LUND UNIVERSITY

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

Kazi, Julhash U.; Rönnstrand, Lars

Link to publication

Highlights

Expression of SOCS2 is elevated in acute myeloid leukemia (AML).

SOCS2 associates with FLT3 in response to FLT3 ligand stimulation in AML cell lines.

SOCS2 interacts with FLT3 through FLT3 phosphotyrosine residues.

SOCS2 SH2 domain is sufficient for this interaction.

SOCS2 increases ubiquitination and degradation of FLT3.

SOCS2 inhibits FLT3 downstream signaling.

SOCS2 inhibits FLT3-ITD-mediated transformation of cells.

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

Julhash U. Kazi and Lars Rönnstrand[*]

Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Wallenberg Laboratory, Skåne University Hospital, 20502 Malmö, Sweden,

[*]Corresponding author:

Lars Rönnstrand

Experimental Clinical Chemistry

Wallenberg Laboratory

Department of Laboratory Medicine

Lund University

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

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.

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 a 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 Ronnstrand, 2009). FLT3 is frequently mutated in acute myeloid leukemia (AML) and the most common mutation is the internal tandem duplication (ITD) in the 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 adaptor 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, 2012a) or SLAP (Kazi and Ronnstrand, 2012b) 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.

4

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 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,

5

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 hours after transfection and then stimulated at 37°C for the indicated time with 100 ng/ml of FL (Prospec Tany). On the other hand Ba/F3 cells were serum-starved four hours in RPMI-1640 medium and then stimulated with 100 ng/ml of FLT3 ligand (FL) at 37°C for five minutes. Cells were then processed for immunoprecipitation and Western blotting as described previously (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 sequence 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 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 dataset 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 after starvation stimulated or not with FL for 5 minutes. 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 performed using PrestoBlue cell viability assays (for detailed method see (Kazi et al., 2012)).

3. Result

3.1. SOCS2-SH2 domain display 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 (Fig. 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 similar to SOCS6, SOCS2 or SOCS5 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 (Fig. 1B). Although critical arginine residues are conserved within the family neither SOCS2 nor SOCS5 displayed a close homology with SOCS6 (Fig. 1C) and thus we were unable to make a conclusion whether SOCS2 or SOCS5 has a probability to interact with FLT3 from sequence alignment.

Then we compared 3D-structure 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 (RMSD-1.7Å) than SOCS5-SH2 (RMSD-1.9Å) with SOCS6-SH2 (Fig. 1F and 1G). The binding site of the phosphotyrosine moiety is known to be created by two arginine and one serine residues shown in figure 1D and 1E (Bullock et al., 2006; Zadjali et al., 2011). Although all three residues in SOCS2-SH2 domain completely showed similar orientation (Fig. 1F), the arginine residue in SOCS5-SH2 domain corresponding to SOCS6-R432

7

displayed different orientation (Fig. 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 corresponding normal tissues using micro-array data. We observed that SOCS2 expression was upregulated in AML, glioblastoma and myeloma patients (Fig. 2A). This observation was further supported by the data that SOCS2 protein expression was upregulated in the AML cell lines Kasumi-1, THP-1, MV4-11 and MOLM-13 as compared to mastocytoma cell line P815 (Fig. 2B). SOCS2 expression did not show any significant difference between different sexes or between patient groups that had complete remission or relapsed (Fig. 2C), but it showed a weak correlation (r^2=0.246) with the age of AML patients (Fig. 2D). Furthermore, SOCS2 expression was not significantly altered in different AML subtypes (Fig. 2E) and also did not show any correlation with overall survival (Fig. 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 (Fig. 2G). This observation was also supported by the recent study that SOCS2 expression was significantly elevated in cell lines as well as 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-WT), while it constitutively interacts with FLT3 in the MV4-11 cell line that expresses FLT3-ITD (Fig. 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 (Fig. 3B). Using the SOCS2-SH2 domain we further showed that this interaction is mediated through SOCS2-

8

SH2 domain (Fig. 3C). Since SH2 domain interacts with phosphotyrosine residue we aimed to detect the phosphotyrosine residues in FLT3 which are required for SOCS2 interaction. Using 12 phospho-peptides corresponding to different tyrosine phosphorylation sites in FLT3, we identified tyrosine 589 and 919 residues as binding sites for SOCS2 (Fig. 3D).

3.4. FL-stimulation induces SOCS2 tyrosine and serine phosphorylation

FLT3 activation induces tyrosine phosphorylation of SOCS6 (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 (Fig. 4A). In addition we observed that SOCS2 remains serine phosphorylated in COS-1 cells and FL-stimulation potentiates SOCS2 serine phosphorylation (Fig. 4B). Although association with certain adaptor proteins, such as Lnk, regulates the receptor tyrosine phosphorylation (Lin et al., 2012), an increased SOCS2 expression did not alter FLT3 tyrosine phosphorylation (Fig. 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 (Fig. 5A) and Western blotting (Fig. 5B). Initially we investigated the subcellular localization of SOCS2 protein in Ba/F3 cells. Cells were stimulated with FL for 5 minutes 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 (Fig. 5C). SOCS2 and FLT3 co-localization was also observed in Ba/F3 cells which further increased significantly in response to FL-stimulation (Fig. 5C and 5D).

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 FLT3-ubiquitination was increased in cells transfected with

SOCS2 while FLT3 tyrosine phosphorylation remained unchanged (Fig. 6A). The difference in receptor ubiquitination was evident by statistical analysis of multiple experiments at the 5 minute time point (Fig 6B).

3.7. SOCS2 increases degradation of FLT3 in proteasome

Receptor ubiquitination leads to degradation of receptor. Thus we tested whether SOCS2 plays a role in FLT3 degradation. We observed that SOCS2 expression significantly increased receptor degradation in Ba/F3 cells (Fig. 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 lysosome inhibitor chloroquine diphosphate inhibited SOCS2-mediated FLT3 degradation (Fig. 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 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 (Fig. 8A). This effect was observed after 5 minute of receptor activation (Fig. 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 cells was verified by flow cytometry (Fig. 9A) and SOCS2 expression was determined by Western blotting (Fig. 9B). SOCS2 expression led to a significant decrease of FLT3-ITD-mediated STAT5 phosphorylation in the Ba/F3 cells (Fig. 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 and colony formation potential in semi-solid culture. SOCS2 expression significantly decreased FLT3-ITD mediated cell prolideration (Fig. 10A). Furthermore, SOCS2 expression led to reduced size of colonies of Ba/F3-FLT3-ITD cells (Fig. 10B). The Ba/F3-FLT3-ITD cells expressing SOCS2 displayed significantly reduced number of colonies in semi-solid culture (Fig. 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 Y^{589} and pY^{919}. The phosphorylated residue pY^{589} is located in the juxtamembrane domain of FLT 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 pY^{919} 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

11

protein Lnk interact with FLT3 through pY[919] and thereby negatively regulate the receptor signaling (Kazi et al., 2012; Lin et al., 2012). Thus the pY[919] 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 event. 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 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 with Elongin C (Haan et al., 2003), but strongly inhibits STAT5 activation (Cacalano et al., 2001). These findings suggests that phosphorylation events regulates stability of SOCS proteins and potentiates its ability of STAT pathways inhibition.

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 but not through the lysosomes which 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 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 (Fig. 11). Thus we suggest that SOCS2 negatively regulates FLT3 signaling by direct interaction and thereby induces receptor ubiquitination and its degradation.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

References

Bayle, J., Letard, S., Frank, R., Dubreuil, P., De Sepulveda, P., 2004. Suppressor of cytokine signaling 6 associates with KIT and regulates KIT receptor signaling. J Biol Chem 279, 12249-12259.

Blume-Jensen, P., Siegbahn, A., Stabel, S., Heldin, C.H., Ronnstrand, L., 1993. Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. EMBO J 12, 4199-4209.

Bullock, A.N., Debreczeni, J.E., Edwards, A.M., Sundstrom, M., Knapp, S., 2006. Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase. Proc Natl Acad Sci U S A 103, 7637-7642.

Cacalano, N.A., Sanden, D., Johnston, J.A., 2001. Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras. Nature cell biology 3, 460-465.

Chen, X.P., Losman, J.A., Cowan, S., Donahue, E., Fay, S., Vuong, B.Q., Nawijn, M.C., Capece, D., Cohan, V.L., Rothman, P., 2002. Pim serine/threonine kinases regulate the stability of Socs-1 protein. Proc Natl Acad Sci U S A 99, 2175-2180.

Choudhary, C., Brandts, C., Schwable, J., Tickenbrock, L., Sargin, B., Ueker, A., Bohmer, F.D., Berdel, W.E., Muller-Tidow, C., Serve, H., 2007. Activation mechanisms of STAT5 by oncogenic Flt3-ITD. Blood 110, 370-374.

De Sepulveda, P., Okkenhaug, K., Rose, J.L., Hawley, R.G., Dubreuil, P., Rottapel, R., 1999. Socs1 binds to multiple signalling proteins and suppresses steel factor-dependent proliferation. EMBO J 18, 904-915.

Greenhalgh, C.J., Rico-Bautista, E., Lorentzon, M., Thaus, A.L., Morgan, P.O., Willson, T.A., Zervoudakis, P., Metcalf, D., Street, I., Nicola, N.A., Nash, A.D., Fabri, L.J., Norstedt, G., Ohlsson, C., Flores-Morales, A., Alexander, W.S., Hilton, D.J., 2005. SOCS2 negatively regulates growth hormone action in vitro and in vivo. The Journal of clinical investigation 115, 397-406.

Haan, S., Ferguson, P., Sommer, U., Hiremath, M., McVicar, D.W., Heinrich, P.C., Johnston, J.A., Cacalano, N.A., 2003. Tyrosine phosphorylation disrupts elongin interaction and accelerates SOCS3 degradation. J Biol Chem 278, 31972-31979.

Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engstrom, U., Wernstedt, C., Heldin, C.H., Ronnstrand, L., 1996. Mutation of a Src phosphorylation site in the PDGF beta-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. EMBO J 15, 5299-5313.

Heiss, E., Masson, K., Sundberg, C., Pedersen, M., Sun, J., Bengtsson, S., Ronnstrand, L., 2006. Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2. Blood 108, 1542-1550.

Horvat, S., Medrano, J.F., 2001. Lack of Socs2 expression causes the high-growth phenotype in mice. Genomics 72, 209-212.

Kazi, J.U., Ronnstrand, L., 2012a. FLT3 signals via the adapter protein Grb10 and overexpression of Grb10 leads to aberrant cell proliferation in acute myeloid leukemia. Molecular oncology, 10.1016/j.molonc.2012.1011.1003.

Kazi, J.U., Ronnstrand, L., 2012b. Src-Like Adaptor Protein (SLAP) Binds to the Receptor Tyrosine Kinase Flt3 and Modulates Receptor Stability and Downstream Signaling. PloS one 7, e53509.

Kazi, J.U., Sun, J., Phung, B., Zadjali, F., Flores-Morales, A., Ronnstrand, L., 2012. Suppressor of Cytokine Signaling 6 (SOCS6) Negatively Regulates Flt3 Signal Transduction through Direct Binding to Phosphorylated Tyrosines 591 and 919 of Flt3. J Biol Chem 287, 36509-36517.

Lennartsson, J., Wernstedt, C., Engstrom, U., Hellman, U., Ronnstrand, L., 2003. Identification of Tyr900 in the kinase domain of c-Kit as a Src-dependent phosphorylation site mediating interaction with c-Crk. Exp Cell Res 288, 110-118.

Levis, M., Small, D., 2003. FLT3: ITDoes matter in leukemia. Leukemia 17, 1738-1752.

Lin, D.C., Yin, T., Koren-Michowitz, M., Ding, L.W., Gueller, S., Gery, S., Tabayashi, T., Bergholz, U., Kazi, J.U., Ronnstrand, L., Stocking, C., Koeffler, H.P., 2012. Adaptor protein Lnk binds to and inhibits normal and leukemic FLT3. Blood 120, 3310-3317.

Masson, K., Liu, T., Khan, R., Sun, J., Ronnstrand, L., 2009. A role of Gab2 association in Flt3 ITD mediated Stat5 phosphorylation and cell survival. Br J Haematol 146, 193-202.

Masson, K., Ronnstrand, L., 2009. Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. Cell Signal 21, 1717-1726.

McBerry, C., Gonzalez, R.M., Shryock, N., Dias, A., Aliberti, J., 2012. SOCS2-induced proteasome-dependent TRAF6 degradation: a common anti-inflammatory pathway for control of innate immune responses. PloS one 7, e38384.

Mehle, A., Goncalves, J., Santa-Marta, M., McPike, M., Gabuzda, D., 2004. Phosphorylation of a novel SOCS-box regulates assembly of the HIV-1 Vif-Cul5 complex that promotes APOBEC3G degradation. Genes & development 18, 2861-2866.

Quentmeier, H., Geffers, R., Jost, E., Macleod, R.A., Nagel, S., Rohrs, S., Romani, J., Scherr, M., Zaborski, M., Drexler, H.G., 2008. SOCS2: inhibitor of JAK2V617F-mediated signal transduction. Leukemia 22, 2169-2175.

Razumovskaya, E., Masson, K., Khan, R., Bengtsson, S., Ronnstrand, L., 2009. Oncogenic Flt3 receptors display different specificity and kinetics of autophosphorylation. Exp Hematol 37, 979-989.

Reddy, P.N., Sargin, B., Choudhary, C., Stein, S., Grez, M., Muller-Tidow, C., Berdel, W.E., Serve, H., Brandts, C.H., Study Alliance, L., 2012. SOCS1 cooperates with FLT3-ITD in the development of myeloproliferative disease by promoting the escape from external cytokine control. Blood 120, 1691-1702.

Schmidt-Arras, D., Bohmer, S.A., Koch, S., Muller, J.P., Blei, L., Cornils, H., Bauer, R., Korasikha, S., Thiede, C., Bohmer, F.D., 2009. Anchoring of FLT3 in the endoplasmic reticulum alters signaling quality. Blood 113, 3568-3576.

Sen, B., Peng, S., Woods, D.M., Wistuba, I., Bell, D., El-Naggar, A.K., Lai, S.Y., Johnson, F.M., 2012. STAT5A-mediated SOCS2 expression regulates Jak2 and STAT3 activity following c-Src inhibition in head and neck squamous carcinoma. Clin Cancer Res 18, 127-139.

Spiekermann, K., Bagrintseva, K., Schwab, R., Schmieja, K., Hiddemann, W., 2003. Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. Clin Cancer Res 9, 2140-2150.

Vesterlund, M., Zadjali, F., Persson, T., Nielsen, M.L., Kessler, B.M., Norstedt, G., Flores-Morales, A., 2011. The SOCS2 ubiquitin ligase complex regulates growth hormone receptor levels. PloS one 6, e25358.

Voytyuk, O., Lennartsson, J., Mogi, A., Caruana, G., Courtneidge, S., Ashman, L.K., Ronnstrand, L., 2003. Src family kinases are involved in the differential signaling from two splice forms of c-Kit. J Biol Chem 278, 9159-9166.

Yoshimura, A., Naka, T., Kubo, M., 2007. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7, 454-465.

Zadjali, F., Pike, A.C., Vesterlund, M., Sun, J., Wu, C., Li, S.S., Ronnstrand, L., Knapp, S., Bullock, A.N., Flores-Morales, A., 2011. Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase. J Biol Chem 286, 480-490.

Figure legends

Fig. 1: 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 SH2 domain 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 SOCS6 and SOCS6 SH2 domains.

Fig. 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-prolymphocytic 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.

Fig. 3: SOCS2 interacts with FLT3.

(A) THP-1, MOLM-13 and MV4-11 cells were stimulated with FL for 5 minutes 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 minutes 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 minutes before lysis. Cell lysates were subjected to immunoprecipitation using either 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.

Fig. 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 minutes, lysed and cell lysates were subjected to Western blotting analysis using 4G10, anti-SOCS2 and anti-FLT3 antibodies.

Fig. 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 grey 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 minutes 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. **, $p < 0.01$.

Fig. 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 minutes 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.

Fig. 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 minutes. Cells were then stimulated with 100 ng/ml of FL for 30 minutes 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 minutes. Cells were then stimulated with 100 ng/ml of FL for 30 minutes 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.

Fig. 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.

Fig. 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 grey 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. **, $p<0.01$.

Fig. 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 hours 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. ***, $p<0.001$.

Fig. 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 receptor to ubiquitination and degradation. This events further inhibits Erk1/2 and STAT5 activation.
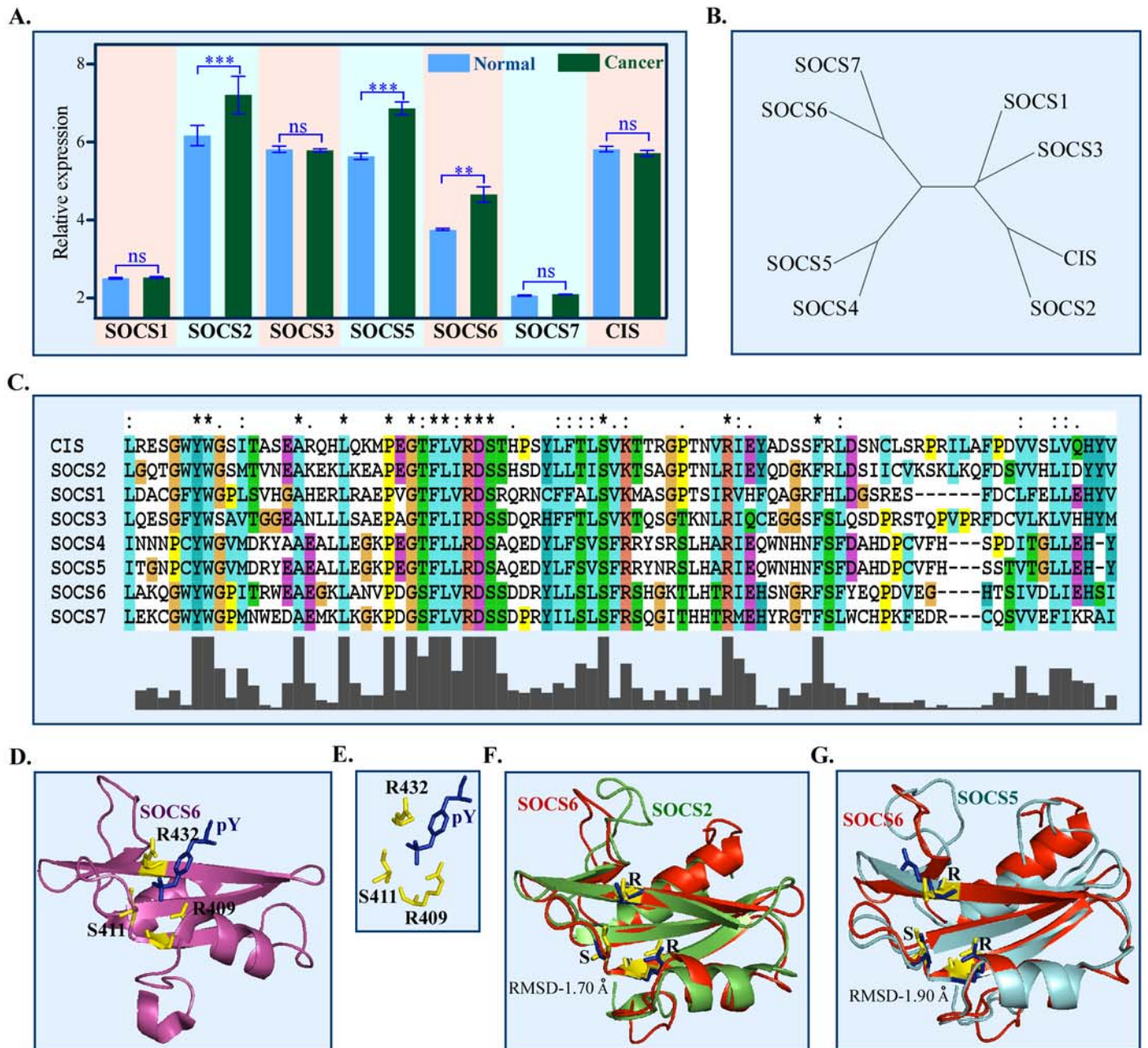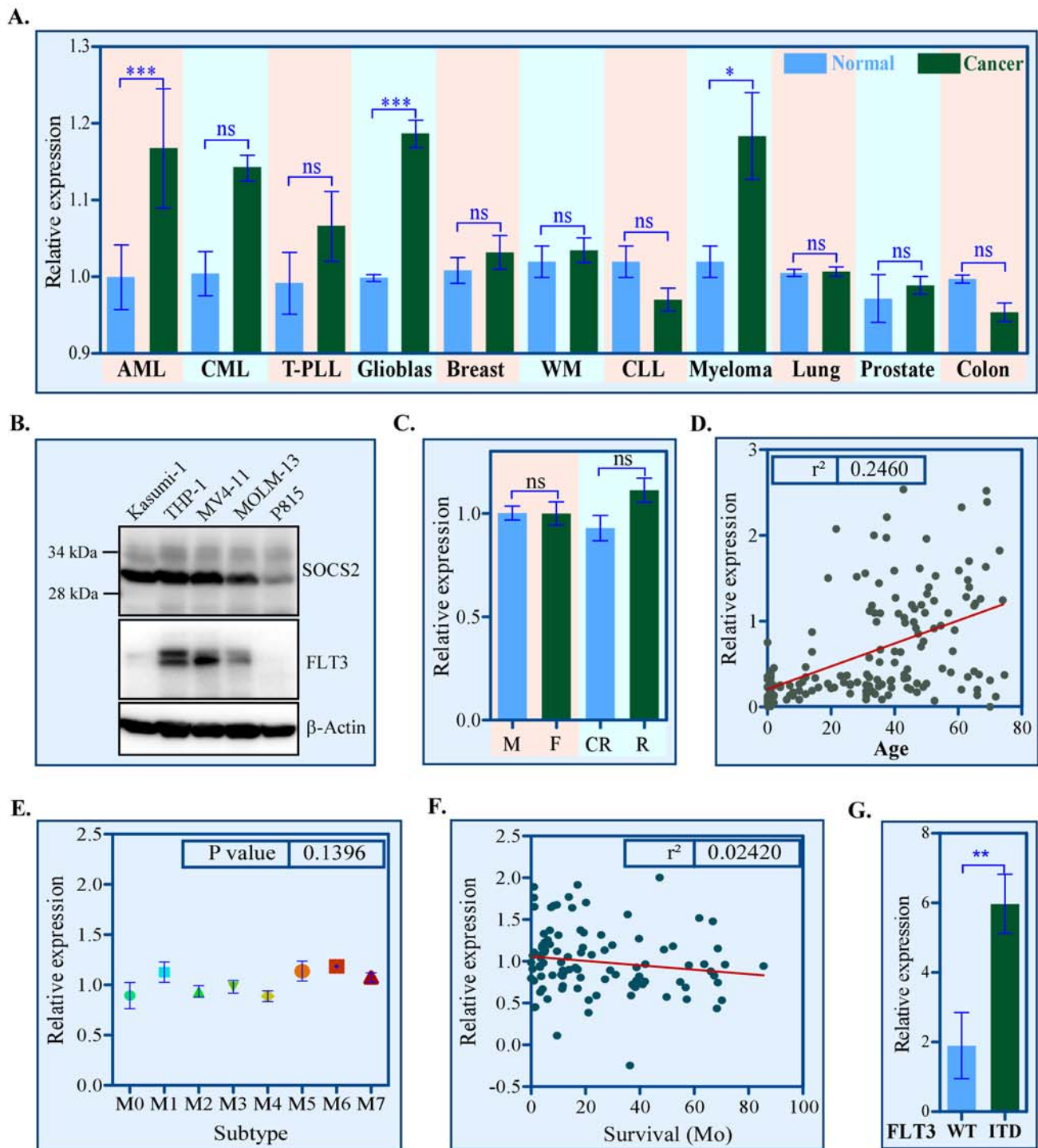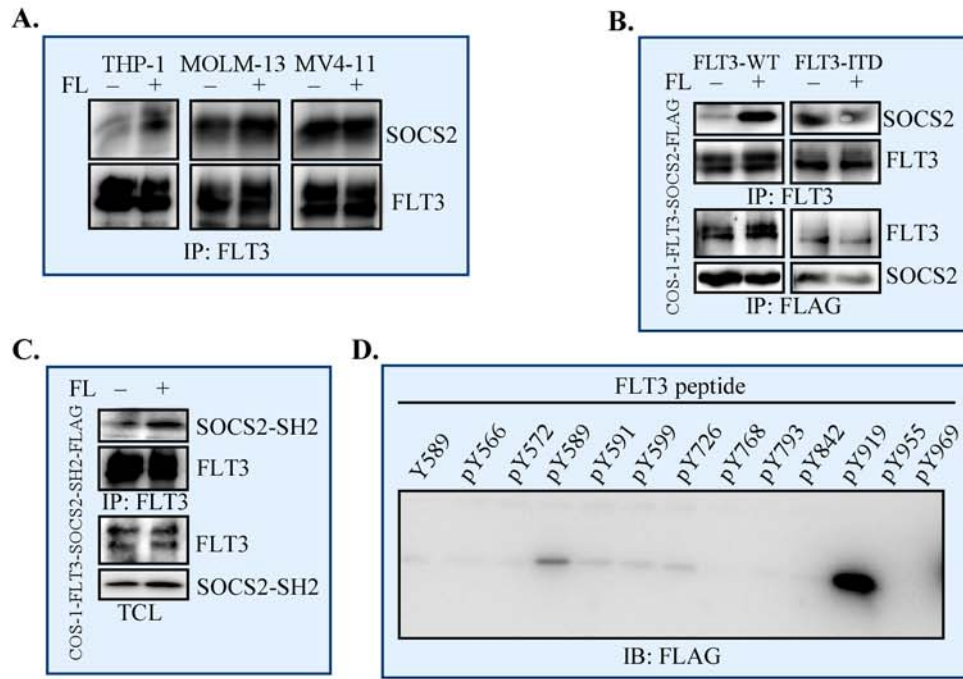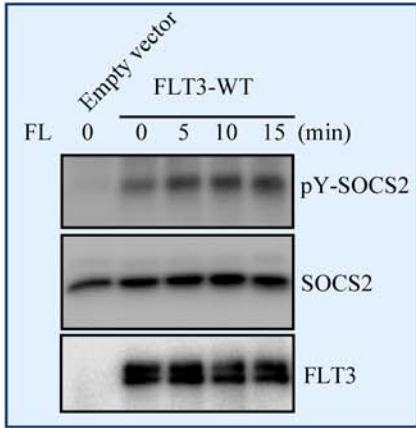
Figure 1

Figure 2

A.

IP: FLT3

B.

IP: FLT3

IP: FLAG

C.

IP: FLT3

TCL

D.

IB: FLAG

Figure 3

A.

FL   0   0 5 10 15 (min)

Empty vector FLT3-WT

pY-SOCS2

SOCS2

FLT3

B.

FL   0   0 5 10 15 (min)

Empty vector FLT3-WT

pSer-SOCS2

SOCS2

FLT3

C.

SOCS2

pY-FLT3

SOCS2

FLT3

Figure 4

A.

FLT3-WT+EV

FLT3-WT+SOCS2

B.

Ba/F3	FLT3-WT	
SOCS2	−	+

SOCS2

IP: FLAG

FLT3

IP: FLT3

C.

FL −

FL +

D.

Figure 5

A.

IP: FLT3

B.

Figure 6

A.

B.

Figure 7

A.

B.

Figure 8

A.

FLT3-ITD+EV

FLT3-ITD+SOCS2

B.

Ba/F3	FLT3-ITD	
SOCS2	-	+

SOCS2

IP: FLAG

FLT3

IP: FLT3

C.

pY-STAT5

STAT5

IP: STAT5

Figure 9

A.

B.

Empty vector

SOCS2

Ba/F3-FLT3-ITD

C.

Figure 10

Figure 11