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KIAA0317 regulates SOCS1 stability to ameliorate colonic inflammation

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Abstract

Dysregulated cytokine signaling is a hallmark of inflammatory bowel diseases. Inflammatory responses of the colon are regulated by suppressor of cytokine signaling (SOCS) proteins. SOCS1 is a key member of this family, and its function is critical in maintaining an appropriate inflammatory response through the JAK/STAT signaling pathway. Dysregulation of SOCS1 protein has been identified as a causal element in colonic inflammatory diseases. Despite this, it remains unclear how SOCS1 protein is regulated. Here, we identify that SOCS1 protein is targeted for degradation by the ubiquitin proteasome system, mediated by the E3 ubiquitin ligase KIAA0317 during experimental colonic inflammation. We characterize the mechanism of protein-protein interaction and ubiquitin conjugation to SOCS1 and demonstrate that modulation of SOCS1 protein level leads to stark effects on JAK/STAT inflammatory signaling. Together, these results provide insight into the regulation of colonic inflammation through a new mechanism of ubiquitin-based control of SOCS1 protein.

Keywords

Signal transducers and activators of transcription 1 (STAT1); E3 ubiquitin ligase; Protein ubiquitination; Suppressor of cytokine signaling 1 (SOCS1); inflammatory bowel disease (IBD)

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AUTHOR CONTRIBUTIONS

B.B.C., Y.L. designed and directed the study. K.C.L., T.B.L., B.B.C. analyzed the data, prepared the figures. K.C.L., T.B.L., B.B.C. wrote the manuscript. K.C.L., T.B.L., S.R., A.C.M., S.R.D., A.N.B., performed all experiments. T.B.L., Y.L., B.B.C. provided funding for the studies.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

Introduction:

SOCS proteins play important roles in the regulation of cytokine signaling. Key among the SOCS family is SOCS1, which is particularly important in regulating JAK-STAT signaling, as it can either facilitate JAK protein stability [1–3] or directly inhibit JAK activity by binding the key JAK domain [4–6], ultimately impairing STAT-dependent signaling and inflammation [7, 8]. Several studies in the past 20 years have shown SOCS1 to be associated with diseases marked by colonic inflammation, such as Ulcerative colitis and Crohn's disease, with SOCS1 providing a 'protective' function [9]. Indeed, loss-of-function mutations in the SOCS1 gene have been associated with development of autoimmunity characterized by amplified JAK-STAT signaling [10]. Given the important role of SOCS1 in modulating JAK-STAT signaling, the regulation of SOCS1 itself has disease-relevant consequences. MicroRNA have been shown to regulate SOCS1 expression, leading to a pro-inflammatory colonic phenotype [11, 12]. Viral protein during Hepatitis B infection interacts with SOCS1 and impairs its activity [13]. Recent studies have noted RNF7 affects SOCS1 protein stability in the context of renal carcinoma [14], and SOCS1 protein stability can be prolonged through de-ubiquitination by JOSD1 [15] or interaction with the protein CUEDC2 in acute myeloid leukemia [16]. Despite these studies, the question of SOCS1 post-translational regulation in colonic inflammation remains unsolved. We sought to elucidate the mechanisms of SOCS1 stability and their effects on JAK-STAT signaling through investigating if colonic epithelial SOCS1 is controlled by the ubiquitin proteasome system (UPS).

Ubiquitination is a process targeting proteins to be degraded by the lysosome or proteosome by tagging them with the small protein ubiquitin. In this enzymatic cascade, three enzymes work to tag a substrate protein with ubiquitin, usually at a target lysine residue [17]. An enzymatic cascade shuttles ubiquitin to target proteins, with the final pathway enzyme, the E3 ubiquitin ligase, playing the critical role for substrate engagement. There are estimated to be more than 600 E3 ligases, with such diversity hypothesized to confer specificity in target selection [17]. This process, and particularly the substrate-targeting E3 ligases, have been implicated in the course of inflammatory diseases, including inflammatory bowel diseases [18].

In this study, we investigated the regulation of SOCS1 stability through the UPS and discovered that induction of colonic inflammation acutely destabilized SOCS1 protein. We also uncovered the E3 ubiquitin ligase KIAA0317 as a key regulator of SOCS1 ubiquitination and degradation and elucidated the key molecular regions of SOCS1 and KIAA0317 required for degradation. Further, we analyzed downstream effects on the JAK/STAT pathway mediated by the SOCS1/KIAA0317 axis upon immune stimulation.

Results:

SOCS1 protein is ubiquitinated and regulated by the proteasome, mediated by E3 ubiquitin ligase KIAA0317

We and others have previously described the protein KIAA0317 as an E3 ligase involved in the degradation of several anti-inflammatory proteins [19–22], including the protein

SOCS2. Due to the homology between SOCS1 and SOCS2 (Fig. 1A), we hypothesized that SOCS1 could also be the substrate of E3 ligase KIAA0317. To test this, we first treated HCT-8 cells with cycloheximide (CHX) – a ribosomal inhibitor, and either Leupeptin, a lysosomal inhibitor, or MG132, a proteasomal inhibitor. SOCS1 stability decreased in both control and leupeptin treatments. Conversely, MG132 co-treatment led to significant SOCS1 preservation, suggesting that SOCS1 is degraded by the proteasome (Fig. 1B). We further tested SOCS1 ubiquitination in cells. A UbiCREST assay was performed following cell treatment with Carfilzomib (Fig. 1C). SOCS1 poly-ubiquitination treated with various de-ubiquitinase enzymes to uncover the character of ubiquitin linkage [23]. SOCS1 poly-ubiquitin signal decreased following treatment with AMSH, suggesting SOCS1 contains K63-linked poly-ubiquitin chains. Finally, KIAA0317 showed sufficiency to catalyze SOCS1 poly ubiquitination in vitro (Fig. 1D).

The Ubiquitin E3 ligase KIAA0317 regulates SOCS1 protein stability and ubiquitination in colonic epithelial cells.

We further investigated the role of the E3 ligase KIAA0317 in SOCS1 ubiquitination in colonic epithelial cells. KIAA0317 expression increased SOCS1 poly-ubiquitination signal upon pulldown from HCT-8 cells (Fig. 2A). To further validate our findings, cells were transfected with either WT KIAA0317 or a catalytically inactive mutant of KIAA0317 (C770S) [19]. WT KIAA0317 expression resulted in a dose dependent decrease in SOCS1 protein levels, without an effect on known complex partners Elongin B or C, as determined by immunoblotting (Fig. 2B). Conversely, SOCS1 protein levels were stable under C770S mutant KIAA0317 expression, further indicating that ligase activity underlies the effect of KIAA0317 on SOCS1 (Fig. 2B). Expression of KIAA0317 C770S resulted in reduced poly-ubiquitinated SOCS1 protein relative to WT (Fig. 2C). Two other HECT-family E3 ligases, UBE3B and HECTD4, were tested as a control; their expression did not affect SOCS1 protein levels, suggesting SOCS1 ubiquitination is not a pan-HECT E3 ligase phenomenon (Fig. 2D). RNAi-based depletion of KIAA0317 led to significantly increased SOCS1 protein level (Fig. 2E). To investigate the effect of KIAA0317 on SOCS1 degradation kinetics, we utilized short hairpin RNA (shRNA) targeting KIAA0317 and treated cells with a CHX time course. Compared to control shRNA treatment, SOCS1 protein levels did not decrease in KIAA0317-depleted cells (Fig. 2F), demonstrating the necessity of KIAA0317 for SOCS1's proteolysis. Conversely, KIAA0317 overexpression accelerated SOCS1 protein degradation (Fig. 2G). These studies suggest that KIAA0317 is an authentic regulator for SOCS1 protein ubiquitination in cells.

Mutational analysis reveals key regions for SOCS1-KIAA0317 interaction

We next sought to elucidate the key sequence regions within SOCS1 needed for its stability and interaction with KIAA0317. Various SOCS1 truncations and point mutations were generated, and we assayed their stabilities and ability to bind KIAA0317 using immunoblotting and immunoprecipitation, respectively (Fig. 3A–E). Several deletion mutations rendered SOCS1 unable to bind KIAA0317, uncovering an N-terminal region between amino acid 54 and 78 within SOCS1 that was critical for binding to KIAA0317 (Fig. 3B). Further, these constructs displayed resistance to degradation in a CHX assay (Fig. 3C). Both the ΔN54 and ΔN78 truncations resulted in little to no binding between the

two proteins, with binding restored with ΔN51: yielding a putative binding region between amino acid 51/54 (Fig. 3A). We introduced point-mutations within this region, and SOCS1 protein levels were assayed to determine effects of these mutations on protein stability. The D52A mutation conferred enhanced stability to SOCS1 protein; other mutations in this region, T53A and H54A, display a time dependent SOCS1 degradation comparable to that of WT SOCS1 (Fig. 3D). This suggests that D52 is a critical residue within SOCS1 for KIAA0317 recognition; indeed, the D52A mutation strongly reduced the interaction as compared to the other two mutations (Fig. 3E). Finally, we investigated the putative ubiquitin-acceptor site within SOCS1. Ubiquitin is typically conjugated to substrates through engagement with a critical substrate lysine site. SOCS1 only has one lysine site at position 118, so we mutated that to an analogous amino acid which is unable to covalently engage ubiquitin. Lys-118-Arg (K118R) resulted in greater SOCS1 stability as compared to WT SOCS1, suggesting this site is critical in modulating SOCS1 ubiquitination (Fig. 3F). These mechanistic studies strengthen our model of KIAA0317 interacting with SOCS1.

Immune stimulation alters SOCS1 stability and downstream JAK/STAT signaling

We next explored the biologic effect of the KIAA0317:SOCS1 axis upon inflammatory signaling in colonic inflammation. KIAA0317 was expressed in increasing doses to HCT-8 cells prior to IL-6 treatment, which resulted in a dose dependent modification of JAK/STAT pathway proteins. STAT1/pSTAT1 and STAT3/pSTAT3 protein levels increased, while SOCS1 protein levels decreased as expected upon overexpression of its E3 (Fig. 4A). These changes in protein expression are consistent with JAK/STAT pathway activation. Similar changes are seen upon IFN-γ (Fig. 4B) stimulation of cells coupled with KIAA0317 overexpression. Next, KIAA0317 was depleted by shRNA expression and IL6 or IFN-γ co-treatment was performed. JAK/STAT pathway activation was assayed, and the results were opposite of those seen upon KIAA0317 overexpression. Silencing of the E3 ligase linked to SOCS1 degradation restored negative feedback on the JAK/STAT pathway as evidenced by decreased expression of key members of the JAK/STAT signaling pathway: pSTAT3 and pSTAT1 (Fig. 4C–D). Taken together, these results indicate that in colonic inflammatory signaling the negative feedback of SOCS1 on JAK/STAT signaling is disrupted, as SOCS1 is degraded by KIAA0317. These results further confirm a key role of this E3 ligase in SOCS1 degradation, and the critical role of SOCS1 in modulating the JAK/STAT signaling pathway.

Discussion:

Research has established SOCS1 as an important target in colonic inflammation. In a DSS model of colonic inflammation, $SOCS^{+/-}$ mice (used due to lethality of complete KO), displayed worsened histopathology, cytokine levels, and survival [24]. Deletion of SOCS1 in the background of TCR KO led to spontaneous severe colitis development in transgenic mice [25]. Further, SOCS1 affects colonic inflammation beyond direct JAK-STAT regulation, as it is involved in regulation of the colonic microbiome. SOCS1 plays a role in controlling immune tolerance to the intestinal microbiome. Murine intestinal colitis induced by genetic ablation of SOCS1 can be rescued by depletion of the microbial population [26]. Further, analysis of the gut microbiota of SOCS1 KO mouse revealed a shift in the microbial species relative to control, including a higher proportion of microbes known

to be associated with colitis. Taken together, these observations place SOCS1 as a primary regulator and preventer of colonic inflammation, whose manipulation has the potential to directly affect multiple pathways tied to colonic inflammation disease course.

This study adds a new layer of control of colonic inflammatory through the proteolytic regulation of SOCS1 protein by the E3 ubiquitin ligase KIAA0317. We observe that SOCS1 protein assembles K63-linked polyubiquitin chains (Fig. 1C). While K48-linked chains are the classic linkage type signaling for proteasomal destruction, several studies have noted a proteasomal fate for K63 linkages [27–29]. More study is needed to definitively characterize SOCS1 ubiquitination patterns. E3 ubiquitin ligases are increasingly appreciated as regulators of intestinal inflammation, such as Pellino, RNF186, and ITCH [18, 30–32]. Given the specificity of E3 ligases to their substrate, they are attractive targets for pharmacological intervention. Targeting of E3 ligases pharmacologically may serve to modulate the abundance of their substrate proteins, ultimately impacting downstream cellular processes. We and other groups have shown that inhibition of E3 ligases leads to positive interventions in inflammatory situations such as in the lung and brain [19, 20, 33], and identification of KIAA0317 as an E3 ligase regulating SOCS1 stability opens a new potential therapeutic avenue.

In this project, we identified SOCS1 as a target of the UPS controlled by the E3 ligase KIAA0317. We elucidated the key regions of SOCS1 protein required for interaction with KIAA0317. Further, we report the pro-inflammatory effects of KIAA0317, whose depletion preserves SOCS1 protein, and dampens JAK/STAT activation in colonic cells. The work presented in this manuscript provides key insights into the mechanisms underlying colonic inflammation related to the KIAA0317/SOCS1 axis.

Materials and Methods

Materials—

QuikChange II XL Site-Directed Mutagenesis Kit (Cat# 200521) was from Aglient. Caco-2 (Cat# HTB-37) and HCT-8 (Cat# CCL-244) were from ATCC. DC Protein Assay Reagent A/B/S (Cat# 500–0113/ 0114/ 0115) and StarBright Blue 700 Goat Anti-Mouse IgG (Cat# 12004159, RRID:AB_2884948) were from BioRad. UbiCRest assay (Cat# K-400) was from BostonBiochem. Carfilzomib (Cat# 17554) and Leupeptin (Cat# 14026) were from Cayman Chemical. Antibodies against Phospho-Stat1 (Tyr701) (58D6) (Cat# 9167, RRID:AB_561284), Phospho-Stat3 (Tyr705) (D3A7) (Cat#9145, RRID:AB_2491009), Stat3 (D3Z2G) (Cat#12640, RRID:AB_2629499), SOCS-1 (A156) (Cat# 3950, RRID:AB_2192983) (Cat# 55313, E3Q4M) and Ubiquitin (Cat# 3933, RRID:AB_2180538) were from Cell Signaling Technology. Human IL-6 Recombinant Protein (14–8069-80) was from ebioscience. DMEM/F-12 (Cat# 11320082), EMEM (Cat# 670086), Fetal Bovine Serum (Cat# 26140079), and Opti-MEM I Reduced Serum Medium (Cat# 31985062) were from Gibco. Phusion polymerase (Cat# M0530), Quick CIP (Cat# M0525), and Quick Ligation Kit (Cat# M2200) were from New England Biolabs. Lullaby siRNA transfection reagent (Cat# LL71000) was from OZ Biosciences. Recombinant Human IFN-γ (Cat# 300–02) was from Peprotech. TnT Quick Coupled Transcription/Translation kit (Cat# L1170) was from Promega. Antibodies against Elongin

B (sc-133090, RRID: AB_2201259), Elongin C (sc-135895, RRID: AB_2303136), STAT1 (C-136, Cat# sc-464, RRID:AB_675899) and SOCS-1 (E-9), Cat# sc-518028) were from Santa Cruz Biotechnologies. KIAA0317 (Cat# SAB2108763) antibody, X-tremeGENE siRNA Transfection Reagent (Cat# 4476115001), and X-tremeGENE HP DNA Transfection Reagent (Cat# 6366244001) were from SigmaAldrich. Antibodies against B-actin (Cat# AM4302, RRID:AB_2536382), Goat anti-Rabbit IgG (H+L) HRP (Cat# 31460, RRID:AB_228341), Goat anti-Mouse IgG (H+L) HRP (Cat# 31430, RRID:AB_228307), HA Tag (2–2.2.14) (Cat# 26183, RRID:AB_10978021), V5 Tag (Cat# R960–25, RRID:AB_2556564), and Dynabeads His-Tag Isolation and Pulldown (Cat# 10103D), Pierce Protease Inhibitor Tablets (Cat# A32963), pcDNA3.1 Directional TOPO Expression Kit (Cat# K490001), Pierce Protein A/G Magnetic Beads (Cat# 88802), Lipofectamine 3000 Transfection Reagents (Cat# L3000015), and TOP10 (Cat# C404010) were from Thermo Fisher Scientific. MG132 (Cat# F1101) was from UBPBio.

Cell Culture—

HCT-8 cells (ATCC) were cultured in RPMI media (Gibco) prepared and filtered in 500 mL supplemented with non-essential amino acids (stock concentration 100X), HEPES (stock concentration 1M), L-Glutamine (stock concentration 200 mM), Penicillin/Streptomycin (stock concentration 10,000U/mL) and 10% FBS. Treatments were conducted with cycloheximide (100μg/mL) and proteasome or lysosome inhibitors (carfilzomib, 1μM; MG132, 1μM; leupeptin, 100μg/mL)

Cytokine Treatments—

HCT-8 cells were treated with pro-inflammatory cytokines associated with colonic inflammatory diseases (IFNγ and IL-6). Briefly, treatment media was prepared in RPMI with 2% FBS to the following final concentrations: IFNγ (50ng/mL), IL-6 (20ng/mL), or as indicated in the figure legend. Cells were treated for 18hr or as indicated before collection and subsequent analysis.

Immunoblotting—

Cells were collected and lysed in RIPA buffer (50mM Tris-HCl, ph 7.6, 150mM NaCl, 1.0% v/v Triton-X-100, 0.1% w/v SDS, 0.5% w/v sodium deoxycholate) supplemented with protease inhibitor cocktail (Pierce). Lysate was sonicated at 20% amplitude for 12 seconds, centrifuged at 12,000 x RCF for 10 minutes at 4° C, and protein concentrations were normalized using Lowry Protein Assay. Lysates were prepared in 3X PSB supplemented with DTT (6.4 mg/mL) for immunoblotting. Sample lysate was resolved using 4–12% acrylamide Invitrogen Bolt Bis-Tris Plus gels and electrophoresed in MOPS or MES buffer. The proteins were then electro-transferred to nitrocellulose membranes. Blots were incubated in 15 ml of blocking buffer for 1 h at room temperature, before incubation in 10 ml of the primary antibody solution (1:1000 dilution) overnight at 4°C. Afterwards, three 10-minute washes were performed in 15 ml Tris-buffered saline + 0.05% Tween-20 (TBST). Blots were then incubated with 10 ml of the secondary antibody solution for 1 h at room temperature. After three 10-minute washes in 15 ml TBST, blots were then developed using West Femto Maximum Sensitivity Substrate from Thermo Scientific, and imaged using

ChemiDoc Imaging System from Bio-Rad. Please see uncropped blots in the supplemental data.

Plasmid Preparation and Cloning—

SOCS1, KIAA0317, other E3 ligases, and deletional mutants were PCR cloned or subcloned into pcDNA3.1D-V5-HIS (Invitrogen) through TOPO cloning. Point mutants (e.g., Lysine to Arginine, etc.) were generated through QuikChange II XL Site-Directed Mutagenesis Kit (Aglient). All plasmid constructs were verified by DNA sequencing (Genewiz).

Protein Half-Life Assay—

To measure protein stability, the ribosomal inhibitor cycloheximide was used to halt protein synthesis, and SOCS1 protein stability was measured through immunoblotting. HCT-8 cells transfected with WT, deletional or point mutant SOCS1 plasmid were treated with cycloheximide (CHX) at 0.1mg/mL for the indicated times before cell lysis and processing for immunoblotting.

Plasmid Transfection—

Plasmid transfections were conducted using lipofection in HCT-8 cells using X-tremeGENE HP DNA transfection reagent or Lipofectamine 3000 transfection reagent was used for plasmid transfections following manufacturer's protocol. Cells were assayed at least 18 hours post-transfection.

HIS-tag pulldown—

SOCS1 WT in pcDNA3.1D-V5-HIS were expressed in HCT-8 cells for 18hr, prior to proteasomal inhibition where indicated, lysis and precipitation with Dynabead HIS-resin (Thermo). Precipitate was eluted in 1x Laemmli Protein Sample Buffer at 95°C for 10 min and resolved through SDS-PAGE immunoblotting.

UbiCRest Assay—

Ubiquitination linkage-type analysis was measured by UbiCRest assay ([23] Boston Biochem). SOCS1-V5-His was expressed in HCT8 for 18hr, then co-treated with Carfilzomib (1μM) and DSS (2% w/v) for 4 hours. Cells were lysed and SOCS1-V5-HIS was purified by HIS-tag pulldown. Pulldown was then subjected to UbiCRest digestion following manufacturer's instructions. After reaction and washing, SOCS1 protein was eluted from resin at 95°C for 10 min and poly-ubiquitin signal was resolved through SDS-PAGE immunoblotting.

In vitro Ubiquitination assay—

SOCS1 in vitro ubiquitination assays were performed as previously described [19, 20]. Briefly, ubiquitination reactions were comprised of a volume of 20 μL containing 50 mM Tris, pH 7.6, 5 mM $MgCl_2$, 0.6 mM DTT, 2 mM ATP, 400 μM MG132, 50 nM Ubiquitin activating enzyme, 0.5 μM UbcH5, 0.5 μM UbcH7, 2 μM ubiquitin, and 1 μM ubiquitin aldehyde. TnT-coupled (Promega) reticulocyte in vitro–synthesized tagless KIAA0317 and

SOCS1-V5 proteins were purified via HisPur Resin (Thermo Fisher Scientific), and reaction products were processed for V5 immunoblotting.

Cell-based Ubiquitination Assay —

SOCS1-V5-HIS WT in pcDNA3.1D was co-expressed with KIAA0317-HA in HCT-8 cells for 18hr, prior to carfilzomib proteasomal inhibition (4hr, 1μM), lysis and precipitation with Dynabead HIS-resin (Thermo). Precipitate was eluted in 1x Laemmli Protein Sample Buffer at 95°C for 10 min and resolved through SDS-PAGE immunoblotting. Alternatively, KIAA0317-V5 WT and C770S in pcDNA3.1D were expressed to BEAS-2B cells for 18hr, prior to carfilzomib proteasomal inhibition (18hr, 0.25μM), lysis and immunoprecipitation of SOCS1 (CST, Cat #55313) and capture with Protein A/G beads. Pulldown was washed and eluted for immunoblotting analysis of SOCS1 ubiquitination.

RNAi knockdown —

KIAA0317 was knocked down using shRNA encoded expression plasmids, transfected as noted above, or with dsiRNA (IDT) transfected with Lullaby (OZ biosciences) for at least 48 hours prior to analysis.

Binding Assays—

Binding assays were conducted as previously described [20]. Briefly, SOCS1 protein were immunoprecipitated from HCT-8 cell lysate using 1:100 antibody dilution (SantaCruz sc-518028). Protein was precipitated in IP buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 0.25 % v/v Triton-X-100) for four hours at +4C, then coupled to protein A/G agarose resin for an additional two hours. Binding mutants were in vitro synthesized using TnT expression kits and allowed to bind overnight. Resin was washed in IP buffer for three rounds of five-minute washes, and protein was eluted in 1x Laemmli buffer at 88°C for five minutes prior to immunoblotting analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical comparisons were performed in GraphPad Prism 9. All statistical details of experiments can be found in the figure legend. Unpaired two-tailed Student's *t*-test was used to compare two groups. Comparisons of more than two groups were tested with one-way or two-way ANOVA with post-hoc test of multiple comparisons. Significance was determined by $p < 0.05$ or greater and is indicated in figure legends.

DATA AND SOFTWARE AVAILABILITY

The published article includes all datasets generated or analyzed during this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ABBREVIATIONS

SOCS1	Suppressor of Cytokine Signaling 1
IP	Immunoprecipitation
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription
Ub	Ubiquitin
CHX	Cycloheximide
IL-6	Interleukin-6
IFNγ	Interferon gamma

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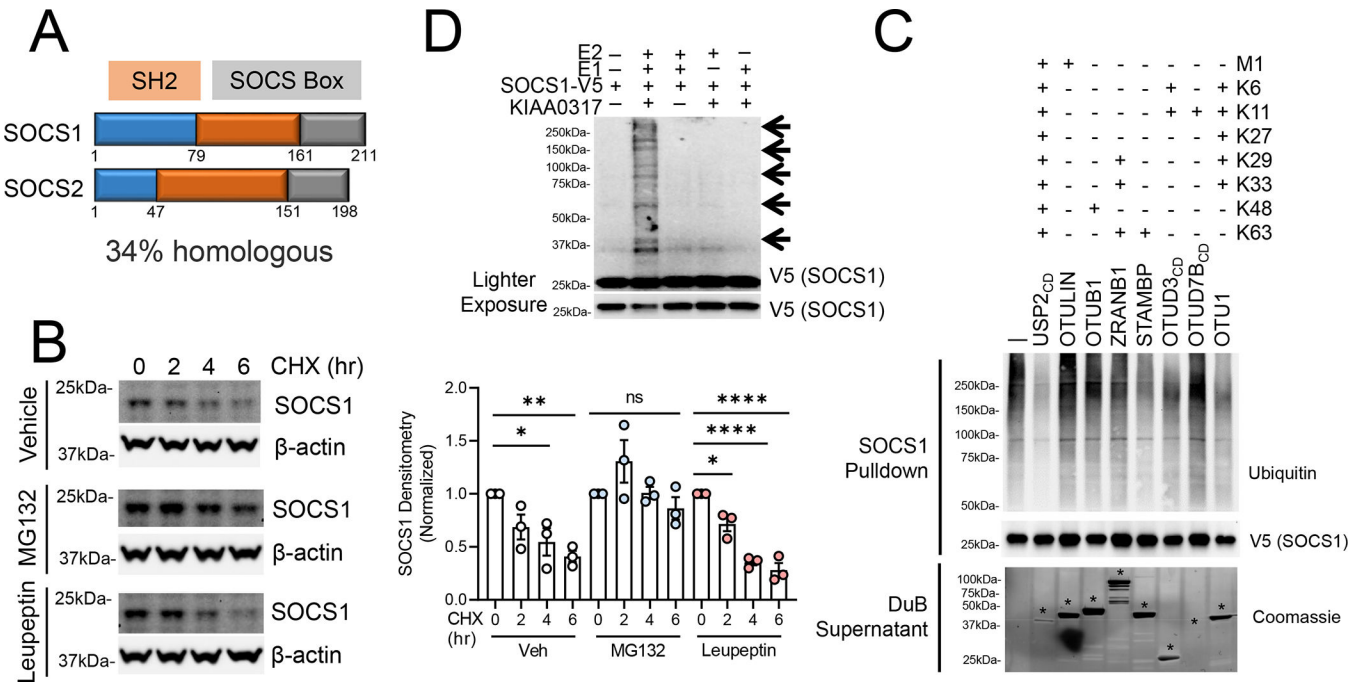

Figure 1: SOCS1 protein is ubiquitinated and regulated by the proteasome, mediated by E3 ubiquitin ligase KIAA0317.

A. Assessment of homology between SOCS2 and SOCS1 to determine a possible role of KIAA0317 in SOCS1 regulation. **B.** Immunoblotting of SOCS1 stability assay in HCT8 cells. Cells were co-treated with cycloheximide (CHX) and either lysosomal (leupeptin) or proteasomal (MG132) inhibition in a time course. Data represent mean ± SEM (n=3). **C.** UbiCRest assay of SOCS1 ubiquitination. HCT8 cells transfected with SOCS1-V5-HIS were treated with carfilzomib (1μM) for 4 hours prior to SOCS1 pulldown and UbiCRest assay. Coomassie gel showing de-ubiquitinase loading, * indicates enzymes. **D.** Immunoblot analysis of *in vitro* ubiquitination assay. Reactions included the full complement or selective omission of ubiquitination machinery, SOCS1, and KIAA0317. Ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$, by one-way ANOVA with Tukey's test for multiple comparisons (B).

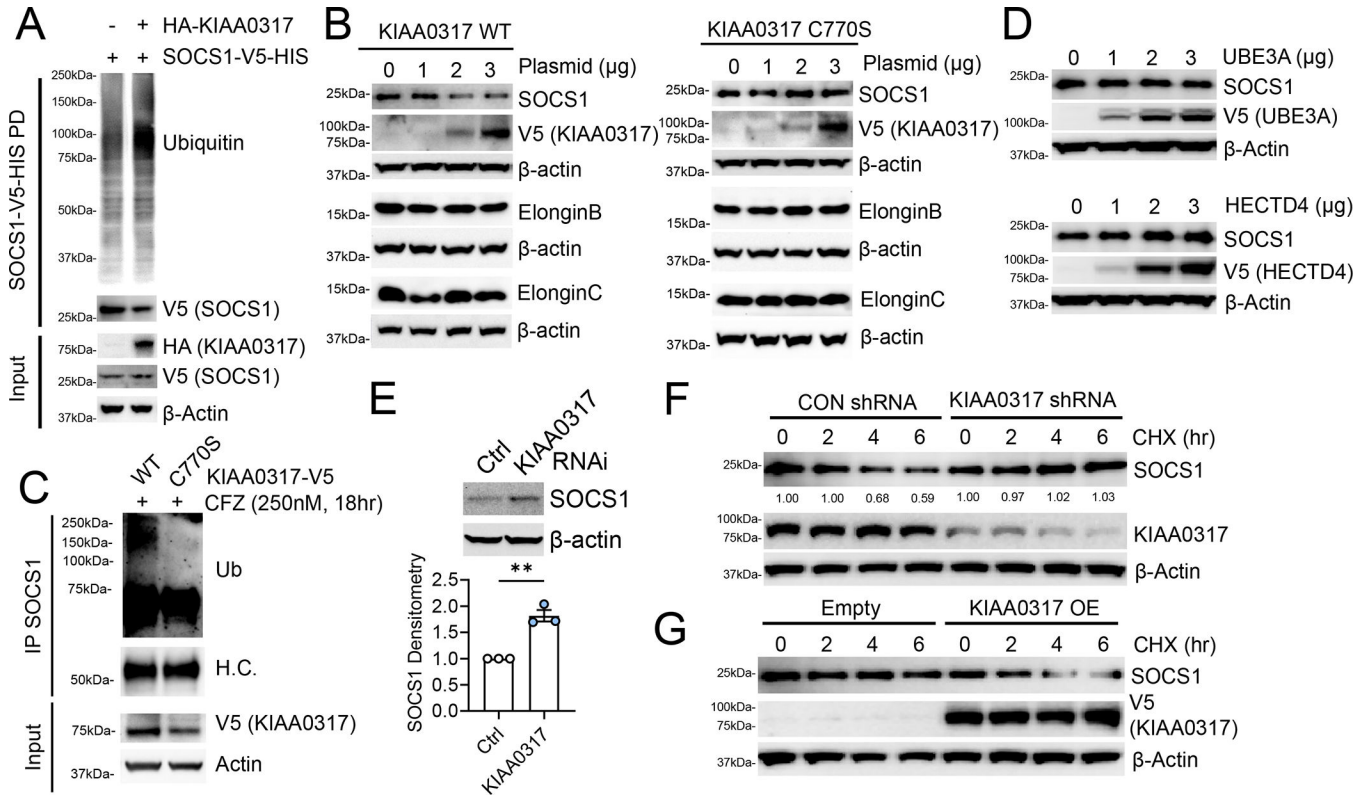

Figure 2: The Ubiquitin E3 ligase KIAA0317 regulates SOCS1 protein stability and ubiquitination in colonic epithelial cells.

A. Immunoblot analysis of KIAA0317 cell-based ubiquitination assay of SOCS1. HCT8 cells were transfected with SOCS1-V5 with or without co-transfection with HA-KIAA. After 18hours, cells were treated with carfilzomib (1μM, 4hr), His-Pulldown was performed, and ubiquitination activity was assayed. **B.** KIAA0317 WT or catalytically inactive C770S mutant were transfected at increasing doses prior to immunoblotting of SOCS1 protein level. **C.** Immunoblotting of BEAS-2B cells expressing KIAA0317 WT or C770S mutants prior to CFZ treatment and SOCS1 immunoprecipitation to measure ubiquitination. H.C., Heavy Chain. **D.** Expression of E3 ligases with high homology to KIAA0317 does not affect SOCS1 protein level. **E.** Immunoblot analysis of HCT-8 cells treated with control of *KIAA0317* esiRNA (IDT). Data represent mean ± SEM (n=3). **F-G.** SOCS1 cycloheximide (CHX) stability assay in HCT-8 cells with either control or KIAA0317 shRNA treatment (D), or control or KIAA0317 plasmid transfection (E). **, p<0.01; by two-sided t-test (E).

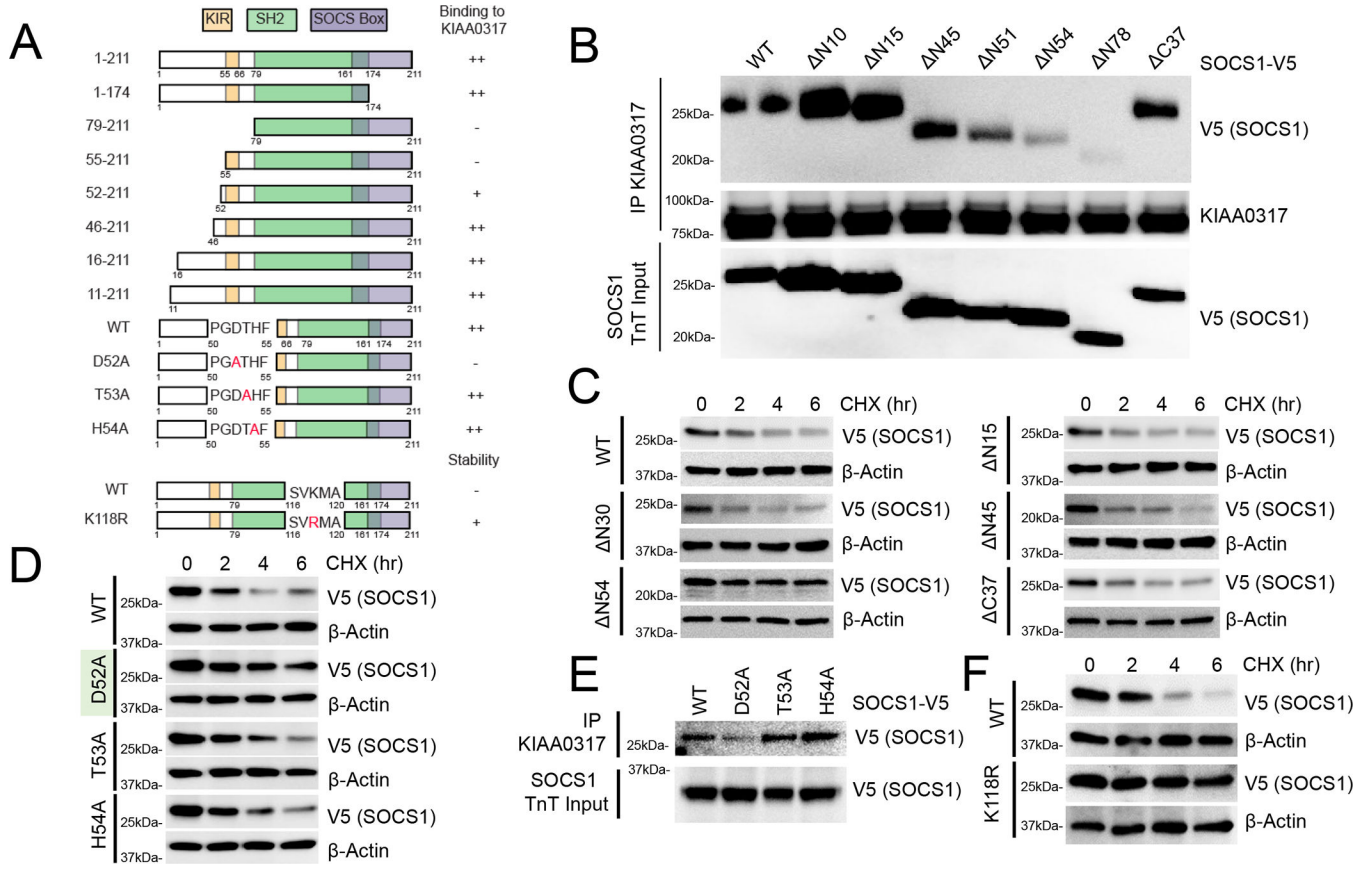

Figure 3: Mutational analysis reveals key regions for SOCS1-KIAA0317 interaction.

A. Generation of N and C terminal mutations, or various point mutations impact the ability of SOCS1 and KIAA0317 to bind each other. **B.** Binding assay between KIAA0317 and SOCS1 deletion mutants. KIAA0317 was immunoprecipitated (IP) from HCT-8 cell lysate prior to incubation with in vitro transcription-and-translation (TnT, Promega) synthesized SOCS1-V5 deletion mutants overnight. Pulldown was washed, eluted, and analyzed for binding by immunoblotting. **C.** Immunoblot analysis of HCT-8 cells transfected with WT and N- or C-terminal SOCS1 deletional mutants prior to cycloheximide (CHX) time course was performed to determine effects of mutation on SOCS1 stability using immunoblotting. **D.** Immunoblotting of SOCS1 stability assay. Cells were transfected with WT or SOCS1 point mutant constructs and a CHX time course was performed. **E.** Binding assay between KIAA0317 and SOCS1 point mutants. KIAA0317 was immunoprecipitated (IP) from HCT-8 cell lysate prior to incubation with in vitro transcription-and-translation (TnT, Promega) synthesized SOCS1-V5 point mutants overnight. Pulldown was washed, eluted, and analyzed for binding by immunoblotting. **F.** SOCS1 Lysine-118 was mutated and effects on SOCS1 stability were assayed using immunoblotting following a CHX time course.

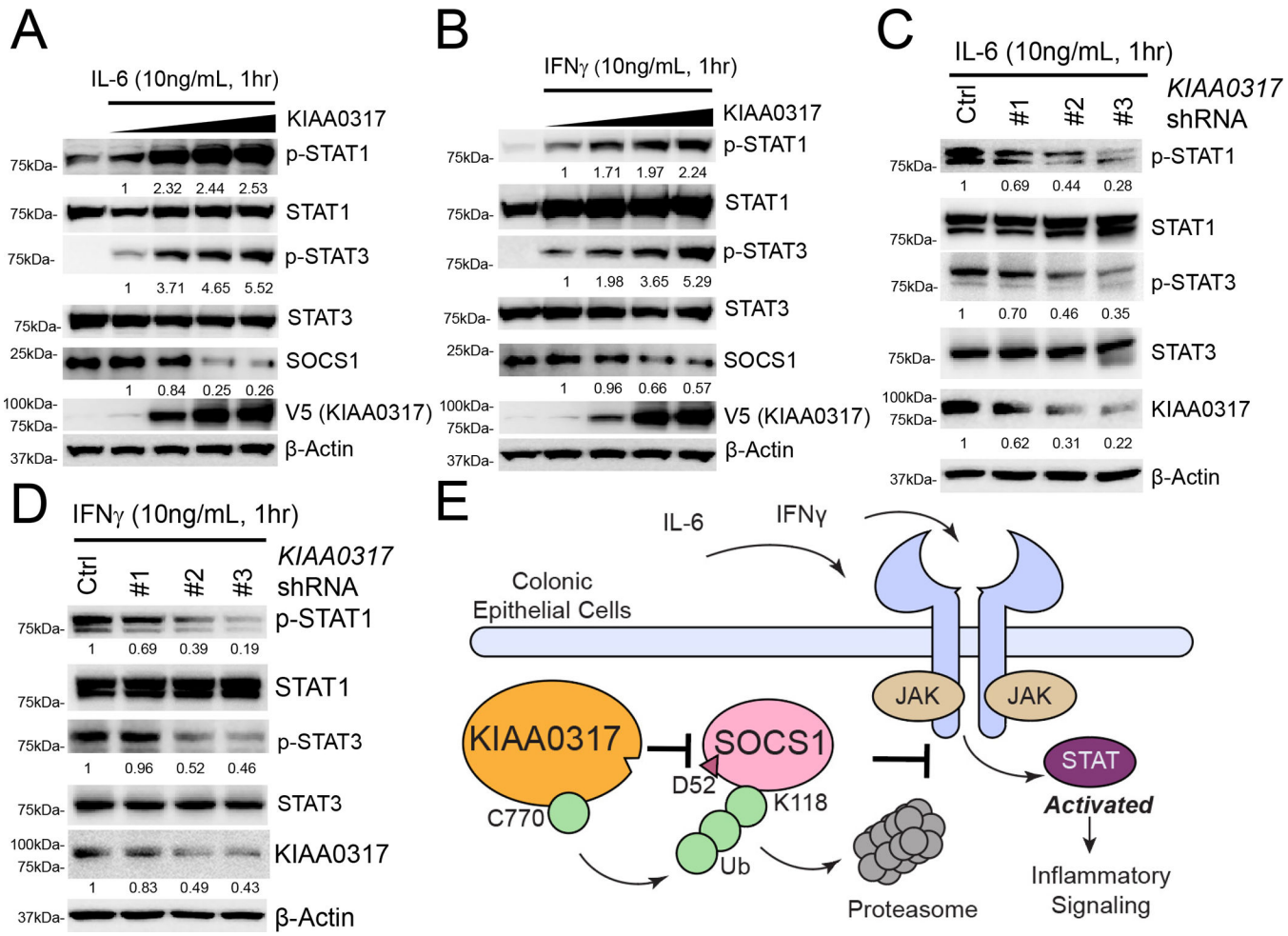

Figure 4: Interactions between various cytokines, SOCS1, and KIAA0317 reveal effects on downstream signaling.

A-B. Immunoblotting of JAK-STAT activation of HCT8 cells transfected with increasing doses of KIAA0317. After 24 hours of expression, cells were treated with IL-6 (10ng/mL) (A) or IFNγ (10ng/mL) (B), for 1hr prior to immunoblotting. Densitometry of phospho-STAT1 corrected to total STAT1, phospho-STAT3 to total STAT3, or SOCS1 protein corrected to β-Actin is noted below immunoblot. **C-D.** Immunoblot analysis of HCT8 cells transfected with KIAA0317 shRNA plasmid to induce knockdown. After 48 hours of knockdown, cells were treated with IL-6 (10ng/mL) (C) or IFNγ (10ng/mL) (D), for 1hr prior to collection. Densitometry of phospho-STAT1 corrected to total STAT1, phospho-STAT3 to total STAT3, or KIAA0317 protein corrected to β-Actin is noted below immunoblot. **E.** Schematic of KIAA0317 mechanism controlling SOCS1 stability and effect on JAK-STAT signaling.