INTRODUCTION

Cyclophilin A (CypA) is a ubiquitously expressed protein that physiologically regulates protein folding, trafficking, and interaction.\(^1\) It is also involved in pathological processes underlying in inflammatory diseases\(^2\)–\(^4\) and cancers.\(^5\) Our previous studies revealed that CypA positively regulates RIG-I-mediated signaling pathway and promotes

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**Abstract**

Cyclophilin A (CypA), a member of the cyclophilin family, plays a vital role in microorganismal infections, inflammatory diseases, and cancers. Interleukin-6 (IL-6) is a pleiotropic cytokine, exerting variety of effects on inflammation, immune response, hematopoiesis, and tumor proliferation. Binding of IL-6 to soluble IL-6 receptor (sIL-6R) induces pro-inflammatory trans-signaling, which has been described to be stronger than anti-inflammatory classic signaling triggered by the binding of IL-6 to membrane-bound IL-6 receptor. Here we found that upon the treatment of IL-6 and sIL-6R, CypA inhibited the ubiquitination-mediated degradation of IL-6 membrane receptor gp130 and enhanced its dimerization, thereby positively regulated the IL-6 trans-signaling and increased the expression of downstream iNOS, IL-6, and CypA. Furthermore, CypA expression could be negatively regulated by suppressor of cytokine signaling 1 (SOCS1). The SH2 and Box domains of SOCS1 interacted with CypA and promoted its K48-linked ubiquitination-mediated degradation, which inhibited the IL-6 trans-signaling pathway. Collectively, our findings reveal an important role of CypA in the positive and negative feedback regulation of the IL-6 trans-signaling pathway.

**KEYWORDS**
cyclophilin A, gp130, interleukin-6, SOCS1, ubiquitination
downstream cytokines production. In addition, CypA contributes to maintain the stability of NF-κB p65 subunit, enhances its nuclear translocation and transcriptional activity, and thereby increases the production of pro-inflammatory cytokines. Moreover, CypA regulates the PRLr/JAK2/STAT5 pathway, which is necessary for mammary differentiation and the pathogenesis of breast cancer. Therefore, understanding the underlying mechanisms of CypA in regulating signaling pathways is important for new drug development against inflammatory diseases, cancers, and other related diseases.

In addition to CypA, interleukin-6 (IL-6) is closely associated with inflammation and tumor disease progression. IL-6 is a master player in the inflammatory response network and is regarded as one of the key biomarkers of the severity and prognosis of cytokine release syndrome. The signal transduction of IL-6 depends on the complex consisting of the IL-6, membrane IL-6 receptor (mIL-6R) or soluble IL-6 receptor (sIL-6R), and the signal transducing receptor subunit gp130. The complex of IL-6 and sIL-6R can bind to gp130 on cells which do not express mIL-6R to activate pro-inflammatory response. This paradigm has been called IL-6 trans-signaling, whereas signaling via the mIL-6R is referred to as classic signaling. The mIL-6R is only present on a few types of cells such as hepatocytes and leucocyte subtypes, which can cleave the receptor protein to generate sIL-6R. However, all cells in the body express gp130, which permits IL-6 to modulate a broad spectrum of cells. The complex of IL-6 and mIL-6R or sIL-6R associates with the gp130 inducing gp130 dimerization to initiate intracellular signaling through the canonical JAK/STAT pathway. In addition, the phosphatase SHP-2 is recruited by tyrosine phosphorylated gp130 dimer and phosphorylated by JAK, which further activates the Ras-Raf-MAPK pathway. It has been reported that CypA is able to regulate the activity of JAK2 and STAT3, which are the key IL-6 signaling proteins. However, the underlying mechanism is unknown and whether CypA involves in the IL-6 trans-signaling remain elusive.

Suppressor of cytokine signaling 1 and 3 (SOCS1, SOCS3) are important members of the classical cytokine signal transduction negative feedback system. They both include the kinase inhibitory region (KIR), an extended SH2 subdomain (ESS) which precedes the central SH2 domain, and the SOCS box. The KIR of SOCS1 and SOCS3 function as a pseudosubstrate to inhibit JAK activity. Additionally, SOCS family proteins can also recruit Elongins B/C, Cullin, and Ring finger proteins (Rbx) through the SOCS Box to form an E3 complex to degrade the substrate bound to the SH2 domain. It is well established that SOCS1 inhibits TLR7-mediated production of type I interferon in human plasmacytoid dendritic cells by targeting IRF7 degradation. SOCS1 interacts with NF-KB P65 subunit to facilitate the ubiquitination and degradation of P65 subunit, and SOCS3 promotes the degradation of phosphorylated gp130 and JAK to prevent signal transduction. Whether SOCS1 exhibits its E3 activity in IL-6 signaling pathway as SOCS3 does is unclear.

The present study reveals that CypA is a key factor in the positive and negative feedback regulation of the IL-6 trans-signaling pathway. CypA is able to promote IL-6 trans-signaling pathway. At the same time, CypA is also a target of SOCS1 to inhibit IL-6 trans-signaling pathway.

2 MATERIALS AND METHODS

2.1 Cell lines and antibodies

ShRNA-based knockdown of CypA in human lung carcinoma A549 cells and human embryonic kidney 293T cells has been described previously. All these cell lines were examined by mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Switzerland). The A549 cells and 293T cells were maintained in Dulbecco’s Modified Eagle Medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) at 37°C in a humidified environment containing 5% CO2. The rabbit polyclonal antibodies to human CypA were generated as previously described. The following antibodies were used for immunoblot analysis: anti-c-Myc (1:1000, sc-40, Santa Cruz), anti-FLAG M2 (1:2000, F3165, Sigma), anti-β-tubulin (1:2000, ab6046, Abcam), anti-GAPDH (1:2000, ab8245, Abcam), anti-HA (1:1000, ab 236632, Abcam), anti-pJAK1 (1:1000, 74129, CST), anti-JAK1 (1:1000, 3344, CST), anti-pTyk2 (1:1000, 68790, CST), anti-Tyk2 (1:1000, bs-6662R, Bioss), anti-pSTAT3 (1:1000, 1010, 9145, CST), anti-JAK1 (1:1000, 3344, CST), anti-pTyk2 (1:1000, 68790, CST), anti-Tyk2 (1:1000, bs-6662R, Bioss), anti-pSTAT3 (1:1000, 9145, CST), anti-STAT3 (1:1000, 1939, CST), anti-p-c-Raf (1:1000, 271928, Santa Cruz), anti-c-Raf (1:1000, 9422, CST), anti-pMEK (1:1000, 9154, CST), anti-MEK (1:1000, 8727, CST), anti-pERK (1:1000, 9101, CST), anti-ERK (1:1000, 4695, CST), anti-IL-6 (1:1000, 12153, CST), anti-SOS (1:1000, 39898, CST), anti-SOCS1 (1:1000, 96870, CST), anti-SOCS3 (1:1000, 3732, CST), anti-mouse IgG, HRP-linked antibody (1:3000, 7074, CST), anti-rabbit IgG, HRP-linked antibody (1:3000, 7074, CST), and rabbit anti-mouse IgG (Light Chain Specific) (D3V2A) mAb (HRP Conjugate) (1:1000, 58802, CST).

2.2 Plasmids

The Flag-CypA, Myc-CypA, and Myc-SOCS1 expression plasmids were maintained in our laboratory. The Flag-SOCS1, Flag-SOCS3, HA-ESB, Flag-ESB, Flag-gp130,
and Myc-gp130IC expression plasmids were synthesized by GENSCRIPT and then cloned into the pCAGGS vector. The HA-Ub, HA-K48-Ub, and HA-K63-Ub expression plasmids were provided by X. Ye (Chinese Academy of Sciences, China).

### 2.3 IL-6 and sIL-6R treatment

To activate the trans-IL-6 signaling pathway, A549 or 293T cells were stimulated with IL-6 (AF-200-06, PeproTech) and sIL-6R (200-06RC, PeproTech) at each concentration of 20 ng/ml for various periods of time.

### 2.4 CHX, MG132, and NH₄Cl treatment

Cells were treated with 100 μg/ml CHX for various periods of time. Then cells were lysed and analyzed by immunoblot. Cells were treated with 10 mM MG132 and 10 mM NH₄Cl at the same time as CHX, then cells were harvested 6 h after treatment for immunoblot analysis.

### 2.5 RNA extraction, cDNA synthesis, and quantitative PCR analysis

Total RNA extraction and cDNA synthesis were performed as previously described. The relative gene expression of the target genes was analyzed by quantitative PCR using TB Green premix (TaKaRa). The Ct values were generated from ABI 7500 and were analyzed by $2^{-\Delta\Delta Ct}$ method. GAPDH was served as an internal control using PCR primers. The PCR primers: hIL-6 forward, 5’-ACTCACCTTCAGAAGCAATGG-3’; hIL-6 reverse, 5’-CCATCTTTGGGAAGGTTCAGGTTG-3’; hiNOS forward, 5’-CGGTGCTGATTTCCTTACGAGGCGAAGAAGG-3’; hiNOS reverse, 5’-GGTGCTGCATTGGAAGGTTCAGGTTG-3’. The expression of the target genes was analyzed by quantitative PCR analysis as previously described.

### 2.7 Native PAGE

Native PAGE was performed with a 6% polyacrylamide gel without SDS on ice. Then, the gel was electrophoresed onto a 0.45-μm PVDF membrane and blocked by TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0) with 5% (w/v) nonfat dry milk and 1% (w/v) BSA for 2 h. The membrane was incubated with primary antibodies at room temperature for 2 h and washed with TBST three times, then the HRP-conjugated specific secondary antibodies were used to bind target primary antibodies for 45 min. After washing with TBST three times, the target protein bands were visible by the ECL western blotting substrate. The expression of the target protein was normalized to GAPDH or β-tubulin. ImageJ was used to quantify the expression of the target proteins.

### 2.8 Nano LC-MS/MS

The 293T cells were transfected with Flag-CypA or control vector. After transfected for 24 h, 293T cells were lysed in lysis buffer supplemented with a complete protease inhibitor cocktail (Roche Diagnostics). The Flag-CypA was purified with anti-Flag M2 affinity gel (Sigma) and eluted by Flag*-peptide (Sigma). Proteins were eluted by Flag*-peptide (Sigma) for 2 h at 4°C. The eluted proteins were digested with trypsin and subjected to nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analysis on EASY-nLC 1000 interfaced via a Nanospray Flex ion source to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, USA) at the Institutional Center for Shared Technologies and Facilities of Institute of Microbiology, Chinese Academy of Sciences. The peptides were loaded onto a trap column (C18, 5 μm particles, 100 μm ID, 3 cm length, Dr Maisch GmbH) and separated using an analytical column (C18, 3 μm particles, 75 μm ID, 15 cm length, Dr Maisch GmbH) at a flow rate of 400 nl/min with a 60-min LC gradient composed of Solvent A (0.1% formic acid) and Solvent B (acetonitrile, 0.1% formic acid). The gradient was 3%-8% B for 5 min, 8%-20% B for 40 min, 20%-35% B for 10 min, 35%-80% B for 3 min, and finally 80% B for 2 min. The mass spectrometer was operated in a data-dependent acquisition mode, in which the precursor MS1 scan (m/z 350–1550) was acquired in the Orbitrap at a resolution setting of 120 000, followed by Orbitrap HCD-MS/MS and ITHCD-MS/MS of the 20 most abundant multiply charged
precursors in the MS1 spectrum. MS2 spectra were acquired at a resolution of 30,000.

MS/MS data were processed using Mascot search engine (v.2.5.1, 2014, http://www.matrixscience.com; Matrix Science Ltd., London, UK). Tandem mass spectra were searched against SWISS-Prot/TrEMBL (http://www.expasy.org/), and Trypsin/P was specified as cleavage enzyme allowing up to two missing cleavages. For precursor ions, the mass error was set to 10 ppm, and for fragment ions, the mass error is set to 0.02 Da. Carbamidomethylation on Cys was specified as fixed modification. Oxidation on Met and ubiquitination on Lys were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide, and modification site were adjusted to <1%. p-value <.05 was considered statistically significant. p-values were corrected for FDR in each dataset. All the other parameters in Mascot were set to default values.

2.9 | Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 software and Microsoft Excel. Data are presented as the mean values ± SD of at least three independent experiments. Comparisons between two groups were performed...
FIGURE 2  CypA maintains the stability and dimerization of gp130. (A) Native PAGE and immunoblot analysis of dimer or monomer forms of gp130 in A549/CypA+ cells and A549/CypA− cells stimulated by IL-6/sIL-6R for the indicated time periods. (B) Immunoblot analysis of lysates in 293T/CypA− cells transfected with Flag-gp130, along with Myc-tagged CypA, prolyl-isomerase-deficient mutant form of CypA (R55A), or control vector for 24 h and then treated with 100 μg/ml CHX for the indicated durations (left). The relative expression levels of Flag-gp130 were quantified (right). (C) Immunoblot analysis of lysates in 293T/CypA− cells transfected with Flag-gp130 for 24 h and then treated for 6 h with 100 μg/ml CHX, along with 10 μM NH₄Cl, 10 μM MG132, or DMSO (left). The relative expression levels of gp130 were quantified (right). (D) Immunoblot analysis of lysates in 293T/CypA+ and 293T/CypA− cells transfected with Myc-gp130 IC for 24 h and then treated with 100 μg/ml CHX for the indicated durations (left). The relative expression levels of Myc-gp130 IC were quantified (right). (E) Immunoblot analysis of lysates in 293T/CypA− cells transfected with Myc-gp130 IC for 24 h and then treated for 6h with 100 μg/ml CHX, along with 10 μM NH₄Cl, 10 μM MG132, or DMSO (left). The relative expression levels of gp130 IC were quantified (right). (F) Immunoblot analysis of lysates in 293T/CypA+ and 293T/CypA− cells transfected for 24 h with HA-K48-Ub and Myc-gp130 IC or control vector, followed by immunoprecipitation with anti-Myc beads. Data are shown as mean ± SD of at least three independent experiments. **p < .01. M, protein molecular weight marker; ns, not significant

using the two-tailed Student’s t-test. p < .05 was considered significant, with *p < .05, **p < .01.

3 | RESULTS

3.1 | CypA positively regulates IL-6 trans-signaling pathway

Several studies have demonstrated that CypA promotes IL-6 expression by regulating the transcriptional activity of NF-κB, but whether and how CypA involves in IL-6 signaling remains elusive. To investigate the impact of CypA on the IL-6 signaling pathway, the trans-IL-6 signaling pathway in CypA-knockdown A549 cells (A549/CypA−) or 293T cells (293T/CypA−) and wild-type (WT) A549 cells (A549/CypA+) or 293T cells (293T/CypA+) was activated with the stimulation of IL-6 and sIL-6R. We observed that CypA knockdown suppressed the phosphorylation of Tyk2 or JAK1, STAT3, Raf, MEK, and ERK both in A549 (Figure 1A) and 293T (Figure S1A) cells, indicating that CypA plays a positive role in the activation of IL-6 trans-signaling. In addition, the mRNA and protein expression levels of downstream cytokines, such as IL-6, iNOS, and CypA, in A549/CypA+ cells were strikingly increased compared with those in A549/CypA− cells (Figure 1B,C). The similar results were obtained in 293T/CypA+ and 293T/CypA− cells (Figure S1B). We also found that overexpressing CypA in 293T/CypA− cells promoted the phosphorylation of STAT3, Raf, MEK, and ERK (Figure 1D). All the results demonstrated that CypA positively regulates IL-6 trans-signaling pathway.
Thus, we sought to determine the impact of CypA on gp130 dimerization. The native PAGE and western blotting results demonstrated that much more dimer and monomer forms of gp130 were observed in A549/CypA+ cells than those in A549/CypA− cells upon IL-6/sIL-6R stimulation (Figure 2A). Next, we attempted to detect whether CypA affects the stability of gp130. The full-length gp130 was transfected in 293T/CypA+ cells, along with CypA, prolyl-isomerase-deficient mutant form of CypA (R55A), or vector, and treated with CHX. The western blotting results showed that CypA inhibited the degradation of gp130 independent of its prolyl-isomerase activity (Figure 2B). We further examined the degradation pathway of gp130. It was observed that the proteasome inhibitor MG132 significantly inhibited the degradation of gp130, whereas the lysosome inhibitor NH4Cl did not (Figure 2C), suggesting that CypA was essential for gp130 stability through inhibition of ubiquitin-mediated proteolysis. As the protein ubiquitination modification usually occurs in the cytoplasm, the effect of CypA on gp130 intracellular domain (gp130 IC) was further investigated (Figure 2D,E), which was consist with Figure 2B,C. In subsequent immunoprecipitation experiments, we verified that CypA significantly reduced the K48-linked ubiquitination of gp130 IC (Figure 2F). These data indicated that CypA positively regulated IL-6 trans-signaling pathway by maintaining the stability and dimerization of gp130.

3.2 CypA maintains the stability and dimerization of gp130

The intracellular domain of IL-6 receptor (IL-6R) is too short to transmit signals alone. The signal transduction of IL-6 also depends on the IL-6 membrane receptor β subunit gp130. The complex of IL-6–IL-6R–gp130 induces gp130 dimerization to initiate the MAPK and JAK/STAT pathways. Thus, we sought to determine the impact of CypA on gp130 dimerization. The native PAGE and western blotting results demonstrated that much more dimer and monomer forms of gp130 were observed in A549/CypA+ cells than those in A549/CypA− cells upon IL-6/sIL-6R stimulation (Figure 2A). Next, we attempted to detect whether CypA affects the stability of gp130. The full-length gp130 was transfected in 293T/CypA+ cells, along with CypA, prolyl-isomerase-deficient mutant form of CypA (R55A), or vector, and treated with CHX. The western blotting results showed that CypA inhibited the degradation of gp130 independent of its prolyl-isomerase activity (Figure 2B). We further examined the degradation pathway of gp130. It was observed that the proteasome inhibitor MG132 significantly inhibited the degradation of gp130, whereas the lysosome inhibitor NH4Cl did not (Figure 2C), suggesting that CypA was essential for gp130 stability through inhibition of ubiquitin-mediated proteolysis. As the protein ubiquitination modification usually occurs in the cytoplasm, the effect of CypA on gp130 intracellular domain (gp130 IC) was further investigated (Figure 2D,E), which was consist with Figure 2B,C. In subsequent immunoprecipitation experiments, we verified that CypA significantly reduced the K48-linked ubiquitination of gp130 IC (Figure 2F). These data indicated that CypA positively regulated IL-6 trans-signaling pathway by maintaining the stability and dimerization of gp130.

3.3 SOCS1 destabilizes gp130 by targeting CypA

The KIR domains of SOCS1 and SOCS3 inhibit JAK kinase activity through direct binding of its KIR domain to the activation loop of JAKs. Moreover, SOCS3 interacts with gp130 and promotes the degradation of phosphorylated gp130 to prevent signal transduction. We sought to investigate whether SOCS1 affected the stability of gp130 as SOCS3 did. The western blotting result demonstrated that the overexpression of SOCS1 facilitated gp130 IC degradation (Figure 3A). We further examined whether SOCS1 interacted with gp130. As a result, no interaction was found between
SOCS1 and gp130 (Figure 3B). Since CypA inhibited the degradation of gp130 (Figure 2B,C), we speculate that SOCS1 might indirectly affect the stability of gp130 through targeting CypA. As we expected, the western blotting result exhibited that SOCS1 showed no effect on the degradation of gp130 IC in 293/ CypA− cells, but inhibited the degradation of gp130 IC when CypA was recovered (Figure 3C). We then examined the interaction between SOCS1 and CypA. Coimmunoprecipitation assays demonstrated that CypA interacted with the transfected SOCS1, but not with SOCS3. In addition, CypA R55A mutation did not alter CypA-SOCS1 interaction (Figure 3D). These results suggested that SOCS1 promoted gp130 degradation by targeting CypA, which did not depend on the prolyl-isomerase activity of CypA.

FIGURE 4 SOCS1 promotes ubiquitin-mediated proteasome degradation of CypA. (A and B) Immunoblot analysis of lysates of the indicated protein in 293T/CypA+ cells transfected for 24 h with Myc-SOCS1 or control vector, followed by stimulated by IL-6/sIL-6R for the indicated time periods (top). The relative expression levels of pSTAT3 and IL-6 (bottom) were quantified. (C) Quantitative PCR analysis of CypA mRNA (top) and immunoblot analysis of SOCS1 (bottom) in 293T/CypA+ transfected with Myc-SOCS1 for the indicated time points. (D) Immunoblot analysis of lysates in 293T/CypA+ cells transfected with Flag-CypA and Myc-SOCS1 or control vector for 24 h and then treated with 100 μg/ml CHX for the indicated durations (top). The relative expression levels of Flag-CypA were quantified (bottom). (E) Immunoblot analysis of lysates in 293T/CypA+ cells transfected with Myc-SOCS1 or control vector for 24 h and then treated with 100 μg/ml CHX for the indicated durations (top). The relative expression levels of Flag-CypA were quantified (bottom). (F) Immunoblot analysis of lysates in 293T/CypA+ cells transfected with Flag-SOCS1, along with Myc-CypA or Myc-R55A for 24 h and then treated with 100 μg/ml CHX for the indicated durations (top). The relative expression levels of Flag-CypA were quantified (bottom). (G) Immunoblot analysis of lysates in 293T/CypA+ cells transfected with Myc-SOCS1 for 24 h and then treated for 9h with 100 μg/ml CHX, along with 10 μM NH4Cl, 10 μM MG132, or DMSO (left). The relative expression levels of CypA were quantified (right). Data are shown as mean ± SD of at least three independent experiments. **p < .01. M, protein molecular weight marker; ns, not significant.
3.4 | SOCS1 promotes the ubiquitin-mediated proteasome degradation of CypA

We considered the possibility that SOCS1 might affect the stability of CypA. We first detected the protein level of CypA when the IL-6 trans-signaling pathway was activated. The results showed that SOCS1 significantly inhibited CypA expression (Figure 4A,B), STAT3 phosphorylation (Figure 4A), and IL-6 expression (Figure 4B). To further determine SOCS1 inhibited CypA expression at translational or transcriptional level, real-time quantitative PCR was performed. SOCS1 showed no effect on the transcription of CypA (Figure 4C). In contrast, SOCS1 facilitated the degradation of both transfected (Figure 4D) and endogenous (Figure 4E) CypA in 293T cells. In addition, CypA R55A mutation did not alter SOCS1-mediated CypA degradation (Figure 4F), indicating that the prolyl-isomerase activity of CypA is not necessary for this process. Next, we investigated which pathway involved in SOCS-mediated CypA degradation. The proteasome inhibitor MG132 significantly inhibited the degradation of CypA, whereas the lysosome inhibitor NH₄Cl did not (Figure 4G), indicating the degradation of CypA by SOCS1 depends on the proteasome-dependent pathway.

3.5 | SOCS1 enhances K48-linked ubiquitination at K154 and K155 of CypA

The effects of SOCS1 on CypA ubiquitination was further investigated. In immunoprecipitation experiments, SOCS1 enhanced the K48-linked ubiquitination of CypA (Figure 5A). So far, no ubiquitination site of CypA has.
been reported. We attempted to identify the K48-linked ubiquitination sites of CypA. Six potential CypA K48-linked ubiquitination sites (K28, K44, K49, K125, K154, and K155) were obtained by using mass spectrometry. Then a serial of CypA mutants (K28R, K44R, K49R, K125R, K154R, and K155R) were constructed. In immunoprecipitation experiments, only the K154R and K155R mutations led to the decreasing of CypA ubiquitination (Figure 5B). Moreover, SOCS1 no longer affected the expression of transfected CypA with the mutation of K154R or K155R (Figure 5C), which was consistent with the results of ubiquitination assay. These data suggested that SOCS1 enhances the K48-linked ubiquitination at K154 and K155 of CypA.

3.6 The SH2 and box domain of SOCS1 negatively regulates the IL-6 trans-signaling pathway by targeting CypA

To determine whether SOCS1 promoted the ubiquitin-mediated proteasome degradation of CypA depending on the domain except for KIR, a truncated SOCS1 (ESB) containing only the ESS, SH2, and SOCS Box domains was constructed. The co-immunoprecipitation results demonstrated that ESB was able to interact with CypA (Figure 6A), facilitated the degradation of CypA (Figure 6B), and significantly promoted the K48-linked ubiquitination of CypA at K154 and K155 (Figure 6C,D), indicating that SOCS1 promoted the ubiquitin-mediated proteasome degradation of CypA independent of its KIR.

Since SOCS1 KIR restrains IL-6 signaling and SOCS1 lacking the KIR basically loses the inhibition of JAK activity,24 we investigated whether ESB could regulate the IL-6 trans-signaling pathway by targeting CypA when KIR was absent. We observed that ESB significantly suppressed the phosphorylation of STAT3, Raf, MEK, and ERK in 293T/CypA+ cells, but not in 293T/CypA− cells (Figure 6E), suggesting that SOCS1 inhibited IL-6 trans-signaling pathway not only through its KIR to block JAK, but also through its SH2 and box domain to degrade CypA. Besides, ESB also exhibited strong inhibition of IL-6 and CypA expression in 293T/CypA+ cells (Figure 6F).
**DISCUSSION**

Both CypA and IL-6 play critical roles in inflammatory diseases and cancers, suggesting that there might be an association between them. It has been reported that CypA contributes to the activation of STAT3, a key IL-6 signaling protein, and STAT3 promotes CypA expression depending on its transcriptional activation. In this study, we demonstrate that upon the treatment of IL-6 and sIL-6R, CypA inhibited the ubiquitination-mediated degradation of IL-6 membrane receptor gp130 and enhanced its dimerization, thereby positively regulated the IL-6 trans-signaling and increased the expression of downstream iNOS, IL-6, SOCS1, and CypA. Furthermore, CypA expression could be negatively regulated by SOCS1, which interacted with CypA and promoted its K48-linked ubiquitination-mediated degradation, then inhibited the IL-6 trans-signaling pathway through degrading CypA, forming a negative feedback loop (Figure 7). Moreover, the prolyl-isomerase activity of CypA is not necessary for its regulation of the IL-6 trans-signaling pathway. Collectively, our data demonstrate that CypA plays an important role in the positive and negative feedback loops of the IL-6 trans-signaling pathway, suggesting a close relationship between CypA and IL-6.

Different types of ubiquitination of proteins is a significant way to determine the fate of the protein and regulate the function of the protein. Gp130 was characterized as the β subunit of the IL-6 receptor. It has been reported that IL-6 stimulation induces lysosome-dependent degradation of gp130, and NEDD4L interacts with gp130 and mediates K27-linked ubiquitination and proteasomal degradation of gp130. CypA is widely distributed in almost all tissues, and our previous research reveals that CypA interacts with many E3 and plays various biological roles.
In this study, CypA stabilized gp130 by inhibiting the K48-linked ubiquitination of gp130, then more gp130 was accumulated on the cell membrane to promote IL-6 trans-signal. We speculate that CypA might stabilize gp130 by binding to certain E3 ligases of gp130, which remains to be further studied.

SOCS1 is a negative feedback regulator of the IL-6 trans-signaling pathway by blocking the substrate-binding groove on JAK, acting as a pseudosubstrate, and the KIR of SOCS1 is essential for this process. In this study, we demonstrated that SOCS1 inhibited the IL-6 trans-signaling pathway not only through its KIR to block JAK, but also through its SH2 and box domain to degrade CypA. Mechanically, SOCS1 interacted with CypA and facilitates its K48-linked ubiquitination-mediated degradation at K154 and K155. These data indicated that the E3 activity of SOCS1 also plays vital roles in regulating the IL-6 trans-signaling pathway.

It has been reported that sIL-6R and IL-6 promote cytokine-chemokine (IL-6, IL-8, and MCP-1) release and intercellular cell adhesion molecule-1 (ICAM-1) expression in endothelial cells. Moreover, LPS and sIL-6R treatment induce a prominent increase in IL-6, IL-8, MCP-1, and the coagulation cascade activator plasminogen activator inhibitor-1 (PAI-1) production in an sIL-6R dose-dependent manner in endothelial cells. These results indicate that the IL-6R trans-signaling is critical for the production of pro-inflammation cytokines. In this study, sIL-6R and IL-6 treatment promoted IL-6 and iNOS expression both in A549 and 293T cells. Whether similar influences come into effect for other pro-inflammation cytokines, such as cytokine-chemokines and adhesion molecules, has not been investigated in these two cell lines, which is the limitation of this study. Although it is well-documented that the JAK/STAT3 and MAPK pathways are critical for inducing pro-inflammation cytokines expression, the precise mechanisms by which IL-6 trans-signaling drives the expression of IL-6, MCP-1, IL-8, and PAI-1 genes is an interesting future direction.

In conclusion, these findings demonstrate the critical roles of CypA in regulating the IL-6 trans-signaling pathway, which expands the biological functions of CypA in signal transduction regulation. Our data also provide a theoretical contribution for the treatment of inflammatory diseases and cancers.

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Lei Sun conceived the study; Lei Sun, Xiaohan Luan, and Wenxian Yang designed the experiments and analyzed the data; Lei Sun and Xiaohan Luan wrote the manuscript; Xiaohan Luan and Wenxian Yang carried out the experiments; Xiaoyuan Bai, Heqiao Li, Huizi Li, He Zhang, and Wenhui Fan provided technical support; Wenjun Liu helped analyze the data and revise the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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