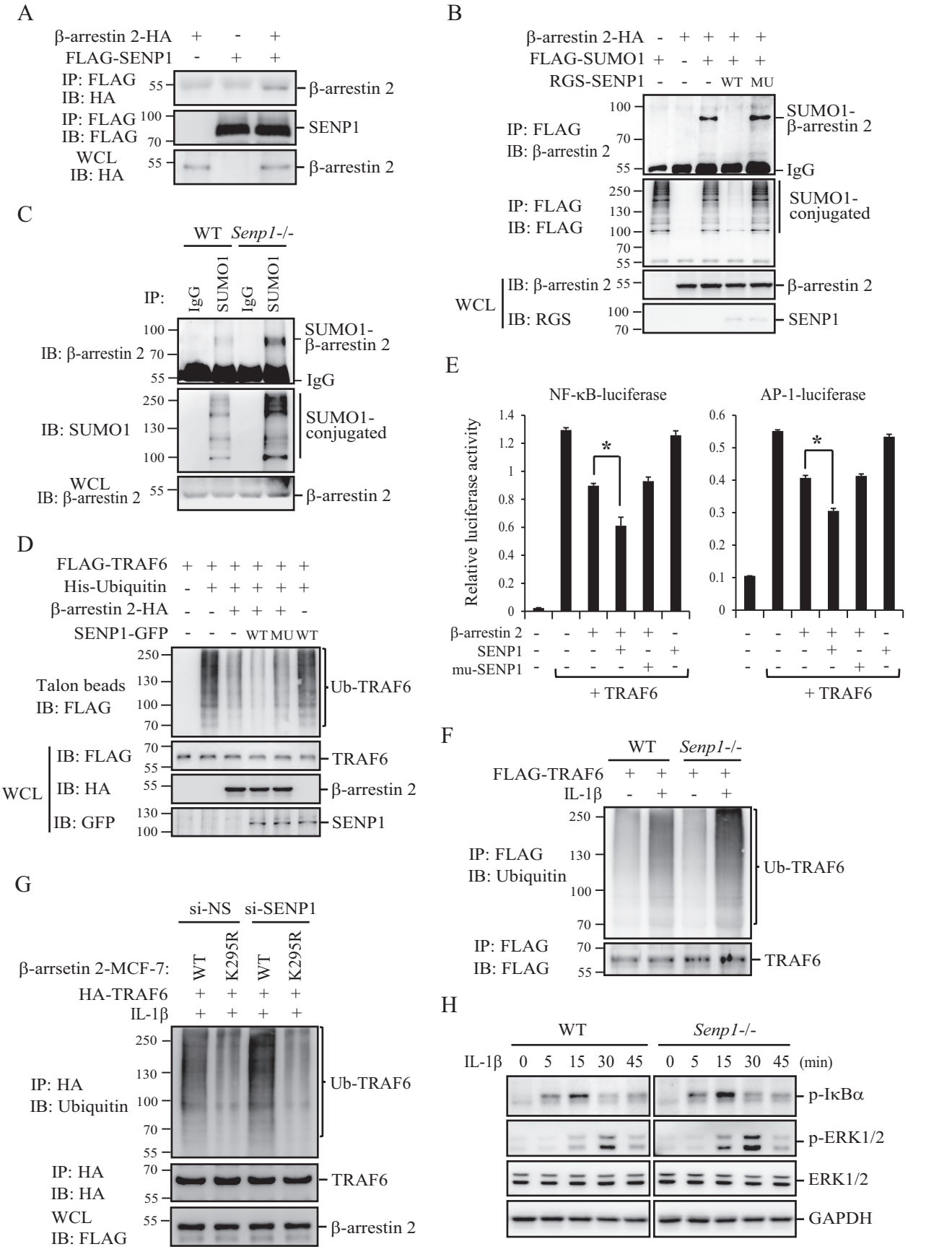
Because oligomerization is necessary for TRAF6 autoubiquitination (8), we reasoned that SUMOylation of β-arrestin 2 would enhance TRAF6 oligomerization by attenuating β-arrestin 2 interaction with TRAF6. To test this, we cotransfected differently tagged TRAF6 into HEK293T cells. The coexpression of β-arrestin 2 reduced the association of GFP-TRAF6 with FLAG-TRAF6 (Fig. 4*C*, *lane 3 versus lane 2*). Interestingly, the β-arrestin 2 K295R-mutant blocked TRAF6 oligomerization more efficiently than β-arrestin 2 wild-type did (Fig. 4*C*, *lane 4 versus lane 3*), suggesting that SUMOylation of β-arrestin 2 could enhance TRAF6 oligomerization through disrupting the interaction between β-arrestin 2 and TRAF6.

Deficiency in SUMOylation Enhances β-Arrestin 2 Inhibition of IL-1β-induced TNFα Expression—To determine the significance of β-arrestin 2 SUMOylation *in vivo*, we compared TNFα expression in β-arrestin 2 (WT)-MCF7 and β-arrestin 2 (K295R)-MCF7 cells. As shown in Fig. 5*A*, the expression of β-arrestin 2 wild-type reduced IL-1β-induced *TNFα* mRNA expression. However, the expression of β-arrestin 2 K295R-mutant showed more of a reduction of the *TNFα* mRNA level than β-arrestin 2 wild-type. Furthermore, the ELISA assay confirmed that IL-1β-treated β-arrestin 2 (K295R)-MCF7 cells produced much less TNFα in culture medium than β-arrestin 2 (WT)-MCF7 cells (Fig. 5*B*). Taken together, these results indicate that SUMOylation attenuates β-arrestin 2 inhibition of IL-1β-induced TNFα expression.

DISCUSSION

In this study, we identify Lys-295 as a major SUMOylation site on human β-arrestin 2. Because this site locates in the TRAF6-binding domain, SUMOylation can attenuate β-arrestin 2 binding to TRAF6. Therefore, SUMOylation of β-arrestin 2 can enhance TRAF6 oligomerization and autoubiquitination and, consequently, activate TRAF6-mediated IL-1R signaling. We also found SENP1 as a specific de-SUMOylation protease of β-arrestin 2 in these processes to enhance β-arrestin 2 inhibition of TRAF6 activation. These data reveal SUMOylation as a novel mechanism to attenuate β-arrestin 2 inhibition of TRAF6 and IL-1R signaling.

As an important adaptor, β-arrestins are widely involved in receptors, especially GPCR family, desensitization, trafficking, signaling, and regulating a growing list of cellular processes such as chemotaxis, apoptosis, inflammatory processes, and metastasis (13, 22). More recently, studies have shown that β-arrestins function as scaffold proteins for many signaling molecules in the cytoplasm and nucleus, thereby regulating gene expression and cellular responses (23). Besides traditional GPCR signaling, β-arrestins participate in the regulation of other signaling pathways, including TLR/IL-1R signaling and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β-arrestin 2-HA + - +
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IB: SUMO1
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IB: β-arrestin 2
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His-Ubiquitin - + + + + +
β-arrestin 2-HA - - + + + -
SENP1-GFP - - - WT MU WT
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Talon beads
IB: FLAG
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TRAF6
IB: HA
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β-arrestin 2
IB: GFP
130
100
SENP1
E
NF-κB-luciferase
AP-1-luciferase
Relative luciferase activity
1.4
1.2
1
0.8
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WT Senp1-/-
FLAG-TRAF6 + + + +
IL-1β - + - +
250
130
100
70
IP: FLAG
IB: Ubiquitin
Ub-TRAF6
IP: FLAG
70
55
IB: FLAG
TRAF6
G
si-NS si-SENP1
β-arrsetin 2-MCF-7: WT K295R WT K295R
HA-TRAF6 + + + +
IL-1β + + + +
250
130
100
70
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IP: HA
IB: Ubiquitin
Ub-TRAF6
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55
IB: HA
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WCL
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ERK1/2
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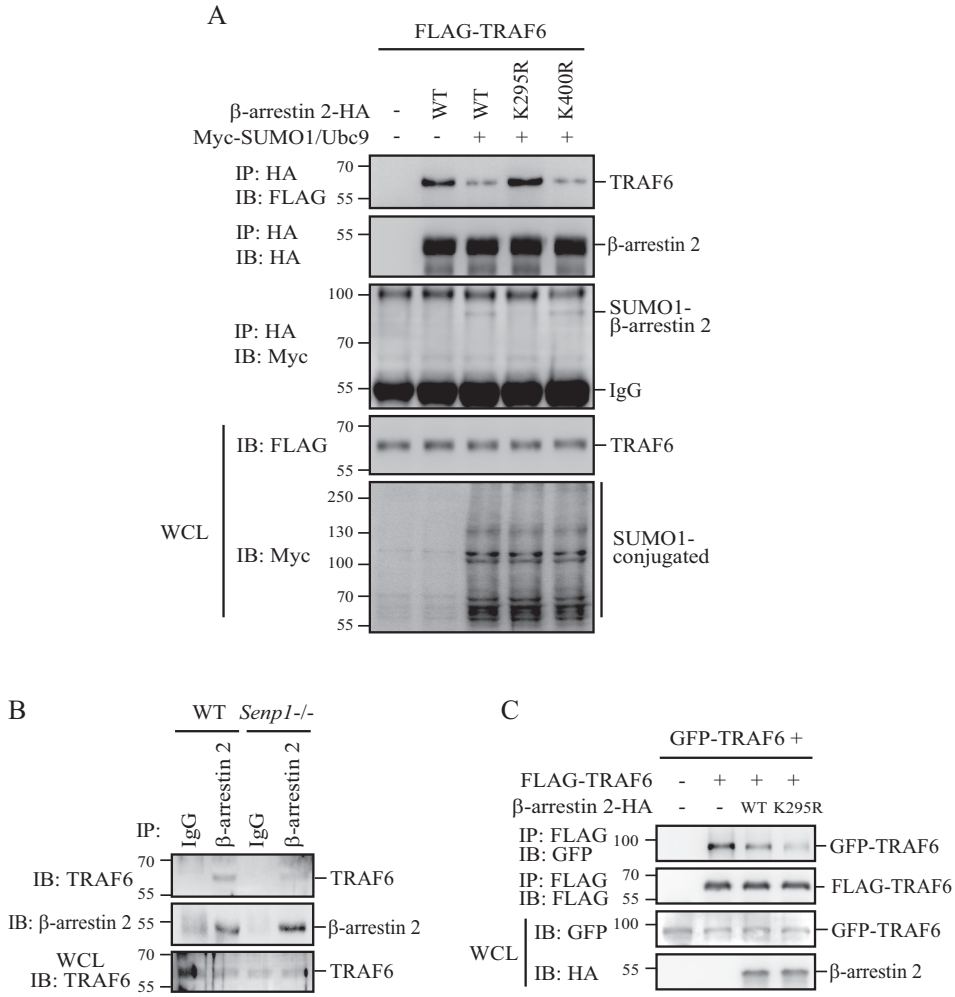

FIGURE 4. **SUMOylation decreases β-arrestin 2 binding to TRAF6.** *A*, HEK293T cells were transfected with the indicated plasmids. The cell lysates were immunoprecipitated (*IP*) with anti-HA-agarose beads. The immunoprecipitates and cell lysates were analyzed by WB with anti-FLAG, anti-HA, or anti-Myc antibodies. *IB*, immunoprecipitation; *WCL*, whole-cell lysate. *B*, *Senp1*$^{-/-}$ MEFs or wild-type MEF cell lysates were immunoprecipitated with anti-β-arrestin 2 antibody or control IgG. Precipitates and cell lysates were analyzed by WB with anti-β-arrestin 2 or anti-TRAF6 antibodies. *C*, GFP-TRAF6 and FLAG-TRAF6 were cotransfected into HEK293T cells with β-arrestin 2-HA wild-type or K295R-mutant, and the transfected cells were harvested 24 h after transfection. The cell lysates were immunoprecipitated with anti-FLAG M2-agarose beads. The precipitates and cell lysates were analyzed by WB with anti-GFP, anti-FLAG, or anti-HA antibodies.

Wnt/β-catenin signaling (17, 24–26). In TLR/IL-1R signaling, β-arrestin 2 has been reported to directly bind to TRAF6 and then reduce TRAF6 autoubiquitination and activation, leading to a negative regulation of TLR/IL-1R signaling. Here we further find β-arrestin 2 SUMOylation, as a negative mechanism, to attenuate β-arrestin 2 inhibition of TRAF6 activation and then promote TRAF6 oligomerization and autoubiquitination.

SUMOylation is an essential posttranslational modification with critical roles in regulating protein functions such as localization, activity, and protein-protein interaction (27–29). Typ-

FIGURE 3. **SENP1 de-SUMOylates β-arrestin 2.** *A*, SENP1 binds to β-arrestin 2. HEK293T cells were transfected with the indicated plasmids, and the transfected cell lysates were immunoprecipitated (*IP*) with anti-FLAG M2-agarose beads, followed by WB analysis with anti-HA or anti-FLAG antibodies. *IB*, immunoblot; *WCL*, whole-cell lysate. *B*, SENP1 deconjugates SUMOylated β-arrestin 2 *in vivo*. HEK293T cells were transfected with the indicated plasmids, and the transfected cell lysates were immunoprecipitated with anti-FLAG M2-agarose beads. The immunoprecipitates and the whole-cell lysates were analyzed by immunoblotting with anti-β-arrestin 2, anti-FLAG, or anti-RGS-his antibodies. *MU*, mutant. *C*, SUMOylated β-arrestin 2 is accumulated in *Senp1*$^{-/-}$ MEFs. MEF cell lysates were immunoprecipitated with anti-SUMO1 antibody or control IgG. The immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-β-arrestin 2 or anti-SUMO1 antibodies. *D*, SENP1 enhances β-arrestin 2 inhibition of TRAF6 autoubiquitination. The indicated plasmids were transfected into HEK293T cells. The cell lysates were purified by Talon beads. The precipitates and cell lysates were immunoblotted using anti-FLAG, anti-HA, or anti-GFP antibodies. *E*, SENP1 enhances β-arrestin 2 inhibition of TRAF6-mediated NF-κB and AP-1 activation. The indicated plasmids were cotransfected into HEK293T cells with an NF-κB- or AP-1-dependent firefly luciferase reporter and a *Renilla* luciferase reporter. The relative luciferase activity was measured 36 h after transfection and normalized on the basis of *Renilla* luciferase activity. *, $p < 0.05$; **, $p < 0.01$. *F*, FLAG-TRAF6-transfected wild-type and *Senp1*$^{-/-}$ MEFs were treated with IL-1β or left untreated before harvest. The cell lysates were immunoprecipitated with anti-FLAG M2-agarose beads, followed by WB analysis with anti-ubiquitin or anti-TRAF6 antibodies. *G*, β-arrestin 2 WT- or β-arrestin 2 K295R-MCF-7 stable cells were transfected with HA-TRAF6 and si-SENP1 or si-NS oligonucleotides. The cells were treated with IL-1β before harvest, the cell lysates were immunoprecipitated with anti-HA-agarose beads, and the immunoprecipitates and the cell lysates were analyzed by immunoblotting with anti-ubiquitin, anti-HA, or anti-FLAG antibodies. *H*, wild-type and S*enp1*$^{-/-}$ MEFs were treated with IL-1β for the indicated time before harvest, followed by WB analysis with the indicated antibodies.

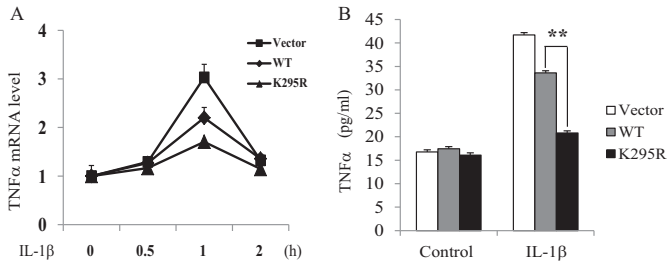

FIGURE 5. **Deficiency in SUMOylation enhances β-arrestin 2 inhibition of IL-1β-induced TNFα expression.** *A*, quantitative real-time RT-PCR analysis showing the relative *TNFα* mRNA level in β-arrestin 2 WT- or β-arrestin 2 K295R-MCF-7 stable cell lines treated with IL-1β for the indicated time. The results are representative of three experiments. *B*, ELISA analysis showing the TNFα concentration in supernatants of β-arrestin 2 WT- or β-arrestin 2 K295R-MCF-7 stable cell lines after IL-1β stimulation. The results are representative of three experiments. **, $p < 0.01$.

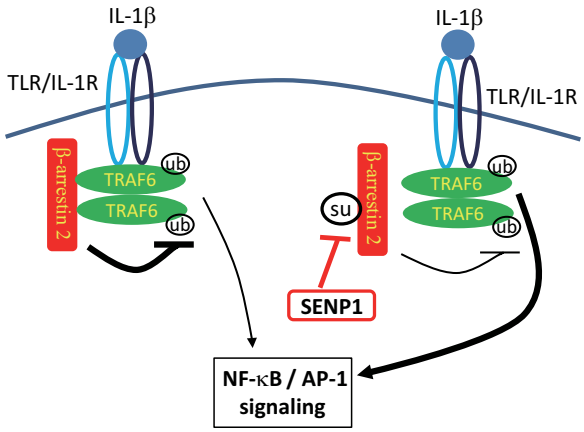

FIGURE 6. **Working model for the regulation of IL-1R-TRAF6 signaling by β-arrestin 2 SUMOylation.**

ically, SUMO-modified proteins contain a SUMO consensus motif defined as ΨK*X*E, where Ψ is a large hydrophobic residue and *X* represents any amino acid (30). Bovine β-arrestin 2 has been shown to be SUMOylated. SUMOylation of bovine β-arrestin 2 does not influence the affinity of receptor interaction but is important for AP-2 interaction and AP-2-mediated receptor internalization (18). However, the Lys-400 residue in the carboxyl-terminal region of human β-arrestin 2 is not a major SUMOylation site because mutation of this site did not significantly affect its SUMOylation status. We identified Lys-295 as a major SUMOylation site on human β-arrestin 2, although there might be other potential SUMOylation sites. This site locates in the TRAF6-binding domain of human β-arrestin 2. Therefore, we observed that SUMOylation of human β-arrestin 2 plays a role in the regulation of TRAF6 activation and TRAF6-mediated signaling. It would be interesting to study whether it is evolution-related that the different SUMOylation sites exist on bovine and human/murine β-arrestin 2. In summary, our study reveals that SUMOylation modulates human β-arrestin 2-mediated inhibition of TRAF6 and TRAF6-mediated IL-1R signaling (Fig. 6).

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