Suppressor Of cytokine signaling 6 (SOCS6) negatively regulates Flt3 signal transduction through direct binding to phosphorylated Tyr 591 and Tyr 919 of Flt3.

Uddin, Kazi; Sun, Jianmin; Phung, Bengt; Zadjali, Fahad; Flores-Morales, Amilcar; Rönnstrand, Lars

Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M112.376111

2012

Citation for published version (APA):
Suppressor Of Cytokine Signaling 6 (SOCS6) negatively regulates Flt3 signal transduction through direct binding to phosphorylated Tyr 591 and Tyr 919 of Flt3

Julhash U. Kazi1, Jianmin Sun1, Bengt Phung1, Fahad Zadjali2, Amilcar Flores-Morales3 and Lars Rönnstrand1*

1Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Wallenberg Laboratory, Skåne University Hospital, 20502 Malmö, Sweden,
2Department of Biochemistry, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat 123, Oman and the
3Novo Nordisk Foundation Center for Protein Research, Faculty of Health Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark

Running title: SOCS6 suppresses Flt3 signaling.

*Corresponding author: Lars Rönnstrand, Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Wallenberg Laboratory, Skåne University Hospital, 20502 Malmö, Sweden, Tel.: +46 40 33 72 22, Fax: +46 40 33 11 04, E-mail: Lars.Ronnstrand@med.lu.se

Keywords: Flt3, Flt3-ITD, SOCS6

Background: Flt3 is an important regulator of hematopoiesis and is often found mutated and constitutively active in patients with acute myeloid leukemia.

Results: SOCS6 is upregulated by Flt3 activation and binds to phosphorylated Flt3.

Conclusion: SOCS6 is a negative regulator of Flt3 signaling.

Significance: Our results provide a role of SOCS6 in Flt3 signaling. Absence of SOCS6 promotes transformation of cells by Flt3-ITD.

SUMMARY

The receptor tyrosine kinase Flt3 is an important growth factor receptor in hematopoiesis, and gain-of-function mutations of the receptor contribute to the transformation of acute myeloid leukemia (AML). The suppressors of cytokine signaling 6 (SOCS6) is a member of the SOCS family of E3 ubiquitin ligases that can regulate receptor tyrosine kinases signal transduction. In this study we analyzed the role of SOCS6 in Flt3 signal transduction. The results show that ligand stimulation to Flt3 can induce association of SOCS6 and Flt3 and tyrosine phosphorylation of SOCS6. Phospho-peptide fishing indicates that SOCS6 binds directly to phospho-tyrosine 591 and 919 of Flt3. By using stable transfected Ba/F3 cells with Flt3 and/or SOCS6, we show that the presence of SOCS6 can enhance ubiquitination of Flt3 as well as internalization and degradation of the receptor. The presence of SOCS6 also induces weaker activation of Erk1/2 but not Akt in transfected Ba/F3 and UT-7 cells, and in OCI-AML-5 cells. The absence of SOCS6 promotes Ba/F3 and UT-7 cell proliferation induced by oncogenic internal-tandem-duplications (ITDs) of Flt3. Taken together, these results suggest that SOCS6 negatively regulates Flt3 activation and downstream Erk signaling pathway and cell proliferation.

INTRODUCTION

Flt3 is a type III receptor tyrosine kinase (RTK) that belongs to the same family as the receptors for PDGF, stem cell factor (SCF) and macrophage colony stimulating factor (M-SCF) (1). Under normal conditions, Flt3 is of great importance for the proliferation and differentiation of hematopoietic stem cells and progenitor cells. Ligand binding leads to dimerization of receptors, activation of their intrinsic tyrosine kinase activity and phosphorylation on tyrosine residues within the receptor intracellular domain, as well as on downstream signal transduction molecules. The phosphorylated tyrosine residues constitute high
affinity binding sites for signal transduction molecules that contains a conserved domain of approximately 100 amino acids, the so-called Src homology 2 (SH2) domain (2). Binding to the phosphorylated tyrosine residues is specified by the three to six amino acids immediately carboxy-terminal to the phosphorylated tyrosine residues, thus defining the specificity of individual SH2 domains. In order to understand the way growth factor receptors signal, it is of utmost importance to define the individual tyrosine residues that are phosphorylated in response to ligand-stimulation.

A number of gain-of-function mutations of Flt3 have been found in leukemia patients and human leukemia-derived cell lines. The internal tandem duplication (ITD) mutations were the first mutation to be identified (3). The ITD mutations are characterized by the duplication of a segment of the juxtamembrane region of Flt3, which leads to ligand-independent constitutive activation of Flt3. The ITD mutations range in size from three to more than 400 bp and always occur in multiple of three with the reading frame maintained. The size of the ITD is negatively correlated with 5 year overall survival of acute myeloid leukemia (AML) patients (4). Furthermore, the alleic ratio ITD/WT-Flt3 is a significant and independent prognostic factor for relapse in pediatric AML (5). Apart from the ITD mutations, point mutations in the kinase domain of Flt3 that can induce constitutive kinase activity have been described in both pediatric and adult AML (6). Among point mutations, the most commonly occurred is mutation at Asp835 of Flt3. Both the ITD mutations and point mutations of Flt3 are primarily found in AML, and a considerable body of evidence is now accumulating regarding their incidence and clinical impact.

The signaling mediated by growth factor induced activation of Flt3 must be tightly regulated. This occurs mainly through the action of specific protein tyrosine phosphatases and ubiquitin ligases. Protein tyrosine phosphatases can terminate the signal transduction by dephosphorylate target proteins. Ubiquitin ligases covalently attach ubiquitin moieties to target proteins which serve as a tag for degradation in either proteasome or, in the case of receptor tyrosine kinases, in the lysosomes. Oncogenic mutations in cancer can render the receptor tyrosine kinases less ubiquitinated, either due to mutations of the binding sites for ubiquitin ligases on the receptors themselves, or through inactivating mutations in the ubiquitin ligases (7). Cbl is the most studied ubiquitin ligase, and loss of function mutations in Cbl have been described in AML and shown to contribute to oncogenic transformation (8).

The suppressors of cytokine signaling (SOCS) is another class of regulator for downstream signal transduction of receptor tyrosine kinases. The SOCS family comprises CIS1 and SOCS1-7 (9). Members of this family are characterized by a SH2 substrate recognition domain and a C-terminal SOCS box which mediates assembly into Elongin B/C-Cullin ubiquitin ligase complexes. CIS1 and SOCS1-3 are well characterized as negative feedback regulators of cytokine receptor signal transduction via the JAK/STAT pathway, but the function of SOCS4-7 is less known. Although originally implicated as regulator of cytokine signaling, they are also involved in regulation of growth factor receptor signal transduction. It has been shown that both SOCS1 and SOCS6 can regulate signal transduction downstream of the stem cell factor receptor/ c-Kit (10,11). SOCS6 was shown to bind the juxtamembrane region of c-Kit and the binding caused a 40% reduction in SCF induced cell proliferation and similar reduction in the activation of Erk and p38 in Ba/F3-Kit cells (11). SOCS1 and SOCS6 can also interact with insulin receptor and inhibit insulin-dependent activation of Erk and protein kinase B (12).

In this study, we present evidence that Flt3 can bind SOCS6 in response to the ligand stimulation. SOCS6 binds directly to phospho-tyrosine residues 591 and 919 of Flt3. Flt3-Ligand (FL) stimulation also up regulates SOCS6 mRNA expression in Ba/F3 cells. The binding of SOCS6 to Flt3 increases receptor ubiquitination, internalization and degradation. Correspondingly, presence of SOCS6 inhibits Flt3 induced Erk and p38 activation and cell proliferation.

**EXPERIMENTAL PROCEDURES**

Reagents and antibodies- Transfection reagent jetPEI was from Polyplus-transfection and Lipofectamine 2000 was from Invitrogen. Cycloheximide was from Sigma. Rabbit polyclonal anti-SOCS6 serum was raised and purified as described (13). The phospho-tyrosine
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antibody 4G10 was from Millipore and ubiquitin antibody was from Covance Research Products. Flt3 antibody was described previously (14). Shc, phospho-p38 and p38 antibodies were from BD Transduction Laboratories. Phospho-Akt antibody was from Epitomics. Polyclonal antibodies against Akt, phospho-Erk and Erk were purchased from Santa Cruz Biotechnology. Phycocerythrin (PE) labeled Flt3 antibody was from BD Biosciences. Horseradish peroxidase-coupled secondary antimouse and anti-rabbit antibodies were from Invitrogen.

Cell culture- Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml recombinant murine interleukin-3 (IL-3). OCI-AML-5 and UT-7 cells were cultured in MEM-alpha supplemented with 20% HI-FBS 100 units/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml recombinant human IL-3. COS-1 and EcoPack cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

Expression constructs- pcDNA3-Flt3-WT, pcDNA3-Flt3-ITD, pMSCV-Flt3-WT and pMSCV-Flt3-ITD constructs were described previously (14). pEF-FLAG-SOCS6-WT, pEF-FLAG-SOCS6-R409E and pEF-FLAG-SOCS6-C504F constructs were described before(15). pMSCV-SOCS6-WT was constructed by subcloning full length open reading frame of murine SOCS6 into pMSCV vector. pcDNA3-Flt3-WT-Y591F/Y919F, pMSCV-Flt3-WT-Y591F/Y919F, pMSCV-Flt3-ITD-Y591F/Y919F constructs were generated by site-directed mutagenesis using QuikChange mutagenesis XL kit (Stratagene, La Jolla, CA). All plasmids were verified by sequencing.

RT-qPCR- Total RNA was isolated from Ba/F3-Flt3-WT cells using the RNeasy mini kit (Qiagen) according to the manufacturer. cDNAs were synthesized with oligo (dT) and random hexamer primers by use of a RevertAid Premium first-strand cDNA synthesis kit (Fermentas) according to the manufacturer's directions. Gene expression was assessed by real-time quantitative PCR (RT-qPCR) using an Applied Biosystems 7900HT Fast Sequence Detection System and gene-specific RT qPCR Primer Assays (SABiosciences). β-Actin was used as endogenous control to normalize expression data. Thermal cycling conditions included 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute according to the RT qPCR Primer Assays protocol. Each sample was analyzed in quadruplicate. The comparative Ct (threshold cycle) method was used to calculate the relative changes in gene expression.

Transient and stable transfection- COS-1 cells were transiently transfected using JetPEI according to the manufacturer. Cells were serum-starved overnight 24 hours after transfection and then stimulated at 37°C for the indicated time with 100 ng/ml FL (Prospec Tany). For transient transfection of OCI-AML-5 and UT-7 cell lines the 4D-nucleofector system (Lonza) was used. In order to establish Ba/F3 and UT-7 cells stably express the wild-type or mutant of Flt3, packaging EcoPack cells were transfected with the corresponding Flt3 construct in pMSCV-puro, and virus-containing supernatants were collected 72 hours after transfection. Retroviral infection of Ba/F3 cells was followed by a 2-week selection in 1.2 μg/ml puromycin. Expression of Flt3 was confirmed by flow cytometry. Flt3 transfected Ba/F3 cells were then further transfected with the SOCS6 construct in pMSCV-neo. Cells were selected with 0.8 mg/ml neomycin sulfate for 2 weeks and SOCS6 expression was verified by western blotting. Ba/F3 cells were serum-starved 4 hours in RPMI-1640 medium without serum and cytokines, and then stimulated at 37°C for the indicated time with 100 ng/ml FL.

Immunoprecipitation and Western blotting- After stimulation, cells were washed once with ice-cold PBS, lysed, and processed for immunoprecipitation and Western blotting as described previously (16). Immunodetection was performed by enhanced chemoluminescence using Immobilon Western chemoluminescent horseradish peroxidase substrate (Millipore Corporation, Milford, MA, USA) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Signal intensity was quantified by Multi-Gauge software (Fujifilm).

Affinity fishing of SOCS6 with immobilized peptides- Peptides corresponding to the tyrosine motifs of Flt3 intracellular domain either phosphorylated or not were synthesized (Y589: CGSSDNEYFYVDFREY, pY566: CHKpYKKNFRYESQLQM, pY572:
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Samples were assessed by SDS-PAGE and Western blotting.

Analysis of SOCS6 expression in human samples- Gene Expression Omnibus (GEO) database was used for expression analysis. Microarray expression data of three individual sets of patient samples for acute promyelocytic leukemia and acute myeloid leukemia, and corresponding matched cells (GSE2550, GSE9476 and GSE2191) were downloaded and used for analysis.

RESULTS

SOCS6 protein interacts with Flt3 under ligand stimulation- It was reported that SOCS6 can associate with c-Kit through its SH2 domain under SCF stimulation (11). Flt3 belongs to the same receptor tyrosine kinase family as c-Kit and contains a similar region as c-Kit where SOCS6 was shown to bind. In order to know whether SOCS6 can also interact with Flt3, SOCS6 and Flt3 were transiently expressed in COS-1 cells, FL stimulation induced strong association of SOCS6 with Flt3 (Fig. 1A). SOCS6 was found to be associated with the oncogenic Flt3-ITD mutant in COS-1 cells (Fig. 1B) and also in Ba/F3 cells (Fig. 1D) as well. Furthermore endogenous SOCS6-Flt3 interaction was detected in OCI-AML-5 cells (Fig. 1C). The kinetics of Flt3-SOCS6 interaction was also studied. Similar as Flt3 activation, Flt3-SOCS6 interaction was rapid and reached the maximum after ligand stimulation for 5 minutes and was stable at least for 1 hour (Fig. 1E). Although we observed a SOCS6 tyrosine phosphorylation in COS-1 cells in response to FL (Fig. 1E), we were unable to detect tyrosine phosphorylation either in Ba/F3 or in OCI-AML-5 cells (data not shown). To determine whether Flt3-SOCS6 interaction is mediated through SOCS6-SH2 domain mutant was able to interact with Flt3, the SOCS6-SH2 domain mutant did not show any interaction with Flt3 (Fig. 1F).

SOCS6 binds directly to phospho-tyrosine 591 and 919 of Flt3- Flt3 intracellular domain contains at least 12 tyrosine residues which are known to be phosphorylated upon activation. To determine the SOCS6 binding sites, phospho and unphospho peptides corresponding to all known
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Flt3 tyrosine phosphorylation residues were synthesized. Peptides were immobilized on UltraLink and subjected to pull down proteins from SOCS6 overexpressing cell lysates. Western blotting with anti-SOCS6 antibody showed that pY591 and pY919 are SOCS6 binding sites (Fig 2A). Interestingly, concomitant phosphorylation of the adjacent tyrosine 589 greatly enhanced the binding of SOCS6 to tyrosine 591 (Fig. 2B). In order to know whether Y591 and Y919 were also SOCS6 binding sites in vivo, we generated pcDNA3-Flt3-Y591F/Y919F. COS-1 cells were cotransfected with either wild type or the double mutant of Flt3 and SOCS6, under FL stimulation, interaction of SOCS6 with Flt3 was significantly reduced when Y591 and Y919 were mutated (Fig. 2C), which indicates that Y591 and Y919 residues of Flt3 are SOCS6 binding sites in vivo.

Activation of Flt3 induces mRNA transcription of SOCS6- In order to assess the influence of Flt3-SOCS6 interaction in hematopoietic cells, we generated Ba/F3-Flt3-WT, Ba/F3-Flt3-WT/SOCS6-WT, Ba/F3-Flt3-ITD and Ba/F3-Flt3-ITD/SOCS6-WT cell lines. Flt3 expression levels of these cell lines were verified by flow cytometry (Fig. 3A) and SOCS6 expression levels were checked by Western blotting (Fig. 3B). It was shown that SCF-stimulation can induce mRNA transcription of SOCS6 (11). We tested whether FL- stimulation can do the same. Ba/F3 cells stably transfected with Flt3 were treated with 100 ng/ml FL for different time points. Total RNAs were extracted and subjected to qPCR analysis, SOCS6 transcription was shown to be linearly increased with the time in response to FL stimulation (Fig. 4A). Furthermore meta-analysis of published microarray data from patient samples revealed that SOCS6 expression significantly increased in acute promyelocytic leukemia expressing Flt3-ITD (Fig. 4B). SOCS6 expression also increased in AML patients (Fig. 4C and 4D).

SOCS6 is involved in ubiquitination, internalization and degradation of Flt3 receptor- Recently it has been shown that SOCS6 promoted ligand-dependent ubiquitination of c-Kit receptor (15). To test ubiquitin ligase activity of SOCS6 on Flt3 receptor we stimulated transfected Ba/F3 cells with FL for different time points. The results clearly demonstrated an increased amount of ubiquitinlated Flt3 in presence of SOCS6 (Fig. 5A and 5B). Internalization of Flt3 following FL stimulation was determined by flow cytometry for the indicated time. Internalization of Flt3 receptor was significantly increased in SOCS6 expressing cells (Fig. 6A). SOCS6 can also promote ligand-induced degradation of Flt3 receptor (Fig 6B).

SOCS6 negatively regulates Erk signaling - Activation of Flt3 is known to regulate several signaling pathways including Ras/Erk pathway, p38 pathway and PI3K-Akt pathway (1). In order to investigate how SOCS6 affected Flt3-mediated signal transduction, activation of Akt, Erk and p38 was examined by Western blotting using phospho-specific antibodies. The presence of SOCS6 in Ba/F3-Flt3 cells significantly inhibited FL-mediated Erk activation (Fig 7A). Although p38 phosphorylation was reduced about 20%, was not statistically significant and Akt activation remained mostly unchanged (Fig 7A). In addition, selective depletion of SOCS6 using siRNA in OCI-AML-5 cells that expresses endogenous Flt3 and SOCS6 (Fig. 3C) significantly increased FL-induced Erk phosphorylation (Fig. 7B). As we observed that SOCS6-R409E mutant was unable to associate with Flt3 (Fig 1F), we compared FL-induced Erk activation in Ba/F3-Flt3 cells expressing SOCS6-WT and SOCS6-R409E. In accordance with interaction data SOCS6-R409E did not block FL-mediated Erk phosphorylation (Fig. 7C). In figure 2C we showed that mutation in Y591 and Y919 residues reduced SOCS6 interaction with Flt3. Thus we transfected UT-7 cells expressing endogenously SOCS6 (Fig 3C) with Flt3-WT and Flt3-WT-Y591F/Y919F constructs. Result suggests that UT-7 cells expressing Flt3-WT-Y591F/Y919F mutant exhibited increased Erk phosphorylation in response to the FL-stimulation in compare to Flt3-WT (Fig. 7D). Shc is a signaling molecule that is upstream of Ras/Erk pathway, similar as Erk activation; a 50% reduction of Shc phosphorylation in SOCS6 expressing Ba/F3-Flt3 cells in response to FL-stimulation was observed (Fig. 7C and D).

SOCS6 negatively regulates FL-induced cell proliferation but not cell survival- Cell proliferation induced by activated RTKs can be suppressed by SOCS family proteins (10,11). Several SOCS proteins also exhibit tumor suppressor function. For example, SOCS6 is down-regulated in a variety of cancer and, ectopic...
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expression of this protein suppresses cell growth and colony formation of gastric cancer cell lines (17). To explore the biological outcome of the Flt3-SOCS6 interaction, we studied the effect of SOCS6 expression on Flt3-dependent cell proliferation. SOCS6 expression led to a 25% decrease of cell proliferation in the Ba/F3-Flt3-ITD cells in response to FL stimulation (Fig. 8A). The effect of SOCS6 expression on cell survival was also examined. By staining the cells with Annexin-V and 7-AAD, it was shown that SOCS6 expression could not alter survival of Ba/F3-Flt3-ITD cells (Fig. 8B). Using PrestoBlue cell viability assays we demonstrated that wild type SOCS6 but not SOCS6-R409E mutant was able to reduce Flt3-ITD mediated cell proliferation (Fig. 8C and D). Furthermore Flt3-ITD-Y591F/Y919F induced cell proliferation was not blocked by endogenous SOCS6 in UT-7 cells (Fig. 8E).

DISCUSSION

It is widely accepted that deregulated activation of Flt3 has important functions in AML pathogenesis. Flt3 is highly expressed in AML. To understand the mechanism that control Flt3 signaling, we analyzed the role of SOCS6 for Flt3 regulation. We identified SOCS6 as a new interaction partner of Flt3. This interaction negatively regulates Flt3-dependent signaling pathways by promoting ubiquitination, internalization and degradation of Flt3 receptor.

The finding that SOCS6 is capable of interacting with Flt3 and c-Kit (11) indicates that SOCS6 may have a role in receptor tyrosine kinase signaling. In both of cases the interaction was dependent on the ligand stimulation. The SH2 domain of SOCS6, but not the SOCS box, is involved in this association suggesting that SOCS6 specifically interacts with phosphotyrosine residues (15,18). Using Flt3 phospho peptides we mapped SOCS6 interaction sites as pY591 and pY919. A previous analysis of binding specificity to phospho-peptide showed that the SOCS6 SH2 domain bound preferentially to motifs containing a valine in the phospho-tyrosine (pY) +1 position and hydrophobic residues in the pY +2 and pY +3 positions (18). The sequence surrounding Y591 in Flt3 fulfils the consensus requirement (YVDF) for binding of SOCS6 and also shows strong similarity with the region of c-Kit where SOCS6 is shown to bind (11). Interestingly, Flt3 pY589 peptide did not associate with SOCS6 but phosphorylation in Y589 residue increased binding affinity dramatically when introduced with pY591 peptide.

The mechanism by which Y589 phosphorylation increases binding affinity to Flt3 pY591 peptide is not clear. One possible explanation is that negative charge at position pY -2 may increase affinity to the pY591. Y591 is located in Flt3 juxtamembrane domain. This domain is of utmost regulatory importance for many growth factor receptors. Y589, Y591 and Y599 in this domain are suggested to be Src binding site (19). Y589 and Y591 have shown to be conserved in related tyrosine kinases c-Kit (20), PDGFRα (21) and PDGFRβ (22). These tyrosine residues are involved in interaction with various signaling proteins including Src family kinases (SFKs), SHP1 and SHP2. Thus Y589/Y591 motif appears to be a major docking site for the protein complexes following type III RTK activation. Another SOCS6 binding site in Flt3 (Y919) is located in the kinase domain which has not been studied well. One report suggests that together with other phospho-tyrosine residues Y919 is involved in activation of Flt3 kinase domain mutant (23).

Expression profiles of Flt3 and SOCS6 overlap. For example, SOCS6 mRNA is expressed in the most of hematopoietic progenitors and bone marrow cells (18). Flt3-positive AML cell line OCI-AML-5 expresses SOCS6 mRNA. In addition, we observed that transcription of SOCS6 mRNA was induced by FL in Ba/F3-Flt3 cell line. This observation suggests that Flt3 signaling might be regulated by SOCS6. Like SOCS1 and SOCS3, SOCS6 associates with Elongins B and C in a SOCS box dependent manner (18,24). Furthermore, SOCS6 promotes ligand-dependent ubiquitination of c-Kit receptor (15) and p56Lck kinase (25). We also observed an increased ligand-dependent ubiquitination of Flt3 receptor in SOCS6 expressing cells. These findings further provide evidence that SOCS6 acts as an E3 ubiquitin ligase in biological systems. Another E3 ligase, Cbl has also been reported involved in ubiquitination and degradation of Flt3 (26). The observation that SOCS6 expressing cells showed accelerated internalization and degradation of Flt3 receptor, can also be explained by increased
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ubiquitination effect of the receptor in the same system.

Loss of SOCS function promotes tumor formation which can occur by several mechanisms including gene deletion, mutation or silencing due to hypermethylation (27). Several SOCS proteins including CIS, SOCS2, SOCS3 and SOCS6 have been implicated in the negative regulation of growth factor signaling. Our data also provide evidence that SOCS6-mediated regulation of Flt3 is biologically important. SOCS6 partially blocked Flt3-ITD-mediated cell proliferation. In addition, SOCS6 partially inhibited ligand-dependent proliferation of Ba/F3-Kit cells but not Ba/F3-EGFR cells (11). Taken together, these observations suggest that SOCS6 controls growth of hematopoietic cells by modulating distinct signaling pathways.

Flt3 physically associates and phosphorylates several signaling proteins including Ras, PLCγ, Grb2, SHP2 and SFKs resulting in further activation of downstream PI3K and MAPK pathways (1,28). Activation of MAPK pathways results in phosphorylation of Erk and p38 kinases while PI3K signaling pathway regulates Akt phosphorylation. We observed that activation of Erk, but not Akt, was significantly inhibited by SOCS6. Bayle et al. (11) also observed very similar inhibition in SOCS6 expressing Ba/F3-Kit cells. These individual observations may suggest that SOCS6 interrupts MAPK signaling pathways but not PI3K pathway. MAPK signaling pathways can be activated by Flt3 in multiple ways. For example, interaction of Grb2 with Flt3 results in Erk phosphorylation (29) and mutation in Src binding sites of Flt3 reduced Erk phosphorylation (19). A protein tyrosine phosphatase SHP2 has also been shown to interact with Flt3 modulating Erk phosphorylation (19). Thus we suggest that interaction of SOCS6 with Flt3 may disrupt binding of signaling proteins with Flt3 resulting in reduced phosphorylation of Erk and p38. Activation of MAPK pathways through EGF, PMA or anisomycin stimulation was not inhibited by SOCS6 (11) which also put further evidence that SOCS6 inhibition takes place in the initial stages. SFKs phosphorylate Shc protein upon activation of RTKs which is an early event in MAPK signaling. One SOCS6 interaction site in Flt3 (Y591) is also a binding site for SFKs (1). We observed that SOCS6 expression inhibited FL-induced phosphorylation of Shc protein. Interaction of SOCS6 with Y591 may partially block SFKs activation followed by reduced Shc phosphorylation. Thus we conclude that SOCS6-Flt3 interaction partially blocked MAPK signaling pathways resulting in reduced cell proliferation in hematopoietic cells.

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Acknowledgments- We thank Susanne Bengtsson, Elena Razumovskaya and Bengt Phung for their help in different experiments. J.U. Kazi was supported by Wenner-Gren Foundations. This research was funded by the Swedish Cancer Society, the Swedish Childrens Cancer Organization, the Swedish Research Council.

FIGURE LEGENDS

Fig. 1: SOCS6 interacts with Flt3 in response to FL-stimulation.

(A) COS-1 cells were co-transfected with SOCS6 and Flt3-WT expression vector. Cells were serum-starved overnight and stimulated by FL or not for 5 minutes before lysis. Cell lysates were subjected to immunoprecipitation using either anti-Flt3 or anti-SOCS6 antibody and analyzed by Western blotting. (B) COS-1 cells were co-transfected with SOCS6 and Flt3-ITD. Cells were then processed as mentioned above. (C) OCI-AML-5 cells were starved overnight and stimulated with FL for 5 minutes. Cells were lysed and processed for immunoprecipitation and western blotting. (D) Ba/F3 cells transfected with Flt3-ITD and SOCS6 were starved for 4 hours followed by 5 minutes FL stimulation. Cells were lysed and processed for immunoprecipitation and western blotting. (E) After overnight serum-starvation
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of transfected COS-1 cells, cells were treated with 100 ng/ml FL for the indicated times before lysis and immunoprecipitated with anti-SOCS6 antibody. (F) COS-1 cells were transfected with Flt3 and SOCS6 mutants. After overnight starvation cells were treated with 100 ng/ml FL for 5 minutes before lysis and immunoprecipitated with anti-SOCS6 antibody.

Fig. 2: Identification of Y591 and Y919 as SOCS6 binding sites.
(A) Phospho-peptides corresponding to 12 known tyrosine phosphorylation sites in Flt3 were synthesized and immobilized on UltraLink. Peptide-bound slurry was incubated with SOCS6-transfected COS-1 cell lysates and pulled-down proteins were then processed for Western blotting using an anti-SOCS6 antibody to screen the SOCS6 binding sites. C-Kit Y568 peptide was used as positive control. (B) Immobilized phospho- and unphospho-peptides were used to pull-down SOCS6 proteins and processed as above to verify whether the binding is dependent on tyrosine phosphorylation. (C) COS-1 cells were co-transfected with SOCS6 and wild type or Y591F/Y919F mutant of Flt3. Cells were serum-starved and stimulated with FL for 5 minutes before lysis. Lysates were immunoprecipitated with anti-Flt3 or anti-SOCS6 antibody and analyzed by Western blotting to show Y591 and Y919 are the SOCS6 binding sites in vivo.

Fig. 3: Expression of Flt3 and SOCS6 in Ba/F3 cells.
(A) Stably transfected Ba/F3 cells were labeled with isotype control or PE-conjugated Flt3 antibody and analyzed by flow cytometry. Faint line indicates cells labeled with isotype control and black line indicates cells labeled with Flt3 antibody. (B) Lysates of stably transfected Ba/F3-Flt3 cells with or without SOCS6 were immunoprecipitated with an anti-SOCS6 antibody and analyzed by Western blotting. (C) Endogenous SOCS6 and Flt3 expressions were detected using specific antibodies in different AML cell lines.

Fig. 4: FL induces SOCS6 gene expression in Ba/F3-Flt3 cells.
(A) Ba/F3-Flt3 cells were serum-starved and stimulated with FL for the indicated times. Cells were lysed and total RNAs were isolated. Expression of SOCS6 mRNA was analyzed by qPCR as described under “Experimental procedures”. (B) SOCS6 expression in acute promyelocytic leukemia patient carrying either Flt3-WT or Flt3-ITD mutation. (C) SOCS6 expression in AML patient and corresponding normal cells. (D) SOCS6 expression in AML patient with complete remission or relapse and corresponding normal cells. * p<0.05; *** p<0.001

Fig. 5: SOCS6 expression increases ubiquitination of Flt3 receptor.
(A) Ba/F3-Flt3 cells with or without SOCS6 were serum-starved and stimulated with FL for the indicated times. Cells were lysed and lysates were immunoprecipitated with an anti-Flt3 antibody followed by Western blotting. (B) Signal intensities were quantified using multi-gauge software to calculate the receptor ubiquitination.

Fig. 6: SOCS6 expression increases internalization and degradation of Flt3 receptor.
(A) Ba/F3-Flt3 cells with or without SOCS6 were serum-starved and stimulated with 100 ng/ml FL for the indicated times. Cells were transferred to ice immediately followed by incubation with PE-conjugated Flt3 antibody. Flt3 surface expression level was analyzed by flow cytometry. Compare with unstimulated cells, internalization of Flt3 was quantified. (B) Ba/F3-Flt3 cells with or without SOCS6 were serum-starved and preincubated with cycloheximide for 4 hours. Cells were then stimulated with 100 ng/ml FL for 30 minutes before lysis. Cell lysates were immunoprecipitated with an anti-Flt3 antibody and analyzed by Western blotting. Signal intensities were quantified using multi-gauge software to calculate the receptor degradation. ** p<0.01
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Fig. 7: Effects of SOCS6 expression on FL induced activation of Erk, p38, Akt and Shc.
(A) Ba/F3-Flt3 cells with or without SOCS6 were treated with 100 ng/ml FL or not for 5 minutes before lysis. Total cell lysates were separated by SDS-PAGE and membranes were probed with phospho-specific antibodies. Membranes were then stripped and reprobed with respective antibodies to show the loading. Signal intensities from three independent experiments were quantified using multi-gauge software to calculate the inhibition. (B) OCI-AML-5 cells were transfected with control siRNA or SOCS6 siRNA using electroporation. One day after transfection cells were starved for overnight and stimulated with FL followed by western blotting analysis. (C) Ba/F3-Flt3 cells transfected with SOCS6-WT or SOCS6-R409E mutant were starved for 4 hours before 5 minutes FL-stimulation. Cells were then processed for western blotting. (D) UT-7 cells transfected with Flt3-WT or Flt3-WT-Y591F/Y919F were starved for overnight before 5 minutes FL-stimulation. Cells were then processed for western blotting. (E) Cell lysates were immunoprecipitated with an anti-Shc antibody and analyzed by Western blotting. Signal intensities were quantified using multi-gauge software to calculate the inhibition. * p<0.05; ** p<0.01; *** p<0.001

Fig. 8: The presence of SOCS6 down regulates cell proliferation of Ba/F3-Flt3-ITD cells.
(A) Ba/F3-Flt3-ITD cells with or without SOCS6 (EV) were grown for 48 hours in the presence or absence of ligand. Viable cells were counted using trypan blue exclusion method. (B) Cells were labeled with Annexin-V and 7-AAD, and living cells were measured by flow cytometry. IL-3 was used as a positive control. (C) Ba/F3-Flt3-ITD/Empty vector cells or Ba/F3-Flt3-ITD/SOCS6-WT cells, (D) Ba/F3-Flt3-ITD/SOCS6-WT cells or Ba/F3-Flt3-ITD/SOCS6-R409E cells, (E) UT-7-Flt3-ITD cells or UT-7-Flt3-ITD-Y591F/Y919F (Flt3-ITD-YYFF) cells were washed to remove IL3 and then seeded in 96-well plates. Cells were then treated with either ligand or not for 48 hours followed by PrestoBlue cell viability assays. * p<0.05; ** p<0.01; *** p<0.001
SOCS6 suppresses Flt3 signaling

Figure 1.

A. FL

B. FL

C. FL

D. FL

E. FL (min)

F. FL
Figure 2.

A. 

<table>
<thead>
<tr>
<th>e-Kit</th>
<th>Flt3</th>
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<tbody>
<tr>
<td>Y568</td>
<td>pY568</td>
</tr>
<tr>
<td>pY1572</td>
<td>Y580/591</td>
</tr>
<tr>
<td>Y580/591</td>
<td>pY2591</td>
</tr>
<tr>
<td>pY280/9/591</td>
<td>Y591</td>
</tr>
<tr>
<td>Y910</td>
<td>pY910</td>
</tr>
</tbody>
</table>

IB: SOCS6

B. 

<table>
<thead>
<tr>
<th>e-Kit</th>
<th>Flt3</th>
</tr>
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<tbody>
<tr>
<td>Y568</td>
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<td>Y910</td>
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</table>

IB: SOCS6

C. 

<table>
<thead>
<tr>
<th>IP</th>
<th>Flt3-WT</th>
<th>SOCS6</th>
<th>Flt3-Y591F/Y919F</th>
<th>SOCS6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>P-Flt3</th>
<th>P-SOCS6</th>
<th>SOCS6</th>
<th>Flt3</th>
</tr>
</thead>
</table>

SOCS6 suppresses Flt3 signaling
Figure 3.

A.

B.

\[
\begin{array}{c|c|c}
\text{Ba/T3} & \text{SOCS6} & \text{Flt3-WT} & \text{Flt3-ITD} \\
& - & + & - & + \\
\hline
\text{IP: SOCS6} & - & + & - & + \\
\text{IB: SOCS6} & \text{SOCS6} & \text{SOCS6} & \text{SOCS6} & \text{SOCS6} \\
\end{array}
\]

C.

\[
\begin{array}{c|c|c|c|c|c}
\text{Mo7e} & \text{PL21} & \text{OCT-AML-5} & \text{THP-1} & \text{UT-7} & \text{SOCS6} \\
& & & & & \text{SOCS6} \\
\text{Flt3} & & & & & \text{Flt3} \\
\end{array}
\]
SOCS6 suppresses Flt3 signaling

Figure 4.

A

P<0.01

Fold of induction

0 1h 4h 8h 24h

FL

B

Relative expression

0 1 2 3 4 5

Flt3-WT Flt3-ITD

Acute promyelocytic leukemia

C

Relative expression

4 6 8

Normal AML

D

Relative expression

0 5 10 15

Normal Remission Relapse

Acute myeloid leukemia
Figure 5.

A.

<table>
<thead>
<tr>
<th>FL (min)</th>
<th>Empty vector</th>
<th>SOCS6</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>10</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

IB: Ubiquitin

IB: Flt3

IP: Flt3

B.

![Graph showing time vs. response with Empty vector and SOCS6 compared](image)

P < 0.01

Time (min)
Figure 6.

A. 

B.
SOCS6 suppresses Flt3 signaling

Figure 7.

A. Empty vector vs. SOCS6

B. Control siRNA vs. SOCS6 siRNA

C. SOCS6 WT vs. R409E

D. Flt3 WT vs. WT-Y911F/Y919F

E. Empty vector vs. SOCS6

Ba/F3-Flt3-WT

UT-7-Flt3-WT
SOCS6 suppresses Flt3 signaling

Figure 8.