# Stony Brook University

# Suppressor of Cytokine Signaling 3 Blocks Breast Tumor Kinase Activation of STAT3 Transcription Factor

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by

**Yiwei Gao**

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Abstract of the Dissertation

**Suppressor of Cytokine Signaling 3 Blocks Breast Tumor Kinase Activation of STAT3 Transcription Factor**

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The Signal Transducers and Activators of Transcription (STATs) are a family of transcription factors that play important roles in cytokine signaling. Following cytokine stimulation they are tyrosine phosphorylated by receptor-associated Janus kinases (JAKs). Phosphorylation promotes their dimerization and this confers their ability to bind DNA consensus sequences and stimulate gene expression.

STAT3 is a member of this family and can be activated by a large number of cytokines, growth factors and hormones. It is also a target of oncogenic tyrosine kinases that commonly link STAT3 closely with cancer. One of these tyrosine kinases is called breast tumor kinase (Brk). Brk is a non-receptor tyrosine kinase that is expressed in more than 60% of breast tumors. We have shown that Brk activates STAT3 and induces transcriptional activation of STAT3. One of the genes induced by tyrosine

phosphorylated STAT3 is a negative regulator of cytokine signaling, the suppressor of cytokine signaling 3 (SOCS3). SOCS3 is known to block signaling mediated by cytokine receptors in a classical feedback loop. We have found that SOCS3 is also induced in response to Brk and it is able to inhibit the ability of Brk to phosphorylate STAT3.

The molecular mechanism by which SOCS3 suppresses Brk activity has been investigated in this study. SOCS3 has several functional domains, a kinase inhibitory region (KIR), followed by an extended SH2 subdomain (ESS), an SH2 domain, and the conserved C-terminal SOCS box. A link to proteosomal degradation was discovered with the association of the SOCS box to components of the E3 ubiquitin ligase complex. It is demonstrated that the primary inhibitory function of SOCS3 on Brk is mediated by the KIR domain. SOCS3 physically associates with Brk and this association is mainly mediated by SH2 domain in SOCS3 and tyrosine kinase domain in Brk. In addition, SOCS3 promotes Brk degradation and the SOCS box is necessary for this effect. These findings identify Brk as a target of SOCS3, and demonstrate the inhibitory mechanism relies on binding to Brk and effecting both kinase activity and protein degradation.

## DEDICATION

To my parents, Hongguang Gao and Shiping Zhu

and

my husband, Nanjun Hu

with LOVE

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## ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| APRE | Acute Phase Response Element |
| ATP | adenosine 5’-triphosphate |
| Brk | Breast Tumor Kinase |
| BSA | Bovine Serum Albumin |
| CBP | CREB-Binding Protein |
| cDNA | complementary deoxyribonucleic acid |
| CIS | Cytokine Inducible SH2-containing protein |
| CNTF | Ciliary Neurotrophic Factor |
| DBD | DNA Binding Domain |
| DNA | deoxyribonucleic acid |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DMSO | dimethyl sulfoxide |
| dsRNA | double stranded ribonucleic acid |
| DTT | Dithiothreitol |
| EDTA | ethylene diaminetetraacetic acid |
| EGF | Epidermal Growth Factor |
| EGFR | Epidermal Growth Factor Receptor |
| ESS | Extended SH2 Subdomain |
| FAK | Focal Adhesion Kinase |
| FBS | fetal bovine serum |
| GAS | Interferon-γ activation sequence |
| GFP | Green Fluorescent Protein |
| GH | Growth Hormone |
| GM-CSF | Granulocyte Macrophage Colony-Stimulating Factor |
| GPCR | G-protein coupled receptor |
| GST | Glutathione S-Transferase |
| HEPES | N-2-hydroxyethylpiperazine N’-2-ethanesulfonic acid |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IGF | Insulin-like Growth Factor |
| IKK | IκB Kinase |

| | |
|---|---|
| IL | Interleukin |
| IPTG | Isopropyl Thio-β-D-Galactoside |
| IRF | Interferon Regulatory Factor |
| IRS | Insulin Receptor Substrate |
| ISGF-3 | Interferon Stimulated Gene Factor 3 |
| ISRE | Interferon Stimulated Response Element |
| JAB | JAK-Binding Protein |
| JAK | Janus Kinase |
| kDa | kilodalton |
| KIR | Kinase Inhibitory Region |
| LB | Luria Broth |
| LIF | Leukaemia Inhibitory Factor |
| LPS | Lipopolysaccharide |
| MEF | mouse embryonic fibroblast |
| μg | microgram |
| μl | microliter |
| mg | milligram |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| NaCl | Sodium cholride |
| NaF | Sodium fluoride |
| ng | nanogram |
| NP40 | Nonidet-P40 |
| OSM | Oncostatin M |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDGF | Platelet-Derived Growth Factor |
| PI3K | Phosphoinositide 3 Kinase |
| PIAS | Protein Inhibitor of Activated STAT |
| PMSF | Phenylmethylsulfonyl fluoride |
| PRL | prolactin |
| PTK | Protein Tyrosine Kinase |
| PTP | Protein Tyrosine Phosphatase |

| | |
|---|---|
| pY | phosphotyrosine |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RT | room temperature |
| RTK | Receptor Tyrosine Kinase |
| RT-PCR | Reverse Transcription Polymerase Chain reaction |
| Sam68 | Src Associated in Mitosis 68 |
| SDS | sodium dodecylsulfate |
| SH2 | Src Homology 2 |
| SH3 | Src Homology 3 |
| SHP | Src Homology 2 domain-containing Phosphatase |
| Sik | Src-related intestinal kinase |
| siRNA | short interfering ribonucleic acid |
| SOCS | Suppressor of Cytokine Signaling |
| SSI | STAT-induced STAT Inhibitor |
| STAT | Signal Transducer and Activator of Transcription |
| SUMO | small ubiquitin-like modifier |
| TAD | Transcriptional Activation Domain |
| TBS | Tris Buffered Saline |
| TLR | Toll-Like Receptor |
| TNF | Tumor Necrosis Factor |
| Tris | tris (hydroxymethyl) aminomethane |
| Tween 20 | Polyoxyethylene-sorbitan-monolaurate |
| U | unit |
| Ub | ubiquitin |
| VEGF | Vascular Endothelial Growth Factor |
| WT | wild-type |

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This dissertation is dedicated to my parents and my husband. My parents give me unconditional support and love. My husband, Nanjun Hu, loves and supports me all the time. He accompanies me during difficult times away from home.

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# Chapter 1

## Introduction

STAT3, a member of the Signal Transducer and Activator of Transcription family, is often associated with tumorigenesis in its active form. STAT3 is activated by tyrosine phosphorylation in response to cytokine signaling or intracellular oncogenic tyrosine kinases. Previously STAT3 was identified as a specific substrate of breast tumor kinase (Brk). The suppressor of cytokine signaling (SOCS) 3 is the primary member induced by STAT3 in response to interleukin-6, and it inhibits Janus kinases (JAKs) that are bound to cytokine receptors. We have found that SOCS3 is able to suppress the ability of Brk to phosphorylate STAT3.

The goal of this dissertation is to investigate the molecular mechanism underlying the inhibition of Brk by SOCS3. The introduction section will present current understanding on cytokine signaling, Brk and SOCS proteins.

The vertebrate immune system provides a defense mechanism against foreign substances, pathogenic organisms and cancer. To achieve effective immune responses, the body requires complex interactions and communication among responsive cells. The mediators of many of these cell-cell communications are a group of small proteins collectively designated cytokines that bind to specific receptors on the membrane of target cells, triggering signal transduction pathways and ultimately altering gene expression. A subset of proteins that sense the extracellular stimuli of specific cytokines

and transmit the signal to the nucleus belong to a family of transcription factors named Signal Transducer and Activator of Transcription (STAT).

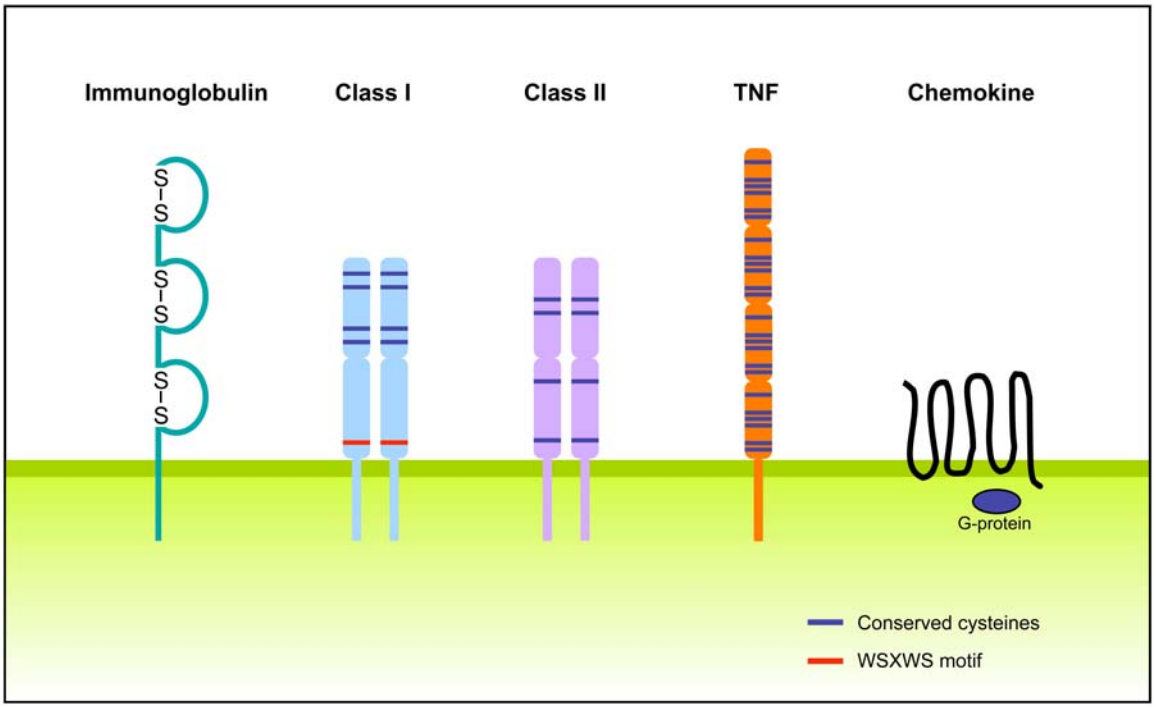
**Cytokines and Cytokine Receptors**

Cytokines are low-molecular-weight regulatory proteins or glycoproteins secreted by specific cells of the immune system in response to different stimuli. They are involved in a broad array of biological activities including innate immunity, adaptive immunity, inflammation and hematopoiesis. The total number of cytokines exceeds 100 and research continues to uncover new ones. Based on their structure, the cytokines characterized so far can be divided into four groups: the hematopoietin (Class I) family, the interferon (Class II) family, the chemokine family, and the tumor necrosis factor (TNF) family.

Cytokines bind to specific cell surface receptors on the target cells with very high affinity to exert their biological effects. Structure studies have shown that cytokine receptors belong to one of five families (Figure 1) [1]:

- Immunoglobulin superfamily receptors
- Class I cytokine receptor family (also known as hematopoietin receptor family)
- Class II cytokine receptor family (also known as interferon receptor family)
- TNF receptor family
- Chemokine receptor family

Most cytokines bind to either Class I or Class II receptors. The Class I cytokine receptors have four positionally conserved cysteine residues (CCCC) and a conserved WSXWS (tryptophan-serine-any amino acid-tryptophan-serine) motif in the extracellular




(Modified from Goldsby R, Immunology, *Fifth Edition*) [1]

**Figure 1: The families of cytokine receptors.**

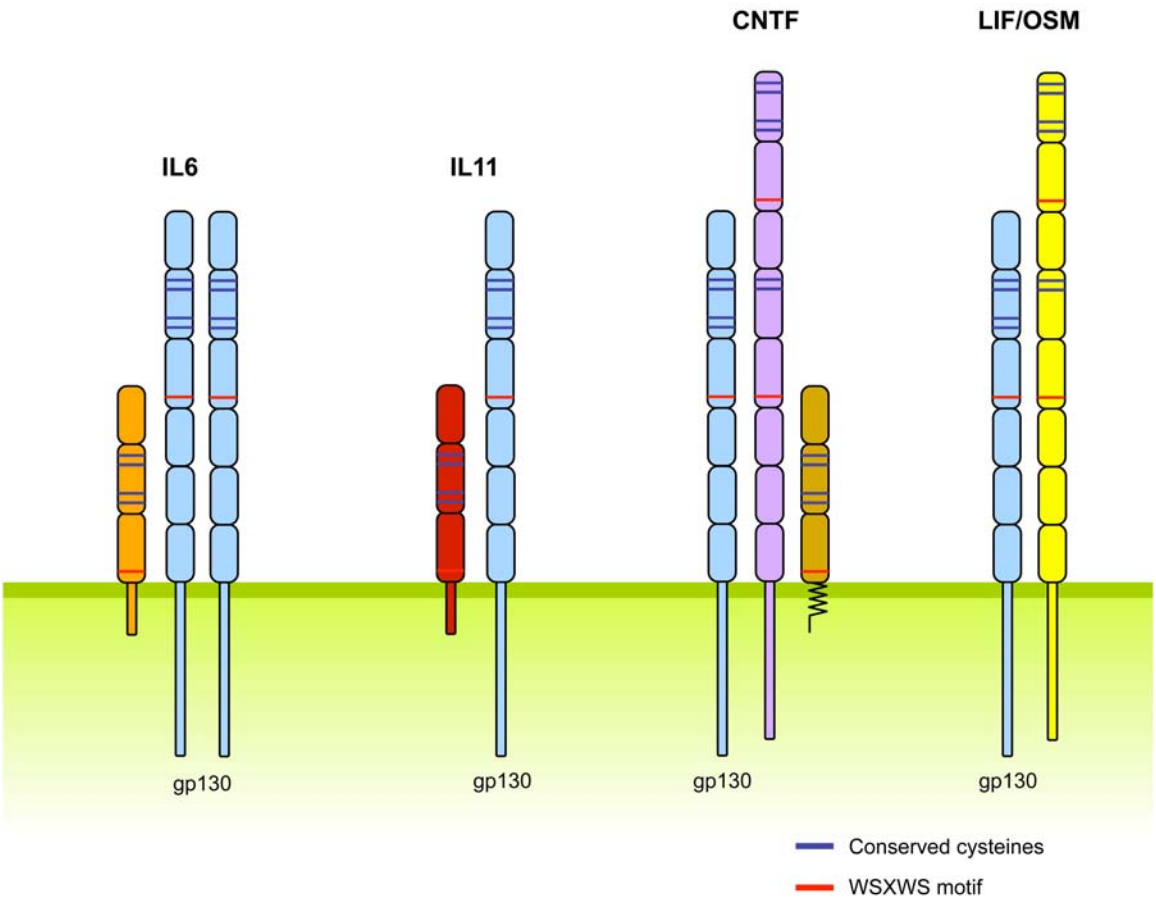
Schematic diagram showing the structural features that define the five families of cytokine receptors to which most cytokines bind.

domain. The Class II cytokine receptors possess the conserved cysteine repeats, but lack the WSXWS motif. Most of Class I and Class II receptors are composed of at least two polypeptide chains, often including a cytokine-specific recognition subunit and a signal-transducing subunit.

Many cytokine receptors share common signal-transducing subunits. Based on the common subunit, the Class I cytokine receptors are classified into three subfamilies: the GM-CSF receptor subfamily that share a common β subunit, the Interleukin 2 (IL-2) receptor subfamily that shares a common γ subunit and the IL-6 receptor subfamily that share a common glycoprotein gp130 subunit (Figure 2). The IL-6 family of cytokines contains IL-6, Leukaemia Inhibitory Factor (LIF), IL-11, Oncostatin M (OSM) and Ciliary Neurotrophic Factor (CNTF). In the case of IL-6, upon IL-6 binding to IL-6 receptor (IL-6R), gp130 is recruited to the receptor complex. After association of the IL-6–IL-6R complex with gp130, homodimerization of gp130 occurs and IL-6 signal transduction starts [2].

**JAK-STAT Signaling Pathway**

Cytokines bind to specific receptors on the membrane of target cells and trigger JAK-STAT signaling pathway that leads to a rapid reprogramming or alteration in the pattern of expressed genes in the target cells. The JAK-STAT signaling pathway transmits information from extracellular signals, through transmembrane receptors, and directly to target gene promoters in the nucleus. The JAK-STAT system consists of three main components: receptor, Janus kinase (JAK) and STAT.




(Adapted from Goldsby R, Immunology, *Fifth Edition*) [1]

**Figure 2: IL-6 receptor subfamily of Class I cytokine receptors.**

Schematic diagram of IL-6 receptor subfamily of Class I cytokine receptors. All members have a common signal-transducing gp130 subunit (blue), but a unique cytokine-specific subunit.

Class I and Class II cytokine receptors lack signaling motifs such as intrinsic tyrosine kinase domains. Instead, they constitutively associate with non-receptor tyrosine kinases, JAKs. In mammalian cells there are four JAK family members: JAK1, JAK2, JAK3 and TYK2, that share ~40% sequence identity and similar domain structure [3,4]. They all have a kinase catalytic domain and a pseudokinase domain that play an important role in regulating their activity. In the absence of cytokine, JAKs keep inactive in the cytoplasm. They are activated by receptor dimerization or oligomerization.

The STAT proteins were first identified as transcription factors that were activated in response to interferon (IFN). In the search for key mediators of IFNα-induced gene expression, a protein complex, IFN-Stimulated Gene Factor 3 (ISGF-3) was identified [5,6]. ISGF-3 complex was assembled in the cytoplasm in response to IFNα stimulation and bound to the highly conserved regulatory DNA element named Interferon-Stimulated Response Element (ISRE). Components of the ISGF-3 complex were proteins with molecular weight of 48kDa, 91kDa and 113kDa, later named Interferon Regulatory Factor 9 (IRF9), Signal Transducer and Activator of Transcription 1 (STAT1) and 2 (STAT2) respectively [7]. STAT1 and STAT2 are the first two members of this new protein family, and later five more mammalian STATs were identified, namely STAT3, STAT4, STAT5a, STAT5b, and STAT6 [8-12]. It was soon demonstrated that the STAT proteins were tyrosine phosphorylated following IFN stimulation [13-15]. To date the STAT proteins are the only documented transcription factors that are activated by tyrosine phosphorylation.

The STAT signaling cascade is initiated upon binding of cytokines to specific cell surface receptors, leading to the dimerization of the receptor subunits and activation of

the receptor-associated JAKs. The activated JAKs create docking sites for the STAT proteins by phosphorylation of specific tyrosine residues on the receptor subunits. While bound to the receptor, the STATs are phosphorylated by JAKs on a specific tyrosine residue, and this modification promotes homo- or hetero-dimerization of the STATs. Subsequently, the phosphorylated STAT dimers undergo the conformational change and translocate into the nucleus where they bind to consensus DNA sequence and induce the expression of specific target genes (Figure 3).

**STATs: Domain, Structure and Biological Functions**

The seven mammalian STATs are composed of 750 to 850 amino acids and share a similar structural arrangement of functional domains (Figure 4). They possess a N-terminal domain, a coil-coil domain that is involved in interactions with distinct proteins, a central DNA binding domain, a Src homology 2 (SH2) domain, a conserved tyrosine residue that is phosphorylated following activation, and a C-terminal transcriptional activation domain. The N-terminal domain strengthens interactions between STAT dimers on adjacent DNA-binding sites [16,17]. The SH2 domain can interact with phosphotyrosine residues on receptors, JAKs or other STAT molecules, thus mediating the recruitment of STATs to receptors and the formation of STAT dimers in a reciprocal manner. The transcriptional activation domain has been shown to interact with CREB-binding protein (CBP)/p300, which acts as transcription adaptors linking a number of transcription activators to the basal transcription apparatus [18-20].

Crystal structures have been solved for both unphosphorylated STATs and tyrosine phosphorylated STAT homodimers bound to DNA (Figure 5). It was initially

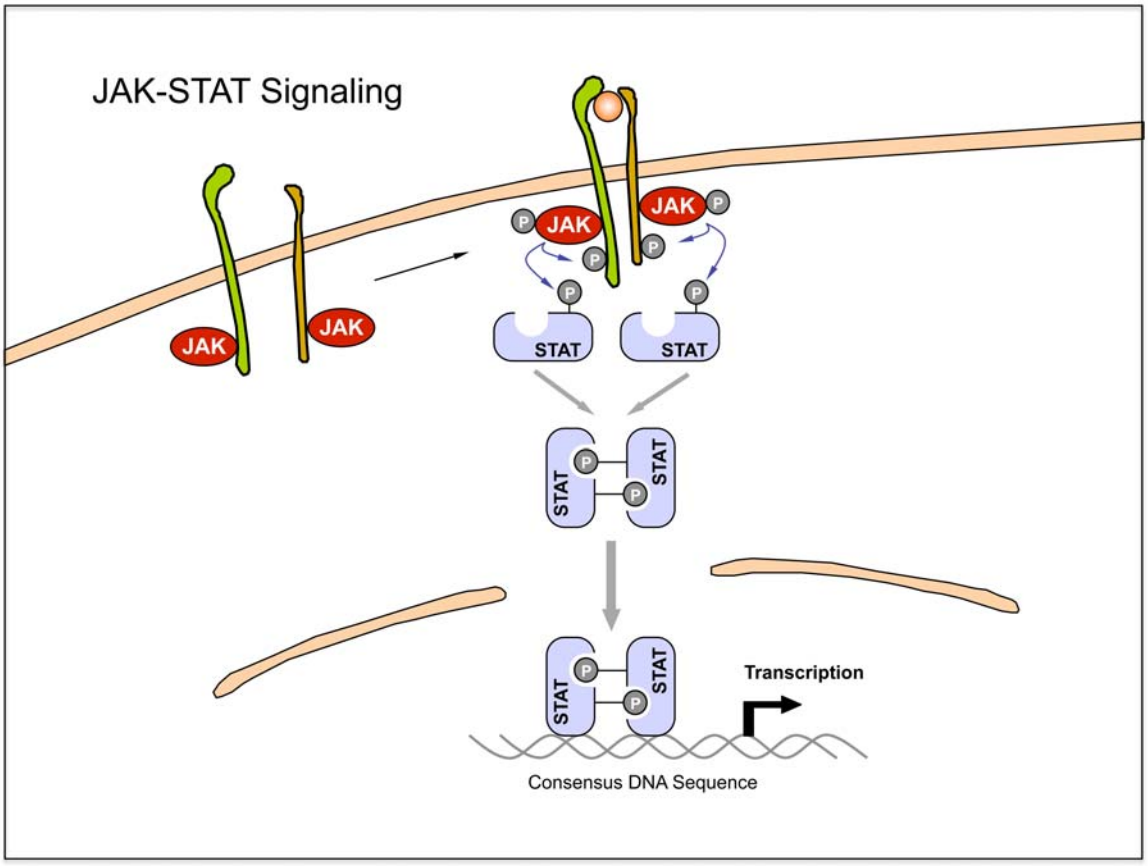



**Figure 3: JAK-STAT signaling pathway.**

Cytokine binding to cell surface receptors activates associated JAKs that phosphorylate cytoplasmic tails of the receptors resulting in STAT recruitment via their SH2 domain. JAKs in turn phosphorylate STATs in the cytoplasm leading to their dimerization, translocation into the nucleus and induction of gene expression.


**Figure 4: A linear depiction of STAT domain organization.**

STAT family members vary from 750 to 850 amino acids in length and have similar functional domains: a conserved N-terminal domain, a coil-coil domain, a central DNA binding domain (DBD), a Src homology 2 (SH2) domain, a single tyrosine residue that is phosphorylated (pY) upon activation, and a C-terminal transcriptional activation domain (TAD).

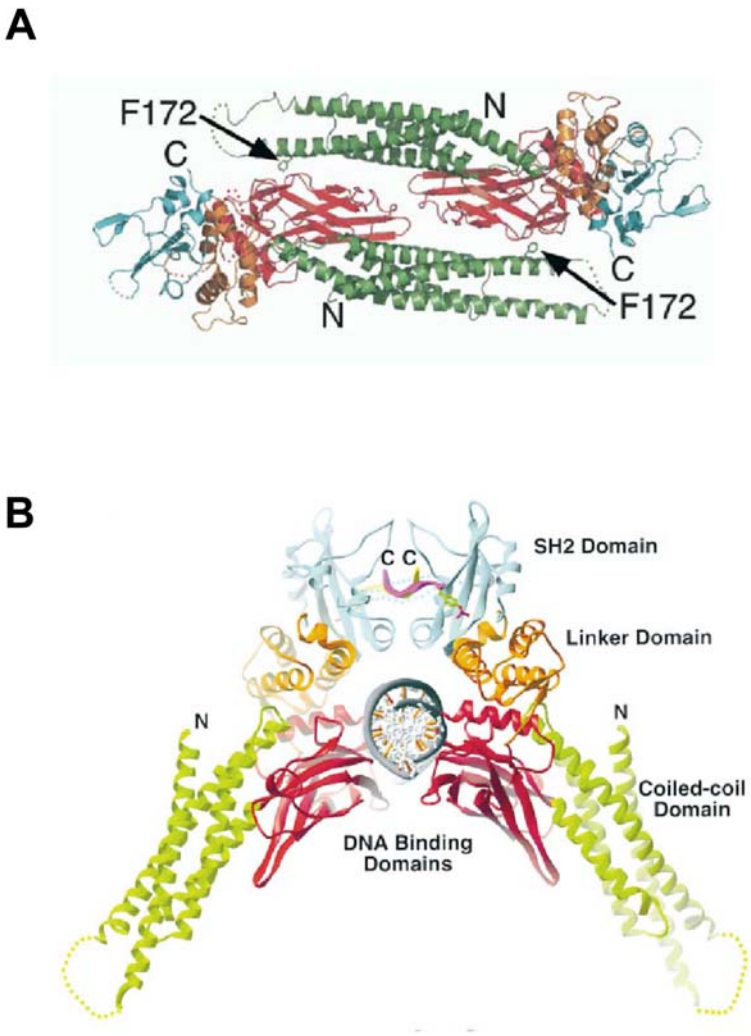



**Figure 5: Crystal structures of unphosphorylated and phosphorylated STAT1 dimer.**

(A) Ribbon diagram of the unphosphorylated STAT1 core dimer (Mao et al., 2005) [21].
(B) Ribbon diagram of the phosphorylated STAT1 core dimer on DNA (Chen et al., 1998) [22].
In both diagrams, the coil-coil domain is shown in green, the DNA-binding domain in red, the linker domain in orange, the SH2 domain in blue and the DNA backbone in gray.

believed that unphosphorylated STATs exist as monomers. However evidence from crystallography shows that the core fragment (lacking the N-terminus and carboxyl transcriptional activation domain) of either unphosphorylated STAT1 or STAT5a is dimeric and they have a very similar structure [21,23]. Following tyrosine phosphorylation, the structure of STAT monomer remains the same, while the STAT dimer adopts a different conformation that is capable of DNA binding. The core structures of tyrosine phosphorylated human STAT1 and mouse STAT3 dimers complexed with DNA show that the STAT dimer forms a contiguous C-shaped clamp embracing the DNA that is stabilized by reciprocal and highly specific interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other. [22,24]. These findings provide the structural basis on which tyrosine phosphorylation serves as a molecular switch to change the conformation and turn on the DNA binding activity of STATs.

Individual STATs are activated by diverse extracellular stimuli and induce transcription of distinct target genes, resulting in different biological outcomes [25,26]. Gene disruption studies in mice have revealed the primary physiological functions of individual STATs (Table 1).

STAT1 or STAT2 knockout mice are highly susceptible to viral infections and other pathological agents because all the physiological functions associated with IFNs are absent [27-29]. STAT3 knockout mice display early embryonic lethality, indicating a critical role for STAT3 in development [30]. Physiological roles of STAT3 revealed by gene disruption in specific tissues will be discussed in the following section. STAT4 is predominantly activated in response to IL-12, a macrophage-derived cytokine that

Table 1 Physiological role of individual STATs

| STATs | Primary activating cytokines | Phenotype of knockout mice |
|---|---|---|
| STAT1 | IFNs | Impaired interferon response; increased susceptibility to viral infections and tumors |
| STAT2 | IFNα/β | Impaired interferon response |
| STAT3 | IL-6 family | Embryonic lethality |
| STAT4 | IL-12 | Deficiency in IL-12 signaling and impaired $T_H1$ cell differentiation |
| STAT5a | Numerous | Impaired prolactin signaling; defective mammary gland development; no lactation |
| STAT5b | Numerous | Impaired growth hormone signaling |
| STAT5a/b | Numerous | Anemia; female infertility; reduction in growth; impaired prolactin and growth hormone signaling; impaired T cell proliferation in response to IL-2 |
| STAT6 | IL-4, IL-13 | Deficiency in IL-4 and IL-13 signaling and impaired $T_H2$ cell differentiation |

mediates T helper 1 ($T_H1$) cell differentiation. The phenotype of STAT4 knockout mice is very similar to that of mice lacking IL-12 or IL-12 receptor [31,32]. STAT6 is primarily activated by IL-4 and the highly related cytokine IL-13. As expected, STAT6 knockout mice lack IL-4 signaling, showing impaired $T_H2$ cell development and the inability of B cells to undergo class switching and produce IgE [33,34]. The two highly related STAT5 proteins, STAT5a and STAT5b, are activated in response to a variety of cytokines, growth factors and hormones. Ablation of STAT5a results in the loss of prolactin-dependent mammary gland development, which is necessary for lactation [35]. STAT5b knockout mice exhibit deficiency in growth hormone response, resulting in the loss of sexually dimorphism [36]. However, STAT5a/b double knockout mice display a much

more severe phenotype [37]. It was reported that STAT5a/b double knockout mice are visibly anemic, female infertile, and have reduced growth and a T cell proliferation defect. These findings suggest that there are both non-redundant as well as redundant functions identified for STAT5 based on the phenotypes of single and double knockout mice.

**The pleiotropic STAT: STAT3**

STAT3 was first described as a DNA-binding factor in IL-6-stimulated hepatocytes, capable of binding to an enhancer element in the promoter of acute-phase genes, known as the acute-phase response element (APRE) [10]. Molecular definiton of this factor demonstrated that it is 53% homology to STAT1 and renamed STAT3. Like all other STATs, STAT3 is activated by tyrosine phosphorylation on a conserved tyrosine residue (Y705). Following activation, STAT3 dimers gain the ability to bind DNA and recognize an inverted repeat of GAA sequence (TTCNNNGAA). This consensus DNA element is usually referred to as a GAS element, reflecting its initial characterization as a γ-interferon Activation Sequence (GAS) [38].

The function of STAT3 has been extensively studied in cell culture systems. IL-6 cytokines evoke a number of distinct responses in different cells, including induction of an acute-phase response in hepatoma cells, stimulation of B lymphocyte proliferation, activation of terminal differentiation and growth arrest in monocytes [39], and maintenance of the pluripotency of mouse embryonic stem cells [40-42]. These findings that STAT3 is involved in seemingly contradictory cell responses are at least in part due to the induction of distinct sets of target genes by STAT3 in different cells [43]. For

example, STAT3 stimulates B cell proliferation through inhibition of apoptosis that is mediated by induction of the anti-apoptotic gene Bcl-2. In contrast, activation of STAT3 in monocytes leads to downregulation of c-myc and c-myb and induction of junB and IRF-1, a pattern of gene regulation consistent with differentiation and growth arrest.

The physiological role of STAT3 in specific tissues has been revealed by conditional gene targeting in mice using the Cre-*loxP* recombination system. Paired *loxP* sites have been targeted into introns surrounding critical regions of STAT3 [42,44]. Following introduction of Cre recombinase, deletion of the DNA segment flanked by the *loxP* sites results in functional inactivation of the *Stat3* gene. To date, this approach has been used to examine STAT3 function in different tissues or cells, including skin, thymic epithelium, T cells, granulocytes, mammary gland, liver, nervous system, cardiomyocytes and endothelium (Table 2) [41-53].

STAT3 is now known as a pleiotropic transcription factor that is activated by a variety of cytokines, hormones and intracellular tyrosine kinases (Figure 6). As introduced previously, the entire family of IL-6 cytokines that signal through the common gp130 subunit can phosphorylate STAT3 through conventional JAK-STAT signaling pathway [39,45]. A number of chemokines signal through G-protein coupled receptors (GPCRs) that associate with JAKs to activate STAT3 [46-48]. Alternatively, STAT3 can be activated in a JAK-independent manner. STAT3 is phosphorylated by growth factors binding to receptor tyrosine kinases such as epidermal growth factor (EGF) receptors and platelet-derived growth factor (PDGF) receptors [49,50]. Upon ligand binding, these receptor tyrosine kinases (RTKs) autophosphorylate multiple tyrosine residues on their cytoplasmic tails and some of them become docking sites for

Table 2 Phenotypes of STAT3 tissue-specific knockout mice

| Target tissue or cells | Phenotypes | Affected functions | Reference |
|---|---|---|---|
| Skin | Impaired second hair cycle, wound repair and keratinocyte migration | Migration | [51] |
| Thymic epithelium | Age-dependent thymic hypoplasia, hypersensitivity to stress | Survival | [52] |
| T lymphocytes | Impaired IL-6-dependent survival, impaired IL-2Rα expression | Survival / proliferation | [44,53] |
| Monocytes and neutrophils | Enhanced inflammatory response, chronic colitis and $T_H1$ differentiation | - | [54] |
| Granulocytes | Enhanced proliferation | Proliferation | [55] |
| Mammary epithelium | Delayed mammary involution | Apoptosis | [56] |
| Endothelium | Increased susceptibility to LPS challenge; exaggerated inflammation and production of proinflammatory cytokines | Anti-inflammation | [57] |
| Liver | Impaired acute-phase response | Gene expression | [58] |
| Cardiomyocytes | Increased susceptibility to cardiac injury; age-related cardiac fibrosis | - | [59] |
| Neurons | Impaired motoneuron survival after injury; enhanced sensory neuronal apoptosis; obesity, diabetes and thermal dysregulation | Survival | [60-62] |

(Modified from Levy D and Lee C, J. Clin. Invest., 109:1143) [63]

STAT3. STAT3 has also been shown to be a target for a number of oncogenic non-receptor tyrosine kinases that commonly link STAT3 closely to cancer.

**STAT3 and Cancer**

As introduced previously, STAT proteins can be phosphorylated and activated by receptor-associated JAKs, protein tyrosine kinases intrinsic to growth factor receptors, and intracellular tyrosine kinases. Since tyrosine kinases are among the most frequently

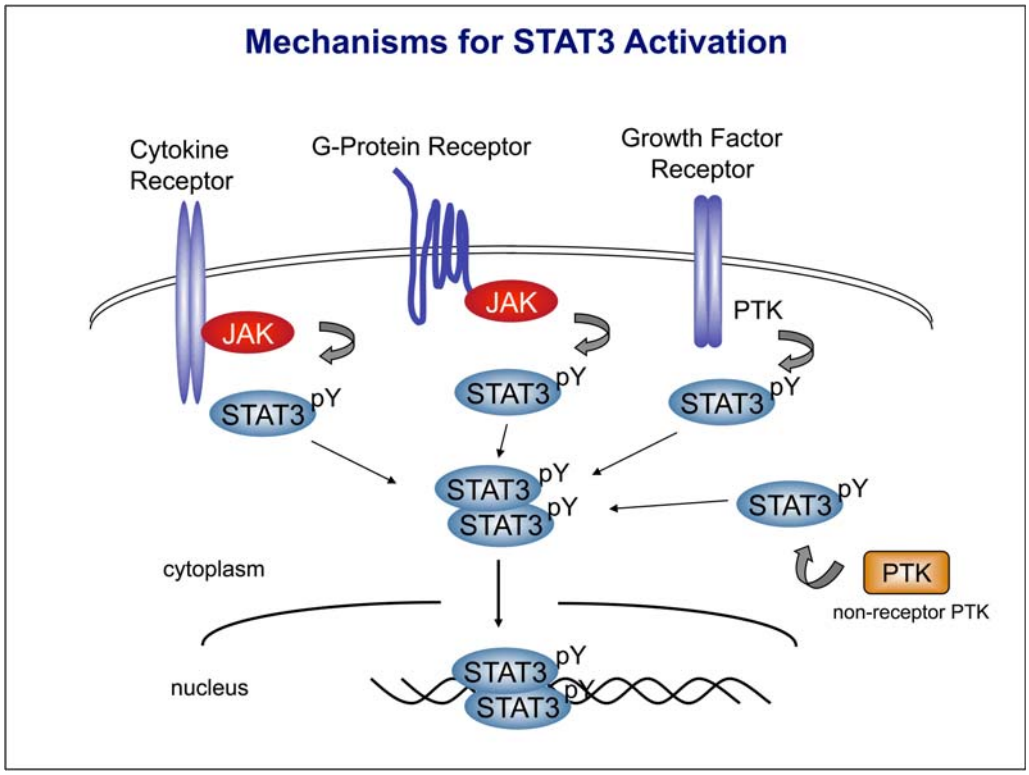



**Figure 6: Multiple mechanisms for STAT3 activation.**

STAT3 can be activated by JAKs that are constitutively associated with cytokine receptors. Some seven transmembrane G-protein coupled receptors also associate with JAKs. These receptors dimerize upon ligand binding, leading to the activation of JAKs and subsequently STAT3. Growth factor receptors that have intrinsic tyrosine kinase activity directly phosphorylate STAT3 following ligand binding. Many non-receptor tyrosine kinases induce STAT3 activation in the absence of extracellular ligands.

activated oncogenic proteins in cancer cells [64], it is not surprising that STATs have been found to be constitutively active in an increasing number of human cancers (Table 3). Among the STATs, STAT3 is most commonly associated with tumorigenesis [65].

Table 3 Constitutive activation of STATs in human cancers

| **Solid tumors** | **Activated STATs** |
|---|---|
| Breast cancer | 1, 3, 5 |
| CNS tumors | 1, 3 |
| Head and neck cancer | 1, 3, 5 |
| Hepatocellular carcinoma | 3 |
| Lung cancer | 1, 3 |
| Melanoma | 3 |
| Ovarian cancer | 3 |
| Pancreatic cancer | 3 |
| Prostate cancer | 3 |

| **Liquid tumors** | **Activated STATs** |
|---|---|
| Multiple Myeloma | 1, 3 |
| *Leukemias:* | |
| Acute lymphoblastic leukemia | 1, 3, 5 |
| Acute myelogenous leukemia (AML) | 1, 3, 5 |
| Chronic lymphocytic leukemia | 1, 3 |
| Chronic myelogenous leukemia (CML) | 5 |
| Erythroleukemia | 1, 5 |
| HTLV-I-dependent leukemia | 3, 5 |
| Large granular lymphocyte leukemia (LGL) | 3 |
| *Lymphomas:* | |
| Anaplastic large-cell lymphoma (ALCL) | 3 |
| Burkitt's lymphoma | 3 |
| Cutaneous T-cell lymphoma | 3 |
| Hodgkins lymphoma | 3 |
| Mycosis fungoides | 3 |
| Non-Hodgkins lymphoma | 3 |

(Modified from Yu H and Jove R, Nat Rev Cancer, 4:97) [66]

It is observed that STAT3 is persistently activated in almost every type of cancer. So far, no natural STAT3 mutations inducing constitutive activation have been identified

in cancer cells. Nevertheless, expression of an engineered constitutively active mutant of STAT3 (STAT3C) on its own can lead to fibroblast transformation and tumor formation in *nude* mice, suggesting that STAT3 is an oncogene [67]. Activated STAT3 has been observed in a variety of experimental malignancies, and its abrogation by use of dominant negative inhibitors or antisense oligonucleotides has led to reversal of the malignant phenotype [68,69]. STAT3 activation is also observed in cells transformed with oncogenic tyrosine kinases such as v-Src, Bcr-Abl and TEL-JAK, and is required for cell transformation by v-Src [70,71]. Moreover, it has been reported that unphosphorylated STAT3 may play a role in oncogenesis [72].

As a transcription factor, STAT3 mediates malignant transformation by induction of genes encoding anti-apoptotic proteins (Bcl-xL, Bcl-2), cell cycle regulators (cyclin D1, cyclin E) and proto-oncoproteins (c-myc) [50,67,73]. Collaboratively these proteins prevent apoptosis, enable cells to escape from the tight cell cycle regulation and promote proliferative processes. STAT3 is also involved in tumor progression through the induction of pro-angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF) [74,75].

**Negative Regulation of STAT Signaling**

The duration of STAT activation is a temporary process, thus within hours the activating signals decay and the STATs are exported back to the cytoplasm. A number of negative regulators of the JAK-STAT signaling pathway have been described, including protein tyrosine phosphatases (PTPs), suppressors of cytokine signaling (SOCSs), and protein inhibitors of activated STAT (PIASs) (Table 4) [76].

Table 4 Negative regulators of JAK-STAT signaling

| Negative regulator | Target proteins |
|---|---|
| Protein tyrosine phosphatases (PTPs) | |
| SHP1 | JAK1, JAK2, JAK3 |
| SHP2 | JAK2, STAT1, STAT5 |
| PTP1B | JAK2, TYK2, STAT5 |
| TC-PTP | JAK1, JAK3, STAT1, STAT3 |
| CD45 | JAK1, JAK3 |
| PTPεC | JAK1, TYK2, STAT3 |
| MKP-1 | STAT1 |
| LMW-PTP | STAT5 |
| Suppressors of cytokine signaling (SOCSs) | |
| CIS | EpoR, PRLR, LeptinR, GHR, IL2R, IL3R, GM-CSFR |
| SOCS1 | JAK1, JAK2, JAK3, TYK2, IFNGR1, GHR, IGF-1R, IRAK1, FAK |
| SOCS2 | GHR, PRLR, IGF-1R, LeptinR, IL2R, IL3R |
| SOCS3 | JAK1, EpoR, GHR, G-CSFR, gp130, IGF-1R, LeptinR, IL-12R |
| SOCS4 | EGFR |
| SOCS5 | |
| SOCS6/7 | IRS-2, IRS-4 |
| Protein inhibitors of activated STATs (PIASs) | |
| PIAS1 | STAT1 |
| PIAS3 | STAT3 |
| PIASx | STAT4 |
| PIASy | STAT1 |

Tyrosine phosphatases are classified into three groups: classical protein tyrosine phosphatases (PTPs); dual-specificity PTPs (dephosphorylate both tyrosine and serine-threonine phosphorylated residues), including mitogen-activated protein kinase phosphatase (MKP) 1; and low-molecular-weight PTPs (LMW-PTPs) [76]. The classical PTPs are divided into two families, the transmembrane receptor-like PTPs, including CD45 and PTPε, and nontransmembrane PTPs, including SH2-domain-containing PTP1 (SHP1), SHP2, PTP1B, and T cell-protein tyrosine phosphatase (TC-PTP). Inhibition of PTP activity by using the PTP inhibitor pervanadate results in constitutive activation of

STATs, which requires JAKs [77]. Several PTPs were studied for their roles in regulation of JAK-STAT signaling, including SHP1, SHP2, PTP1B, TC-PTP, CD45, PTPεC, MKP-1, and LMW-PTP. For instance, SHP1 associates with JAK2 via the N-terminal domain, not the SH2 domain, and dephosphorylates JAK2 [78]. SHP1 also downregulates IL-2-induced tyrosine phosphorylation of JAK1 and JAK3 [79]. PTP1B, a cytosolic PTP, dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b [80]. PTP1B also dephosphorylates TYK2 and JAK2, which contain the (E/D)-pY-pY-(R/K) consensus [81]. CD45 is the most studied transmembrane PTP that serves as a JAK phosphatase [82]. It inhibits IL-4-induced JAK1 and JAK3 phosphorylation and negatively regulates class-switch recombination to IgE.

SOCS proteins are inducible inhibitors of cytokine signaling, which are induced after cytokine stimulation and inhibit cytokine signaling. They were discovered by three groups using distinct approaches to search for proteins that inhibit cytokine responses, interact with JAKs, and are homologous to STAT SH2 domain [83-85]. Thus SOCSs are also known as JAK-binding proteins (JAB), STAT-induced STAT inhibitors (SSI), and cytokine inducible SH2-containing proteins (CIS). The SOCS family consists of eight members, SOCS1 to 7 and CIS. The structure and physiological role of SOCS proteins and mechanisms of JAK-STAT inhibition will be discussed in the following section.

The PIAS proteins are nuclear proteins termed proteins that inhibit activated STATs. They are a group of five STAT-interacting proteins, PIAS1, PIAS3, PIASy, PIASxα, and PIASxβ. The first member, PIAS1, was discovered in a yeast two-hybrid system screening for STAT-interacting proteins [86]. Other family members were identified on the basis of sequence homology to PIAS1. Studies in cultured cells indicate

that PIAS1 and PIAS3 directly interact with tyrosine phosphorylated STAT1 and STAT3, respectively and inhibit their binding to DNA [86,87]. In contrast, PIASx does not block STAT DNA-binding activity. It was shown to bind to activated STAT4 complexes after IL-12 stimulation and inhibit STAT4-dependent gene induction [88]. In addition, a novel mechanism by which PIAS proteins regulate STAT activity has been discovered. The PIAS proteins contain a conserved RING-like domain and can function as E3-like SUMO (small ubiquitin-like modifier) ligases in mammals. SUMOylation is a reversible posttranslational modification and has been implicated in the regulation of protein-protein interactions, protein stability, protein localization, and protein activity. It was shown that STAT1 can be SUMOylated on lysine 703 and this is promoted by PIAS1, PIAS3, and PIASx [89,90]. PIAS1 has also been shown to target SUMO to other proteins, including the androgen receptor, p53, c-Jun and NF-κB [91-93]. The E3 ligase function of PIAS1 is required for its ability to repress transcription mediated by NF-κB and STAT1, suggesting that SUMOylation is needed for the transcriptional repression to occur [94].

Our previous studies on STAT3 cellular localization have demonstrated that STAT3 maintains prominent nuclear presence independent of tyrosine phosphorylation, suggesting that STAT3 may be a target of tyrosine kinases in the nucleus. The search for a nuclear tyrosine kinase for STAT3 has led us to an intracellular tyrosine kinase, Brk.

**Breast Tumor Kinase (Brk)**

Breast tumor kinase (Brk) also known as protein tyrosine kinase 6 (PTK6) is a non-receptor tyrosine kinase evolutionarily related to the Src family of tyrosine kinases. Brk was originally isolated from a human metastatic breast tumor [95], and shortly thereafter its murine homolog Sik (Src-related intestinal kinase) was cloned from the mouse gastrointestinal tract [96]. Brk expression was detected in approximately two-thirds of human breast tumors, but was low or undetectable in normal mammary tissue and benign lesions [97]. A recent analysis of 250 samples on a human breast tissue microarray revealed Brk protein expression in 86% of invasive ductal breast tumors [98]. Brk expression was also found in other cancers including metastatic melanomas, T-cell lymphomas, head and neck squamous cell carcinomas (SCC), ovarian cancer cells, prostate and colon tumors [99-104]. In normal tissues, Brk is expressed in a variety of epithelial linings, including the oral epithelium, the skin, and the epithelial cells of the small intestine and the prostate lumen [99,104-106].

Brk is a 451 amino acid protein that displays 46% sequence homology and similar domain arrangement to the Src kinase (Figure 7). Brk consists of a Src Homology 3 (SH3), a Src Homology 2 (SH2) and a tyrosine kinase domain, but lacks an N-terminal myristoylation site for membrane association. Similar to the Src kinase, Brk is regulated by autophosphorylation and autoinhibition [107]. Brk autophosphorylates itself at tyrosine residue 342 within the kinase activation loop, which increases its catalytic activity. Mutation of a C-terminal tyrosine residue 447 increases enzyme activity and SH2 domain accessibility, indicating a role for this residue in autoinhibition. Interactions between the SH3 domain and the proline-rich linker region are also involved in

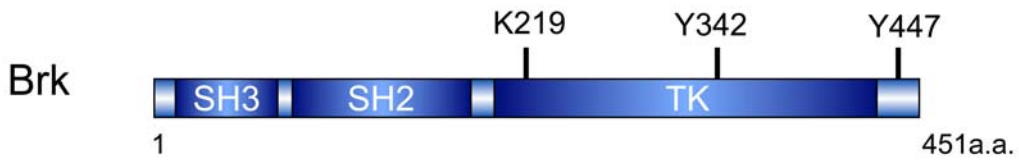



**Figure 7: Brk linear domain arrangement.**

Brk has a structural arrangement of SH3, SH2 and tyrosine kinase (TK) domain. The major autophosphorylation site of Brk is on tyrosine 342 (Y342). Phosphorylation of tyrosine 447 (Y447) appears to exert an autoinhibitory effect on the enzymatic activity. Mutation of the lysine at 219 (K219) inhibits ATP binding and renders the kinase dead.

maintaining an inactive form of Brk [107-109]. In contrast to Src, an intramolecular interaction between SH2-kinase linker region and kinase domain appears essential for its catalytic activity [110].

The SH3 domain of Brk is mainly composed of β-sheets and retains a unique and folded conformation at the neutral pH condition [111]. Aside from intramolecular interactions, the SH3 domain plays a major role in substrate interactions [112-114]. The SH2 domain of Brk contains a consensus α/β-fold and a phosphotyrosine binding surface. Two of the α-helices are located on opposite faces of the central β-sheet and the arrangement of a central four-stranded antiparallel β-sheet differs from that of other Src family members [115,116]. It has been shown that the SH2 domain is important for protein-protein interactions [114,117,118] as well as the regulation of enzymatic activity [107].

**Brk Substrates and Binding Partners**

A rapidly growing number of Brk substrates and interacting proteins are being discovered (Table 5 and 6). To date, substrates include AKT [119], β-catenin [120], KAP3A [121], p190RhoGAP [122], Paxillin [123], PSF [112], Sam68 [114,124,125], SLM1 and SLM2 [126], BKS/STAP2 [117,127], STAT3 [128], and STAT5b [129]. Other potential substrates that have not been fully validated at this time include β-tubulin, FLJ39441/SPTY2D1, GNAS [121], and an unidentified 100 kDa STAP2 associated protein [117]. A recent study on screening Brk substrate specificity revealed the preferred target sequence X-(E/I/L/N)-Y-(D/E)-(D/E), where X can be any amino acid [130].

Table 5 Brk substrates

| Protein | Association | Substrate target | Proposed function/role | Reference |
|---|---|---|---|---|
| AKT | Yes | Yes | Growth regulation | [119] |
| β-catenin | Yes | Y64, Y331/333, Y142 | Growth regulation | [120] |
| β-tubulin | No | Yes | Not validated | [121] |
| FLJ39441 | No | Yes | Not validated | [121] |
| GNAS | No | Yes | Not validated | [121] |
| KAP3A | Yes | C-terminal tyrosines | Migration | [121] |
| p190RhoGAP | Yes | Y1105 | Growth and migration | [122] |
| Paxillin | Yes | Y31, Y118 | Migration and invasion | [123] |
| PSF | Brk SH3 | C-terminal tyrosines | Growth regulation | [112] |
| Sam68 | Brk SH2 and SH3 | Y435; Y440; Y443 | Inhibition of Sam68 | [20,30,31] |
| SLM1; SLM2 | N/D | Yes | Inhibition of SLM1 and SLM2 | [126] |
| STAP2 | STAP2 SH2 | Y250 | STAT3 activation | [117,127] |
| STAT3 | N/D | Y705 | Growth regulation | [128] |
| STAT5b | N/D | Y699 | Growth regulation | [129] |
| 100kDa protein | (STAP2-mediated) | Likely | Protein not identified | [117] |

N/D=Not determined

(Modified from Brauer P, Biochim. Biophys. Acta 2010) [131]

Table 6 Brk binding partners

| Protein | Association | Substrate target | Proposed function/role | Reference |
|---|---|---|---|---|
| ADAM-15A and B | Yes | N/D | Not validated | [132] |
| ErbB1 | Yes | N/D | Growth regulation | [133] |
| ErbB2 | Yes | N/D | Growth regulation | [134,135] |
| ErbB3 | Yes | N/D | Growth regulation | [136] |
| ErbB4 | Yes | N/D | Not validated | [135] |
| GapA (p65) | Brk SH2 | Not likely | Differentiation | [136] |
| IRS-4 | Brk SH2 and SH3 | N/D | Growth and migration | [118] |
| MAPK | Yes | N/D | Not validated | [135] |
| PTEN | Yes | N/D | Not validated | [135] |
| 23kDa protein | Yes | N/D | Protein not identified | [137] |

N/D=Not determined

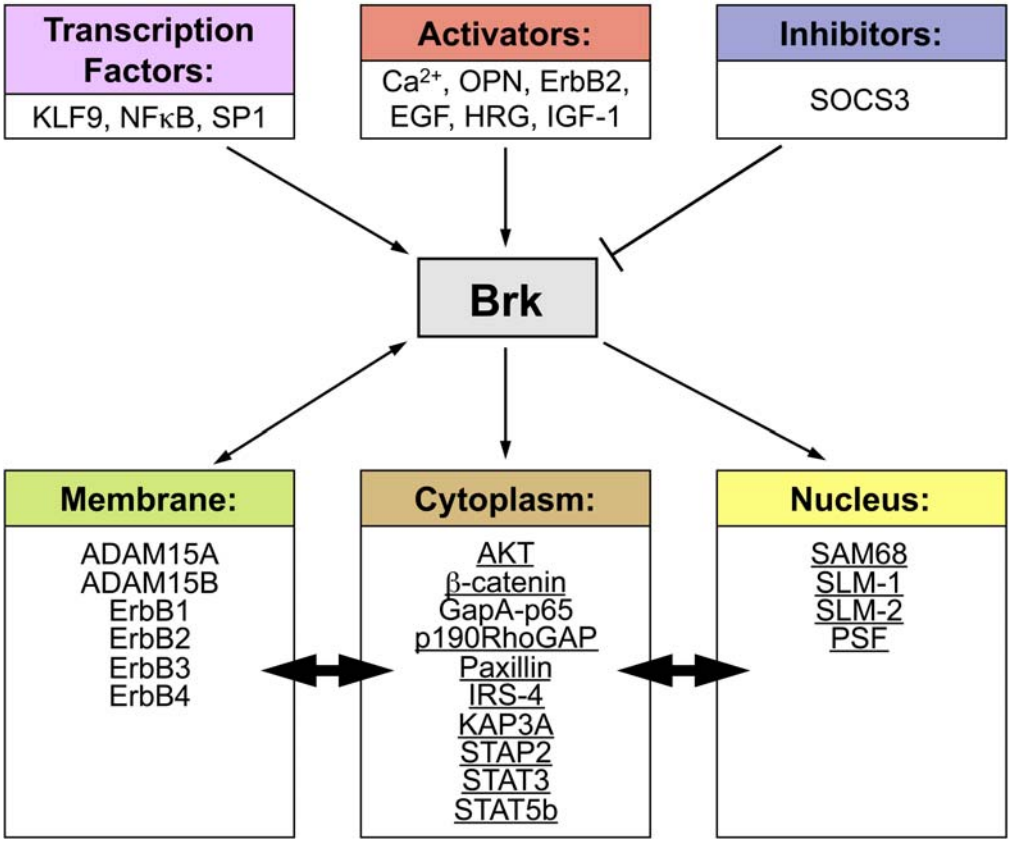
(Modified from Brauer P, Biochim. Biophys. Acta 2010) [131]

Brk has been shown to associate with a number of proteins that are likely upstream of Brk in various signaling pathways, or for which Brk may play an adaptor-like role. These proteins include ADAM-15A and ADAM-15B [132], ErbB1 [133], ErbB2 [134,135], GapA-p65 [136] and IRS-4 [118]. Some data also indicate other potential binding partners including ErbB4, MAPK, PTEN [135], and an unidentified 23 kDa protein that also associates with Src [137]. The signaling pathways that involve Brk substrates and binding partners will be discussed further below.

**Brk Signaling Pathways**

Brk is regulated by a variety of factors (Figure 8). It has been shown to stimulate ErbB receptor tyrosine kinase signaling in cancer cells. Experimental expression of Brk in human mammary epithelial cells, at pathologically relevant levels, leads to sensitization to the mitogenic effect of EGF and enhances the EGF-induced phosphoinositide 3 (PI3) kinase recruitment to ErbB3 and subsequent phosphorylation of AKT [133,138]. In addition to EGF, other ErbB receptor ligands such as heregulin, can activate Brk kinase activity [98]. In breast cancer cells, heregulin stimulation results in activation of ERK5, p38 MAPK and MEF2, which is abrogated by Brk shRNA. Brk has also been reported to coamplified and coexpressed with ErbB2 in human breast cancers [134]. ErbB2 interacts with Brk and enhances its kinase activity. Expression of Brk increases the ErbB2-induced activation of Ras/MAPK signaling and cyclin E/cdk2 activity to induce cell proliferation.

The studies of Brk functions in cell regulation were performed by using both overexpression and knockdown systems. Brk expression in human mammary epithelial cells potentiates anchorage-independent growth, and partially transforms mouse embryonic fibroblasts [133]. On the other hand, knockdown of Brk expression by RNA interference in breast carcinoma cells results in a significant suppression of cell proliferation [139]. Brk was shown to contribute to migration and proliferation by phosphorylating its substrate p190RhoGAP, which promotes association with p120RasGAP, leading to RhoA inactivation and Ras activation [122]. EGF stimulation activates Brk to phosphorylate the focal adhesion protein paxillin. The phosphorylation of paxillin promotes the activation of Rac1 via the function of CrkII, thereby promoting cell




(Adapted from Brauer P, Biochim. Biophys. Acta 2010) [131]

**Figure 8: Brk upstream and downstream signaling.**

Brk is regulated by a number of activators and inhibitors. Substrates (underline) and binding partners may be localized to different cellular compartments (as indicated by double headed arrows) and Brk may have effects on substrates and interacting proteins in different cellular compartments. (OPN: osteopontin; HRG: heregulin).

migration and tumor invasion [123]. In addition, it has been shown that kinesin-2 subunit KAP3A is a physiological substrate of Brk and is required in modulation of Brk-induced migration [121]. Collectively, these findings suggest that Brk plays important roles in promoting proliferation, anchorage-independent growth, cell migration, and tumor growth.

The STAT family members are important for different biological processes, including inflammation, development, cell proliferation and survival. Two STAT members, STAT3 and STAT5b, have been reported to be direct substrates of Brk. Brk activates STAT3 and STAT5b to promote proliferation, and this may be facilitated by adaptor protein STAP2 [127-129]. The suppressor of cytokine signaling 3 (SOCS3), which acts as a negative regulator of JAK-STAT signaling, inhibits Brk kinase activity [128]. Other factors that activate Brk include calcium [136], IGF-1 [118], and osteopontin [140]. IGF-1 is an activator of Brk that binds to IRS-4, suggesting a potential role of Brk in the IGF-1 induced cell proliferation. Osteopontin was shown to trigger VEGF-dependent tumor progression and angiogenesis by activating Brk signaling cascade in breast cancer system.

There are only a few studies focused on Brk signaling in normal physiological context. Disruption of the *brk* gene in the mouse resulted in increased growth and impaired enterocyte differentiation [141]. An increased AKT activity was detected, accompanied by decreased nuclear localization of the AKT substrate FoxO1, indicating that Brk inhibits AKT activity in normal tissues. High levels of nuclear β-catenin were also observed in the small intestine of Brk-deficient mice. While Brk directly phosphorylates β-catenin on several tyrosine residues, mutation of these sites does not

abrogate the ability of Brk to inhibit β-catenin transcriptional activity [120]. However, Brk expression increases levels of T-cell factor 4 (TCF4) and the transcriptional co-repressor TLE/Groucho. The ability of Brk to negatively regulate β-catenin/TCF transcription by modulating levels of TCF4 and TLE/Groucho may contribute to its growth-inhibitory activities. In addition, Brk targets a number of nuclear RNA-binding proteins including Sam68 (Src-associated in mitosis 68), the first identified Brk substrate, and the Sam68-like mammalian proteins SLM1 and SLM2 [114,126]. Tyrosine phosphorylation of these proteins by Brk leads to inhibition of their RNA-binding activities. The recent finding that Sam68 haploinsufficiency impedes mammary tumor onset *in vivo* may arouse interests in studying the crosstalk between Brk and Sam68 in mammary gland tumorigenesis [142]. Brk also phosphorylates and associates with the nuclear protein PSF (Polypyrimidine tract-binding protein-associated Splicing Factor) [112]. The phosphorylation of PSF promotes its cytoplasmic relocalization and impairs its binding to polypyrimidine RNA, leading to cell cycle arrest.

**Brk as a Therapeutic Target**

Brk expression in greater than 60% of breast tumor cases is significantly higher than the incidences of other common molecular alterations: p53 mutation (around 20%) or ErbB2 overexpression (18-25%) [143]. In addition to its status as a “prognostic marker” of breast cancer, there is growing evidence that Brk promotes the proliferation of carcinoma cells, suggesting that Brk is a potential target for the development of novel cancer therapies based on specifically or selectively interfering with its function. ErbB2 is a prominent therapeutic target in breast cancer and both small molecule inhibitors and

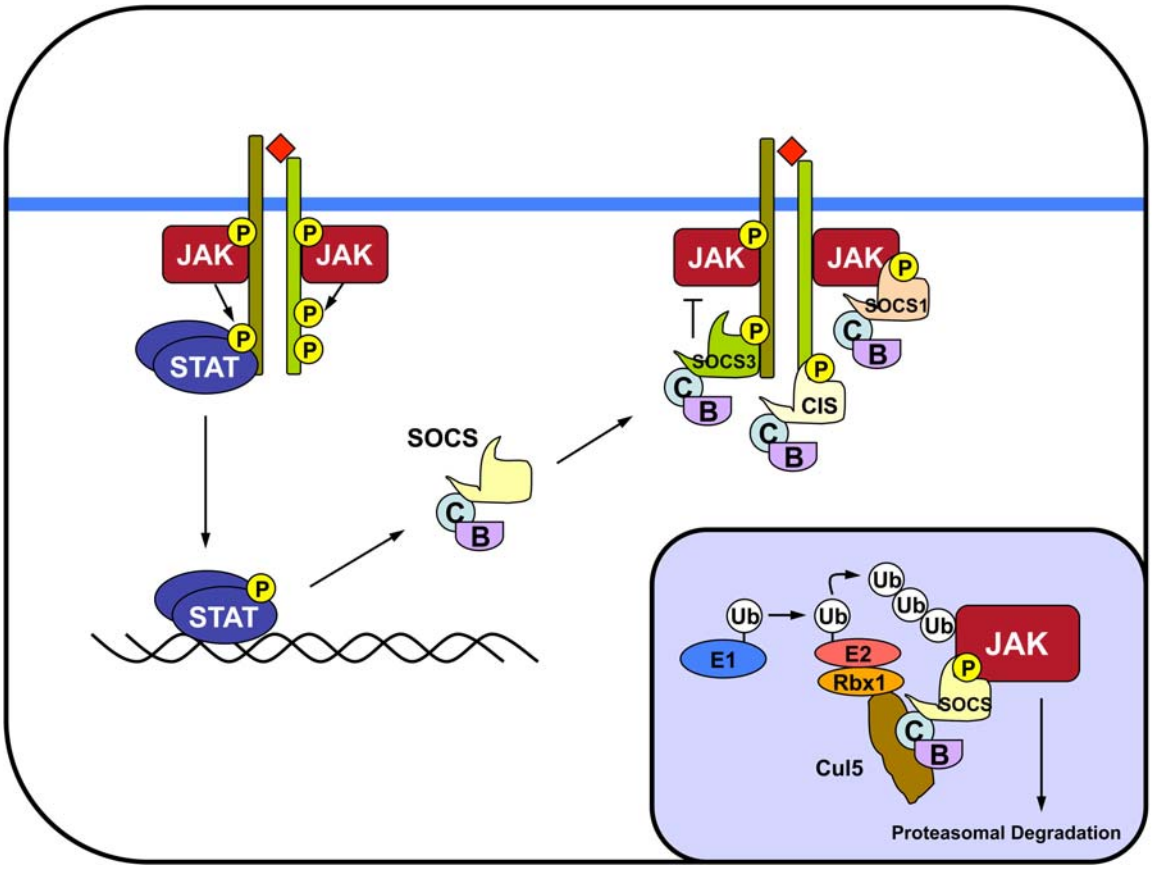
monoclonal antibodies have been developed for clinical trials. The correlation between Brk and ErbB2 overexpression in human invasive ductal breast tumors [98] and the finding that Brk cooperates with ErbB2 to promote cell growth in breast cancer [134] raise the possibility that targeting Brk along with ErbB2 may offer a significant therapeutic advantage. Brk-directed therapies also can target breast cancers that do not overexpress ErbB2 as well as a broad range of other cancers in which Brk is expressed.

Our previous studies have identified SOCS3 as an inhibitor of Brk, which blocks Brk-induced STAT3 phosphorylation and transcriptional activity [128]. The following section will discuss the structure and function of SOCS family proteins.

**Mechanism of SOCS Action**

Cytokine signaling is stringently controlled by a number of regulatory processes. Prolonged cytokine responses can be detrimental. Dysregulation of cytokine signaling is associated with many diseases, including several cancers, disorders in hematopoiesis and autoimmune diseases [144]. A key family involved in the negative regulation of cytokine signaling is the SOCS family.

The expression of SOCS proteins is induced by cytokine stimulation and STAT activation, and they serve to interfere with signaling from the inducing cytokine in a classic negative feedback loop. Upon cytokine binding, SOCS genes are rapidly induced in a STAT-dependent manner, and their protein products block further signaling by inactivating the JAK-STAT pathway, thus regulating the strength and duration of the cytokine responses (Figure 9). Different SOCS proteins inhibit signaling in different modes. SOCS1 directly binds to JAKs and inhibit cytokine receptor phosphorylation by JAKs and STAT activation [145]. SOCS3 binds to phosphotyrosine residues on receptor chains followed by inhibition of JAK kinase activity [146]. CIS and SOCS2 also bind to receptor phosphotyrosine residues and inhibit signaling by competing with STATs for phosphorylated docking sites on receptors [147]. In addition, SOCS proteins can inhibit the cytokine receptor signaling by ubiquitination and subsequent proteasomal degradation of JAKs and receptors. However, SOCS-mediated ubiquitination can also direct the internalization and lysosomal routing of cytokine receptors, such as the growth hormone (GH) and granulocyte-colony stimulating factor (G-CSF) receptors [148,149].




(Modified from Croker B, Semin Cell Dev Bio., 19:414) [150]

**Figure 9: SOCS proteins inhibit JAK-STAT signaling by different mechanisms.**

Cytokine binding to cell surface receptors results in activation of JAK-STAT pathway and induction of SOCS gene transcription in a STAT-dependent manner. The SOCS proteins then inhibit signaling either by direct inhibition of JAK kinase activity (SOCS1), binding to phosphotyrosine residues on receptor cytoplasmic tails, followed by inhibition of JAK activity (SOCS3), or by competition with STAT SH2 domains for specific receptor phosphotyrosine residues (CIS, SOCS2). An additional level of regulation is provided by an E3 ubiquitin ligase complex bound to the SOCS box, which ubiquitinates the associated proteins, targeting them for proteasomal degradation (inset).

**Structure of SOCS Proteins**

The SOCS family consists of eight proteins, SOCS1-7 and CIS. They share a similar three-part architecture, including a variable N-terminal region, a central SH2 domain and a conserved C-terminal domain known as the SOCS box (Figure 10). Only SOCS1 and SOCS3 possess a kinase inhibitory region (KIR) in the N-terminal domain. This 12 amino acid region is proposed to act as a pseudosubstrate. It mimics the activation loop found in kinases such as JAK2 and FGF receptor kinase, lodging in the catalytic cleft to prevent substrate access to the kinases [145,151]. This hypothesis is supported by KIR point mutations that abrogate SOCS action without affecting SH2 domain binding. Recently a SOCS1-KIR peptide and Tkip, a SOCS1 analogue, have been shown to interact directly with the JAK autophosphorylation loop and inhibit IFNγ signaling in primary cells [152,153].

Immediately following the KIR domain is the extended SH2 subdomain (ESS), a sequence preceding the SH2 domain, which is critical for phosphotyrosine binding. Crystal structures of SOCS2, SOCS3 and SOCS4 SH2 domain reveal that the ESS forms a 15-residue α-helix, which directly interacts with the phosphotyrosine-binding loop and determines its orientation [154-156]. For example, in SOCS3 the conserved Val38 and Leu41 form strong bonds with residues adjacent to the phosphotyrosine-binding loop, Ile70 and Phe80 (Figure 11A). The SH2 domain of SOCS proteins is responsible for binding to phosphotyrosine residues on the cytokine receptors and/or the JAKs. SOCS3 SH2 domain contains an unstructured PEST motif, which is not necessary for STAT inhibition, but affects SOCS3 stability [154].


**Figure 10: A linear diagram of SOCS3 domain structure.**

SOCS3 has a kinase inhibitory region (KIR), an extended SH2 subdomain (ESS), an SH2 domain, and a C-terminal SOCS box. The SH2 domain is interrupted by a 35 amino acid PEST motif.

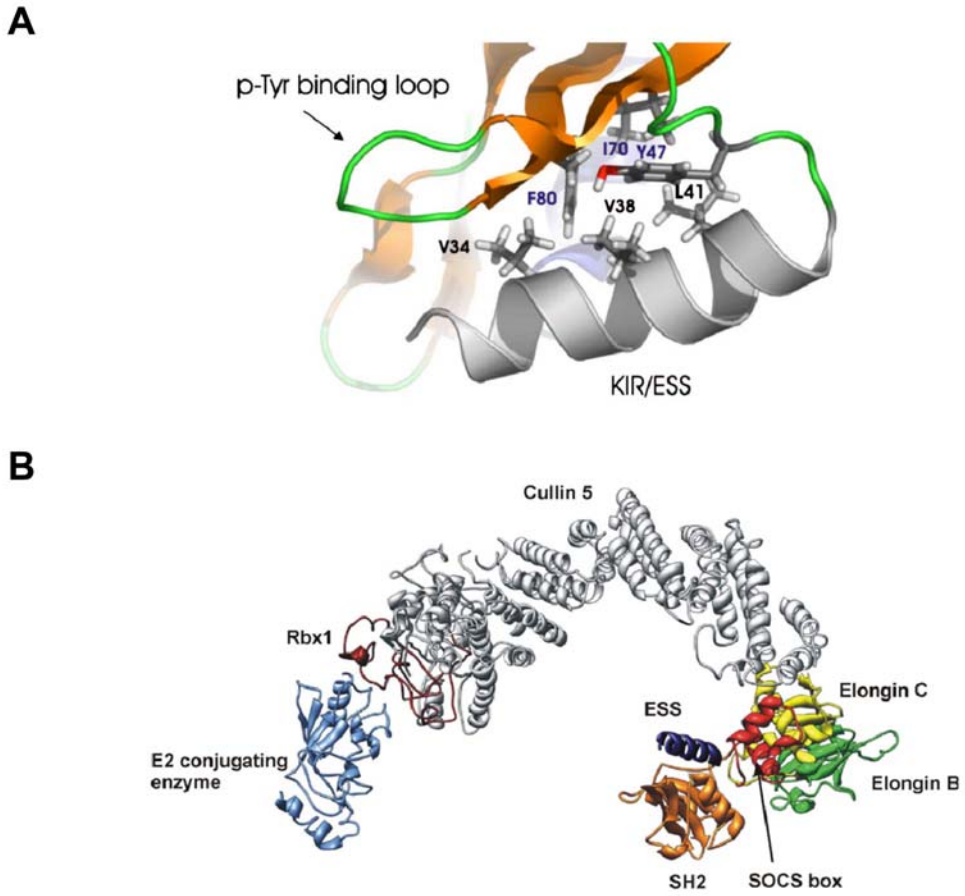



**Figure 11: Structures of ESS and SOCS box.**

(A) Residues in the SOCS3 ESS help to determine the geometry of the p-Tyr binding pocket. A view of SOCS3 shows that residues from the ESS, in particular Val34, Val38, and Leu41 (black labels), interact with Ile70 and Phe80 (blue labels) (Babon et al., 2006) [154].
(B) Homology model for the SOCS E3 complex. SOCS proteins bind to a substrate via their SH2 domain. The SOCS box interacts with Elongin C and B and additional recruitment of Cullin5 and Rbx1 completes the formation of an E3 ligase complex. Rbx1 binds an E2 ubiquitin-conjugating enzyme, allowing ubiquitin conjugation to the substrate (Piessevaux et al., 2008) [157].

The conserved C-terminal SOCS box has been shown to directly bind Elongin C and B and form Elongin C-Cullin-SOCS box (ECS)-type complexes that function as E3 ubiquitin ligases (Figure 11B). The SOCS box consists of three α-helices. The first helix mediates an interaction with Elongin C, while Elongin B stabilizes the complex making limited contacts with the loop region between helix 2 and 3 [155,156,158]. Elongin B binds Elongin C and this dimer acts as a linker that bridges the substrate recognized by the SOCS proteins to a Cullin5 scaffold protein. Cullin5 in turns recruits a RING-finger-containing protein Rbx1, thereby completing the assembly of the E3 ligase complex [159]. Therefore, SOCS proteins can target bound signaling molecules for proteasome-mediated degradation.

The SOCS proteins have been demonstrated to promote ubiquitination and degradation of a wide range of proteins. SOCS1 promotes the SOCS box-dependent ubiquitination and turnover of JAK2 and the TEL-JAK2 oncoprotein [160-162]. SOCS3 can target receptors for proteasomal degradation, such as CD33 and sialic acid-binding immunoglobulin-like lectin (Siglec) 7, a member of the Siglec receptor family [163,164]. The proteolytic activity of SOCS proteins is not restricted to the components of cytokine signaling, for instance, receptor complexes or associated JAKs, but also targets a variety of substrates, including the Toll-like receptor (TLR) adaptor Mal, the guanine nucleotide exchange factor VAV, the p65/RelA subunit of NF-κB, and the E7 protein of human papilloma viruses (HPV) in case of SOCS1 [165-168]. SOCS1 and SOCS3 can also promote degradation of insulin receptor substrate (IRS)-1 or IRS-2 and of focal adhesion kinase (FAK) [169,170]. An essential role for the SOCS box of SOCS1 and SOCS3 in the inhibition of cytokine action was also demonstrated *in vivo*. Transgenic mice

expressing a SOCS box deletion mutant of SOCS1 have an increased responsiveness to IFNγ and slowly develop a fatal inflammatory disease [171]. Transgenic mice with a truncated SOCS3 protein lacking SOCS box are hyperresponsive to G-CSF signaling and show altered response to inflammatory stimuli [172].

**The Complexities of SOCS Biology**

The importance of SOCS proteins in regulating cytokine responses has emerged from SOCS knockout mice. SOCS1 knockout mice die within 3 weeks of birth as a result of severe lymphopenia, liver damage and macrophage infiltration of major organs [173,174]. This was shown to primarily result from uncontrolled IFNγ signaling, as crossing SOCS1$^{-/-}$ and IFNγ$^{-/-}$ mice rescues the SOCS1$^{-/-}$ phenotype, although these mice eventually exhibit a range of inflammatory diseases [175]. SOCS1 has also been shown to play a role in regulating TLR signaling [176]. SOCS1 is rapidly induced by lipopolysaccharide (LPS) and negatively regulates LPS signaling. The SOCS1$^{-/-}$IFNγ$^{-/-}$ mice are more sensitive to LPS-induced lethal effects.

SOCS2 knockout mice develop gigantism characterized by increased body weight, bone length and organ size [177,178]. The phenotype of SOCS2 knockout mice resembles that of growth hormone (GH)-transgenic mice, suggesting that SOCS2 is a physiological negative regulator of the GH-STAT5b signaling pathway. Paradoxically, SOCS2 transgenic mice also display the same phenotype as SOCS2 knockout mice [179]. This is likely to be a consequence of the ability of SOCS2 to inhibit the expression of other SOCS proteins that normally block GH signaling [180].

SOCS3 has been shown to negatively regulate phosphorylation of STAT3 stimulated by IL-6 family cytokines that bind to the common gp130 receptor subunits [181]. SOCS3 knockout mice die during the embryonic stage of development due to placental defects [182,183]. This is thought to be a consequence of enhanced LIF activation as inactivation of the LIF receptor rescues the SOCS3 knockout phenotype. The loss of SOCS3 alters gp130 signaling by prolonging IL-6 activation of STAT3, contributing to the progression of chronic inflammatory diseases, such as arthritis, Crohn's disease and inflammatory bowel disease [184,185]. These findings suggest that SOCS3 could suppress inflammatory reactions in which IL-6-related cytokines play important progressive roles.

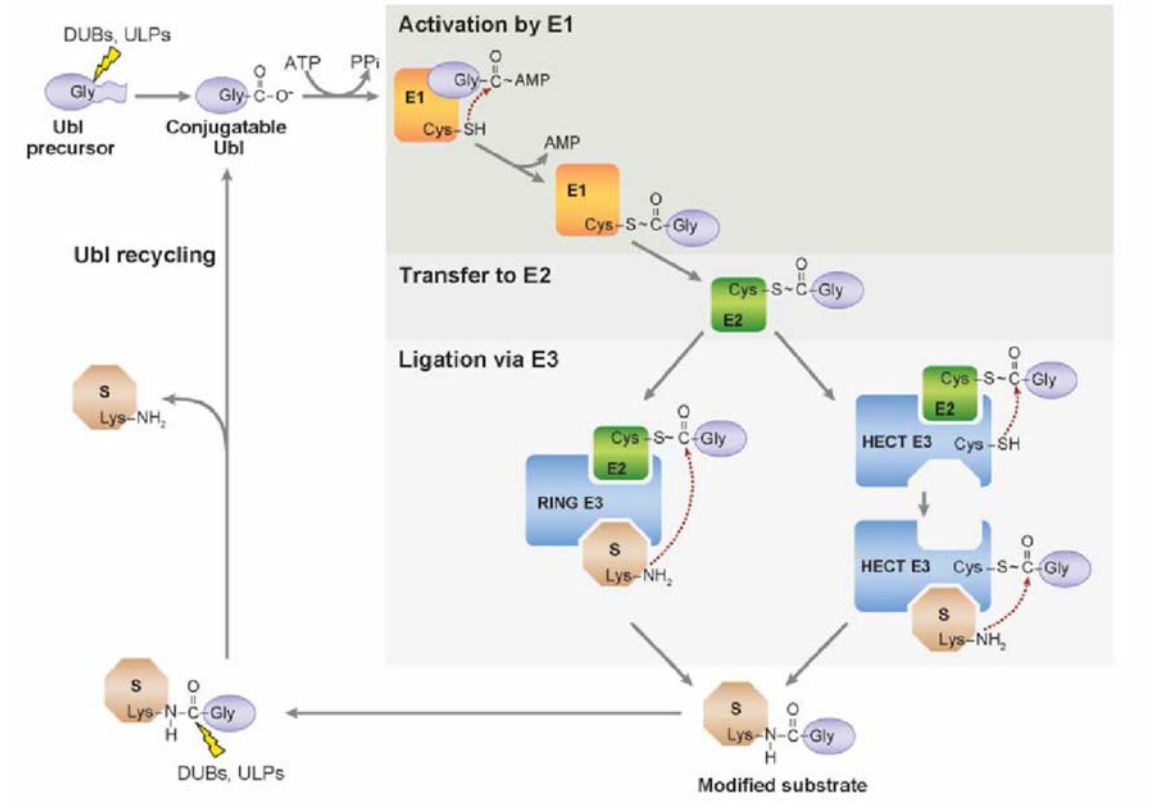
Dysregulation of the JAK-STAT signaling pathway has also been implicated in malignant progression. Many human cancers including hepatocellular carcinoma (HCC), non-small-cell lung cancer, acute myeloid leukemia, head and neck squamous cell carcinoma (HNSCC), cholangiocarcinoma, and Barrett's adenocarcinoma, demonstrate constitutive STAT phosphorylation [186-191]. This is frequently accompanied by transcriptional silencing of one or more SOCS genes due to hypermethylation. These observations strongly suggest that SOCS proteins may be tumor suppressors. Consistent with this notion, experimental overexpression of SOCS proteins in cancer cells reduces STAT activity, inhibits proliferation and induces apoptosis of these cells [187,190,191]. In addition, some mutations and deletions in SOCS proteins have been found in cancer cells. For example, a biallelic mutation in SOCS1 resulting in a defective SOCS box was observed in the primary mediastinal large B-cell lymphoma cell line, MedB-1 [192]. It is thought that impaired SOCS1-mediated JAK2 degradation results in sustained JAK2

activation and low turnover of JAK2 protein leads to lymphomas. While originally only considered as classical negative regulators of cytokine signaling, SOCS proteins are now widely regarded as important tumor suppressors and cancer therapies with SOCS proteins will have promise.

**Ubiquitination**

Ubiquitin, a 76-residue polypeptide, is highly conserved among eukaryotes but is absent from bacteria and archaea. Ubiquitin and its kin (ubiquitin-like proteins, or Ubls) share similarity, not only in structure, but also in the way of action [193,194]. The C terminus of this class of proteins (glycine residue G76) can be ligated to the ε-amino group of a lysine residue or α-amino group of the N-terminal amino acid in a substrate protein. Following ubiquitin conjugation to the substrate as a polymer, the polyubiquitin chain is then recognized by specific receptors within the proteasome or by adaptor proteins that subsequently bind the proteasome for degradation [195].

Although the mechanism of different Ubl conjugation reactions may vary in detail, a common theme has emerged that these reactions are all carried out with the aid of a set of enzymes that usually include an E1-activating enzyme, a E2-conjugating enzyme and a E3 ligase (Figure 12). Ubiquitin and most of the Ubls are synthesized as inactive precursors that are processed by either deubiquitinating enzymes (DUBs) or Ubl-specific proteases (ULPs) to expose a C-terminal glycine in the mature Ubl. The processed Ubl is now conjugation competent and can be activated with ATP by E1. The E1 adenylates the Ubl C-terminal carboxyl group, forming a high-energy Ubl-AMP intermediate. This intermediate is attacked and covalently bound by the catalytic cysteine




(Adapted from Kerscher O, Annu. Rev. Cell Dev. Biol., 22:159) [194]

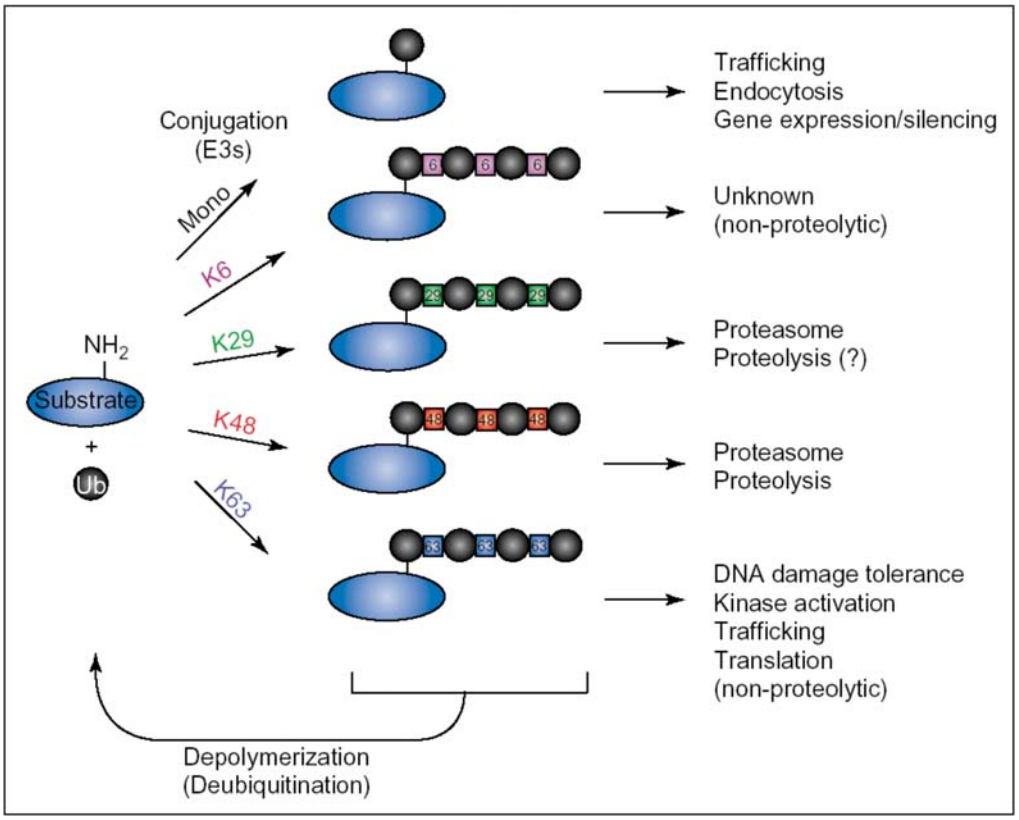
**Figure 12: A generalized Ubl-conjugation pathway.**

The C-terminal carboxyl group of Ubls is covalently ligated to the active cysteine residue of the E1 enzyme. Subsequently, the Ubls are transferred to the active cysteine residue in an E2 enzyme, which in turn relays the Ubl molecules to substrates in the presence of an E3 ligase. The DUBs and ULPs can remove Ubls from substrates.

of the E1, creating a thioester linkage and releasing AMP. From the E1, the modifier is transferred to the active-site cysteine of the E2 via a transthiolation reaction. Finally, the modifier is conjugated to its substrate with the aid of an E3 ligase, resulting in the covalent isopeptide linkage of the modifier's C-terminus to the ε-amino group of a lysine in the substrate.

Multiply-modified proteins can result either when ubiquitin molecules are individually conjugated to different residues on a substrate or when they are attached to each other to form a chain that is conjugated to the substrate at a single site or several sites. Ubiquitin molecules in these chains are linked to one another in the same way in which they are usually linked to substrate proteins. In particular, the C-terminus of the more distal ubiquitin molecule is attached to the ε-amino group of a lysine residue in the previously attached ubiquitin molecule.

Ubiquitin has seven lysine residues, all of which are potentially involved in chain formation, although ubiquitin-Lys48 and -Lys63 are the best-characterized residues involved in polyubiquitination [196]. It has been well known that polyubiquitin chains bearing different linkages convey distinct structural and functional information (Figure 13) [197]. A well-accepted doctrine in the field is that ubiquitin chains linked by Lys48 target a conjugated substrate to the proteasome for degradation. In contrast, Lys63-linked chains represent a distinct linkage topology and perform non-proteolytic functions, including DNA damage repair, cellular signaling, intracellular trafficking and ribosomal biogenesis. Functionally, both types of polyubiquitin chain attachment can be distinguished from monoubiquitination, which is involved in processes such as endocytosis and transcriptional regulation.




(Adapted from Pickart C, Curr. Opin. Chem. Biol., 8:610) [197]

**Figure 13: Influence of signal structure on the consequences of ubiquitination.**

Polyubiquitin chains with different linkages might represent functionally distinct signals. The differently colored squares denote linkage of the corresponding polyubiquitin chains through different ubiquitin lysine residues.

# Chapter 2

## Materials and Methods

### Cell culture and reagents

HeLa and COS1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum. Hep3B cells were cultured in Minimum essential medium with 10% fetal bovine serum. Recombinant human IL-6 (Invitrogen) was used at 20ng/ml and human IL-6 soluble receptor (R&D Systems) was used at 10ng/ml. Recombinant human IFNα (gift from Hoffman-LaRoche, Nutley, NJ) was used at 1000U/ml. Doxycycline (Sigma) was used at 2μg/ml. MG132 (Sigma) was resolved in DMSO and used at 5μM. DNA transfections were performed with *Trans*IT-LT1 reagent (Mirus) according to manufacturer's instructions. Anti-phosphotyrosine STAT3 (B7, Santa Cruz), anti-STAT3 (H190, Santa Cruz), anti-Brk (C18, Santa Cruz), anti-c-Myc (9E10, Santa Cruz), anti-GFP (Roche) and anti-4G10 (Upstate Biotech) were used at a 1:1,000 dilution for Western Blot. Anti-V5 (Invitrogen), anti-Flag (M2, Sigma) and anti-αtubulin (Sigma) were used at a 1:5,000 dilution for Western Blot.

### Site-directed mutagenesis

Site-directed mutagenesis was performed according to the instruction manual for the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The method is performed using high-fidelity *PfuTurbo* DNA polymerase and a temperature cycler. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) plasmid as PCR

template and two complementary synthetic oligonucleotides containing the desired mutation at the central region as primers. A 50μl reaction contains 50ng template plasmid DNA, 125ng of each primer, 0.1mM dNTP, *PfuTurbo* buffer and 1 unit *PfuTurbo*. The mixture was subjected to 20 thermal cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 16 minutes. The reaction was then incubated with 10 units of Dpn I endonuclease at 37°C for 4~6 hours. Dpn I is specific for methylated DNA and is used to digest the parental plasmid template. The resulting product was transformed into TAM1 competent E. coli (Active Motif) and plasmids from single colonies were prepared and screened for the desired mutations by DNA sequencing.

**Plasmid vectors**

pEGFP-C2 was purchased from Clontech and pcDNA3 was purchased from Invitrogen. pcDNA4/TO/*myc*-His C (tetracycline-inducible expression vector, Invitrogen) and pcDNA6/TR (tetracycline repressor, Invitrogen) were gifts from Molecular Cloning Facility (Stony Brook University). pGEX-KG vector for bacterial expression of proteins was gift from Dr. Patrick Hearing (Stony Brook University). p3xFLAG-CMV-7.1 (Sigma), CMV/Brk and CMV/Brk K219M plasmids were gifts from Dr. W. Todd Miller (Stony Brook University). STAT3-GFP and Brk-V5 plasmids were generated by Ling Liu Ph.D., a former graduate student of Dr. Nancy Reich's laboratory (Stony Brook University) and have been described previously [128,198]. Flag-SOCS3 plasmid was gift from Dr. Douglas J. Hilton (The Walter and Eliza Hall Institute for Medical Research, Australia) and has been described previously [83]. pEF-Flag-I vector was generated by excising SOCS3 cDNA from Mlu I sites of Flag-SOCS3 and self-ligating the empty

vector. The His-tagged octameric ubiquitin plasmid (His-$Ub_8$) was gift from Dr. Michael Hayman (Stony Brook University) and has been described previously [199].

**Plasmid constructs**

cDNA encoding Brk or Brk K219M was generated by polymerase chain reaction (PCR) with primers containing EcoR I and BamH I sites. The PCR products were cloned into the pEGFP-C2 vector to generate GFP-Brk WT or K219M verified by sequencing. SOCS3 deletion mutants were generated by PCR and cloned into the pEF-Flag-I vector within Mlu I sites. Point mutants of SOCS3 were generated by the above site-directed mutagenesis protocol and confirmed by sequencing. SOCS3 cDNA was cloned into the pGEX-KG vector in frame with GST for bacterial expression and purification. The 3xFLAG-Brk ΔSH3 and ΔSH2 were generated by subcloning Brk ΔSH3 and ΔSH2 (gifts from Dr. W. Todd Miller, Stony Brook University) [113] into the p3xFLAG-CMV-7.1 vector using "cut and paste" method. The 3xFLAG-Brk ΔTK were generated by cloning Brk aa1-190 into the p3xFLAG-CMV-7.1 vector between EcoR I and BamH I sites. Brk Y251F and Δ249-256 in GFP-Brk or Brk-V5 plasmid were generated by site-directed mutagenesis. The TO/Brk-myc (tetracycline-inducible Brk) was generated by PCR and cloned into the pcDNA4/TO/*myc*-His C vector between EcoR I and Not I sites and His tag was then removed by site-directed mutagenesis. The His-tagged single-copy ubiquitin plasmid (His-Ub) was generated by PCR with the template of His-$Ub_8$ and cloned into the pcDNA3 vector. The His-Ub K0 and K0R63K plasmids were generated by PCR with the templates of HA-Ub K0 and K0R63K as gifts from Dr. Bar-Sagi (New York

University) [200] and cloned into the pcDNA3 vector. The His-Ub K0R48K was generated by site-directed mutagenesis in the template of His-Ub K0.

**DNA transfection into mammalian cells**

Cells were plated 18-24 hours before transfection so that they would reach about 50%-70% confluent at the time of transfection. All transient transfection was performed with *Trans*IT-LT1 (Mirus) following manufacturer's instructions. In brief, desired amount of *Trans*IT-LT1 was pipetted into 50μl (for cells in a well of 12-well plate), 100μl (for cells in a well of 6-well plate or one 60mm tissue culture dish) or 200μl (for cells on one 100mm or 150mm dish) serum-free DMEM, mixed completely and incubated at RT for 5 minutes. Desired amount of DNA (0.5μg/μl *Trans*IT-LT1) was then pipetted in, mixed completely and incubated at RT for 15-30 minutes. The *Trans*IT-LT1:DNA complex in medium was then dropwise added to cells. The amount of DNA and *Trans*IT-LT1 to use in the transfection was as follows:

| Type of tissue culture dishes | Total DNA | Volume of *Trans*IT |
|---|---|---|
| well / 12-well plate | 0.25μg | 0.5μl |
| 35mm dish or well / 6-well plate | 0.5μg | 1μl |
| 60mm dish | 1μg | 2μl |
| 100mm dish | 2μg | 4μl |
| 150mm dish | 4μg | 8μl |

**Western blot analysis**

Cells were lysed in 1% NP40 lysis buffer (50mM Tris pH7.5, 150mM NaCl, 5% glycerol, 5mM EDTA and 1% NP40) with protease inhibitors (1mM PMSF, 1mM DTT and 1X protease inhibitor cocktail, Sigma) and optional phosphatase inhibitors (1mM sodium vanadate, 10mM NaF and 1mM glycerophosphate) at 4°C for 30 minutes. Cell lysates were centrifuged at 14,000rpm, 4°C for 20 minutes to pellet debris, and the supernatants were mixed with SDS sample buffer and boiled for 5 minutes. The lysates were resolved on 8%-10% polyacrylamide gels and proteins were transferred to Immobilon-P membranes (Millipore). After transfer the membranes were incubated in blocking solution at RT for 1 hour with gentle agitation. Depending on different primary antibodies different blocking solutions were used. For anti-STAT3 phosphotyrosine antibody 3%BSA in TBS-Tween buffer (25mM Tris pH7.5, 155mM NaCl, 0.05% Tween 20) was used and 5% non-fat milk in TBS-Tween was used for all other antibodies. The membranes were incubated with primary antibodies diluted in blocking solution at 4°C overnight. Membranes were then washed three times with TBS-Tween buffer for 10 minutes each time, followed by incubation with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase 1:5,000 diluted in blocking solutions at RT for 1-2 hours with gentle agitation. The membranes were washed three times and subjected to enhance chemiluminescence for detection of specific proteins. Membranes sometimes were stripped and re-probed with different primary antibodies. To strip antibodies off the membrane stripping buffer (62.5mM Tris pH6.8, 2% SDS, 100mM β-mercaptoethanol) was incubated with the membrane at 50°C for 30 minutes. The membrane was then washed in TBS-Tween for 3-4 times and blocked and probed as above.

**Luciferase reporter assay**

Cells were seeded in 12-well plates ($1\times10^5$cells/well) and transfected at a 1:2 DNA:*Trans*IT-LT1 ratio. All cells were transfected in duplicates with protein expression plasmids, GAS luciferase reporter plasmid and a promoterless Renilla luciferase plasmid (pRL-null) for transfection control. Transfected cells were serum-starved for 24 hours before lysis. Preparation of cell lysates and measurement of light units were performed using the Dual-Luciferase Reporter Assay System (Promega) following manufacturer's instructions. Relative Light Units were calculated as (firefly luciferase activity/Renilla luciferase activity)×1000.

**Co-immunoprecipitation**

COS1 cells seeded on 100mm tissue culture dishes were co-transfected with equal amount of GFP-tagged Brk or Flag-tagged SOCS3 expression plasmids. Following 24-hour transfection cells were harvested and resuspended in Medium salt NP40 buffer (50mM Tris pH 8.0, 280mM NaCl, 5mM EDTA, 0.5% NP40, 1mM PMSF, 1X protease inhibitor cocktail, 1mM sodium vanadate and 10mM NaF). After incubation at 4°C for 30 minutes with gentle agitation cell debris were removed by centrifugation. 400μg of total proteins were used in each immunoprecipitation reaction and incubated with 1μg anti-SOCS3 (H103, Santa Cruz) antibody or control rabbit IgG (Santa Cruz) at 4°C for 4 hours with gentle agitation. 20μl protein G agarose beads (Invitrogen) were added to each reaction and the incubation was continued overnight. Immunocomplexes collected on protein G beads were eluted by SDS loading dye and analyzed by SDS-PAGE and Western blot.

**Bacterial expression and purification of GST fusion proteins**

Plasmid encoding GST-SOCS3 was transformed into BL21 codon plus (Stratagene) E. coli and a single colony was inoculated into 100ml LB with ampicillin and chloramphenicol and grew at 37°C overnight. 40ml overnight culture was then added into 800ml fresh LB with ampicillin and chloramphenicol and grew at 37°C until the absorbance at 600nm reached 0.6 when 0.2mM IPTG was added to induce the expression of protein. The proteins were induced at 23°C for 16-20 hours. Cells were then harvested and frozen at -80°C until ready to purify. For purification, cells were resuspended in cold PBS containing 5mM EDTA, 1% TritonX-100, 1mM PMSF and 1mg/ml lysozyme, incubated on ice for 30 minutes and French-pressed at 600psi twice to rupture the cells. Cell debris were then removed by centrifugation at 18,000rpm for 30 minutes at 4°C. The GST-SOCS3 proteins were purified from the cleared lysates by incubating with pre-equilibrated 1.5ml glutathione agarose beads (Sigma) at 4°C with gentle agitation for 3 hours and the beads were subsequently washed three times with 10ml cold lysis buffer without lysozyme. Bound proteins were eluted with 20mM reduced glutathione in 50mM Tris pH~7.0 three times. The eluates were dialyzed twice (overnight, then 3 hours) against 500ml dialysis buffer (20mM Hepes pH7.9, 50mM NaCl, 1mM EDTA, 15% glycerol, 0.2mM PMSF and 1mM β-mercaptoethanol) to remove free glutathione. Purified proteins were aliquoted into 200μg vials, frozen in liquid nitrogen and stored in -80°C.

**GST pull-down assay**

15 μg of purified recombinant GST-SOCS3 protein or GST protein were used in each pull-down reaction. Proteins were immobilized on glutathione agarose beads pre-blocked overnight with 3%BSA/PBS and then equilibrated in binding buffer. GFP-Brk wide-type or deletion mutants were expressed in COS1 cells. Cells were lysed in RIPA buffer (50mM Tris pH7.6, 150mM NaCl, 5mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 1X protease inhibitor cocktail, 1mM sodium vanadate and 10mM NaF) and cytosol was collected following centrifugation. Lysates containing 400μg total protein were then incubated with GST-SOCS3 proteins immobilized on GST beads at 4°C with gentle agitation for 3 hours. The beads were washed four times, and bound proteins were eluted and detected by SDS-PAGE and Western blot with specific antibodies.

**Reverse Transcription PCR**

RNA extraction was performed with SurePrep TrueTotal RNA Purification Kit (Fisher) and cDNA was synthesized with M-MLV Reverse Transcriptase (Promega). PCRs were performed with *Taq* DNA polymerase (Invitrogen) for 25 cycles of a 45-second 95°C, a 30-second 55°C and a 1-minute 72°C incubation with the following primers at their optimal condition:

SOCS3 forward 5′-CAGCTGGTGGTGAACGCAGTG

SOCS3 reverse 5′-GATGTAATAGGCTCTTCTGGG

GAPDH forward 5′-GGAGCCAAAAGGGTCATCATCTC

GAPDH reverse 5′-AGTGGGTGTCGCTGTTGAGTC

**His-ubiquitination assay**

Cells were co-transfected with His-tagged ubiquitin plasmid, TO/Brk-myc and pcDNA6/TR with or without Flag-SOCS3 or SOCS3 Δbox (aa1-185) for 24 hours and then treated with doxycycline with or without MG132 for 24 hours. Cells were harvested and resuspended in Buffer A (6M Guanidine-HCl, 0.1M $NaH_2PO_4$, 10mM imidazole, pH adjusted to 8.0 with NaOH). Cell suspension was viscous and subjected to sonication using microtip (maximum microtip limit; 8 pulses of 1 second each). Nickel charged resins (Ni-NTA agarose beads, Qiagen) were pre-equilibrated in Buffer A and incubated with cell lysates at RT with gentle agitation for 3 hours to capture histidine-tagged proteins. The Nickel beads were then washed in Buffer A twice, in Buffer A/TI (1 volume Buffer A mixed with 3 volumes TI) twice and in Buffer TI (25mM Tris pH6.8 and 20mM imidazole) once. Bound proteins were eluted by SDS loading dye and myc-tagged Brk was detected by SDS-PAGE and Western blot.

# Chapter 3

## Results

### SOCS3 inhibits Brk phosphorylation of STAT3

STAT3 is activated conventionally by JAKs in response to cytokine signaling, but it is also a target of oncogenic tyrosine kinases such as Brk [128]. STAT3 tyrosine phosphorylation has been detected in breast tumor cell lines expressing Brk, and exogenous expression of Brk induces the tyrosine phosphorylation and transcriptional activation of STAT3. The proteins of the SOCS family are best characterized as negative regulators of cytokine signaling. SOCS3 is the primary member induced by STAT3 in response to IL-6 signaling, and an increasing number of reports have indicated a tumor suppressor role of SOCS proteins in the treatment of cancer [187,191]. For these reasons I evaluated the potential effect of SOCS3 on the enzymatic activity of Brk.

To evaluate the effect of SOCS3 expression on STAT3 phosphorylation induced by Brk, Western blot was performed by using a specific STAT3 phosphotyrosine antibody (Figure 14). COS1 cells were co-transfected with plasmids encoding STAT3-GFP, Brk, with or without SOCS3, and serum-starved for 24 hours to minimize the potential STAT3 phosphorylation by growth factors in the media. Cell lysates were separated by SDS-PAGE and STAT3 phosphorylation was detected by Western Blot with phospho-STAT3 antibody. The results indicated that SOCS3 expression effectively inhibited Brk-induced STAT3 phosphorylation (lanes 5, 6). A comparative positive control was included to demonstrate the effect of SOCS3 on STAT3 phosphorylation in

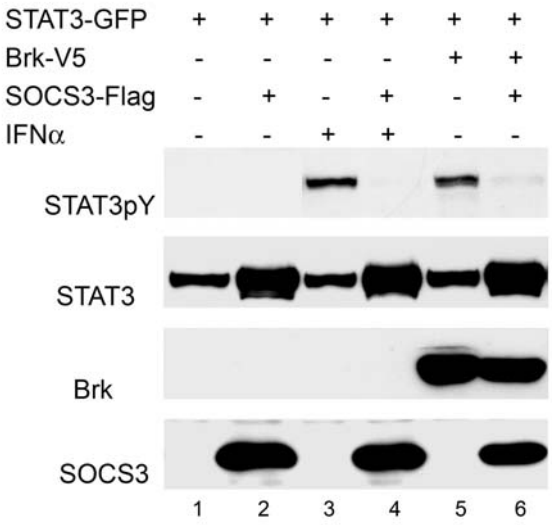



**Figure 14: SOCS3 expression inhibits Brk-induced STAT3 phosphorylation.**

COS1 cells were co-transfected with STAT3-GFP, Brk, with or without SOCS3 and serum-starved for 24 hours. Control cells were transfected with STAT3-GFP and SOCS3 and serum-starved for 24 hours followed by 30 min IFNα treatment. Lysates were prepared and analyzed by Western Blot with individual specific STAT3 phosphotyrosine, GFP (to detect total STAT3), V5 (to detect Brk) and Flag (to detect SOCS3) antibodies on the same blotting membrane.

cytokine signaling (lanes 3, 4). Cells were co-transfected with genes encoding STAT3-GFP, with or without SOCS3, and serum-starved for 24 hours followed by a 30-minute IFNα treatment. It clearly showed that SOCS3 inhibited STAT3 phosphorylation stimulated by IFNα in the JAK-dependent pathway.

The effect of SOCS3 on STAT-dependent gene expression was also evaluated using luciferase reporter assays (Figure 15). Tyrosine phosphorylated STAT dimers bind to a consensus DNA sequence designated γ-interferon Activation Sequence (GAS) that was initially identified in IFN responsive genes [38]. Since Brk specifically phosphorylates STAT3 but not other STAT members [128], the induced gene expression in response to Brk is only attributed to STAT3 activation. COS1 cells were transfected with a luciferase reporter gene regulated by a GAS element, Brk, with or without SOCS3 and lysed to measure luciferase activity. Consistent with the suppression of tyrosine phosphorylation, expression of SOCS3 completely abolished GAS-luciferase gene induction in response to Brk or IFNα, indicating that SOCS3 inhibited Brk or IFNα induced transcription of the STAT3-dependent genes.

**SOCS3 gene is induced in response to Brk**

SOCS3 is a target gene of STAT3 and is rapidly induced upon IL-6 binding to the IL-6 receptors. Studies have shown that STAT3 activates the transcription of SOCS3 mRNA by associating with the 5' promoter region of the SOCS3 gene [201]. Since Brk activates STAT3, I examined whether the SOCS3 gene is induced by Brk expression. The studies on the kinetics of SOCS3 mRNA in response to IL-6 showed that SOCS3 mRNA was induced by IL-6 very rapidly, with maximal levels detected 30 minutes and declined

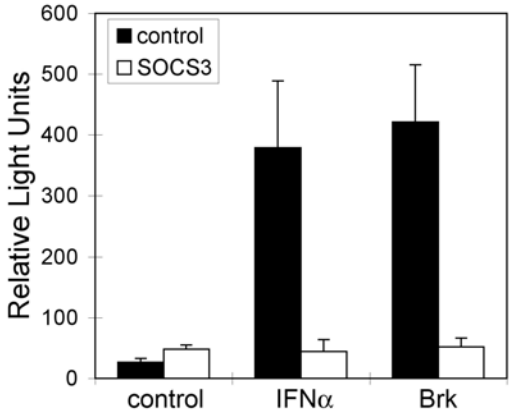



**Figure 15: SOCS3 suppresses STAT3-dependent gene expression induced by Brk.**

COS1 cells were co-tranfected with a GAS site-driven reporter construct, Brk, with or without SOCS3 and serum-starved for 36 hours. Control cells were transfected with the reporter construct with or without SOCS3, and serum-starved for 24 hours followed by IFNα treatment for 12 hours. Lysates were prepared and used to measure luciferase activity. The presented data represent two independent experiments.

60% by 1 hour [202]. For this reason I established a tetracycline-inducible Brk expression system to perform the time-course analysis of SOCS3 gene induction. Hep3B cells were transfected with plasmids encoding tetracycline-inducible Brk and tetracycline repressor and treated with doxycycline to induce Brk expression. Cells were then harvested at 4, 6, 8, 12 and 24 hours after doxycycline treatment. RNA was extracted from the cells, reverse transcribed into cDNA, and SOCS3 mRNA level was detected by RT-PCR by using primers specific to SOCS3 (Figure 16). GAPDH primers were used to standardize the amount of cDNA used in each reaction. A small portion of cells were lysed and subjected to SDS-PAGE and Western blot to evaluate the levels of STAT3 phosphorylation and Brk expression (Figure 17). The results indicated that SOCS3 mRNA was induced by Brk expression with a peak at 6 hour and gradually declined to near basal level after 24 hours. SOCS3 mRNA was also strongly induced by IL-6 after a 30-minute stimulation. However, compared to the positive control of IL-6 treatment, the magnitude of SOCS3 mRNA induced by Brk was greatly reduced (Figure 16). This can be explained by the facts that IL-6 stimulates all cells whereas Brk transiently transfects small percentage of cells. Western blotting showed that Brk was not expressed in the absence of doxycycline. Both the expression of Brk and STAT3 phosphorylation were detected after 4-hour doxycycline treatment, earlier than the induction of SOCS3 gene. Brk maintained at a high level utill 24 hours and phosphorylated STAT3 increased gradually in the presence of Brk (Figure 17). Equal levels of total STAT3 were verified by reprobing the membrane with a STAT3 antibody. Collectively, these results demonstrated that SOCS3 gene is induced in response to Brk.

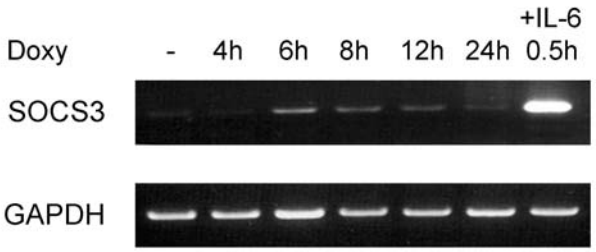



**Figure 16: SOCS3 mRNA is induced by Brk expression.**

Hep3B cells were transfected with tetracycline-inducible Brk and tetracycline repressor at ratio of 1:6 for 24 hours and treated with doxycycline (Doxy) for 4, 6, 8, 12 and 24 hours. Control cells were serum-starved for 24 hours followed by 30-minute IL-6 treatment. Total RNA was prepared and SOCS3 mRNA was detected by RT-PCR using specific primers. GAPDH mRNA was amplified as internal control.

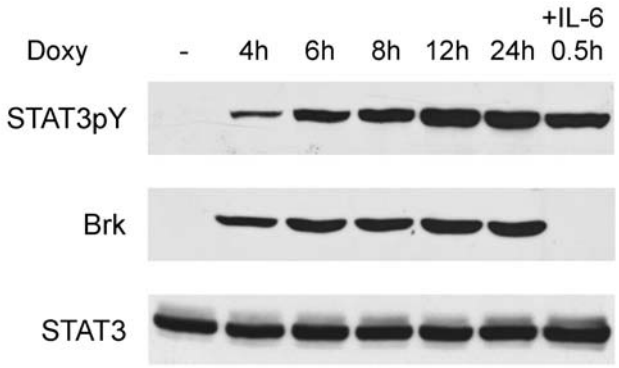



**Figure 17: Doxycycline induces Brk expression and STAT3 phosphorylation.**

Hep3B cells were transfected with tetracycline-inducible Brk and tetracycline repressor at ratio of 1:6 for 24 hours and treated with doxycycline (Doxy) for 4, 6, 8, 12 and 24 hours. Control cells were serum-starved for 24 hours followed by 30-minute IL-6 treatment. Cell lysates were prepared and analyzed by SDS-PAGE and Western blot with specific STAT3 phosphotyrosine, c-Myc (to detect Brk) and STAT3 antibodies.

**The KIR domain in SOCS3 is required for inhibition of Brk**

SOCS3 consists of a small N-terminal domain, kinase inhibitory region (KIR), extended SH2 subdomain (ESS), SH2 domain, and C-terminal SOCS box (Figure 18A). To determine which domain in SOCS3 is required for the inhibition of Brk kinase activity, I generated several SOCS3 deletion mutants and tested their abilities to inhibit STAT3 phosphorylation induced by Brk. COS1 cells were transfected with STAT3-GFP and Brk, with or without co-transfected SOCS3 full-length and deletion mutants. Proteins from cell lysates were separated on a SDS-PAGE and STAT3 phosphorylation was analyzed by Western blot using antibodies specific to STAT3 phosphotyrosine. The SOCS3 deletion mutants amino acid 34-225 and 46-225 both of which lack KIR domain were found unable to suppress Brk-induced STAT3 phosphorylation, suggesting a critical role of KIR domain in the inhibition of Brk kinase activity (Figure 18B). However, similar to the full-length SOCS3, the SOCS3 Δbox (amino acid 1-185) expression effectively inhibited Brk phosphorylation of STAT3, indicating that the SOCS box is not essential for Brk inhibition.

Among the SOCS family members, only SOCS1 and SOCS3 possess the KIR domain in which several amino acids are conserved, including Leucine 22 and Phenylalanine 25 (Figure 19A). The SOCS3 KIR substitution point mutants, such as L22D and F25A, have been reported to abrogate the ability of SOCS3 to inhibit cytokine signaling [151]. Therefore, I generated SOCS3 KIR single mutants L22D and F25A, and double mutant L22D/F25A (LF) and tested whether these point mutants could inhibit Brk phosphorylation of STAT3. As shown in Figure 19B, both SOCS3 L22D and F25A partially blocked STAT3 phosphorylation by Brk, whereas the double mutant LF almost

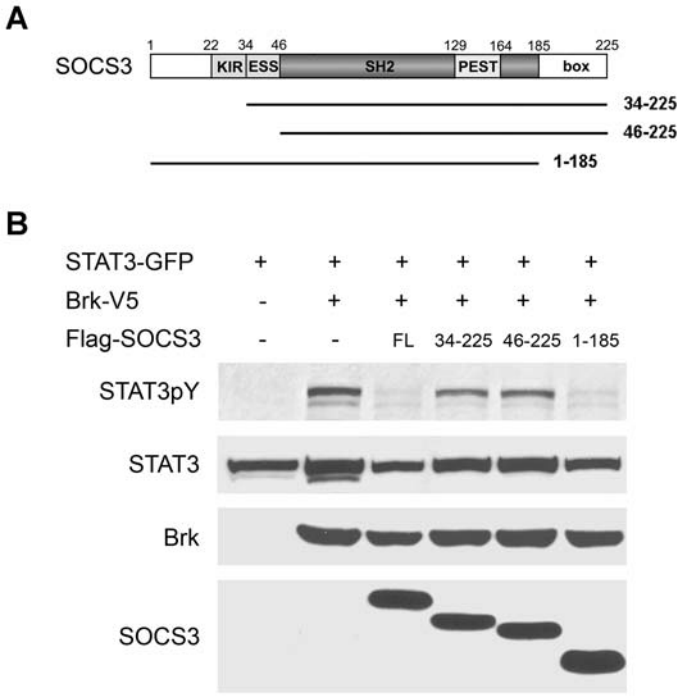



**Figure 18: Requirement of SOCS3 KIR domain for Brk inhibition.**

(A) Linear diagram of SOCS3 deletion mutants.
(B) COS1 cells were co-transfected with STAT3-GFP, Brk-V5 with or without Flag-SOCS3 full-length (FL) or SOCS3 deletion mutants and serum-starved for 24 hours. Cell lysates were subjected to SDS-PAGE and Western blot with individual specific STAT3 phosphotyrosine, GFP (to detect total STAT3), V5 (to detect Brk) and Flag (to detect SOCS3 and deletion mutants) antibodies.

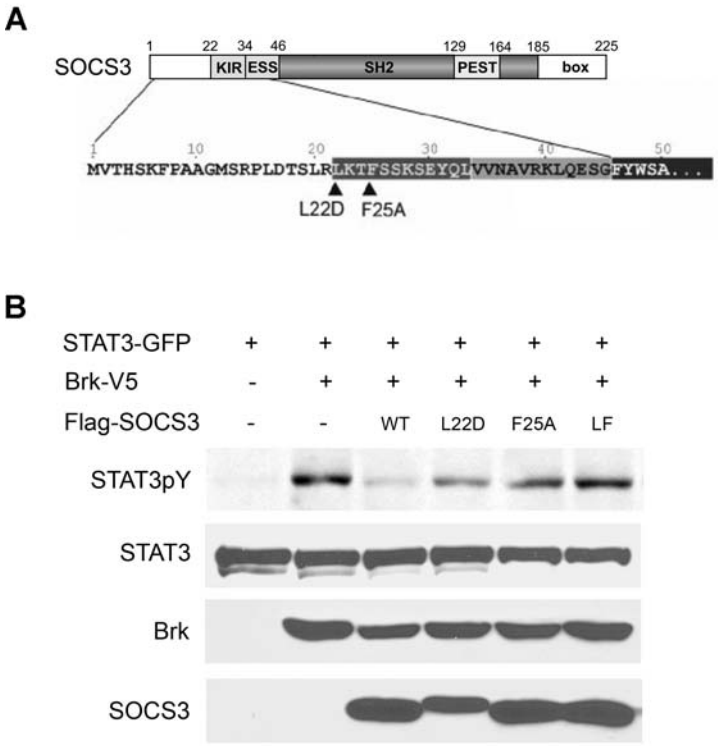



**Figure 19: SOCS3 KIR point mutants are not able to inhibit Brk activity.**

(A) Amino acid sequences of the N-terminal region of SOCS3.
(B) COS1 cells were co-transfected with STAT3-GFP, Brk-V5 with or without Flag-SOCS3 wide-type (WT) or SOCS3 L22D, F25A or double mutant L22D/F25A (LF) and serum-starved for 24 hours. Lysates were prepared and analyzed by Western blot with specific STAT3 phosphotyrosine, GFP (to detect total STAT3), V5 (to detect Brk) and Flag (to detect SOCS3 and point mutants) antibodies.

completely abolished the ability of SOCS3 to inhibit Brk activity. This result confirms the requirement of KIR domain for Brk inhibition. Taken together, these data suggest that the KIR domain in SOCS3 is responsible for the SOCS3 inhibitory function on Brk.

**SOCS3 binds to Brk**

SOCS3 inhibits IL-6 signaling by binding to the phosphotyrosine residues on the cytoplasmic domain of the gp130 receptor, followed by inhibition of JAK activity [146]. SOCS3 has also been shown to directly interact with JAKs to inhibit kinase activity in other cytokine signaling pathways, such as IL-2 and Oncostatin M [203,204]. To determine the mechanisms of Brk inhibition by SOCS3, co-immunoprecipitation was performed to assess whether SOCS3 binds to Brk to block its kinase activity (Figure 20). COS1 cells were transiently transfected with GFP-tagged Brk and Flag-tagged SOCS3 or empty vector. Cell lysates were subjected to immunoprecipitation with anti-SOCS3 antibody or with rabbit IgG as control, and the presence of Brk was determined by Western blot using specific Brk antibody. Brk was detected in the SOCS3 immunocomplex, whereas no Brk was found in the immunocomplex precipitated with a control IgG antibody. In addition, no Brk was immunoprecipitated with anti-SOCS3 antibody in the cells expressing Brk alone. These results indicated that SOCS3 physically associates with Brk *in vivo*.

I further performed a GST pull-down assay to test the interaction between SOCS3 and Brk *in vitro* (Figure 21). Equal amounts of bacterially purified GST or GST-SOCS3 fusion proteins were immobilized on glutathione agarose beads as estimated from Commassie blue staining (data not shown). As a source of Brk protein, mammalian cells

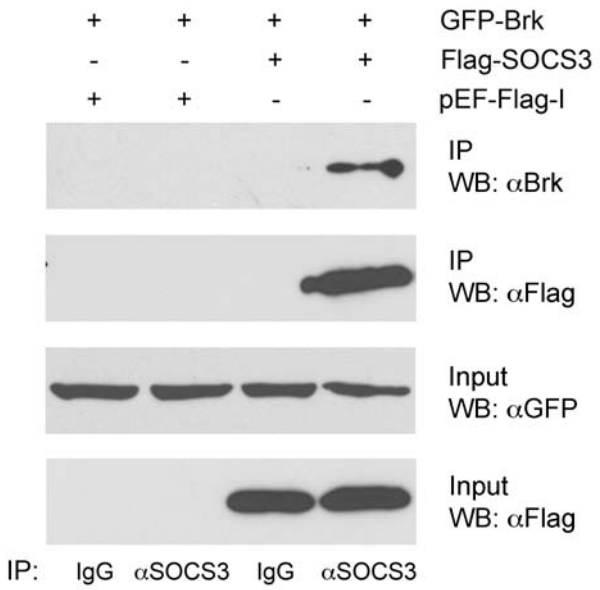



**Figure 20: The interaction between SOCS3 and Brk.**

COS1 cells were co-transfected with GFP-Brk and Flag-SOCS3 or empty vector. Forty-eight hours after transfection, the cells were lysed and immunoprecipitated with anti-SOCS3 antibody or control rabbit IgG. The immunoprecipitates were resolved by SDS-PAGE and Western blot was performed with anti-Brk or anti-Flag antibody. An aliquot of each input was analyzed by Western blot with anti-GFP or anti-Flag antibody.

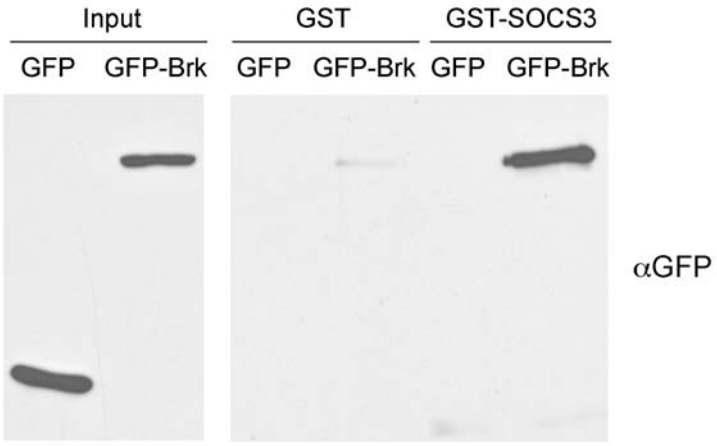



**Figure 21: The *in vitro* binding between SOCS3 and Brk.**

GST-SOCS3 or GST proteins were bound to glutathione agarose beads and incubated with lysates from COS1 cells expressing GFP-Brk or GFP. Bound Brk proteins were detected by Western blot with anti-GFP antibody.

were transfected with GFP-tagged Brk for 48 hours. Cell lysates were incubated with the GST-SOCS3 beads, bound proteins were eluted, and the presence of Brk was detected by Western blot with GFP antibody. The results showed that Brk was pulled down by GST-SOCS3 protein but not GST alone, indicating the *in vitro* binding between SOCS3 and Brk.

**Interaction between SOCS3 SH2 domain and Brk**

SOCS3 contains a central SH2 domain that recognizes the phosphotyrosine residues and is often involved in protein-protein interactions. To determine whether SH2 domain of SOCS3 mediates its binding to Brk, I generated two SOCS3 SH2 mutants (Figure 22A). The SOCS3 amino acid 46-185 encodes the entire SH2 domain including a 35 amino acid PEST motif insertion. The SOCS3 amino acid 130-225 only contains the PEST motif, C-terminal part of SH2 domain and the SOCS box and was generated as a negative control. Following transfection of COS1 cells with expression vectors for GFP-tagged Brk and Flag-tagged SOCS3 full-length (FL) or SOCS3 SH2 mutants, cells were lysed and lysates were immunoprecipitated with anti-SOCS3 antibody. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blot with anti-Brk antibody (Figure 22B). A significant interaction between SOCS3 46-185 and Brk was observed and as expected, the control SOCS3 130-225 was not able to bind to Brk. These results clearly indicated that the binding between SOCS3 and Brk is mediated by SOCS3 SH2 domain.

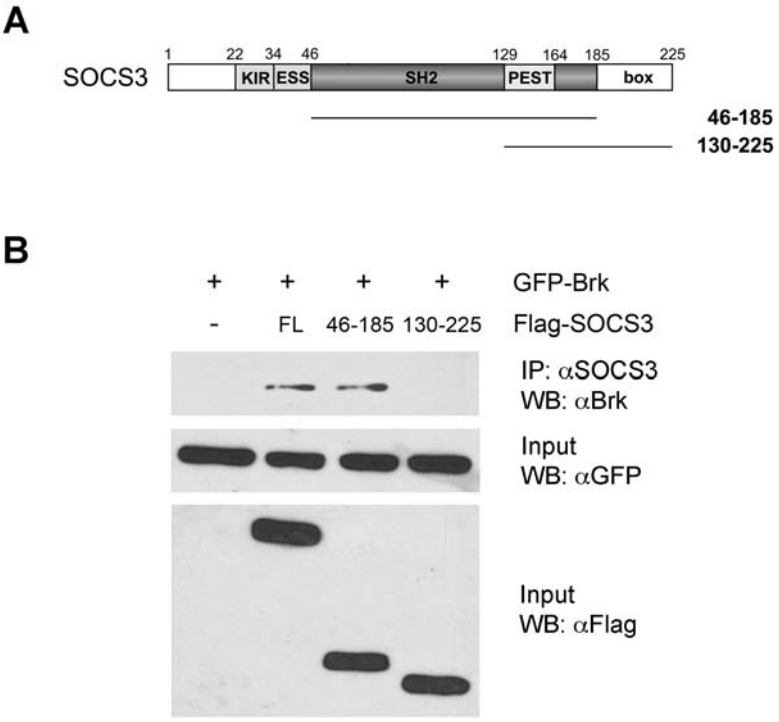



**Figure 22: SOCS3 SH2 domain mediates SOCS3-Brk interaction.**

(A) Linear diagram of SOCS3 SH2 mutants.
(B) COS1 cells were co-transfected with GFP-Brk and Flag-SOCS3 full-length (FL) or SOCS3 SH2 mutants (amino acid 46-185 and 130-225) for 48 hours. Cells lysates were immunoprecipitated with anti-SOCS3 antibody and the immunoprecipitates were analyzed by Western blot with anti-Brk antibody. An aliquot of each input was subjected to Western blot with anti-GFP or anti-Flag antibody.

Mutagenesis experiments have shown that mutation of a critical arginine 71 within the SOCS3 SH2 domain abolishes its binding to phosphotyrosine residues on the receptors [151]. To study the role of arginine 71 in the SOCS3-Brk interaction, I mutated arginine 71 to alanine or glutamic acid in full-length SOCS3 to generate SOCS3 R71A or R71E. The crystal structure of SOCS3 in complex with a phosphotyrosine-containing peptide from the gp130 receptor reveals that the phosphotyrosine is located close to a positively charged patch on the surface of the domain formed by arginine 71 and arginine 94 [154]. Therefore, a triple SH2 mutant SOCS3 R71E/L93D/R94E (RLR) was generated. Moreover, both mutagenesis studies and crystal structure of SOCS3 have revealed the critical role of extended SH2 subdomain (ESS) in phosphotyrosine binding [151,154]. The conserved valine 38 and leucine 41 in ESS directly interact with the phosphotyrosine-binding loop and mutation of either residue has been shown to abolish phosphotyrosine binding. For this reason, I generated a SOCS3 ESS/SH2 double mutant, SOCS3 L41R/R71A (LR). All of these SOCS3 point mutants were used to perform an immunoprecipitation with Brk (Figure 23). The results indicated that all of the tested SOCS3 SH2 or ESS/SH2 point mutants were able to associate with Brk, suggesting that the properties of SOCS3-Brk interaction may be different from those of the binding between SOCS3 and cytokine receptors.

The KIR domain in SOCS3 is required for the inhibition of cytokine signaling. Mutation of essential residues in the KIR, or its deletion, affects JAK kinase inhibition without affecting phosphotyrosine binding [151]. I have found that SOCS3 KIR point mutants abrogate SOCS3 inhibition on Brk; therefore, I further tested the ability of these KIR mutants to bind Brk (Figure 24). The SOCS3 KIR mutant, SOCS3 L22D/F25A (LF)

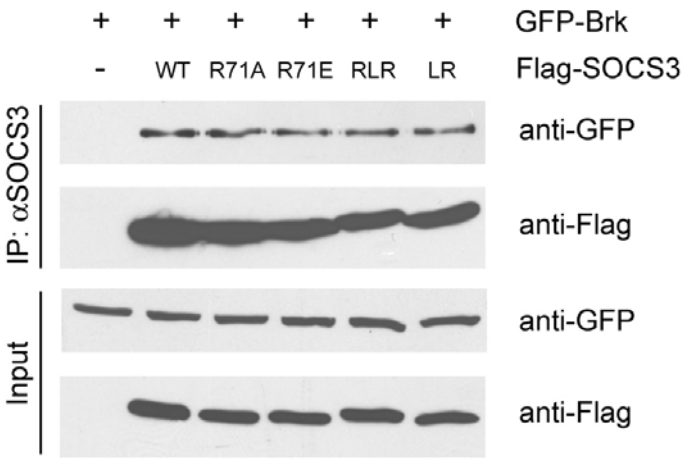



**Figure 23: Mutation of critical arginine 71 in SOCS3 SH2 domain does not abrogate Brk binding.**

COS1 cells were co-transfected with GFP-Brk and Flag-SOCS3 wide-type (WT) or SOCS3 R71A, R71E, R71E/L93D/R94E (RLR) and L41R/R71A (LR) for 48 hours. Cells lysates were immunoprecipitated with anti-SOCS3 antibody and the presence of Brk was determined by Western blot with anti-GFP antibody. An aliquot of each input was analyzed by Western blot with anti-GFP or anti-Flag antibody.

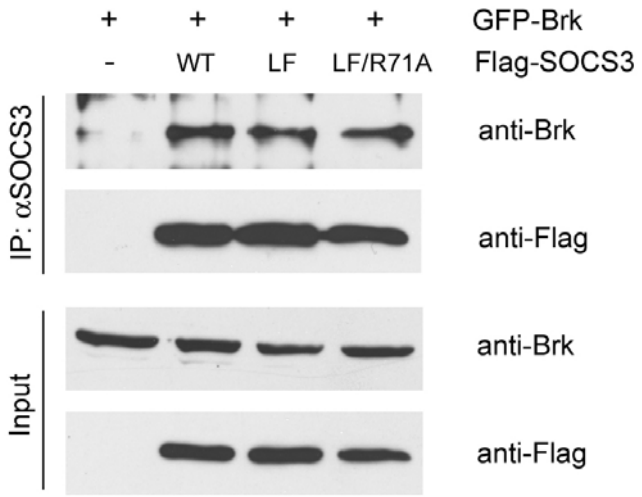



**Figure 24: SOCS3 KIR mutants do not affect SOCS3-Brk interaction.**

COS1 cells were co-transfected with GFP-Brk and Flag-SOCS3 wide-type (WT) or SOCS3 KIR mutant L22D/F25A (LF), or KIR/SH2 double mutant L22D/F25A/R71A (LF/R71A) for 48 hours. Cells lysates were immunoprecipitated with anti-SOCS3 antibody and immunoblotted with anti-Brk or anti-Flag antibody. An aliquot of each input was analyzed by Western blot with anti-Brk or anti-Flag antibody.

and KIR/SH2 double mutant, SOCS3 L22D/F25A/R71A (LF/R71A) were used to perform a co-immunoprecipitation. COS1 cells were co-transfected with GFP-tagged Brk and Flag-tagged SOCS3 wide-type (WT) or KIR mutants. Cell lysates were immunoprecipitated with anti-SOCS3 antibody and the presence of Brk in the immunocomplexes was determined by Western blot with anti-Brk antibody. It was found that neither SOCS3 KIR mutant nor KIR/SH2 double mutant affected Brk binding. These results are consistent with the findings on KIR action in cytokine signaling, suggesting that KIR domain may inhibit Brk and JAKs in a similar way, likely acting as a pseudosubstrate.

To further study the role of SOCS3 SH2 domain in Brk binding, I mutated arginine 71 in SH2 domain to generate SOCS3 46-185 R71E and 46-129 R71E (Figure 25A). These mutants were used for a co-immunoprecipitation to test their ability to bind to Brk (Figure 25B). COS1 cells expressing GFP-tagged Brk and Flag-tagged SOCS3 mutants were lysed for an immunoprecipitation using anti-SOCS3 antibody. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot with anti-GFP antibody. Compared to SOCS3 46-185 (SH2 domain), the arginine mutant SOCS3 46-185 R71E was found to weakly bind to Brk. A significant interaction between SOCS3 46-129 (major fragment of SH2 domain) and Brk was observed. However, the arginine mutant SOCS3 46-129 R71E completely abolished its binding to Brk. These results suggest that arginine 71 is not indispensable for SH2 domain binding to Brk and other part of SH2 domain (sequence aa130-185) may coordinate with arginine 71 to mediate this interaction.

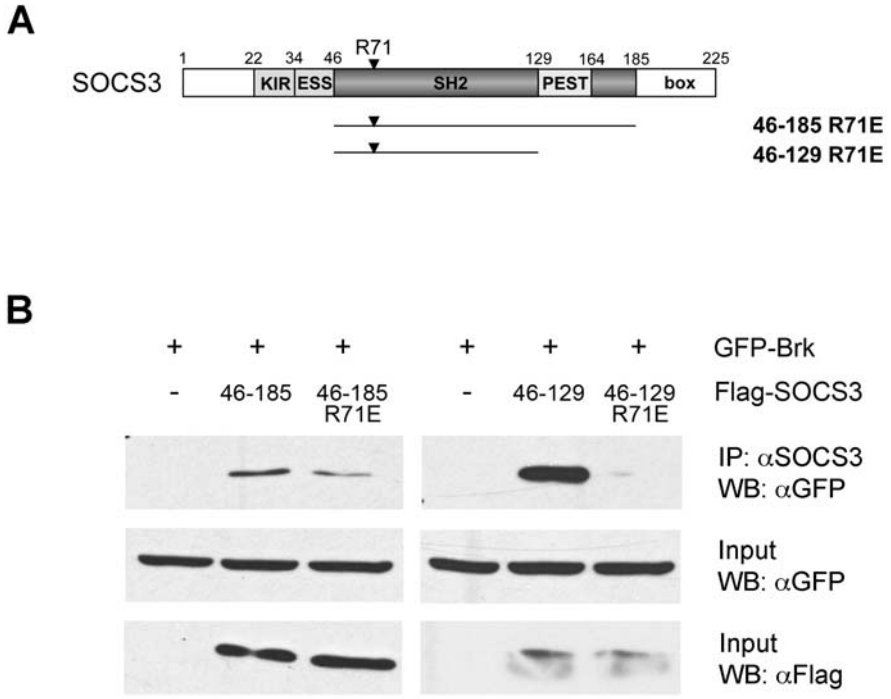


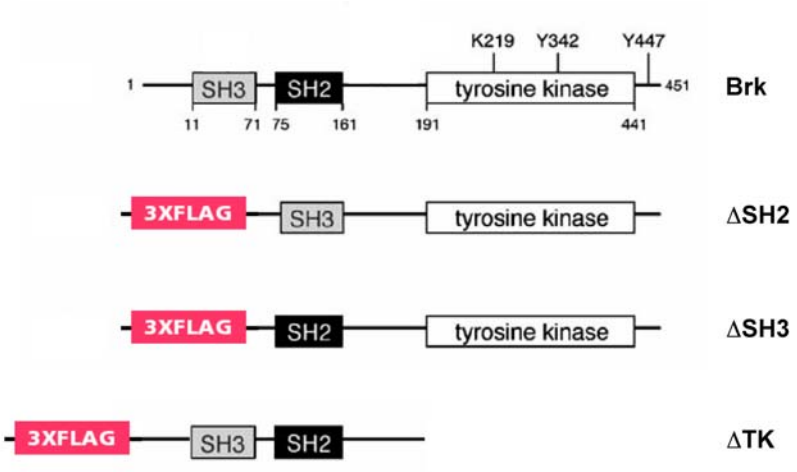

**Figure 25: Mutation of arginine 71 in SOCS3 aa46-129 abolishes Brk binding.**

COS1 cells were co-transfected with GFP-Brk and Flag-SOCS3 mutants (46-185, 46-185 R71E, 46-129, 46-129 R71E) for 48 hours. Cells lysates were prepared and immunoprecipitated with anti-SOCS3 antibody. The presence of Brk was detected by Western blot with anti-GFP antibody. An aliquot of each input was immunoblotted with anti-GFP or anti-Flag antibody.

**SOCS3 binds to the tyrosine kinase domain of Brk**

Brk consists of an SH3 domain, an SH2 domain and a tyrosine kinase (TK) domain. Molecular characterization reveals that both the SH3 and SH2 domains are involved in intramolecular interactions and enzyme regulation, whereas the SH3 domain plays a major role in substrate recognition [107,113]. To determine which domain in Brk is involved in the association with SOCS3, I assessed the ability of Brk domain deletion mutants (ΔSH3, ΔSH2 and ΔTK) to bind to SOCS3. The ΔSH3, ΔSH2 and ΔTK mutants have complete deletions in these domains (Figure 26). To perform a GST pull-down assay, the SOCS3 protein was produced as a GST fusion and bound to glutathione agarose beads. As a source of Brk truncation proteins, COS1 cells were transfected with Flag-tagged Brk ΔSH3, ΔSH2 and ΔTK. Cell lysates were incubated with the GST-SOCS3 beads, and bound proteins were eluted and analyzed by Western blot using anti-Flag antibody. As shown in Figure 27, both Brk ΔSH3 and ΔSH2 bound to SOCS3 potently, however, Brk ΔTK failed to bind to SOCS3. These findings suggest that the TK domain of Brk is required for the interaction with SOCS3.

Lysine 219 is required for the enzymatic activity of Brk, and the K219M mutation renders the kinase dead with a substitution in the ATP-binding site [114,133]. Brk autophosphorylates itself at several tyrosine residues, but K219M shows no autophosphorylation [107]. This finding was confirmed by immunoprecipitation experiments (Figure 28). To test for autophosphorylation, Brk wide-type (WT) or K219M were immunoprecipitated from COS1 cell lysates with anti-Brk antibody and analyzed by SDS-PAGE with anti-phosphotyrosine (4G10) Western blotting. As expected, no autophosphorylation was detected in Brk K219M. Therefore, K219M can be considered




(Modified from Qiu H, Oncogene, 23:2216)

**Figure 26: Schematic structure of Brk and Brk domain deletion mutants.**

The top diagram shows the domain structure of wild-type Brk. The Brk domain deletion mutants are shown below the wild-type Brk.

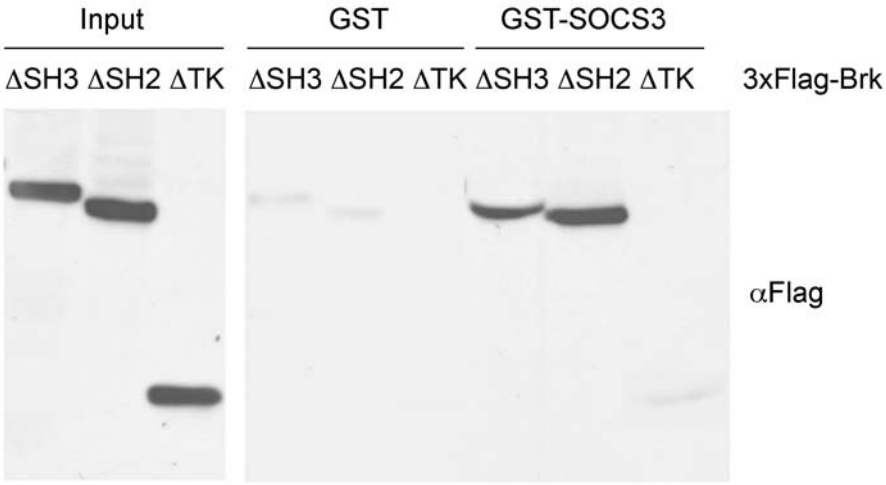



**Figure 27: The tyrosine kinase (TK) domain in Brk is essential for the interaction between SOCS3 and Brk.**

GST or GST-SOCS3 fusion proteins were immobilized on glutathione agarose beads and incubated with lysates from COS1 cells expressing 3xFLAG-Brk ΔSH3, ΔSH2 and ΔTK. Bound Brk proteins were detected by Western blot with anti-Flag antibody. Cell lysates were analyzed as input controls.

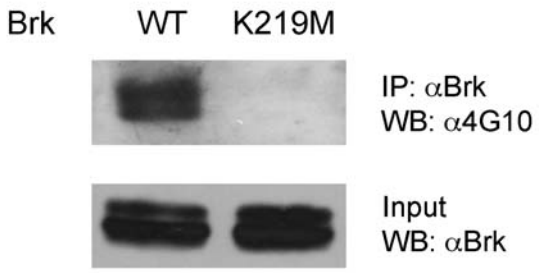



**Figure 28: Brk K219M shows no autophosphorylation.**

COS1 cells were transfected with plasmids encoding Brk wide-type (WT) or K219M for 48 hours. Lysates were prepared and immunoprecipitated with anti-Brk antibody. The immunoprecipitated proteins were subjected to SDS-PAGE and Western blot using anti-phosphotyrosine (4G10) antibody. An aliquot of each input was analyzed with anti-Brk antibody.

as an unphosphorylated form of Brk.

To determine whether Brk K219M binds to SOCS3, a co-immunoprecipitation was performed (Figure 29). Following transfection of COS1 cells with expression vectors for GFP-tagged Brk wide-type (WT) or K219M and Flag-tagged SOCS3, cells were lysed and lysates were immunoprecipitated with anti-SOCS3 antibody. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blot with anti-GFP antibody. As an unphosphorylated form of Brk, K219M showed severely impaired binding to SOCS3. Together with the findings that SOCS3 SH2 domain and Brk tyrosine kinase (TK) domain are involved in their interaction and SH2 domain commonly recognizes the phosphotyrosine residues, this result suggests that SOCS3 uses its SH2 domain to bind to the phosphotyrosine residue(s) in Brk TK domain. The specific phosphotyrosine residue(s) in Brk TK domain that are involved in SOCS3-Brk interaction need to be determined.

**The sequence 249-256 in Brk TK domain is critical for SOCS3-Brk interaction**

The studies on mapping the phosphopeptide binding preferences of the SH2 domain from SOCS3 have identified a consensus ligand binding motif for SOCS3: pY-(S/A/V/Y/F)-hydrophobic-(V/I/L)-hydrophobic-(H/V/I/Y) (Figure 30) [205]. The most selective position appears to be pY+3, where approximately 65% of the selected sequences have either valine, isoleucine, or leucine [205]. The crystal structure of SOCS3 in complex with a phosphopeptide from the gp130 receptor have confirmed the significance of residues Val+3 and Val+4, as both of these valines show significant hydrophobic contacts with the internal surface of the BG loop [154]. The BG loop is

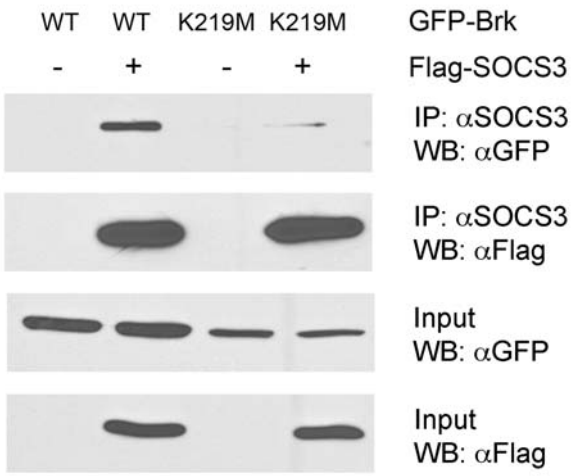



**Figure 29: Brk K219M is defective in binding to SOCS3.**

COS1 cells were co-transfected with GFP-Brk wide-type (WT) or K219M, with or without Flag-SOCS3 for 48 hours. Cells lysates were immunoprecipitated with anti-SOCS3 antibody and the immunocomplexes were run on SDS-PAGE. Western blot analysis was performed using anti-GFP or anti-Flag antibody. An aliquot of each input was analyzed with anti-GFP or anti-Flag antibody.

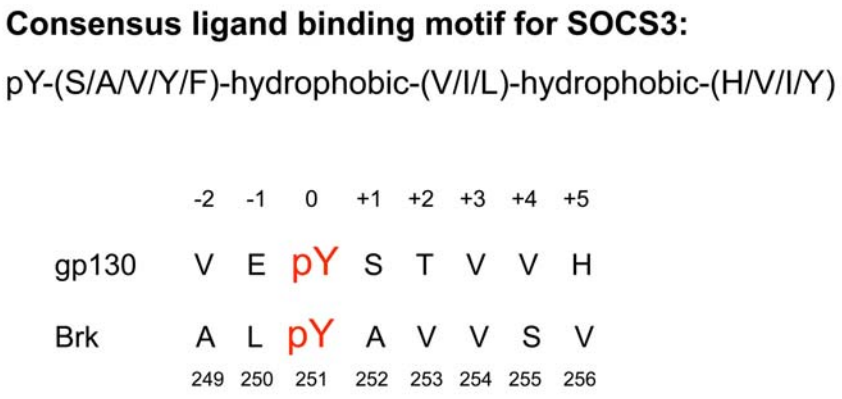



**Figure 30: Consensus ligand binding motif for SOCS3.**

The diagram shows a putative consensus ligand binding motif for SOCS3. The sequence of Brk aa249-256 is compared with that of phosphopeptide from the gp130 receptor.

located downstream of the PEST insert. In Brk protein sequence there are seventeen tyrosine residues in total. Screening the entire Brk sequence, I found that the sequence aa249-256 matched the SOCS3 consensus binding motif very well (Figure 30). This sequence is located in the Brk tyrosine kinase domain and has a tyrosine 251 followed by a valine at pY+3. Therefore, this tyrosine 251 may be recognized by SH2 domain of SOCS3.

Two Brk mutants were generated to study the roles of tyrosine 251 and the sequence 249-256 in SOCS3-Brk interaction. The tyrosine 251 was substituted with phenylalanine to create Brk Y251F. The sequence 249-256 was deleted from full-length Brk to generate an internal deletion mutant, Brk Δ249-256. GST pull-down assay was used to examine the ability of these two mutants to bind to SOCS3 (Figure 31). Equal amounts of purified GST or GST-SOCS3 fusion proteins were immobilized on glutathione agarose beads and incubated with lysates from COS1 cells expressing GFP-Brk wide-type (WT), Y251F or Δ249-256. The bound proteins were eluted and analyzed by Western blot with anti-Brk antibody. The results indicated that Brk tyrosine mutant Y251F showed greatly reduced binding to SOCS3 and the internal deletion mutant Δ249-256 abolished its binding to SOCS3 completely. These preliminary data suggest that tyrosine 251 in Brk TK domain may be one of the tyrosines that are involved in the binding of SOCS3 SH2 domain. The sequence flanking tyrosine 251 appears essential for the recognition by SH2 domain of SOCS3. However, this tyrosine 251 is not identified as an autophosphorylation site by mass spectrometry [107]. Further investigation needs to be performed to determine whether tyrosine 251 is autophosphorylated.

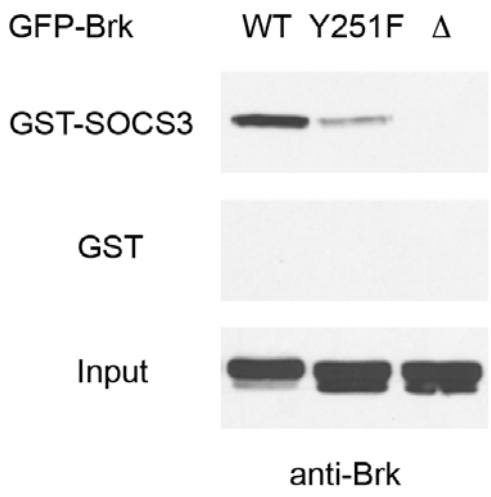



**Figure 31: Preliminary evidence that amino acid sequence 249-256 in Brk is required for association with SOCS3.**

GST-SOCS3 or GST proteins were bound to glutathione agarose beads and incubated with lysates from COS1 cells expressing GFP-Brk wide-type (WT), Y251F or Δ249-256 (Δ). Bound Brk proteins were detected by Western blot with anti-Brk antibody. Cell lysates were analyzed as input controls.

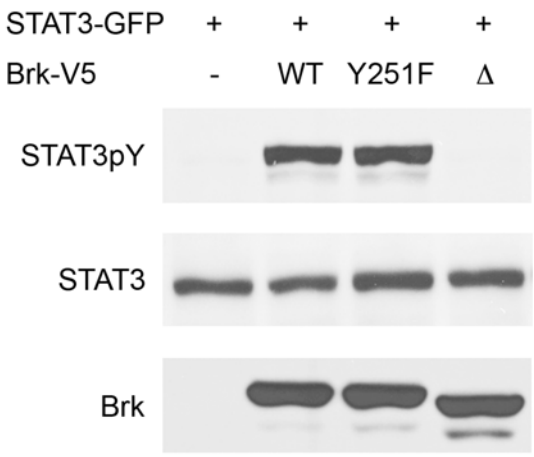


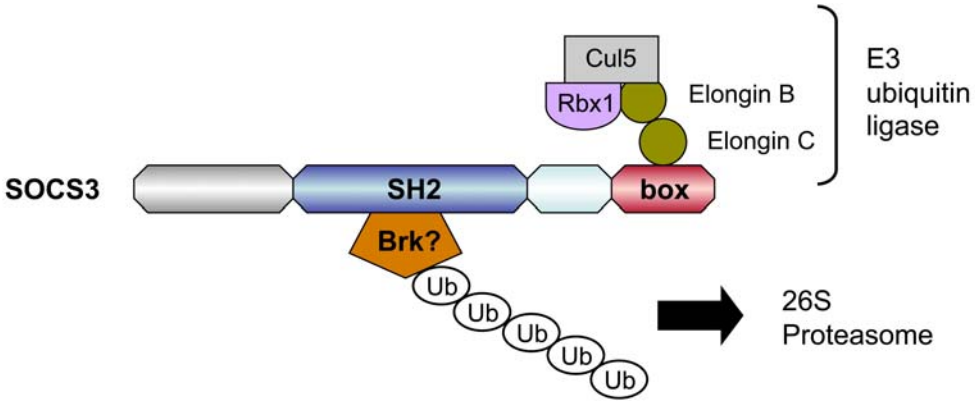

**Figure 32: Brk Δ249-256 is inactive in kinase activity.**

COS1 cells were transfected with STAT3-GFP and Brk-V5 wide-type (WT), Y251F, or Δ249-256 (Δ) and serum-starved for 24 hours. Cell lysates were subjected to SDS-PAGE and Western blot analysis with specific STAT3 phosphotyrosine, GFP (to detect total STAT3) and V5 (to detect Brk) antibodies.

I next evaluated the kinase activities of these two mutants and STAT3 phosphorylation was a read-out (Figure 32). Cells were co-transfected with STAT3-GFP and Brk wide-type (WT), Y251F or Δ249-256 and serum-starved for 24 hours. Lysates were subjected to SDS-PAGE and Western blot with specific STAT3 phosphotyrosine antibody. As shown in Figure 32, Brk Y251F stimulated STAT3 tyrosine phosphorylation at the same level as wide-type whereas the internal deletion mutant Δ249-256 clearly failed to induce any detectable phosphorylation of STAT3.

**SOCS3 promotes Brk degradation via SOCS box**

Accumulated data suggest that proteins that bind to SOCS molecules are targeted for proteasomal degradation. This is resulting from the function of the SOCS box itself. The SOCS box has been shown to directly interact with Elongin C and B and form an E3 ligase complex with additional recruitment of Cullin5 and Rbx1 (Figure 33). SOCS3 has been reported to target receptors for proteasomal degradation, including CD33 and Siglec 7, and promote destruction of insulin receptor substrate (IRS) 1 or IRS 2 and of focal adhesion kinase (FAK) [163,164,169,170]. To determine whether SOCS3 promotes proteasomal degradation of Brk, I performed a preliminary experiment to evaluate whether the expression of SOCS3 affects Brk protein levels (Figure 34). Cells were transfected with SOCS3 and treated with doxycycline to induce Brk expression. Brk levels were determined by Western blot and tubulin as an internal control. The results indicated that Brk levels decreased about 30% in the presence of SOCS3, suggesting that SOCS3 may promote the degradation of Brk.




(Modified from Johnston J, J Leukoc Biol., 75:743) [206]

**Figure 33: Structure of the SOCS3-Elongin-E3 ligase interaction.**

The SOCS box region binds to Elongin C and Elongin B, which in turn bind the Cullin family member, Cul5, and a ring finger-containing protein Rbx1. This acts as an E3 ligase, which ligates ubiquitin to the target substrate.

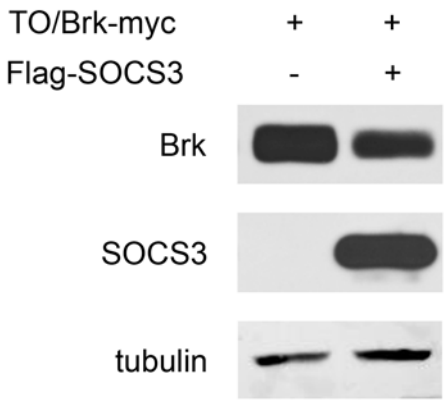



**Figure 34: The expression of SOCS3 decreases Brk protein levels.**

Cells were transfected with tetracycline-inducible Brk, tetracycline repressor with or without Flag-SOCS3 for 24 hours and treated with doxycycline for 24 hours. Cell lysates were analyzed by SDS-PAGE and Western blot with c-Myc (to detect Brk), Flag (to detect SOCS3) and αtubulin antibodies.

Proteins that are targeted for proteasomal degradation commonly undergo ubiquitin conjugation and the polyubiquitin chain is then recognized by receptors within the proteasome. To determine whether SOCS3 promotes the ubquitination of Brk, a His-ubiquitination assay was performed to evaluate the protein and ubiquitination levels of Brk in the presence of SOCS3 (Figure 35). His-tagged ubiquitin construct that is used for this assay has an octameric ubiquitin precursor expressed from the CMV promoter. Each ubiquitin unit contains a $His_6$ tag at its N-terminus. The precursor is expressed and efficiently processed by cellular ubiquitin-C-terminal hydrolases [199]. Cells were transfected with His-$Ub_8$ and SOCS3, and treated with doxycycline to induce Brk expression. The ubiquitinated Brk was captured by nickel beads, eluted and analyzed by Western blot. The protein levels of Brk were detected in the whole cell lysates. The results showed that Brk alone was extensively ubquitinated in cells. SOCS3 expression decreased the ubiquitination of Brk, but promoted the degradation of Brk, reducing the Brk level by approximately 50%. To ensure that the reduced levels of Brk are due to the effects of SOCS3 and rule out the possibilities that SOCS3 expression affects the transfection of Brk, a proteasome inhibitor MG132 was used to treat cells. As shown in Figure 34, the MG132 treatment restored the Brk protein level and highly increased the ubiquitination of Brk.

To further investigate whether the SOCS3-induced Brk degradation is SOCS box-dependent, I performed a similar His-ubiquitination assay using a SOCS box deletion mutant, SOCS3 Δbox (aa1-185). As shown in Figure 36, the SOCS3 Δbox restored Brk protein level and ubiquitination level, indicating that the SOCS box is necessary for this effect.

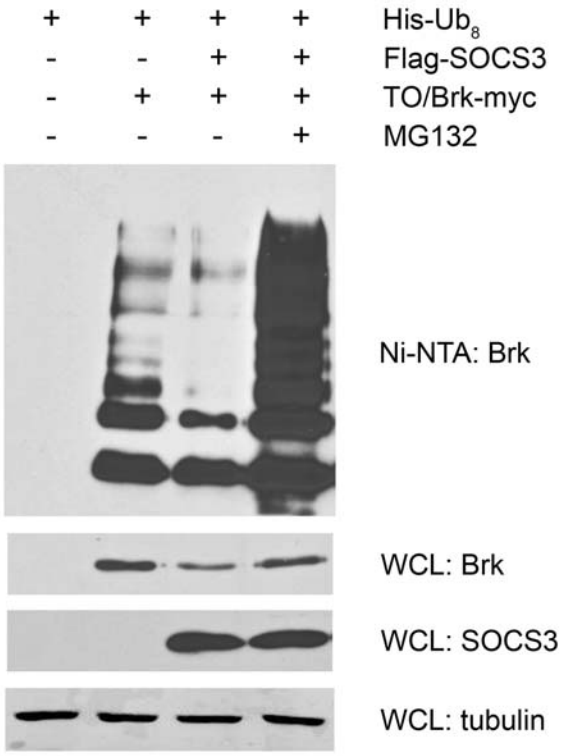



**Figure 35: SOCS3 promotes Brk degradation.**

Cells were transfected with tetracycline-inducible Brk, tetracycline repressor and His-$Ub_8$ with or without Flag-SOCS3 for 24 hours and treated with doxycycline with or without MG132 for 24 hours. Histidine-tagged proteins were purified using Ni-NTA agarose beads and Myc-tagged Brk proteins were detected by Western blot with anti-c-Myc antibody. An aliquot of each whole cell lysate (WCL) was analyzed with anti-c-Myc, anti-Flag or anti-αtubulin antibody.

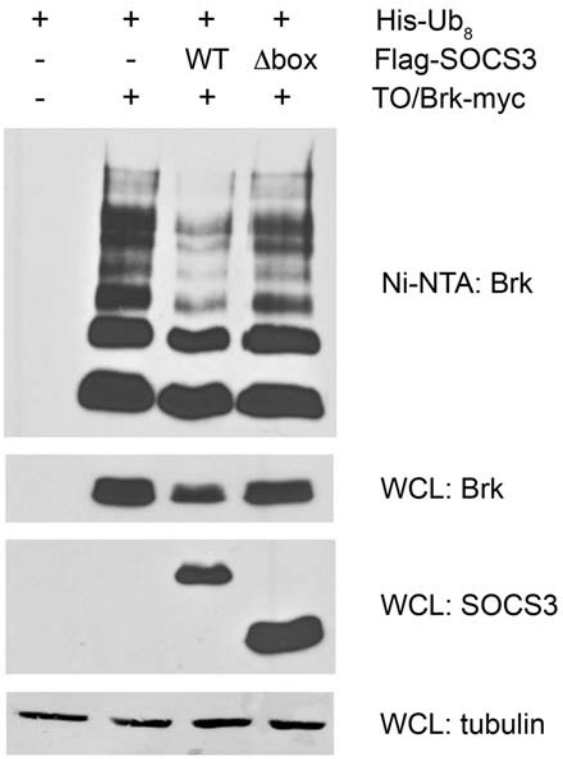



**Figure 36: SOCS box is required for Brk degradation.**

Cells were transfected with tetracycline-inducible Brk, tetracycline repressor and His-$Ub_8$ with or without Flag-SOCS3 wide-type (WT) or Δbox (aa1-185) for 24 hours and treated with doxycycline for 24 hours. Histidine-tagged proteins were purified using Ni-NTA agarose beads and Myc-tagged Brk proteins were detected by Western blot with anti-c-Myc antibody. An aliquot of each whole cell lysate (WCL) was analyzed with anti-c-Myc, anti-Flag or anti-αtubulin antibody.

**Brk polyubiquitin chains are mainly linked by ubiquitin lysine 63**

Ubiquitin lysine 48 and lysine 63 that are involved in chain formation are the best characterized residues involved in polyubiquitination. It is well known that polyubiquitin chains bearing different linkages represent distinct functional signals [197]. Ubiquitin chains linked by lysine 48 target a conjugated substrate for proteasomal degradation, whereas lysine 63-linked chains perform a variety of non-proteolytic functions, including cellular signaling, intracellular trafficking and DNA damage repair.

Ubiquitin lysine mutants are often used to study ubiquitin linkage of the protein of interest. It is not practical to mutate lysine residues in His-$Ub_8$ plasmid that contains eight tandem copies of ubiquitin. Therefore, I generated a His-Ub construct that only has one copy of ubiquitin. His-ubiquitination assay was carried out to compare the pattern of Brk ubiquitination in the presence of His-Ub or His-$Ub_8$ (Figure 37). His-Ub or His-$Ub_8$ was expressed with the inducible Brk system, and ubiquitinated proteins were captured by nickel beads. Brk was detected by Western blot. As shown in Figure 37, a nice ladder pattern of ubiquitinated Brk was observed when His-$Ub_8$ was expressed. The single-copy ubiquitin construct His-Ub was conjugated to Brk at a much lower level.

To evaluate the ubiquitin linkage with Brk, three ubiquitin mutants were generated in the template of His-Ub (Figure 38). Ub K0 encoded ubiquitin in which all of its seven lysine residues were substituted with arginines so that it can only support monoubiquitination. Ub K0R63K was generated by mutating arginine 63 in Ub K0 back to lysine and therefore ubiquitination can occur only through lysine 63 linkage. Ub K0R48K is an ubiquitin mutant by which polyubiquitin chains can only be extended through lysine 48. Cells were transfected with His-Ub wide-type (WT) or mutants, and

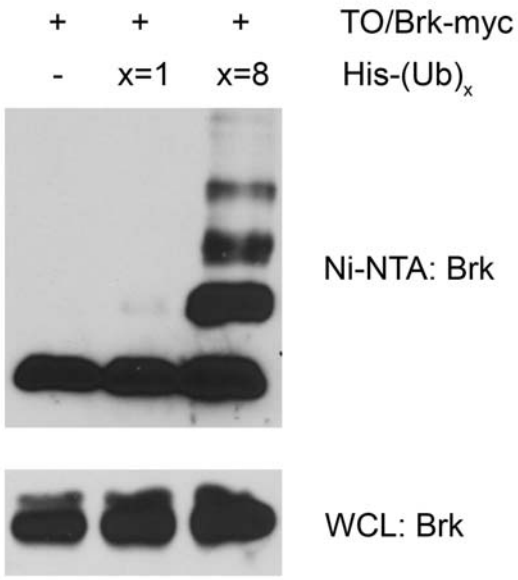



**Figure 37: The pattern of Brk ubiquitination with the expression of His-Ub or His-$Ub_8$.**

Cells were transfected with tetracycline-inducible Brk, tetracycline repressor and His-Ub or His-$Ub_8$ for 24 hours and treated with doxycycline for 24 hours. Histidine-tagged proteins were purified using Ni-NTA agarose beads and Myc-tagged Brk proteins were detected by Western blot with anti-c-Myc antibody. An aliquot of each whole cell lysate (WCL) was analyzed with anti-c-Myc antibody.


**Figure 38: The sequence of ubiquitin wide-type and mutants.**

The amino acid sequence of ubiquitin wide-type (WT) and mutants (K0, K0R63K and K0R48K) are shown.

treated with doxycycline to induce Brk expression. The ubiquitinated Brk was purified by nickel beads and detected by Western blot. The results indicated that the ubiquitin chains of Brk are linked both by lysine 48 and lysine 63, but mainly through lysine 63 (Figure 39). The data suggest that this major lysine 63 linkage may direct Brk for other cellular responses. The K0 mutant only mediates monoubiquitination. However, multiply-ubiquitinated Brk was detected with K0 expression. This may occur when single ubiquitin molecule is individually conjugated to different lysine residues on Brk.

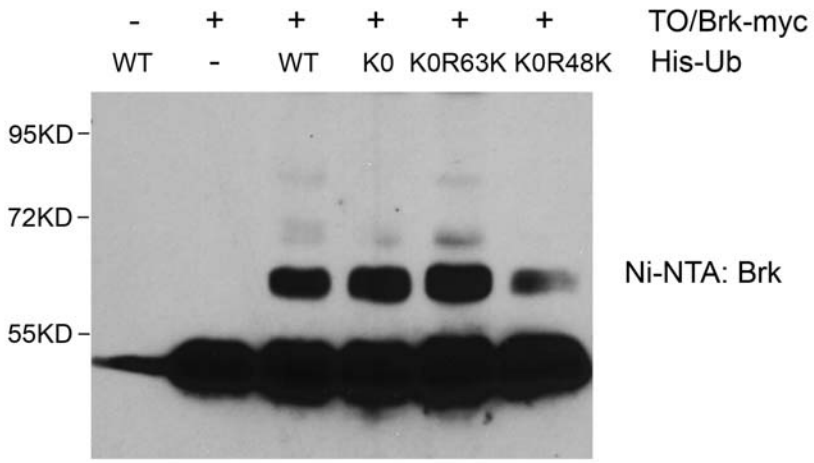



**Figure 39: Brk is polyubiquitinated mainly via lysine 63.**

Cells were transfected with tetracycline-inducible Brk, tetracycline repressor and His-Ub wide-type (WT), K0, K0R63K or K0R48K for 24 hours and treated with doxycycline for 24 hours. Histidine-tagged proteins were captured by Ni-NTA agarose beads and Myc-tagged Brk proteins were detected by Western blot using anti-c-Myc antibody.

# Chapter 4

## Discussion

Brk appears to have conflicting roles in cancer and normal tissues. In tumor cells, Brk promotes proliferation and cell migration. In contrast, expression of Brk in normal epithelia correlates with cell cycle exit, differentiation and growth suppression. Disruption of the mouse Brk gene leads to increased growth and delayed differentiation of the small intestine with the discovery of increased AKT signaling and the Wnt pathway [131,141]. Brk may have context- and tissue-specific functions and it is regulated by a variety of factors. The positive or negative regulation of Brk could have a profound impact on its effects. Therefore it is critical to understand the molecular mechanisms underlying Brk regulation and this may help to develop new therapeutic strategies for Brk targeting in cancer.

We identified the STAT3 transcription factor as one of the substrates of Brk [128]. Persistent tyrosine phosphorylation of STAT3 has been observed in almost every type of cancer and it induces the expression of genes encoding anti-apoptotic proteins, cell cycle regulators and proto-oncoproteins [66,67,207]. The SOCS3 gene is induced in response to activated STAT3, and this led to the finding that SOCS3 can inhibit the ability of Brk to phosphorylate STAT3 [128]. In this study we demonstrate that expression of Brk can induce the endogenous SOCS3 gene, suggesting this is a negative feedback mechanism for Brk activity. The results support the reasoning that Brk activates STAT3 which in turn induces the expression of SOCS3 by binding to a target site in the

promoter of the SOCS3 gene [208]. SOCS3 is the only negative regulator of Brk that has been identified.

**Mechanisms of Brk inhibition by SOCS3**

Our studies demonstrate a physical interaction between SOCS3 and Brk. Analysis of various mutations revealed the association appears to be mediated primarily by the SH2 domain of SOCS3 and the tyrosine kinase domain of Brk. Brk kinase activity is required for this interaction since SOCS3 binding to a kinase dead mutant is dramatically reduced. The binding of SOCS3 to Brk is not sufficient to inhibit its activity. Inhibition requires the kinase inhibitory region (KIR) that is present at the amino terminal region of SOCS3. Point mutations of the KIR domain abrogate the ability of SOCS3 to inhibit Brk. In the regulation of cytokine signaling the SOCS KIR domain appears to act as a pseudosubstrate. It mimics the activation loop found in kinases such as JAKs, lodging in the catalytic cleft to prevent substrate access to the kinases [145,151]. This hypothesis is supported by KIR point mutations that abrogate SOCS action without affecting SH2 domain binding. Considering the results that SOCS3 KIR domain is required for Brk inhibition but not required for binding to Brk, it is possible that KIR domain acts on Brk and JAKs in a similar manner.

Mutation of critical arginine 71 in SOCS3 SH2 domain has been shown to completely abolish inhibition of STAT3 activation and phosphotyrosine binding to receptors in the cytokine signaling [151]. It is likely that the guanidine moiety of this conserved arginine directly contacts the negative phosphate group on the phosphotyrosine itself. We have found that SOCS3 R71E mutant still has ability to bind to Brk, suggesting

that arginine 71 may not be the only region involved in SOCS3 binding to Brk. Further mutation of arginine 71 in SOCS3 constructs containing 46-185 amino acids or 46-129 amino acids revealed that the binding of SOCS3 46-185 R71E to Brk is diminished, however, the arginine mutation in SOCS3 46-129 R71E completely abolishes its binding to Brk. The results suggest that the region 130-185 amino acids may be involved in SOCS3 SH2 domain binding to Brk. Mutation or deletion of either arginine 71 or the region 130-185 is not sufficient to disrupt the interaction, however when both are mutate or deleted, SOCS3 is not able to bind to Brk.

All SOCS proteins have a conserved carboxyl terminal SOCS box. The SOCS box is able to bind to elongin C, a component of the ECS-type E3 ubiquitin ligase complex [157]. SOCS proteins can thereby target associated proteins for ubiquitination. Polyubiquitination via lysine 48 on ubiquitin can target proteins for proteosomal degradation, and SOCS proteins have been found to regulate the ubiquitination and half-life of particular JAKs, receptors, and signaling molecules. For this reason we evaluated the effect of SOCS3 on Brk ubiquitination and degradation. Expression of SOCS3 with Brk was found to reduce the protein levels of Brk by approximately two-fold. This reduction in Brk protein was dependent on the presence of the SOCS box in SOCS3. A two-fold reduction in the level of Brk protein is not sufficient for the dramatic inhibitory effect of SOCS3 on Brk activity. Therefore the primary inhibitory effect of SOCS3 on Brk activity appears to be mediated by the KIR domain. The results in this study suggest a primary a mechanism of action of SOCS3 on Brk activity (Figure 40). The SH2 domain of SOCS3 appears to bind to the autophosphorylated Brk tyrosine kinase domain. This binding positions the SOCS3 KIR domain so that it inhibits the ability of Brk to

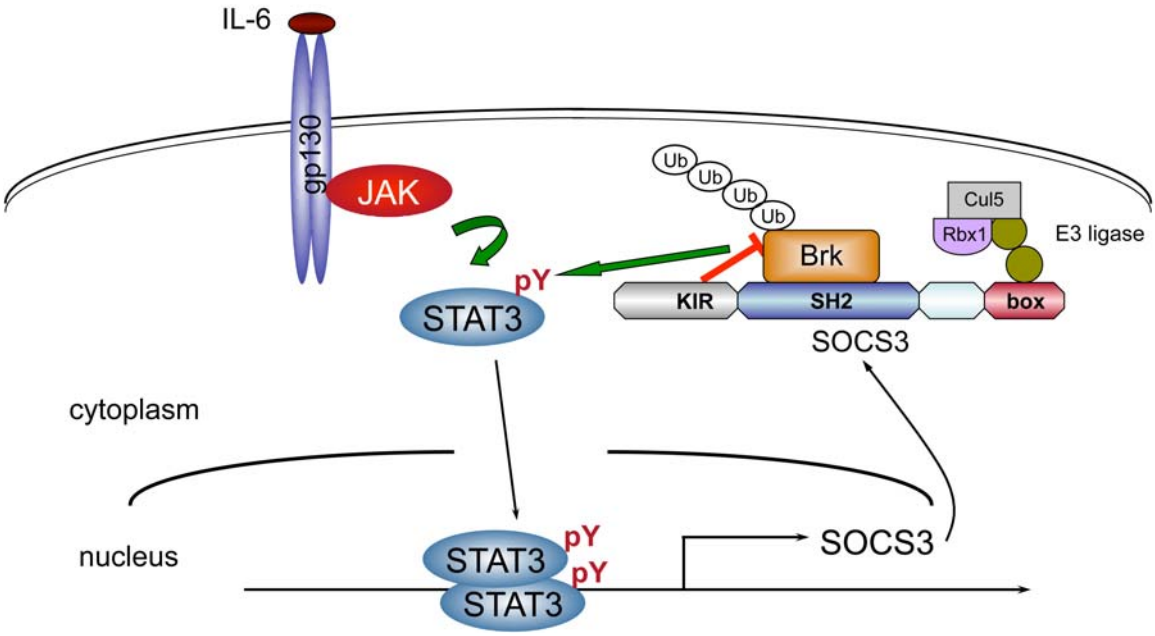



**Figure 40: The proposed mechanism by which SOCS3 inhibits Brk activity.**

Brk activates STAT3 which in turn induces the SOCS3 gene expression. SOCS3 proteins bind to Brk tyrosine kinase domain via its SH2 domain and this binding positions KIR domain to inhibit Brk kinase activity. In addition, the SOCS box binds to E3 ligase to promote Brk degradation.

phosphorylate STAT3. The KIR domain may help to facilitate binding to Brk, but its primary role is inhibition of kinase activity, likely acting as a pseudosubstrate. The SOCS box of SOCS3 is not necessary for inhibition of Brk although it appears to play a modest role in Brk stability. In addition to JAKs, SOCS3 has been shown to inhibit the activity of focal adhesion kinase (FAK) [170]. Distinct from inhibition of Brk, the primary mechanism of SOCS3 action on FAK appeared to be ubiquitin-mediated degradation.

**Ubiquitination of Brk**

Ubiquitination can be formed by isopeptide linkages with seven different lysines on ubiquitin, can occur as homotypic chains or mixed chains, and can occur as polymers or monomers [209]. Surprisingly, Brk was found to be highly ubiquitinated without SOCS3. By using ubiquitin mutants that only contain lysine 48 or lysine 63, it became apparent that both lysine 48 and lysine 63 are involved in the formation of Brk ubiquitin chains whereas lysine 63 linkage is the prominent modification (Figure 39). The ‘canonical’ lysine 48-linked chains usually signal proteasome proteolysis, therefore the degradation of Brk induced by SOCS3 may be connected to lysine 48 linkage. Lysine 63 is the major linkage in ubiquitinated Brk and this modification may mediate signaling functions such as protein-protein interactions with Brk. It has been shown that lysine 63-linked chains are recognized by zinc-finger domains within specific kinase adaptor proteins, in turn leading to polyUb-dependent IKK activation [210]. In this case the lysine 63-linked chain seems to act as a scaffolding element for the assembly of a competent signaling complex. Future studies are needed to explore the roles of lysine 63 linkage in Brk signaling and evaluate how this modification modulates properties of Brk.

Brk may undergo multiple monoubiquitinations as multiply-modified Brk was detected when an ubiquitin mutant that lacks all lysine residues and only supports monoubiquitination was expressed (Figure 39). There are twenty lysine residues located in all three domains of Brk. Preliminary experiments to study the ubiquitination of Brk domain deletion mutants showed no differences in the levels of ubiquitination among three domains (data not shown). Mutagenesis studies along with mass spectrometry can be carried out further to study which lysine residues in Brk are conjugated to ubiquitin.

Moreover, advances in mass spectrometry provide a powerful tool for accurate, direct determination of ubiquitinated lysines. The method is based on the detection of a signature Ub-derived tryptic peptide in which the linking lysine residue is attached to a GG or LRGG 'remnant' derived from next Ub in the chain [211,212]. This approach can be used to identify other potential linkages in Brk.

**SOCS and cancer**

Accumulating evidence suggests the SOCS proteins manifest the signs of tumor suppressors [213]. The transcriptional silencing of one or more SOCS genes due to hypermethylation and mutations and deletions in SOCS proteins has been found in many types of cancer. In addition, aberrant phosphorylation of SOCS proteins in some cancers has been shown to inhibit the ability of SOCS proteins to associate with Elongin C and the E3 ligase complex. Originally discovered to inhibit JAK-STAT cytokine signaling, the SOCS proteins appear to have a more global role in the regulation of proliferation. The negative effect of SOCS3 on Brk may maintain a normal balance that is necessary to

inhibit uncontrolled proliferative signals and provide new insights into breast cancer therapies with SOCS proteins.

**Future directions**

Future studies on functional interactions among Brk, STAT3 and SOCS3 can be carried out in cell culture system or *in vivo* mouse model.

Brk and STAT3 have been shown to play a role in growth and proliferation. By using MEF cells stably expressing Brk or breast cancer cells in which Brk is highly expressed (such as MDA-MB-435s and T47D), the effects of SOCS3 in cellular proliferation can be investigated by performing MTT assay, [$^{3}$H]-thymidine incorporation assay or fluorescence activated cell sorting (FACS) analysis. Colony formation in soft agar can be used to study the effects of SOCS3 in cell transformation. It has been reported that Brk enhances ErbB2-induced reinitiation of proliferation in 3D epithelia acini [134]. By introducing SOCS3 into this 3D culture system, we can investigate whether SOCS3 inhibits the proliferation in epithelia acini.

Some mouse cancer models can be used to test the effects of SOCS3 *in vivo*. We can test the effect of overexpression of SOCS3 on tumorigenesis by introducing SOCS3 via retroviral or lentiviral transduction into breast cancer cells. The transduced cells will be adoptively transferred into mice and evaluated for tumor development. We can also introduce SOCS3 into EμMyc transgenic mice that develop spontaneous B lymphoma to test whether SOCS3 inhibits tumor formation.

In this dissertation we describe the distinct ability of SOCS3 to inhibit the activity of Brk and evaluate the mechanisms by which SOCS3 suppresses Brk. The results demonstrate the inhibitory mechanism of SOCS3 relies on binding to Brk to affect both kinase activity and protein degradation. Originally studies identified SOCS proteins as classical negative regulators of JAK-STAT signaling, but this study characterizes SOCS3 as a suppressor of Brk.

## References


1. Goldsby R, Kindt T, Osborne B, Kuby J: **Immunology**. *Fifth Edition* 2003:W. H. Freeman and Company.
2. Gerez J, Bonfiglio J, Sosa S, Giacomini D, Acuna M, Nagashima AC, Perone MJ, Silberstein S, Renner U, Stalla GK, et al.: **Molecular transduction mechanisms of cytokine-hormone interactions: role of gp130 cytokines**. *Exp Physiol* 2007, **92**:801-806.
3. Yeh TC, Pellegrini S: **The Janus kinase family of protein tyrosine kinases and their role in signaling**. *Cell Mol Life Sci* 1999, **55**:1523-1534.
4. Yamaoka K, Saharinen P, Pesu M, Holt VE, 3rd, Silvennoinen O, O'Shea JJ: **The Janus kinases (Jaks)**. *Genome Biol* 2004, **5**:253.
5. Levy DE, Kessler DS, Pine R, Darnell JE, Jr.: **Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro**. *Genes Dev* 1989, **3**:1362-1371.
6. Dale TC, Imam AM, Kerr IM, Stark GR: **Rapid activation by interferon alpha of a latent DNA-binding protein present in the cytoplasm of untreated cells**. *Proc Natl Acad Sci U S A* 1989, **86**:1203-1207.
7. Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE, Jr.: **The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction**. *Proc Natl Acad Sci U S A* 1992, **89**:7840-7843.
8. Azam M, Erdjument-Bromage H, Kreider BL, Xia M, Quelle F, Basu R, Saris C, Tempst P, Ihle JN, Schindler C: **Interleukin-3 signals through multiple isoforms of Stat5**. *EMBO J* 1995, **14**:1402-1411.
9. Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, McKnight SL: **An interleukin-4-induced transcription factor: IL-4 Stat**. *Science* 1994, **265**:1701-1706.
10. Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T: **Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway**. *Cell* 1994, **77**:63-71.
11. Zhong Z, Wen Z, Darnell JE, Jr.: **Stat3 and Stat4: members of the family of signal transducers and activators of transcription**. *Proc Natl Acad Sci U S A* 1994, **91**:4806-4810.
12. Wakao H, Gouilleux F, Groner B: **Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response**. *EMBO J* 1995, **14**:854-855.
13. Schindler C, Fu XY, Improta T, Aebersold R, Darnell JE, Jr.: **Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha**. *Proc Natl Acad Sci U S A* 1992, **89**:7836-7839.
14. Shuai K, Schindler C, Prezioso VR, Darnell JE, Jr.: **Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein**. *Science* 1992, **258**:1808-1812.

15. Gutch MJ, Daly C, Reich NC: **Tyrosine phosphorylation is required for activation of an alpha interferon-stimulated transcription factor**. *Proc Natl Acad Sci U S A* 1992, **89**:11411-11415.
16. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JE, Jr.: **DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites**. *EMBO J* 1996, **15**:5616-5626.
17. Xu X, Sun YL, Hoey T: **Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain**. *Science* 1996, **273**:794-797.
18. Bhattacharya S, Eckner R, Grossman S, Oldread E, Arany Z, D'Andrea A, Livingston DM: **Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha**. *Nature* 1996, **383**:344-347.
19. Zhang JJ, Vinkemeier U, Gu W, Chakravarti D, Horvath CM, Darnell JE, Jr.: **Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling**. *Proc Natl Acad Sci U S A* 1996, **93**:15092-15096.
20. McDonald C, Reich NC: **Cooperation of the transcriptional coactivators CBP and p300 with Stat6**. *J Interferon Cytokine Res* 1999, **19**:711-722.
21. Mao X, Ren Z, Parker GN, Sondermann H, Pastorello MA, Wang W, McMurray JS, Demeler B, Darnell JE, Jr., Chen X: **Structural bases of unphosphorylated STAT1 association and receptor binding**. *Mol Cell* 2005, **17**:761-771.
22. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Jr., Kuriyan J: **Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA**. *Cell* 1998, **93**:827-839.
23. Neculai D, Neculai AM, Verrier S, Straub K, Klumpp K, Pfitzner E, Becker S: **Structure of the unphosphorylated STAT5a dimer**. *J Biol Chem* 2005, **280**:40782-40787.
24. Becker S, Groner B, Muller CW: **Three-dimensional structure of the Stat3beta homodimer bound to DNA**. *Nature* 1998, **394**:145-151.
25. Akira S: **Functional roles of STAT family proteins: lessons from knockout mice**. *Stem Cells* 1999, **17**:138-146.
26. Ihle JN: **The Stat family in cytokine signaling**. *Curr Opin Cell Biol* 2001, **13**:211-217.
27. Durbin JE, Hackenmiller R, Simon MC, Levy DE: **Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease**. *Cell* 1996, **84**:443-450.
28. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, et al.: **Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway**. *Cell* 1996, **84**:431-442.
29. Park C, Li S, Cha E, Schindler C: **Immune response in Stat2 knockout mice**. *Immunity* 2000, **13**:795-804.
30. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S: **Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality**. *Proc Natl Acad Sci U S A* 1997, **94**:3801-3804.

31. Kaplan MH, Sun YL, Hoey T, Grusby MJ: **Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice**. *Nature* 1996, **382**:174-177.
32. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, et al.: **Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells**. *Nature* 1996, **382**:171-174.
33. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T, Akira S: **Essential role of Stat6 in IL-4 signalling**. *Nature* 1996, **380**:627-630.
34. Shimoda K, van Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, Chu C, Quelle FW, Nosaka T, Vignali DA, et al.: **Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene**. *Nature* 1996, **380**:630-633.
35. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L: **Stat5a is mandatory for adult mammary gland development and lactogenesis**. *Genes Dev* 1997, **11**:179-186.
36. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW: **Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression**. *Proc Natl Acad Sci U S A* 1997, **94**:7239-7244.
37. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN: **Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses**. *Cell* 1998, **93**:841-850.
38. Decker T, Lew DJ, Mirkovitch J, Darnell JE, Jr.: **Cytoplasmic activation of GAF, an IFN-gamma-regulated DNA-binding factor**. *EMBO J* 1991, **10**:927-932.
39. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L: **Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway**. *Biochem J* 1998, **334 ( Pt 2)**:297-314.
40. Niwa H, Burdon T, Chambers I, Smith A: **Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3**. *Genes Dev* 1998, **12**:2048-2060.
41. Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T: **STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells**. *EMBO J* 1999, **18**:4261-4269.
42. Raz R, Lee CK, Cannizzaro LA, d'Eustachio P, Levy DE: **Essential role of STAT3 for embryonic stem cell pluripotency**. *Proc Natl Acad Sci U S A* 1999, **96**:2846-2851.
43. Hirano T, Ishihara K, Hibi M: **Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors**. *Oncogene* 2000, **19**:2548-2556.
44. Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S: **Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice**. *J Immunol* 1998, **161**:4652-4660.

45. Taga T, Kishimoto T: **Gp130 and the interleukin-6 family of cytokines**. *Annu Rev Immunol* 1997, **15**:797-819.
46. Mellado M, Rodriguez-Frade JM, Manes S, Martinez AC: **Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation**. *Annu Rev Immunol* 2001, **19**:397-421.
47. Vila-Coro AJ, Rodriguez-Frade JM, Martin De Ana A, Moreno-Ortiz MC, Martinez AC, Mellado M: **The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway**. *FASEB J* 1999, **13**:1699-1710.
48. Wong M, Fish EN: **RANTES and MIP-1alpha activate stats in T cells**. *J Biol Chem* 1998, **273**:309-314.
49. Zhong Z, Wen Z, Darnell JE, Jr.: **Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6**. *Science* 1994, **264**:95-98.
50. Bowman T, Broome MA, Sinibaldi D, Wharton W, Pledger WJ, Sedivy JM, Irby R, Yeatman T, Courtneidge SA, Jove R: **Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis**. *Proc Natl Acad Sci U S A* 2001, **98**:7319-7324.
51. Sano S, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, Yoshikawa K, Akira S, Takeda J: **Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis**. *EMBO J* 1999, **18**:4657-4668.
52. Sano S, Takahama Y, Sugawara T, Kosaka H, Itami S, Yoshikawa K, Miyazaki J, van Ewijk W, Takeda J: **Stat3 in thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival**. *Immunity* 2001, **15**:261-273.
53. Akaishi H, Takeda K, Kaisho T, Shineha R, Satomi S, Takeda J, Akira S: **Defective IL-2-mediated IL-2 receptor alpha chain expression in Stat3-deficient T lymphocytes**. *Int Immunol* 1998, **10**:1747-1751.
54. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S: **Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils**. *Immunity* 1999, **10**:39-49.
55. Lee CK, Raz R, Gimeno R, Gertner R, Wistinghausen B, Takeshita K, DePinho RA, Levy DE: **STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation**. *Immunity* 2002, **17**:63-72.
56. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, Takeda K, Akira S, Clarke AR, Watson CJ: **Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3**. *Genes Dev* 1999, **13**:2604-2616.
57. Kano A, Wolfgang MJ, Gao Q, Jacoby J, Chai GX, Hansen W, Iwamoto Y, Pober JS, Flavell RA, Fu XY: **Endothelial cells require STAT3 for protection against endotoxin-induced inflammation**. *J Exp Med* 2003, **198**:1517-1525.
58. Alonzi T, Maritano D, Gorgoni B, Rizzuto G, Libert C, Poli V: **Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver**. *Mol Cell Biol* 2001, **21**:1621-1632.

59. Jacoby JJ, Kalinowski A, Liu MG, Zhang SS, Gao Q, Chai GX, Ji L, Iwamoto Y, Li E, Schneider M, et al.: **Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age**. *Proc Natl Acad Sci U S A* 2003, **100**:12929-12934.
60. Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W, Musiani P, Poli V, Davies AM: **Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons**. *Mol Cell Neurosci* 2001, **18**:270-282.
61. Schweizer U, Gunnersen J, Karch C, Wiese S, Holtmann B, Takeda K, Akira S, Sendtner M: **Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult**. *J Cell Biol* 2002, **156**:287-297.
62. Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY: **Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation**. *Proc Natl Acad Sci U S A* 2004, **101**:4661-4666.
63. Levy DE, Lee CK: **What does Stat3 do?** *J Clin Invest* 2002, **109**:1143-1148.
64. Levitzki A: **Protein tyrosine kinase inhibitors as novel therapeutic agents**. *Pharmacol Ther* 1999, **82**:231-239.
65. Yu H, Jove R: **The STATs of cancer--new molecular targets come of age**. *Nat Rev Cancer* 2004, **4**:97-105.
66. Yu H, Pardoll D, Jove R: **STATs in cancer inflammation and immunity: a leading role for STAT3**. *Nat Rev Cancer* 2009, **9**:798-809.
67. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr.: **Stat3 as an oncogene**. *Cell* 1999, **98**:295-303.
68. Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R: **Stat3 activation by Src induces specific gene regulation and is required for cell transformation**. *Mol Cell Biol* 1998, **18**:2545-2552.
69. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, Tweardy DJ: **Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro**. *J Clin Invest* 1998, **102**:1385-1392.
70. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE, Jr.: **Stat3 activation is required for cellular transformation by v-src**. *Mol Cell Biol* 1998, **18**:2553-2558.
71. Spiekermann K, Pau M, Schwab R, Schmieja K, Franzrahe S, Hiddemann W: **Constitutive activation of STAT3 and STAT5 is induced by leukemic fusion proteins with protein tyrosine kinase activity and is sufficient for transformation of hematopoietic precursor cells**. *Exp Hematol* 2002, **30**:262-271.
72. Yang J, Chatterjee-Kishore M, Staugaitis SM, Nguyen H, Schlessinger K, Levy DE, Stark GR: **Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation**. *Cancer Res* 2005, **65**:939-947.
73. Hsieh FC, Cheng G, Lin J: **Evaluation of potential Stat3-regulated genes in human breast cancer**. *Biochem Biophys Res Commun* 2005, **335**:292-299.
74. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, et al.: **Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis**. *Oncogene* 2002, **21**:2000-2008.

75. Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, et al.: **Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways**. *Oncogene* 2005, **24**:5552-5560.
76. Chen W, Daines MO, Khurana Hershey GK: **Turning off signal transducer and activator of transcription (STAT): the negative regulation of STAT signaling**. *J Allergy Clin Immunol* 2004, **114**:476-489; quiz 490.
77. Haque SJ, Wu Q, Kammer W, Friedrich K, Smith JM, Kerr IM, Stark GR, Williams BR: **Receptor-associated constitutive protein tyrosine phosphatase activity controls the kinase function of JAK1**. *Proc Natl Acad Sci U S A* 1997, **94**:8563-8568.
78. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, Yi T: **Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1**. *Mol Cell Biol* 1996, **16**:6985-6992.
79. Migone TS, Rodig S, Cacalano NA, Berg M, Schreiber RD, Leonard WJ: **Functional cooperation of the interleukin-2 receptor beta chain and Jak1 in phosphatidylinositol 3-kinase recruitment and phosphorylation**. *Mol Cell Biol* 1998, **18**:6416-6422.
80. Aoki N, Matsuda T: **A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b**. *J Biol Chem* 2000, **275**:39718-39726.
81. Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, Barford D, Tonks NK: **TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B**. *J Biol Chem* 2001, **276**:47771-47774.
82. Irie-Sasaki J, Sasaki T, Matsumoto W, Opavsky A, Cheng M, Welstead G, Griffiths E, Krawczyk C, Richardson CD, Aitken K, et al.: **CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling**. *Nature* 2001, **409**:349-354.
83. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, et al.: **A family of cytokine-inducible inhibitors of signalling**. *Nature* 1997, **387**:917-921.
84. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, et al.: **A new protein containing an SH2 domain that inhibits JAK kinases**. *Nature* 1997, **387**:921-924.
85. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, et al.: **Structure and function of a new STAT-induced STAT inhibitor**. *Nature* 1997, **387**:924-929.
86. Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K: **Inhibition of Stat1-mediated gene activation by PIAS1**. *Proc Natl Acad Sci U S A* 1998, **95**:10626-10631.
87. Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, Shuai K: **Specific inhibition of Stat3 signal transduction by PIAS3**. *Science* 1997, **278**:1803-1805.
88. Arora T, Liu B, He H, Kim J, Murphy TL, Murphy KM, Modlin RL, Shuai K: **PIASx is a transcriptional co-repressor of signal transducer and activator of transcription 4**. *J Biol Chem* 2003, **278**:21327-21330.

89. Rogers RS, Horvath CM, Matunis MJ: **SUMO modification of STAT1 and its role in PIAS-mediated inhibition of gene activation**. *J Biol Chem* 2003, **278**:30091-30097.
90. Ungureanu D, Vanhatupa S, Kotaja N, Yang J, Aittomaki S, Janne OA, Palvimo JJ, Silvennoinen O: **PIAS proteins promote SUMO-1 conjugation to STAT1**. *Blood* 2003, **102**:3311-3313.
91. Nishida T, Yasuda H: **PIAS1 and PIASxalpha function as SUMO-E3 ligases toward androgen receptor and repress androgen receptor-dependent transcription**. *J Biol Chem* 2002, **277**:41311-41317.
92. Schmidt D, Muller S: **Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity**. *Proc Natl Acad Sci U S A* 2002, **99**:2872-2877.
93. Liu B, Yang R, Wong KA, Getman C, Stein N, Teitell MA, Cheng G, Wu H, Shuai K: **Negative regulation of NF-kappaB signaling by PIAS1**. *Mol Cell Biol* 2005, **25**:1113-1123.
94. Liu B, Yang Y, Chernishof V, Loo RR, Jang H, Tahk S, Yang R, Mink S, Shultz D, Bellone CJ, et al.: **Proinflammatory stimuli induce IKKalpha-mediated phosphorylation of PIAS1 to restrict inflammation and immunity**. *Cell* 2007, **129**:903-914.
95. Mitchell PJ, Barker KT, Martindale JE, Kamalati T, Lowe PN, Page MJ, Gusterson BA, Crompton MR: **Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours**. *Oncogene* 1994, **9**:2383-2390.
96. Siyanova EY, Serfas MS, Mazo IA, Tyner AL: **Tyrosine kinase gene expression in the mouse small intestine**. *Oncogene* 1994, **9**:2053-2057.
97. Barker KT, Jackson LE, Crompton MR: **BRK tyrosine kinase expression in a high proportion of human breast carcinomas**. *Oncogene* 1997, **15**:799-805.
98. Ostrander JH, Daniel AR, Lofgren K, Kleer CG, Lange CA: **Breast tumor kinase (protein tyrosine kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells**. *Cancer Res* 2007, **67**:4199-4209.
99. Derry JJ, Prins GS, Ray V, Tyner AL: **Altered localization and activity of the intracellular tyrosine kinase BRK/Sik in prostate tumor cells**. *Oncogene* 2003, **22**:4212-4220.
100. Easty DJ, Mitchell PJ, Patel K, Florenes VA, Spritz RA, Bennett DC: **Loss of expression of receptor tyrosine kinase family genes PTK7 and SEK in metastatic melanoma**. *Int J Cancer* 1997, **71**:1061-1065.
101. Kasprzycka M, Majewski M, Wang ZJ, Ptasznik A, Wysocka M, Zhang Q, Marzec M, Gimotty P, Crompton MR, Wasik MA: **Expression and oncogenic role of Brk (PTK6/Sik) protein tyrosine kinase in lymphocytes**. *Am J Pathol* 2006, **168**:1631-1641.
102. Lin HS, Berry GJ, Fee WE, Jr., Terris DJ, Sun Z: **Identification of tyrosine kinases overexpressed in head and neck cancer**. *Arch Otolaryngol Head Neck Surg* 2004, **130**:311-316.
103. Schmandt RE, Bennett M, Clifford S, Thornton A, Jiang F, Broaddus RR, Sun CC, Lu KH, Sood AK, Gershenson DM: **The BRK tyrosine kinase is expressed in**

**high-grade serous carcinoma of the ovary**. *Cancer Biol Ther* 2006, **5**:1136-1141.

104. Llor X, Serfas MS, Bie W, Vasioukhin V, Polonskaia M, Derry J, Abbott CM, Tyner AL: **BRK/Sik expression in the gastrointestinal tract and in colon tumors**. *Clin Cancer Res* 1999, **5**:1767-1777.
105. Vasioukhin V, Serfas MS, Siyanova EY, Polonskaia M, Costigan VJ, Liu B, Thomason A, Tyner AL: **A novel intracellular epithelial cell tyrosine kinase is expressed in the skin and gastrointestinal tract**. *Oncogene* 1995, **10**:349-357.
106. Petro BJ, Tan RC, Tyner AL, Lingen MW, Watanabe K: **Differential expression of the non-receptor tyrosine kinase BRK in oral squamous cell carcinoma and normal oral epithelium**. *Oral Oncol* 2004, **40**:1040-1047.
107. Qiu H, Miller WT: **Regulation of the nonreceptor tyrosine kinase Brk by autophosphorylation and by autoinhibition**. *J Biol Chem* 2002, **277**:34634-34641.
108. Kim H, Jung J, Lee ES, Kim YC, Lee W, Lee ST: **Molecular dissection of the interaction between the SH3 domain and the SH2-Kinase Linker region in PTK6**. *Biochem Biophys Res Commun* 2007, **362**:829-834.
109. Ko S, Ahn KE, Lee YM, Ahn HC, Lee W: **Structural basis of the auto-inhibition mechanism of nonreceptor tyrosine kinase PTK6**. *Biochem Biophys Res Commun* 2009, **384**:236-242.
110. Kim H, Lee ST: **An intramolecular interaction between SH2-kinase linker and kinase domain is essential for the catalytic activity of protein-tyrosine kinase-6**. *J Biol Chem* 2005, **280**:28973-28980.
111. Koo BK, Kim MH, Lee ST, Lee W: **Purification and spectroscopic characterization of the human protein tyrosine kinase-6 SH3 domain**. *J Biochem Mol Biol* 2002, **35**:343-347.
112. Lukong KE, Huot ME, Richard S: **BRK phosphorylates PSF promoting its cytoplasmic localization and cell cycle arrest**. *Cell Signal* 2009, **21**:1415-1422.
113. Qiu H, Miller WT: **Role of the Brk SH3 domain in substrate recognition**. *Oncogene* 2004, **23**:2216-2223.
114. Derry JJ, Richard S, Valderrama Carvajal H, Ye X, Vasioukhin V, Cochrane AW, Chen T, Tyner AL: **Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability**. *Mol Cell Biol* 2000, **20**:6114-6126.
115. Hong E, Shin J, Bang E, Kim MH, Lee ST, Lee W: **Complete sequence-specific 1H, 13C and 15N resonance assignments of the human PTK6 SH2 domain**. *J Biomol NMR* 2001, **19**:291-292.
116. Hong E, Shin J, Kim HI, Lee ST, Lee W: **Solution structure and backbone dynamics of the non-receptor protein-tyrosine kinase-6 Src homology 2 domain**. *J Biol Chem* 2004, **279**:29700-29708.
117. Mitchell PJ, Sara EA, Crompton MR: **A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK**. *Oncogene* 2000, **19**:4273-4282.
118. Qiu H, Zappacosta F, Su W, Annan RS, Miller WT: **Interaction between Brk kinase and insulin receptor substrate-4**. *Oncogene* 2005, **24**:5656-5664.

119. Zhang P, Ostrander JH, Faivre EJ, Olsen A, Fitzsimmons D, Lange CA: **Regulated association of protein kinase B/Akt with breast tumor kinase**. *J Biol Chem* 2005, **280**:1982-1991.
120. Palka-Hamblin HL, Gierut JJ, Bie W, Brauer PM, Zheng Y, Asara JM, Tyner AL: **Identification of beta-catenin as a target of the intracellular tyrosine kinase PTK6**. *J Cell Sci* **123**:236-245.
121. Lukong KE, Richard S: **Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration**. *Cell Signal* 2008, **20**:432-442.
122. Shen CH, Chen HY, Lin MS, Li FY, Chang CC, Kuo ML, Settleman J, Chen RH: **Breast tumor kinase phosphorylates p190RhoGAP to regulate rho and ras and promote breast carcinoma growth, migration, and invasion**. *Cancer Res* 2008, **68**:7779-7787.
123. Chen HY, Shen CH, Tsai YT, Lin FC, Huang YP, Chen RH: **Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin**. *Mol Cell Biol* 2004, **24**:10558-10572.
124. Coyle JH, Guzik BW, Bor YC, Jin L, Eisner-Smerage L, Taylor SJ, Rekosh D, Hammarskjold ML: **Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK**. *Mol Cell Biol* 2003, **23**:92-103.
125. Lukong KE, Larocque D, Tyner AL, Richard S: **Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression**. *J Biol Chem* 2005, **280**:38639-38647.
126. Haegebarth A, Heap D, Bie W, Derry JJ, Richard S, Tyner AL: **The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2**. *J Biol Chem* 2004, **279**:54398-54404.
127. Ikeda O, Miyasaka Y, Sekine Y, Mizushima A, Muromoto R, Nanbo A, Yoshimura A, Matsuda T: **STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation**. *Biochem Biophys Res Commun* 2009, **384**:71-75.
128. Liu L, Gao Y, Qiu H, Miller WT, Poli V, Reich NC: **Identification of STAT3 as a specific substrate of breast tumor kinase**. *Oncogene* 2006, **25**:4904-4912.
129. Weaver AM, Silva CM: **Signal transducer and activator of transcription 5b: a new target of breast tumor kinase/protein tyrosine kinase 6**. *Breast Cancer Res* 2007, **9**:R79.
130. Shin DS, Kim YG, Kim EM, Kim M, Park HY, Kim JH, Lee BS, Kim BG, Lee YS: **Solid-phase peptide library synthesis on HiCore resin for screening substrate specificity of Brk protein tyrosine kinase**. *J Comb Chem* 2008, **10**:20-23.
131. Brauer PM, Tyner AL: **Building a better understanding of the intracellular tyrosine kinase PTK6 - BRK by BRK**. *Biochim Biophys Acta* **1806**:66-73.
132. Zhong JL, Poghosyan Z, Pennington CJ, Scott X, Handsley MM, Warn A, Gavrilovic J, Honert K, Kruger A, Span PN, et al.: **Distinct functions of natural ADAM-15 cytoplasmic domain variants in human mammary carcinoma**. *Mol Cancer Res* 2008, **6**:383-394.
133. Kamalati T, Jolin HE, Mitchell PJ, Barker KT, Jackson LE, Dean CJ, Page MJ, Gusterson BA, Crompton MR: **Brk, a breast tumor-derived non-receptor**

**protein-tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor**. *J Biol Chem* 1996, **271**:30956-30963.
134. Xiang B, Chatti K, Qiu H, Lakshmi B, Krasnitz A, Hicks J, Yu M, Miller WT, Muthuswamy SK: **Brk is coamplified with ErbB2 to promote proliferation in breast cancer**. *Proc Natl Acad Sci U S A* 2008, **105**:12463-12468.
135. Aubele M, Walch AK, Ludyga N, Braselmann H, Atkinson MJ, Luber B, Auer G, Tapio S, Cooke T, Bartlett JM: **Prognostic value of protein tyrosine kinase 6 (PTK6) for long-term survival of breast cancer patients**. *Br J Cancer* 2008, **99**:1089-1095.
136. Vasioukhin V, Tyner AL: **A role for the epithelial-cell-specific tyrosine kinase Sik during keratinocyte differentiation**. *Proc Natl Acad Sci U S A* 1997, **94**:14477-14482.
137. Bae CS, Lee ST: **The human PTK6 interacts with a 23-kDa tyrosine-phosphorylated protein and is localized in cytoplasm in breast carcinoma T-47D cells**. *J. Biochem. Mol. Biol.* 2000, **34**:33-38.
138. Kamalati T, Jolin HE, Fry MJ, Crompton MR: **Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation**. *Oncogene* 2000, **19**:5471-5476.
139. Harvey AJ, Crompton MR: **Use of RNA interference to validate Brk as a novel therapeutic target in breast cancer: Brk promotes breast carcinoma cell proliferation**. *Oncogene* 2003, **22**:5006-5010.
140. Chakraborty G, Jain S, Kundu GC: **Osteopontin promotes vascular endothelial growth factor-dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms**. *Cancer Res* 2008, **68**:152-161.
141. Haegebarth A, Bie W, Yang R, Crawford SE, Vasioukhin V, Fuchs E, Tyner AL: **Protein tyrosine kinase 6 negatively regulates growth and promotes enterocyte differentiation in the small intestine**. *Mol Cell Biol* 2006, **26**:4949-4957.
142. Richard S, Vogel G, Huot ME, Guo T, Muller WJ, Lukong KE: **Sam68 haploinsufficiency delays onset of mammary tumorigenesis and metastasis**. *Oncogene* 2008, **27**:548-556.
143. Harvey AJ, Crompton MR: **The Brk protein tyrosine kinase as a therapeutic target in cancer: opportunities and challenges**. *Anticancer Drugs* 2004, **15**:107-111.
144. Elliott J, Johnston JA: **SOCS: role in inflammation, allergy and homeostasis**. *Trends Immunol* 2004, **25**:434-440.
145. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, Wakioka T, Ohtsuka S, Imaizumi T, Matsuda T, Ihle JN, et al.: **The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop**. *EMBO J* 1999, **18**:1309-1320.
146. Nicholson SE, De Souza D, Fabri LJ, Corbin J, Willson TA, Zhang JG, Silva A, Asimakis M, Farley A, Nash AD, et al.: **Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130**. *Proc Natl Acad Sci U S A* 2000, **97**:6493-6498.

147. Ram PA, Waxman DJ: **SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms**. *J Biol Chem* 1999, **274**:35553-35561.
148. Landsman T, Waxman DJ: **Role of the cytokine-induced SH2 domain-containing protein CIS in growth hormone receptor internalization**. *J Biol Chem* 2005, **280**:37471-37480.
149. Irandoust MI, Aarts LH, Roovers O, Gits J, Erkeland SJ, Touw IP: **Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor**. *EMBO J* 2007, **26**:1782-1793.
150. Croker BA, Kiu H, Nicholson SE: **SOCS regulation of the JAK/STAT signalling pathway**. *Semin Cell Dev Biol* 2008, **19**:414-422.
151. Sasaki A, Yasukawa H, Suzuki A, Kamizono S, Syoda T, Kinjyo I, Sasaki M, Johnston JA, Yoshimura A: **Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain**. *Genes Cells* 1999, **4**:339-351.
152. Waiboci LW, Ahmed CM, Mujtaba MG, Flowers LO, Martin JP, Haider MI, Johnson HM: **Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: implications for the development of a SOCS-1 antagonist**. *J Immunol* 2007, **178**:5058-5068.
153. Flowers LO, Johnson HM, Mujtaba MG, Ellis MR, Haider SM, Subramaniam PS: **Characterization of a peptide inhibitor of Janus kinase 2 that mimics suppressor of cytokine signaling 1 function**. *J Immunol* 2004, **172**:7510-7518.
154. Babon JJ, McManus EJ, Yao S, DeSouza DP, Mielke LA, Sprigg NS, Willson TA, Hilton DJ, Nicola NA, Baca M, et al.: **The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability**. *Mol Cell* 2006, **22**:205-216.
155. Bullock AN, Rodriguez MC, Debreczeni JE, Songyang Z, Knapp S: **Structure of the SOCS4-ElonginB/C complex reveals a distinct SOCS box interface and the molecular basis for SOCS-dependent EGFR degradation**. *Structure* 2007, **15**:1493-1504.
156. Bullock AN, Debreczeni JE, Edwards AM, Sundstrom M, Knapp S: **Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase**. *Proc Natl Acad Sci U S A* 2006, **103**:7637-7642.
157. Piessevaux J, Lavens D, Peelman F, Tavernier J: **The many faces of the SOCS box**. *Cytokine Growth Factor Rev* 2008, **19**:371-381.
158. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, Simpson RJ, Moritz RL, Cary D, Richardson R, Hausmann G, et al.: **The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation**. *Proc Natl Acad Sci U S A* 1999, **96**:2071-2076.
159. Kamura T, Burian D, Yan Q, Schmidt SL, Lane WS, Querido E, Branton PE, Shilatifard A, Conaway RC, Conaway JW: **Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase**. *J Biol Chem* 2001, **276**:29748-29753.

160. Kamizono S, Hanada T, Yasukawa H, Minoguchi S, Kato R, Minoguchi M, Hattori K, Hatakeyama S, Yada M, Morita S, et al.: **The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2**. *J Biol Chem* 2001, **276**:12530-12538.
161. Frantsve J, Schwaller J, Sternberg DW, Kutok J, Gilliland DG: **Socs-1 inhibits TEL-JAK2-mediated transformation of hematopoietic cells through inhibition of JAK2 kinase activity and induction of proteasome-mediated degradation**. *Mol Cell Biol* 2001, **21**:3547-3557.
162. Ungureanu D, Saharinen P, Junttila I, Hilton DJ, Silvennoinen O: **Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1**. *Mol Cell Biol* 2002, **22**:3316-3326.
163. Orr SJ, Morgan NM, Buick RJ, Boyd CR, Elliott J, Burrows JF, Jefferies CA, Crocker PR, Johnston JA: **SOCS3 targets Siglec 7 for proteasomal degradation and blocks Siglec 7-mediated responses**. *J Biol Chem* 2007, **282**:3418-3422.
164. Orr SJ, Morgan NM, Elliott J, Burrows JF, Scott CJ, McVicar DW, Johnston JA: **CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover**. *Blood* 2007, **109**:1061-1068.
165. Mansell A, Smith R, Doyle SL, Gray P, Fenner JE, Crack PJ, Nicholson SE, Hilton DJ, O'Neill LA, Hertzog PJ: **Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation**. *Nat Immunol* 2006, **7**:148-155.
166. De Sepulveda P, Ilangumaran S, Rottapel R: **Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation**. *J Biol Chem* 2000, **275**:14005-14008.
167. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP: **Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA**. *Mol Cell* 2003, **12**:1413-1426.
168. Kamio M, Yoshida T, Ogata H, Douchi T, Nagata Y, Inoue M, Hasegawa M, Yonemitsu Y, Yoshimura A: **SOCS1 [corrected] inhibits HPV-E7-mediated transformation by inducing degradation of E7 protein**. *Oncogene* 2004, **23**:3107-3115.
169. Rui L, Yuan M, Frantz D, Shoelson S, White MF: **SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2**. *J Biol Chem* 2002, **277**:42394-42398.
170. Liu E, Cote JF, Vuori K: **Negative regulation of FAK signaling by SOCS proteins**. *EMBO J* 2003, **22**:5036-5046.
171. Zhang JG, Metcalf D, Rakar S, Asimakis M, Greenhalgh CJ, Willson TA, Starr R, Nicholson SE, Carter W, Alexander WS, et al.: **The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo**. *Proc Natl Acad Sci U S A* 2001, **98**:13261-13265.
172. Boyle K, Egan P, Rakar S, Willson TA, Wicks IP, Metcalf D, Hilton DJ, Nicola NA, Alexander WS, Roberts AW, et al.: **The SOCS box of suppressor of cytokine signaling-3 contributes to the control of G-CSF responsiveness in vivo**. *Blood* 2007, **110**:1466-1474.

173. Naka T, Matsumoto T, Narazaki M, Fujimoto M, Morita Y, Ohsawa Y, Saito H, Nagasawa T, Uchiyama Y, Kishimoto T: **Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice**. *Proc Natl Acad Sci U S A* 1998, **95**:15577-15582.
174. Starr R, Metcalf D, Elefanty AG, Brysha M, Willson TA, Nicola NA, Hilton DJ, Alexander WS: **Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1**. *Proc Natl Acad Sci U S A* 1998, **95**:14395-14399.
175. Metcalf D, Mifsud S, Di Rago L, Nicola NA, Hilton DJ, Alexander WS: **Polycystic kidneys and chronic inflammatory lesions are the delayed consequences of loss of the suppressor of cytokine signaling-1 (SOCS-1)**. *Proc Natl Acad Sci U S A* 2002, **99**:943-948.
176. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, Yoshida H, Kubo M, Yoshimura A: **SOCS1/JAB is a negative regulator of LPS-induced macrophage activation**. *Immunity* 2002, **17**:583-591.
177. Metcalf D, Greenhalgh CJ, Viney E, Willson TA, Starr R, Nicola NA, Hilton DJ, Alexander WS: **Gigantism in mice lacking suppressor of cytokine signalling-2**. *Nature* 2000, **405**:1069-1073.
178. Greenhalgh CJ, Bertolino P, Asa SL, Metcalf D, Corbin JE, Adams TE, Davey HW, Nicola NA, Hilton DJ, Alexander WS: **Growth enhancement in suppressor of cytokine signaling 2 (SOCS-2)-deficient mice is dependent on signal transducer and activator of transcription 5b (STAT5b)**. *Mol Endocrinol* 2002, **16**:1394-1406.
179. Greenhalgh CJ, Metcalf D, Thaus AL, Corbin JE, Uren R, Morgan PO, Fabri LJ, Zhang JG, Martin HM, Willson TA, et al.: **Biological evidence that SOCS-2 can act either as an enhancer or suppressor of growth hormone signaling**. *J Biol Chem* 2002, **277**:40181-40184.
180. Tannahill GM, Elliott J, Barry AC, Hibbert L, Cacalano NA, Johnston JA: **SOCS2 can enhance interleukin-2 (IL-2) and IL-3 signaling by accelerating SOCS3 degradation**. *Mol Cell Biol* 2005, **25**:9115-9126.
181. Lehmann U, Schmitz J, Weissenbach M, Sobota RM, Hortner M, Friederichs K, Behrmann I, Tsiaris W, Sasaki A, Schneider-Mergener J, et al.: **SHP2 and SOCS3 contribute to Tyr-759-dependent attenuation of interleukin-6 signaling through gp130**. *J Biol Chem* 2003, **278**:661-671.
182. Roberts AW, Robb L, Rakar S, Hartley L, Cluse L, Nicola NA, Metcalf D, Hilton DJ, Alexander WS: **Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3**. *Proc Natl Acad Sci U S A* 2001, **98**:9324-9329.
183. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, Ihle JN: **SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation**. *EMBO J* 2003, **22**:372-384.
184. Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, Hanada T, Takeda K, Akira S, Hoshijima M, et al.: **IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages**. *Nat Immunol* 2003, **4**:551-556.
185. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, et al.: **CIS3/SOCS3/SSI3 plays a negative**

**regulatory role in STAT3 activation and intestinal inflammation**. *J Exp Med* 2001, **193**:471-481.
186. Tischoff I, Hengge UR, Vieth M, Ell C, Stolte M, Weber A, Schmidt WE, Tannapfel A: **Methylation of SOCS-3 and SOCS-1 in the carcinogenesis of Barrett's adenocarcinoma**. *Gut* 2007, **56**:1047-1053.
187. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM: **SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer**. *Proc Natl Acad Sci U S A* 2003, **100**:14133-14138.
188. Watanabe D, Ezoe S, Fujimoto M, Kimura A, Saito Y, Nagai H, Tachibana I, Matsumura I, Tanaka T, Kanegane H, et al.: **Suppressor of cytokine signalling-1 gene silencing in acute myeloid leukaemia and human haematopoietic cell lines**. *Br J Haematol* 2004, **126**:726-735.
189. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ: **Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing**. *Gastroenterology* 2007, **132**:384-396.
190. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC, Herman JG: **SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity**. *Nat Genet* 2001, **28**:29-35.
191. Weber A, Hengge UR, Bardenheuer W, Tischoff I, Sommerer F, Markwarth A, Dietz A, Wittekind C, Tannapfel A: **SOCS-3 is frequently methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition**. *Oncogene* 2005, **24**:6699-6708.
192. Barth TF, Melzner I, Wegener S, Bucur AJ, Bruderlein S, Dorsch K, Hasel C, Leithauser F, Moller P: **[Biallelic mutation of SOCS-1 impairs JAK2 degradation and sustains phospho-JAK2 action in MedB-1 mediastinal lymphoma line]**. *Verh Dtsch Ges Pathol* 2005, **89**:234-244.
193. Welchman RL, Gordon C, Mayer RJ: **Ubiquitin and ubiquitin-like proteins as multifunctional signals**. *Nat Rev Mol Cell Biol* 2005, **6**:599-609.
194. Kerscher O, Felberbaum R, Hochstrasser M: **Modification of proteins by ubiquitin and ubiquitin-like proteins**. *Annu Rev Cell Dev Biol* 2006, **22**:159-180.
195. Young P, Deveraux Q, Beal RE, Pickart CM, Rechsteiner M: **Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a**. *J Biol Chem* 1998, **273**:5461-5467.
196. Haglund K, Dikic I: **Ubiquitylation and cell signaling**. *EMBO J* 2005, **24**:3353-3359.
197. Pickart CM, Fushman D: **Polyubiquitin chains: polymeric protein signals**. *Curr Opin Chem Biol* 2004, **8**:610-616.
198. Liu L, McBride KM, Reich NC: **STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3**. *Proc Natl Acad Sci U S A* 2005, **102**:8150-8155.
199. Treier M, Staszewski LM, Bohmann D: **Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain**. *Cell* 1994, **78**:787-798.
200. Jura N, Scotto-Lavino E, Sobczyk A, Bar-Sagi D: **Differential modification of Ras proteins by ubiquitination**. *Mol Cell* 2006, **21**:679-687.

201. Auernhammer CJ, Bousquet C, Melmed S: **Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter**. *Proc Natl Acad Sci U S A* 1999, **96**:6964-6969.
202. Wormald S, Zhang JG, Krebs DL, Mielke LA, Silver J, Alexander WS, Speed TP, Nicola NA, Hilton DJ: **The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling**. *J Biol Chem* 2006, **281**:11135-11143.
203. Cohney SJ, Sanden D, Cacalano NA, Yoshimura A, Mui A, Migone TS, Johnston JA: **SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation**. *Mol Cell Biol* 1999, **19**:4980-4988.
204. Stross C, Radtke S, Clahsen T, Gerlach C, Volkmer-Engert R, Schaper F, Heinrich PC, Hermanns HM: **Oncostatin M receptor-mediated signal transduction is negatively regulated by SOCS3 through a receptor tyrosine-independent mechanism**. *J Biol Chem* 2006, **281**:8458-8468.
205. De Souza D, Fabri LJ, Nash A, Hilton DJ, Nicola NA, Baca M: **SH2 domains from suppressor of cytokine signaling-3 and protein tyrosine phosphatase SHP-2 have similar binding specificities**. *Biochemistry* 2002, **41**:9229-9236.
206. Johnston JA: **Are SOCS suppressors, regulators, and degraders?** *J Leukoc Biol* 2004, **75**:743-748.
207. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mule J, Kerr WG, et al.: **Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity**. *Nat Med* 2005, **11**:1314-1321.
208. He B, You L, Uematsu K, Matsangou M, Xu Z, He M, McCormick F, Jablons DM: **Cloning and characterization of a functional promoter of the human SOCS-3 gene**. *Biochem Biophys Res Commun* 2003, **301**:386-391.
209. Ikeda F, Dikic I: **Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series**. *EMBO Rep* 2008, **9**:536-542.
210. Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ: **TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains**. *Mol Cell* 2004, **15**:535-548.
211. Nishikawa H, Ooka S, Sato K, Arima K, Okamoto J, Klevit RE, Fukuda M, Ohta T: **Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase**. *J Biol Chem* 2004, **279**:3916-3924.
212. Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP: **A proteomics approach to understanding protein ubiquitination**. *Nat Biotechnol* 2003, **21**:921-926.
213. Elliott J, Hookham MB, Johnston JA: **The suppressors of cytokine signalling E3 ligases behave as tumour suppressors**. *Biochem Soc Trans* 2008, **36**:464-468.