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Suppressor of cytokine signaling 2 (SOCS2) associates with FLT3 and negatively regulates downstream signaling

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ABSTRACT

The suppressor of cytokine signaling 2 (SOCS2) is a member of the SOCS family of E3 ubiquitin ligases. SOCS2 is known to regulate signal transduction by cytokine receptors and receptor tyrosine kinases. The receptor tyrosine kinase FLT3 is of importance for proliferation, survival and differentiation of hematopoietic cells and is frequently mutated in acute myeloid leukemia. We observed that SOCS2 associates with activated FLT3 through phosphotyrosine residues 589 and 919, and co-localizes with FLT3 in the cell membrane. SOCS2 increases FLT3 ubiquitination and accelerates receptor degradation in proteasomes. SOCS2 negatively regulates FLT3 signaling by blocking activation of Erk 1/2 and STAT5. Furthermore, SOCS2 expression leads to a decrease in FLT3-ITD-mediated cell proliferation and colony formation. Thus, we suggest that SOCS2 associates with activated FLT3 and negatively regulates the FLT3 signaling pathways.

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1. Introduction

The suppressor of cytokine signaling 2 (SOCS2) is a member of the SOCS family of ubiquitin E3 ligases. Members of this family are CIS1 and SOCS1–7 (Yoshimura et al., 2007). The important features of this family proteins include presence of an SH2 domain and a C-terminal SOCS box. The SOCS box mediates assembly into Elongin B/C-Cullin complexes to facilitate the ubiquitination processes, while the SH2 domain mediates interaction with phosphotyrosine residues. SOCS family proteins are mainly characterized as negative feedback regulators of cytokine receptor signal transduction via the JAK/STAT pathway and have recently been implicated in receptor tyrosine kinase signaling (Bayle et al., 2004; De Sepulveda et al.,

1999; Kazi et al., 2012). SOCS2 depleted mice displayed high-growth phenotype suggesting a role of SOCS2 in growth control (Horvat and Medrano, 2001). SOCS2 interacts with the growth hormone receptor (GHR) through phosphorylated tyrosine residues and negatively regulates receptor signaling (Greenhalgh et al., 2005). This regulation is mediated through ubiquitination and proteasomal degradation of the receptor (Vesterlund et al., 2011).

The type III receptor tyrosine kinase FLT3 is under normal conditions of great importance for the proliferation, survival and differentiation of hematopoietic stem cells and progenitor cells (Masson and Rönnstrand, 2009). FLT3 is frequently mutated in acute myeloid leukemia (AML) and the most common mutation is the internal tandem duplication (ITD) in the

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juxtamembrane domain (Levis and Small, 2003). This mutation leads to ligand-independent activation of receptor as well as downstream signaling. The signaling mediated by activated FLT3 is tightly regulated by adapter proteins. For example, interaction with SOCS6 (Kazi et al., 2012) and Lnk (Lin et al., 2012) negatively regulates downstream signaling while association with Grb2 (Masson et al., 2009), Grb10 (Kazi and Ronnstrand, 2013) or SLAP (Kazi and Ronnstrand, 2012) results in activation of downstream signaling.

Here we present evidence that SOCS2 interacts with both normal and oncogenic FLT3. This association is mediated through phosphotyrosine residues 589 and 919 of FLT3. SOCS2 co-localizes with FLT3 in Ba/F3 cells and increases receptor ubiquitination followed by degradation in the proteasomes. Furthermore SOCS2 expression suppresses Erk 1/2 and STAT5 signaling. SOCS2 expression inhibits FLT3-ITD-mediate colony formation in semi-solid culture.

2. Materials and methods

2.1. Reagents and antibodies

Rabbit polyclonal anti-SOCS2 serum was raised and purified as described before (Blume-Jensen et al., 1993). The 4G10 and ubiquitin antibodies were from Millipore and Covance Research Products, respectively. The anti-FLT3 antibody was described previously (Razumovskaya et al., 2009). Phycoerythrin (PE)-conjugated FLT3 antibody was from BD Biosciences. The anti-phospho-Akt and other antibodies were from Epitomics and Santa Cruz Biotechnology, respectively. The RbX anti-phosphoserine antibody was from Millipore.

2.2. Expression constructs

The pcDNA3-FLT3-WT, pcDNA3-FLT3-ITD, pMSCV-FLT3-WT and pMSCV-FLT3-ITD constructs were described previously (Razumovskaya et al., 2009). The pCMV5-Myc-FLAG-SOCS2-WT plasmid was purchased from OriGene. The pcDNA3-SOCS2-SH2-FLAG plasmid was generated by sub-cloning of FLAG-tagged SOCS2-SH2 domain into the pcDNA3 vector.

2.3. Cell culture, transient and stable transfection

The COS-1 cells (cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS) were transiently transfected using JetPEI (Polyplus-transfection) according to the manufacturer's instructions. Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS and 10 ng/ml recombinant murine interleukin-3 (IL-3). Ba/F3-FLT3-WT and Ba/F3-FLT3-ITD cells were generated using retroviral transduction as described before (Kazi et al., 2012). Cells were further transfected with pCMV5-Myc-FLAG-SOCS2-WT construct using 4D-nucleofector system (Lonza) followed by two weeks selection with 0.8 mg/ml G-418. Expression of FLT3 was verified by flow cytometry and Western blotting, while SOCS2 expression was checked by Western blotting. Ba/F3-FLT3-WT cells were cultured in same medium used for Ba/F3 and Ba/F3-FLT3-ITD, Kasumi-1, THP-1, MV4-11 and MOLM-13 cells were cultured in RPMI 1640 medium

supplemented with 10% heat inactivated FBS. P815 cells were cultured in the same medium as was used for COS-1 cells.

2.4. Immunoprecipitation and Western blotting

The COS-1 cells were serum-starved overnight 6 h after transfection and then stimulated at 37 °C for the indicated time with 100 ng/ml of FLT3 ligand (FL) (Prospec Tany). Ba/F3 cells were serum-starved 4 h in RPMI-1640 medium and then stimulated with 100 ng/ml of FL at 37 °C for 5 min. Cells were then processed for immunoprecipitation and Western blotting as previously described (Kazi et al., 2012; Voytyuk et al., 2003).

2.5. Affinity fishing of SOCS2 with immobilized peptides

Peptides corresponding to the tyrosine motifs of FLT3 intracellular domain either phosphorylated or not were used for affinity fishing (for sequences see (Kazi et al., 2012; Lin et al., 2012)). Peptides were immobilized to UltraLink beads (Thermo Scientific) according to the manufacturer's instructions. The SOCS2 construct was transfected into the COS-1 cells. Twenty four hours after transfection cells were lysed and 50 µl of peptide beads slurry was added to each ml of cell lysates. Then proteins pulled down by peptide were processed for Western blotting.

2.6. Analysis of SOCS2 expression in patient samples

The SOCS2 expression was retrieved from microarray datasets for human patient samples from different cancers (GSE9476, GSE5550, GSE5788, GSE4290, GSE2223, GSE20437, GSE6691, GSE4115, GSE6919 and GSE8671) available through the NCBI GEO site.

2.7. Co-localization study

Ba/F3-FLT3-WT-SOCS2-FLAG cells were stimulated or not with FL for 5 min after starvation. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X 100. After blocking with goat serum cells were stained with fluorophore-conjugated anti-FLT3 and anti-FLAG antibodies as well as with DAPI. Cellular localization of FLT3 and SOCS2 was analyzed by a Carl Zeiss LSM 710 Laser Scanning Microscope. Co-localization was measured with CoLocalizer Pro 2.7.1 software.

2.8. Colony formation and cell proliferation assay

Ba/F3-FLT3-ITD cells transfected with SOCS2 or empty control vector were cultured in semisolid methylcellulose medium (MethoCult M3231, Stem Cell Technologies) for 5 days. Cell proliferation was measured using PrestoBlue cell viability assays (for detailed method, see Kazi et al., 2012).

3. Result

3.1. SOCS2-SH2 domain displays closest structural homology to the SOCS6-SH2 domain

Recently we reported that SOCS6 is overexpressed in AML (Kazi et al., 2012). To understand the role of other SOCS family

members in AML, we compared mRNA expression from micro-array data of AML patient samples with that of healthy donors. We observed that expression of SOCS2, SOCS5 and SOCS6 was upregulated in AML (Figure 1A). In the same report we also provided evidence that SOCS6 associates with FLT3 and negatively regulates FLT3 downstream signaling (Kazi et al., 2012). Since expressions of SOCS2 and SOCS5 are also deregulated in AML, and FLT3 is frequently mutated in this disease, we hypothesized that SOCS2 or SOCS5, similar to SOCS6, might also play a role in FLT3 signaling. SOCS6 associates with FLT3 through FLT3-pY residues and the SOCS6-SH2 domain (Kazi et al., 2012). Thus we initially compared SH2 domains of SOCS proteins using protein sequences. We observed that SOCS-SH2 domains are divided into four subgroups (Figure 1B). Although critical arginine residues are conserved within the family neither SOCS2 nor SOCS5 displayed a close homology with SOCS6 (Figure 1C) and thus we were unable to draw a conclusion whether SOCS2 or SOCS5 has a probability to interact with FLT3 from sequence alignment.

Then we compared 3D-structures of SOCS2-SH2 and SOCS5-SH2 domain with that of SOCS6-SH2 domain. We

aligned SOCS2-SH2-SOCS6-SH2 and SOCS5-SH2-SOCS6-SH2 domain pairs using SPAlign. The SOCS2-SH2 domain displayed closer structural similarity to SOCS6-SH2 (RMSD-1.7Å) than to SOCS5-SH2 (RMSD-1.9Å) (Figure 1F and G). The binding site of the phosphotyrosine moiety is known to be created by two arginine and one serine residues shown in Figure 1D and E (Bullock et al., 2006; Zadjali et al., 2011). Although all three residues in SOCS2-SH2 domain completely showed similar orientation (Figure 1F), the arginine residue in SOCS5-SH2 domain corresponding to SOCS6-R432 displayed a different orientation (Figure 1G). Thus we suggest that SOCS2, but not SOCS5, has a higher probability to interact with FLT3.

3.2. SOCS2 expresses in AML

We then compared SOCS2 expression in 11 different cancers with the corresponding normal tissues using micro-array data. We observed that SOCS2 expression was upregulated in AML, glioblastoma and myeloma patients (Figure 2A). This observation was further supported by the data that SOCS2

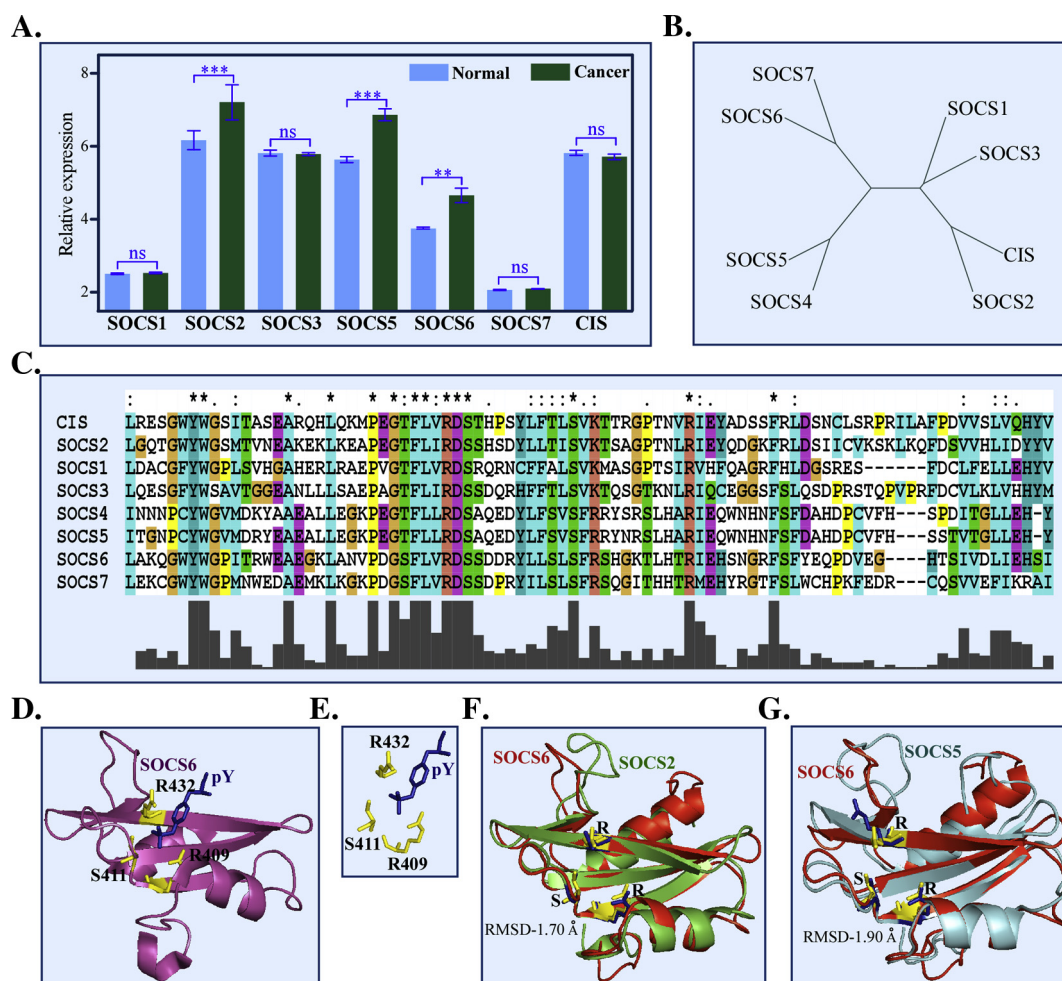


Figure 1 – The family of SOCS proteins. (A) Expression of different SOCS family members was analyzed from microarray data of AML patient samples and corresponding healthy donors. **, $p < 0.01$; ***, $p < 0.001$. (B) Phylogenetic tree of the SH2 domains of SOCS family members was created by CLUSTALW and visualized by Hypertree. (C) Alignment of SOCS-SH2 domains was created by CLUSTALW. (D) SOCS6-SH2 domain (PDB-2VIF) with phosphotyrosine residue. (E) Three critical residues of SOCS6-SH2 domain with phosphotyrosine residue. (F) Aligned structure of SOCS2 and SOCS6 SH2 domains. (G) Aligned structure of SOCS5 and SOCS6 SH2 domains.

protein expression was upregulated in the AML cell lines Kasumi-1, THP-1, MV4-11 and MOLM-13 as compared to the mastocytoma cell line P815 (Figure 2B). SOCS2 expression did not show any significant difference between different sexes or between patient groups that had complete remission or relapsed (Figure 2C), but it showed a weak correlation ($r^2 = 0.246$) with the age of AML patients (Figure 2D). Furthermore, SOCS2 expression was not significantly altered in different AML subtypes (Figure 2E) and also did not show any correlation with overall survival (Figure 2F). We showed in our previous report that SOCS6 expression was elevated in response to FLT3 ligand (FL) stimulation and also in patients expressing FLT3-ITD mutant (Kazi et al., 2012). Thus we analyzed whether SOCS2 expression was also elevated in patients carrying FLT3-ITD mutation. We observed that similar

to SOCS6, SOCS2 expression was significantly upregulated in patients having FLT3-ITD mutation (Figure 2G). This observation was also supported by the recent study that SOCS2 expression was significantly elevated in cell lines as well as in murine bone marrow cells expressing FLT3-ITD (Reddy et al., 2012).

3.3. SOCS2 interacts with FLT3 through SOCS2-SH2 domain

Since we observed that SOCS6 associates with FLT3 in a phosphorylation dependent manner (Kazi et al., 2012), we tested whether SOCS2 also associates with the receptor tyrosine kinase FLT3. SOCS2 associates with FLT3 in response to FL stimulation in THP-1 and MOLM-13 cell lines (which express FLT3-

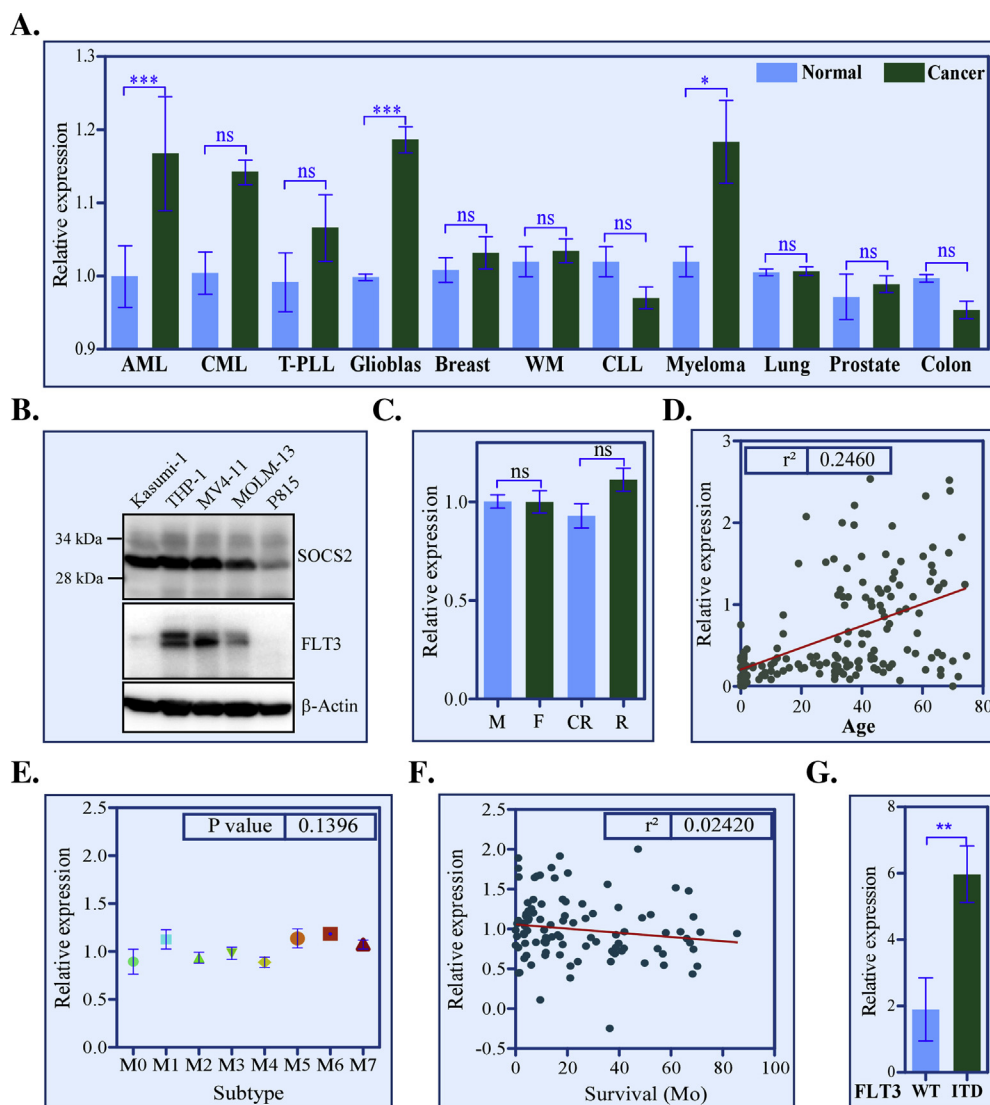


Figure 2 – SOCS2 expression in cancer. (A) SOCS2 expression was analyzed from microarray data of different patient samples and corresponding healthy donors. AML, acute myeloid leukemia; CML, chronic myeloid leukemia; APL, acute promyelocytic leukemia; T-PLL, T-cell-lymphocytic leukemia; WM, Waldenström's macroglobulinemia; CLL, chronic lymphocytic leukemia. (B) Kasumi-1, THP-1, MV4-11, MOLM-13 and P815 cell lines were lysed and total cell lysates were subjected to Western blotting analysis. (C) Relative SOCS2 expression in AML patients. M, male; F, female; CR, complete remission; R, relapse. (D) Relative SOCS2 expression with AML patients' age. (E) Relative SOCS2 expression in different AML subtypes. (F) Relative SOCS2 expression with overall survival (months) of AML patients. (G) Relative SOCS2 expression in AML patients carrying FLT3-WT or FLT3-ITD. **, $p < 0.01$.

WT), while it constitutively interacts with FLT3 in the MV4-11 cell line that expresses FLT3-ITD (Figure 3A). Moreover, by co-transfecting COS-1 cells with SOCS2-FLAG and FLT3-WT or FLT3-ITD we further provide evidence that SOCS2 interacts with both FLT3-WT and FLT3-ITD (Figure 3B). Using the SOCS2-SH2 domain we further showed that this interaction is mediated through SOCS2-SH2 domain (Figure 3C). Since SH2 domain interacts with phosphotyrosine residues we aimed to detect the phosphotyrosine residues in FLT3 which are required for SOCS2 interaction. Using 12 phosphopeptides corresponding to different tyrosine phosphorylation sites in FLT3, we identified tyrosine 589 and 919 residues as binding sites for SOCS2 (Figure 3D).

3.4. FL-stimulation induces SOCS2 tyrosine and serine phosphorylation

FLT3 activation induces tyrosine phosphorylation of SOCS2 (Kazi et al., 2012). Thus we analyzed whether FLT3 activation also initiates SOCS2 tyrosine phosphorylation. We transfected COS-1 cells with FLT3-WT and SOCS2-FLAG constructs and after overnight starvation cells were stimulated with FL for different periods of time. FL-stimulation initiated SOCS2 tyrosine phosphorylation in FLT3 transfected cells (Figure 4A). In addition we observed that SOCS2 remains serine phosphorylated in COS-1 cells and FL-stimulation potentiates SOCS2 serine phosphorylation (Figure 4B). Although association with certain adapter proteins, such as Lnk, regulates the

receptor tyrosine phosphorylation (Lin et al., 2012), an increased SOCS2 expression did not alter FLT3 tyrosine phosphorylation (Figure 4C).

3.5. SOCS2 co-localizes with FLT3 in the cell membrane

We generated Ba/F3-FLT3-WT-Empty vector and Ba/F3-FLT3-WT-SOCS2-FLAG cell lines to test the role of SOCS2 in hematopoietic cells. FLT3 and SOCS2 proteins levels were verified by flow cytometry (Figure 5A) and Western blotting (Figure 5B). Initially we investigated the subcellular localization of SOCS2 protein in Ba/F3 cells. Cells were stimulated with FL for 5 min after starvation and fixed for immunostaining. Fluorophore-conjugated anti-FLAG and anti-FLT3 antibodies were used to detect localization of SOCS2 and FLT3 respectively. SOCS2 was found to be localized mainly in the cytosol and plasma membrane (Figure 5C). SOCS2 and FLT3 co-localization was also observed in Ba/F3 cells which further increased significantly in response to FL-stimulation (Figure 5C and D).

3.6. SOCS2 increases ubiquitination of the FLT3 receptor

Since SOCS2 exhibits ubiquitin ligase activity (Vesterlund et al., 2011), we tested FLT3-ubiquitination in SOCS2 expressing cells. After stimulation of transfected Ba/F3 cells with FL for different time points, we observed that ubiquitination of FLT3 was increased in cells transfected with SOCS2 while FLT3 tyrosine phosphorylation remained unchanged

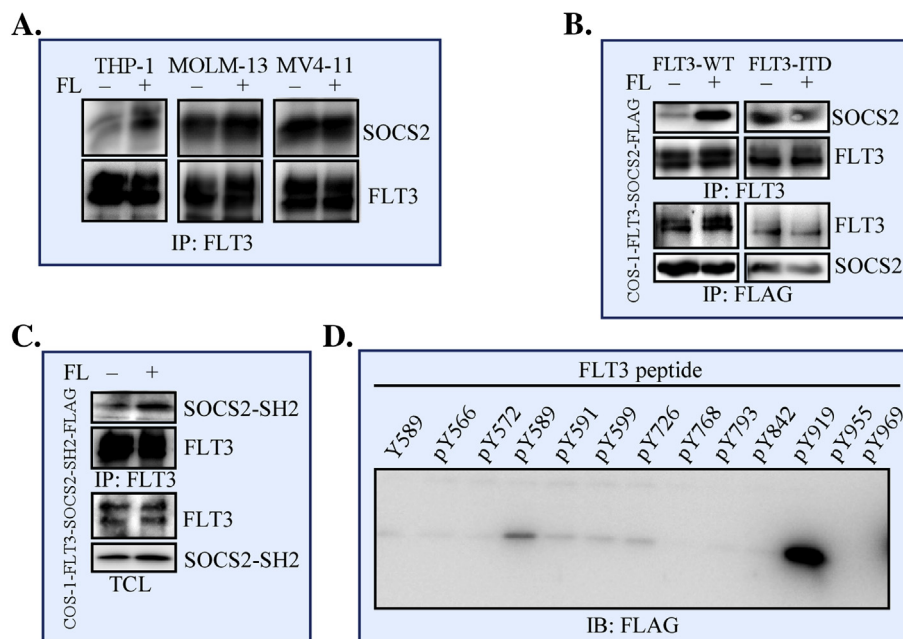


Figure 3 – SOCS2 interacts with FLT3. (A) THP-1, MOLM-13 and MV4-11 cells were stimulated with FL for 5 min before lysis. Cell lysates were subjected to immunoprecipitation using anti-FLT3 antibody followed by Western blotting analysis. (B) COS-1 cells were co-transfected with SOCS2-FLAG and FLT3-WT or FLT3-ITD expression vectors. Cells were serum-starved overnight and stimulated by FL for 5 min before lysis. Cell lysates were subjected to immunoprecipitation using either anti-FLT3 or anti-FLAG antibody followed by Western blotting analysis. (C) COS-1 cells were co-transfected with SOCS2-SH2-FLAG and FLT3-WT expression vectors. Cells were serum-starved overnight and stimulated by FL for 5 min before lysis. Cell lysates were subjected to immunoprecipitation using anti-FLT3 antibody followed by Western blotting analysis. (D) Phospho-peptides immobilized on UltraLink were incubated with SOCS2-FLAG-transfected COS-1 cell lysates and pulled-down proteins were then processed for Western blotting.

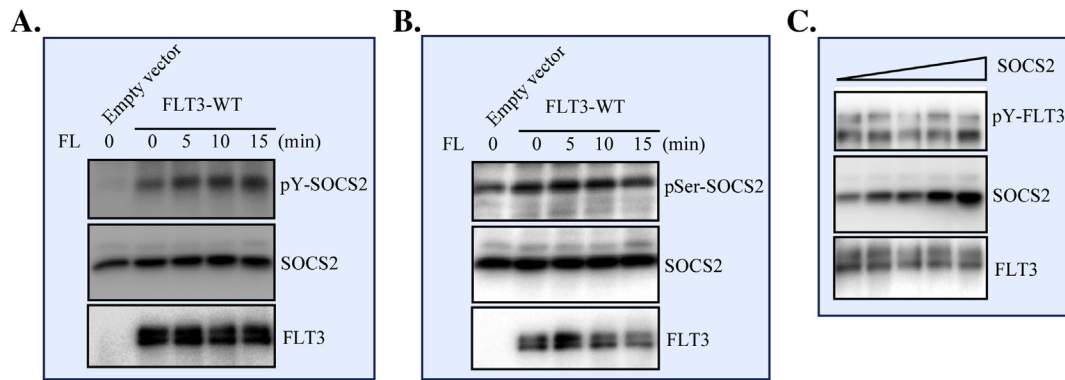


Figure 4 – SOCS2 and FLT3 phosphorylation. (A) COS-1 cells were co-transfected with SOCS2-FLAG and FLT3-WT. Cells were serum-starved overnight, stimulated, lysed and cell lysates were subjected to Western blotting analysis using 4G10, anti-SOCS2 and anti-FLT3 antibodies. (B) Cell lysates from experiment A were subjected Western blotting analysis using pSer, anti-SOCS2 and anti-FLT3 antibodies. (C) COS-1 cells were co-transfected with FLT3-WT and increased amount of SOCS2-FLAG. Cells were serum-starved overnight, stimulated for 5 min, lysed and cell lysates were subjected to Western blotting analysis using 4G10, anti-SOCS2 and anti-FLT3 antibodies.

(Figure 6A). The difference in receptor ubiquitination was evident by statistical analysis of multiple experiments at the 5 min time point (Figure 6B).

3.7. SOCS2 increases degradation of FLT3 in proteasome

Receptor ubiquitination leads to degradation of receptors. Thus we tested whether SOCS2 plays a role in FLT3 degradation. We observed that SOCS2 expression significantly increased receptor degradation in Ba/F3 cells (Figure 7A). Ubiquitin ligases are known to direct the receptor degradation either to proteasomes or to the lysosomes. Using selective

proteasome and lysosome specific inhibitors we tested which pathway is initiated by SOCS2. The proteasome inhibitor MG132, but not the lysosome inhibitor chloroquine diphosphate, inhibited SOCS2-mediated FLT3 degradation (Figure 7B).

3.8. SOCS2 selectively regulates Erk signaling

FLT3 activation leads to activation of several downstream signaling pathways. Major pathways include the PI3K-Akt pathway and the Ras/Erk pathway (Masson and Ronnstrand, 2009). To understand the effect of SOCS2 on FLT3 downstream

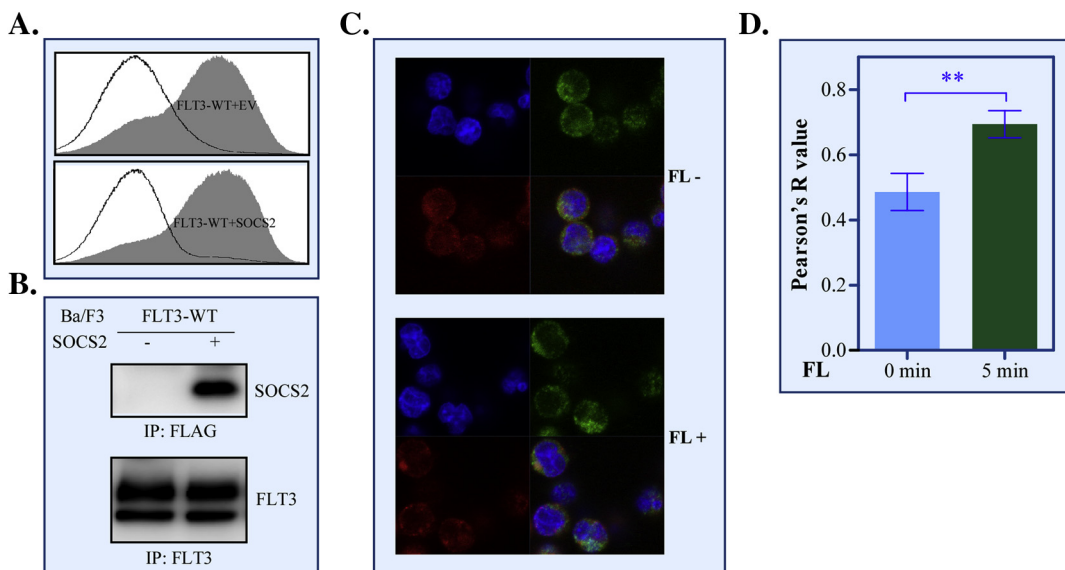


Figure 5 – SOCS2 localizes to the cell membrane. (A) Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were labeled with isotype control or PE-conjugated FLT3 antibody and analyzed by flow cytometry. Black lines represent cells labeled with isotype control and gray area indicates cells labeled with FLT3 antibody. (B) Lysates of Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were immunoprecipitated with an anti-FLAG or anti-FLT3 antibody and analyzed by Western blotting. (C) Ba/F3-FLT3-WT-SOCS2-FLAG cells were stimulated with FL for 5 min and processed for immunostaining followed by confocal imaging. Blue, nuclear staining; Green, SOCS2; Red, FLT3. (D) Co-localization of multiple cells from different experiments was quantified using CoLocalizer Pro. **, $p < 0.01$.

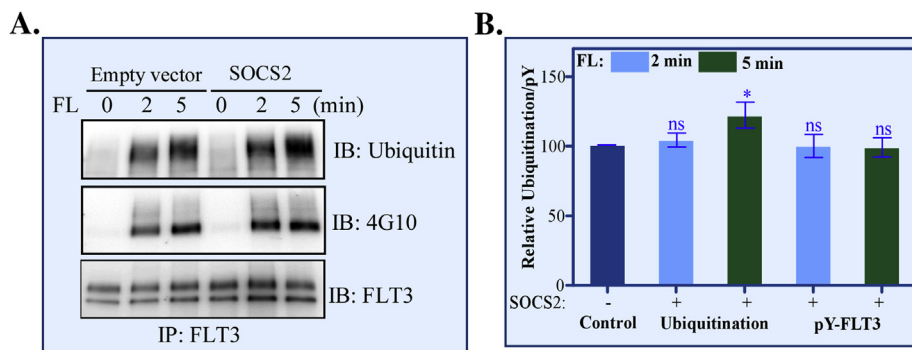


Figure 6 – SOCS2 expression increases ubiquitination of FLT3. (A) Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were serum-starved, preincubated with MG132 and chloroquine diphosphate for 30 min and stimulated with 100 ng/ml of FL for the indicated period of times. Cells were then washed with cold PBS, lysed and immunoprecipitated with an anti-FLT3 antibody followed by Western blotting analysis. (B) Signal intensities of blots from multiple experiments were quantified using Multi-Gauge software (Fujifilm) to calculate the receptor ubiquitination and phosphorylation. *, $p < 0.05$; ns, not significant.

signaling we tested Akt and Erk 1/2 activation following FL-stimulation using phospho-specific antibodies. Ba/F3-FLT3 cells expressing SOCS2 displayed decreased Erk 1/2 phosphorylation without affecting Akt phosphorylation (Figure 8A). This effect was observed after 5 min of receptor activation (Figure 8B). Thus, since the effect is selective for Erk phosphorylation, it cannot be fully explained by increased degradation of FLT3.

3.9. SOCS2 negatively regulates FLT3-ITD-mediated STAT5 phosphorylation

SOCS2 is a negative regulator of JAK2/STAT5 signaling (Quentmeier et al., 2008). Since only FLT3-ITD but not FLT3-WT activates STAT5 (Choudhary et al., 2007), we generated Ba/F3-FLT3-ITD-empty vector and Ba/F3-FLT3-ITD-SOCS2-FLAG cells. Surface expression of FLT3-ITD in transfected

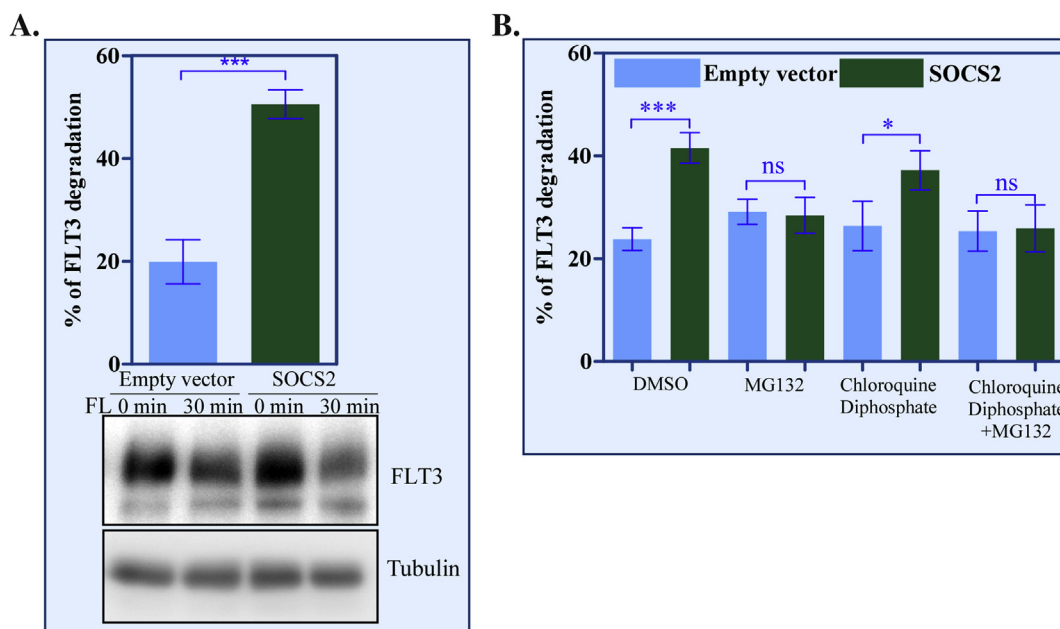


Figure 7 – SOCS2 expression increases to FLT3 degradation. (A) Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were serum-starved and preincubated with cycloheximide for 30 min. Cells were then stimulated with 100 ng/ml of FL for 30 min in presence of cycloheximide before lysis. Cell lysates were analyzed by Western blotting. Signal intensities from multiple experiments were quantified using multi-gauge software to calculate the receptor degradation. **, $p < 0.01$ (B) Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were serum-starved and preincubated with cycloheximide and DMSO or MG132 or chloroquine diphosphate or MG132 + chloroquine diphosphate for 30 min. Cells were then stimulated with 100 ng/ml of FL for 30 min in presence of inhibitors before lysis. Cell lysates were analyzed by Western blotting. Signal intensities from multiple experiments were quantified using multi-gauge software to calculate the receptor degradation. ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

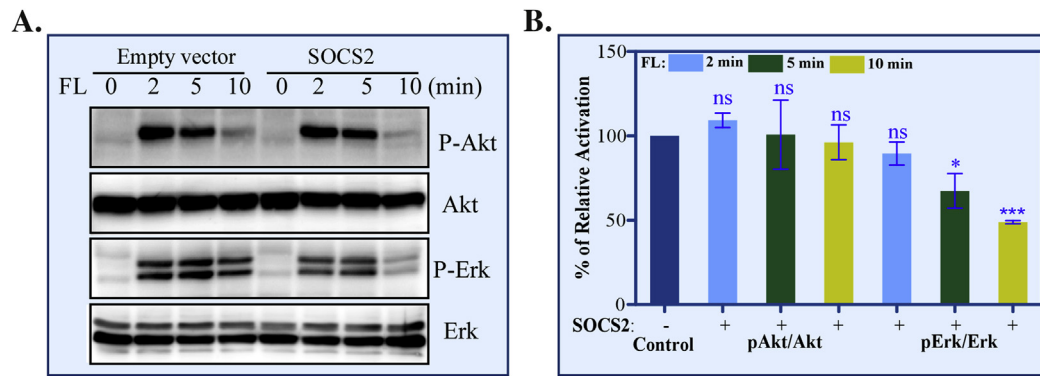


Figure 8 – SOCS2 expression suppresses Erk signaling. (A) Ba/F3-FLT3-WT-empty vector or Ba/F3-FLT3-WT-SOCS2-FLAG cells were treated with 100 ng/ml of FL for different time points before lysis. Total cell lysates were analyzed by Western blotting using phospho-specific antibodies. (B) Blots from multiple experiments were quantified using multi-gauge software to calculate the inhibition. ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

cells was verified by flow cytometry (Figure 9A) and SOCS2 expression was determined by Western blotting (Figure 9B). SOCS2 expression led to a significant decrease of FLT3-ITD-mediated STAT5 phosphorylation in the Ba/F3 cells (Figure 9C).

3.10. SOCS2 negatively regulates FLT3-ITD-mediated cell proliferation and colony formation in semi-solid culture

To elucidate the biological outcome of SOCS2 expression in Ba/F3 cells we tested FLT3-ITD-mediated cell proliferation

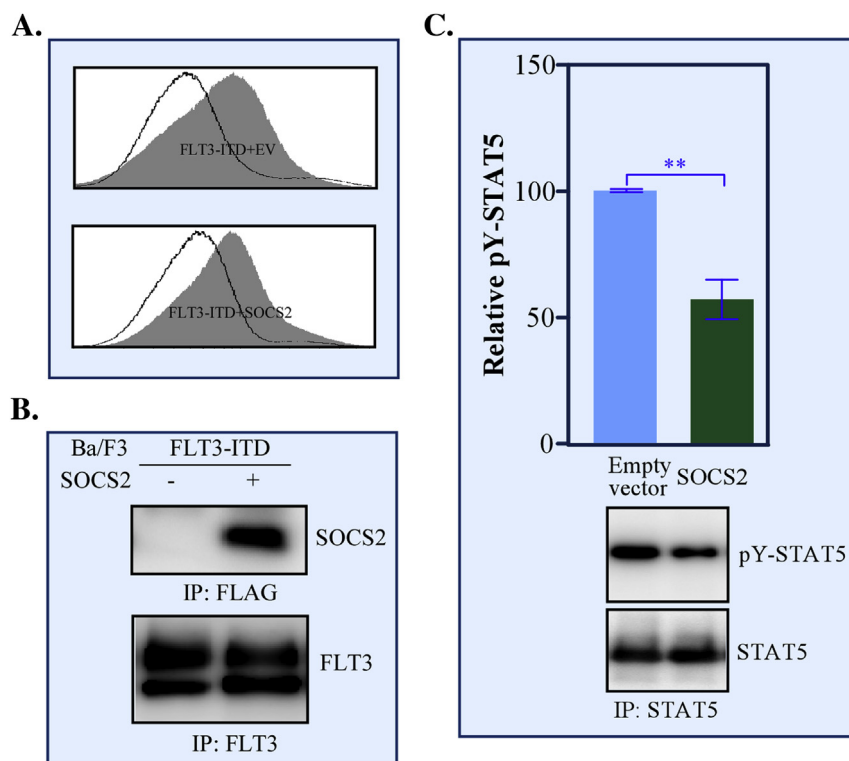


Figure 9 – SOCS2 expression decreases STAT5 phosphorylation. (A) Ba/F3-FLT3-ITD-empty vector or Ba/F3-FLT3-ITD-SOCS2-FLAG cells were labeled with isotype control or PE-conjugated FLT3 antibody and analyzed by flow cytometry. Black lines represent cells labeled with isotype control and gray area indicates cells labeled with FLT3 antibody. (B) Lysates of Ba/F3-FLT3-ITD-empty vector or Ba/F3-FLT3-ITD-SOCS2-FLAG cells were immunoprecipitated with an anti-FLAG or anti-FLT3 antibody and analyzed by Western blotting. (C) Ba/F3-FLT3-ITD-empty vector or Ba/F3-FLT3-ITD-SOCS2-FLAG cells were lysed and immunoprecipitated using an anti-STAT5 antibody followed by Western blotting analysis using 4G10 and STAT5 antibodies. Blots from multiple experiments were quantified using multi-gauge software to calculate the inhibition. **, $p < 0.01$.

and colony formation potential in semi-solid culture. SOCS2 expression significantly decreased FLT3-ITD mediated cell proliferation (Figure 10A). Furthermore, SOCS2 expression led to reduced size of colonies of Ba/F3-FLT3-ITD cells (Figure 10B). The Ba/F3-FLT3-ITD cells expressing SOCS2 also displayed significantly reduced number of colonies in semi-solid culture (Figure 10C).

4. Discussion

Signaling through receptor tyrosine kinases is tightly regulated. Protein kinases, phosphatases and ubiquitin ligases play critical roles in this regulation. This report suggests a mechanism of FLT3 regulation by the SOCS2 ubiquitin ligase. SOCS2 associates with FLT3 through phosphotyrosine residues 589 and 919 in FLT3 and this interaction increases FLT3 ubiquitination which is followed by degradation of the receptor as well as a decrease in Erk 1/2 and STAT5 phosphorylation. Thus SOCS2 exhibits a negative role on FLT3 signaling.

SOCS2 binding to wild-type FLT3 was found to be dependent on ligand stimulated phosphorylation of Y⁵⁸⁹ and pY⁹¹⁹. The phosphorylated residue pY⁵⁸⁹ is located in the juxtamembrane domain of FLT3 and has previously been shown to be of importance for activation of Src family kinases (SFKs) (Heiss et al., 2006). Thus, there is likely to be a competition between SOCS2 and SFKs for binding to this site, which might explain some of the effects on signaling. The role of the phosphorylated residue pY⁹¹⁹ has not been studied in detail. In the

related receptor c-Kit the corresponding site pY900 was found to mediate recruitment of the adapter protein Crk through the p85 subunit of PI3-kinase (Lennartsson et al., 2003). In the PDGF β -receptor the corresponding site, pY934, is involved in regulating the activity of PLC- γ (Hansen et al., 1996). Recently we showed that SOCS6 and the adapter protein Lnk interact with FLT3 through pY⁹¹⁹ and thereby negatively regulate the receptor signaling (Kazi et al., 2012; Lin et al., 2012). Thus the pY⁹¹⁹ residue seems to be the site in FLT3 which interacts with several negative regulators of signaling.

SOCS2 constitutively interacts with FLT3-ITD and is predominantly localized to the cell membrane indicating that oncogenic FLT3 signaling might also be partially regulated by SOCS2 and this regulation takes place when receptor localizes to the cell membrane. Similar to SOCS6 FLT3 ligand induces SOCS2 tyrosine phosphorylation. In addition, SOCS2 remains serine phosphorylated in unstimulated COS-1 cells and FLT3 activation potentiates this phosphorylation. The exact biological role of SOCS2 phosphorylations is yet to be defined. SOCS2 shares serine residue in SOCS-box with S144 of Vif proteins (Mehle et al., 2004). Phosphorylation of S114 of Vif led to a decrease of Elongin C binding without altering the ability of substrate degradation. Phosphorylation of SOCS1 by PIM serine/threonine kinases stabilizes SOCS1 thus inhibiting STAT6 tyrosine phosphorylation (Chen et al., 2002). Tyrosine phosphorylated SOCS3 displays impaired binding to Elongin C (Haan et al., 2003), but strongly inhibits STAT5 activation (Cacalano et al., 2001). These findings suggests that phosphorylation events regulate the stability of SOCS proteins and potentiates their ability of inhibiting STAT pathways.

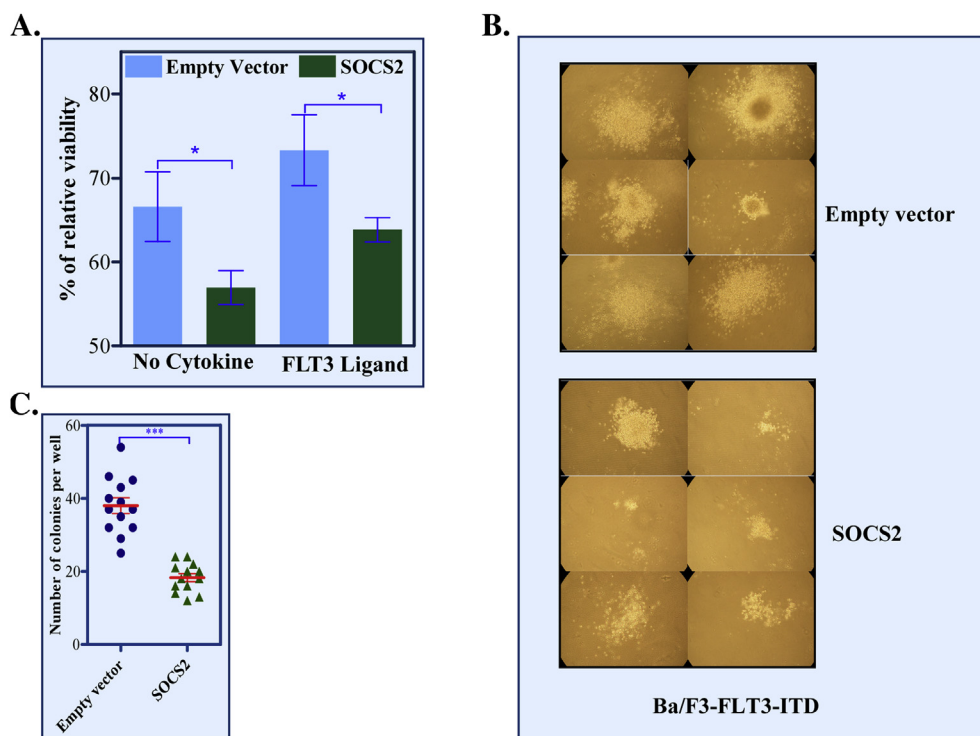


Figure 10 – SOCS2 expression decreases FLT3-ITD-mediated colony formation in semi-solid culture. (A) Ba/F3-FLT3-ITD-empty vector or Ba/F3-FLT3-ITD-SOCS2-FLAG cells were cultured in presence or absence of FL for 24 h followed by PrestoBlue assay. *, $p < 0.05$. (B) Ba/F3-FLT3-ITD-empty vector or Ba/F3-FLT3-ITD-SOCS2-FLAG cells were cultured in semi-solid medium for 5 days. (C) Number of colonies was counted from different experiments. ***, $p < 0.001$.

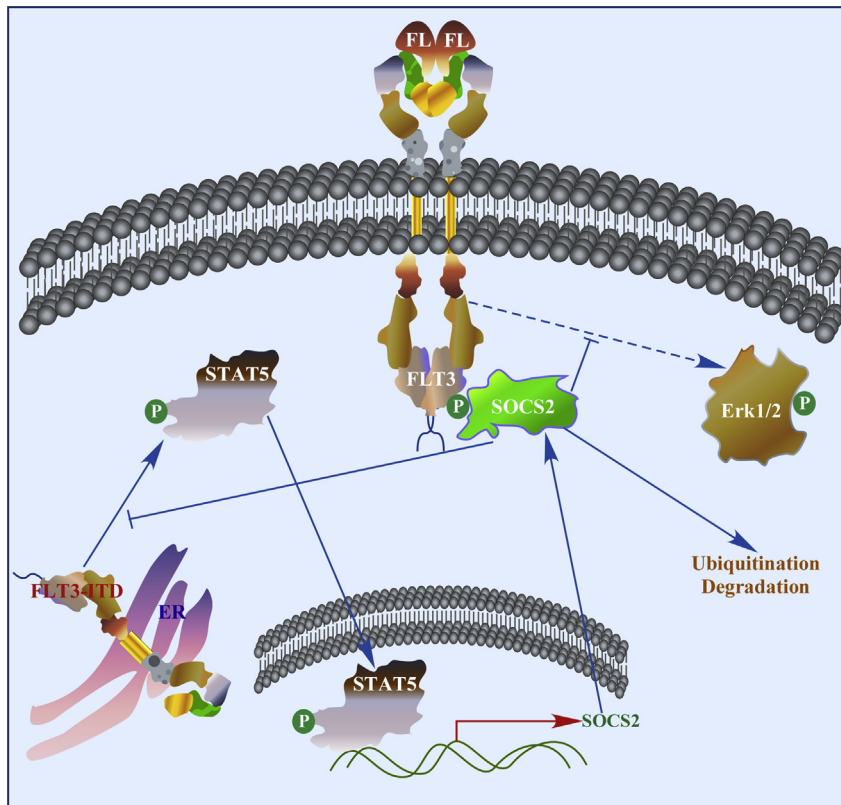


Figure 11 – Model of SOCS2 mediated regulation of FLT3 signaling. Oncogenic FLT3 activates STAT5 leading to nuclear translocation and expression of SOCS2 that associates with tyrosine phosphorylated FLT3 and directs the receptor for ubiquitination and degradation. This events further inhibits Erk1/2 and STAT5 activation.

SOCS2 increases ligand-induced ubiquitination and accelerates degradation of FLT3 which is in line with the observations that SOCS2 regulates the stability of interacting proteins by recruiting the ubiquitination machinery (McBerry et al., 2012; Vesterlund et al., 2011). SOCS2-mediated receptor degradation was blocked by MG132 but not by chloroquine suggesting that SOCS2 promotes FLT3 degradation through proteasomes and not through the lysosomes, as was also observed with GHR (Vesterlund et al., 2011).

SOCS2 also regulates receptor downstream signaling and displays a degree of specificity in regulation of receptor downstream signaling. Since SOCS2 has the ability to induce receptor degradation, it is expected that SOCS2 expression could negatively regulate downstream signaling. However, to our surprise we found that SOCS2 selectively regulates Erk signaling without affecting the Akt pathway. This is similar to what we previously have found regarding SOCS6 and FLT3 signaling (Kazi et al., 2012). Thus, the reduced activation of Erk can't solely be explained by degradation of FLT3 since that would also have affected Akt phosphorylation.

The STAT5 activation by FLT3-ITD is supposed to take place in the endoplasmic reticulum (ER) (Schmidt-Arras et al., 2009) and it is known that ligand stimulation does not induce STAT5 activation in cells expressing FLT3-WT (Spiekermann et al., 2003). The observation that STAT5 phosphorylation is blocked by SOCS2 expression suggests that constitutive FLT3-ITD-SOCS2 association disrupts STAT5 phosphorylation mediated by FLT3-ITD. SOCS2 expression

partially blocked FLT3-ITD-mediated colony formation which further provides evidence that SOCS2 blocks FLT3-ITD oncogenic signaling by blocking phosphorylation of STAT5, an event known to be essential for FLT3-ITD-mediated transformation. SOCS2 mRNA expression is regulated by STAT5 (Sen et al., 2012) and we observed that SOCS2 mRNA expression is upregulated in cells expressing oncogenic FLT3. Since oncogenic FLT3 directly activates STAT5, we suggest that elevated SOCS2 expression is mediated through STAT5 activation by oncogenic FLT3 that further regulates FLT3 signaling (Figure 11). Thus we suggest that SOCS2 negatively regulates FLT3 signaling by a direct interaction and thereby induces receptor ubiquitination and its degradation.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

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